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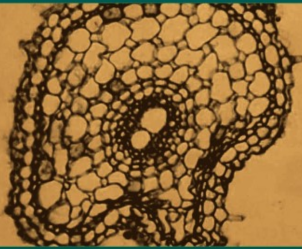
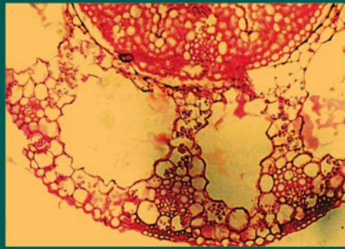
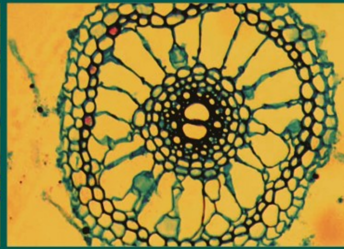

Disease Management

Bacteria in Agrobiology: Disease Management

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Editor

Bacteria in Agrobiology: Disease Management

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Cover illustration: Optical micrograph showing cross sections of intercellular colonization rice calli and regenerated plantlets by A. *caulinodans*: CS view of root uninoculated control; magnified cross section view of leaf colonized by A. *caulinodans* in regenerated rice plant; possible sites of infection and colonization of rice root (from left to right); see also Fig. 3.1 in "Endophytic Bacteria – Perspectives and Applications in Agricultural Crop Production", Senthilkumar M, R. Anandham, M. Madhaiyan, V. Venkateswaran, Tong Min Sa, in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

Background: Positive immunofluorescence micrograph showing reaction with cells of the biofertilizer strain used in autecological biogeography studies; see also Fig. 10.6 in "Beneficial Endophytic Rhizobia as Biofertilizer Inoculants for Rice and the Spatial Ecology of this Bacteria-Plant Association", Youssef Garas Yanni, Frank B. Dazzo, Mohamed I. Zidan, in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

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Preface

For burgeoning population while arable land and most other natural resources continue to decrease, and as our environment becomes further congested and stressed, the need for management of crop plant diseases effectively and safely has become one of the most basic requirements for feeding the hungry billions of our increasing overpopulated world.

Although diverse group of microorganisms exhibit to impart interference with the growth of disease causing phytopathogens, but only few of these microbial antagonists have achieved success in providing protection against deleterious pathogens resulting in growth promotions and in crop yield enhancements. Such antagonists are one of the important groups of plant growth promoting bacteria (PGPB), which act as potential biocontrol agents for the management of plant diseases. The combination of PGPB rhizospheric proficiency along with suppressiveness of diseases and pests are considered as contemporary research themes to a great extent. Numerous bacterial genera have now been analysed for their efficiency against soil and seed borne diseases causing pathogens, but their replication in field has been chimerical to a great extent.

Contents of the present book discuss various facts of advancement of disease management in sustainable manner, is suitably described in the 18 chapters contributed by eminent experts of their area of research. Bacteria in general and PGPR in particular in disease management is followed by effect of various factors influencing their efficacy in biological control of pre- and post-harvest disease of roots, tuber croups, cereals and other wide range of crop plants followed by well-established phenomenon of induced systemic resistance in plant diseases that leads to healthy plant growth. A due account is provided on PGPR plant interaction in disease management and suppressiveness of phytopathogens. For such purpose the involvement of antifungal substance of bacterial origin cannot be ruled out. The beneficial bacteria produce certain antagonistic molecules that are not limited to act against harmful bacteria and fungi, but their application has proved a better insight in to the management of plant parasitic nematodes and disease complex with fungi in suitable manner.

This book will be useful not only for the students, teacher and researchers but also for those interested to strengthen their knowledge in Agricultural Microbiology, Phytopathology and Plant Protection, Environmental Management, Crop Science and Agronomy.

My heartfelt gratitude is to all the expert contributors for their overwhelming support and co-operation. I acknowledge with thanks the assistance rendered by my research students Dr. Abhinav, Dr. Rajat and Narendra. I am thankful to University Grant Commission (UGC), New Delhi, and Uttarakhand Council of Science and Technology (UCOST), Dehradun, Uttarakhand, India, for their financial support in the form of research grants that indeed served as a prelude to lay foundation for compilation of book like this.

I wish to record my special thanks to Dr. Jutta Lindenborn from Springer for her valuable support in multivarious ways. In the last, the cooperation and moral support from my wife, Dr. Sadhana Maheshwari, and son, Er. Ashish, for inspiration and encouragements.

Haridwar, Uttarakhand, India Dinesh K. Maheshwari

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Chapter 1
Advances in Plant Growth Promoting Rhizobacteria for Biological Control of Plant Diseases

Kui-Jae Lee, Byung-Taek Oh, and Kamala-Kannan Seralathan

1.1 Introduction

Plant disease control is an important need for agriculture in the twenty-first century. The increasing demand for a healthy food supply by a growing human population will require controlling of plant diseases that reduce yield of economically important crops. Different approaches are used to prevent, mitigate, or control plant diseases in economically important crops. Beyond good agronomic and horticulture practice, farmers often rely heavily on synthetic pesticides and fungicides. Such inputs to the agriculture have contributed significantly to the spectacular improvements in yield over the past few decades. However, the excessive application and misuse of pesticides and fungicides have led to extensive pollution in the agriculture field. These pesticides and fungicides directly or indirectly enter the aquatic ecosystem and are subsequently biomagnified in food chain, endangering ecosystem and public health (Robison et al. 2006). Furthermore, the growing cost of pesticides, revised environmental safety regulations, and public concern about synthetic pesticides in foods have reduced the application of chemical-based synthetic pesticides and fungicides that effectively control several plant diseases. Consequently, researchers have focused their efforts on developing new methods that provide successful control over plant diseases without any negative effect for human health and environment (Gerhardson 2002).

Biological control of plant diseases has emerged as a powerful alternative to synthetic pesticides and fungicides. The term “biological control” applies to the use of microbial antagonist to suppress plant disease, and the organism that suppresses the pathogen is referred to as a biological control agent (BCA). Increasing the population of a BCA in the vicinity of a plant can suppress the incidence of diseases

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without producing negative effects on the rest of the microbial community as well as to other organisms in the ecosystem. The involvement of different mechanisms of disease suppression by a single microorganism, the complex interaction between the organisms, and the survival of BCA in the environment in which they are used, all contribute to the belief that biological control of plant diseases will be more durable than synthetic chemicals (Compant et al. 2005; Lee et al. 2008).

Plant growth promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria capable of promoting plant growth. In last few decades, several bacteria including the species of *Bacillus*, *Pseudomonas*, *Azospirillium*, *Azotobacter*, *Klebsiella*, *Burkholderia*, and *Serratia* have been reported as PGPR (El-Khawas and Adachi 1999; Gray and Smith 2005; Jaleel et al. 2007; Lee et al. 2008; Joe et al. 2009). As reviewed by Gray and Smith (2005), a lot of work has been done on the mechanisms and principles of plant growth promotion, which was accepted widely as rhizosphere effect. Gray and Smith (2005) also reported that some of these PGPR can also enter into plant root interior and establish endophytic bacterial population. The survival and colonization of these bacteria in different parts of the host plants reflect the ability of the bacteria to adapt themselves to these selective ecological niches (Gray and Smith 2005). Consequently, intimate association between bacteria and host plants can be formed without harming the mechanism of the plant.

Despite their different ecological niches, most of the PGPR use the same mechanism to control phytopathogens. The most commonly recognized mechanisms are competition for root colonization or a substrate, production of inhibitory substances, and inducing systemic resistance (ISR) in the host plants to a broad spectrum of pathogens (Bloemberg and Lugtenberg 2001). This review surveys the research focusing on the principles and mode of action of PGPR, and their use for the biological control of plant diseases.

1.2 Competition for Nutrients and Colonization Potential of PGPR

The root surface and surrounding rhizosphere are significant nutrient sinks. Thus, the root surface and rhizoplane attract diverse microorganisms, including phytopathogens. Competition for these nutrients is the fundamental mechanism by which the PGPR protect plants from phytopathogens (Rovira 1965; Duffy 2001). While it is difficult to prove directly, much indirect evidence suggests that competition is the important factor for limiting disease incidence and severity. As an example, soil-borne pathogens such as *Fusarium* sp. and *Pythium* sp. that infect the host plant through mycelial contact are more susceptible to competition from other soil- and plant-associated microbes than those pathogens that germinate directly on the plant surface and infect through appressoria and infection pegs.

Root colonization of PGPR is another fundamental mechanism for plant growth promotion and biological control. The PGPR colonize root surfaces by active

motility facilitated by flagella and are guided by chemotactic response. Several chemical substances including organic acids, amino acids, and specific sugars are present in the root exudates, which play an important role in chemotaxis (Compant et al. 2005). However, the degree of chemotactic response to each of these compounds differs among the bacterial strains. Some bacteria may be uniquely equipped to sense chemoattractants; the best example is the exudates from rice plants which have stronger chemotactic response to endophytic bacteria than other bacteria present in that environment (Bacilio-Jimenez et al. 2003). Moreover, some exudates can also have antimicrobial activity and thus give ecological niche advantage to organisms that have resistance to antimicrobial compounds. The quantity and the composition of the root exudates are influenced by genetic and environmental factors (Bais et al. 2004). This indicates that PGPR competence highly depends either on their ability to take advantage of a specific environment or on their ability to adapt to environmental conditions.

In addition to chemotactic factors, bacterial lipopolysaccharides and pili can also contribute an important role in root colonization of PGPR. The importance of lipopolysaccharide might be depending upon the O-antigenic side chain of the bacterial strain. As an example, the O-antigenic side chain of *Pseudomonas fluorescens* WCS374 does not involve in potato root adhesion, whereas the O-antigenic side chain of *P. fluorescens* PCL1205 contributes a major role in tomato root colonization (De Weger et al. 1989; Dekkers et al. 1998). Like lipopolysaccharides, pili also contribute a major role in root colonization. Several studies reported the involvement of type IV pili in plant colonization of *Azoarcus* sp (Strom and Lory 1993; Dorr et al. 1998; Steenhoudt and Vanderleyden 2000).

Soil temperature plays a significant role in rhizosphere colonization of PGPR. Bowers and Parke (1993) reported that the increase in temperature of soil was negatively correlated with the density of bacterial population of roots between depths of 0 and 1 cm, but positively correlated with root colonization at deeper soil layers (1–2 cm). Similarly, the adsorption of the microbial cells to the soil particles depends on soil pH, proportions of organic matter, clay contents, and microbial extracellular polymers (Gammack et al. 1992). For example, in artificial soils, the survival of the inoculated bacteria was positively correlated with the percentage of clay, organic matter and nitrogen content, but was negatively correlated with the percentage of sand and calcium carbonate content. No correlation was found between bacterial viability and soil pH level, phosphate or potassium, or loam percentage.

1.3 Role of Siderophores in Biological Control of Plant Diseases

Iron is an essential element for almost all living organisms. Despite being one of the most abundant elements in the earth's crust, the bioavailability of iron in rhizosphere soil is extremely limited. In highly oxidized and aerated soil, the iron element is present in Fe^{3+} form, which is insoluble and the concentration may be as low as 10^{-18} M (Lindsay 1979). This concentration is too low for the growth of the microorganisms, which generally need concentrations of

10^{-6} M. To survive in such an environment, PGPR produce low molecular weight compounds called siderophores to competitively sequester iron from the micro-environment.

Siderophores are a group of low molecular weight compounds produced by microorganisms that bind ferric ion extracellularly to form a stable chelate for transport into the cell. A total of approximately 50 different siderophore structures have been described so far which mainly consists of the basic catecholate ligand structures. Although various types of bacterial siderophores are reported, each type differs in its ability to sequester iron; in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper and Henkels 1999). Kloepper et al. (1980) first reported the importance of siderophore production in biological control of *Erwinia carotovora* by plant growth promoting *P. fluorescens* strains A1, BK1, TL3B1, and B10. Elad and Baker (1985) established the correlation between the siderophore synthesis in *Pseudomonas* sp and their capacity to inhibit *Fusarium oxysporum* spore germination.

Siderophore synthesis in bacterial system is regulated by iron-sensitive Fur proteins; global regulators such as GacS and GacA; sigma factors such as RpoS, PvdS, and FpvI; quorum sensing molecule such as *N*-acyl homoserine lactone; and site-specific recombinases (Cornelis and Matthijs 2002; Ravel and Cornelis 2003). However, some studies have reported that GacS and RpoS are not involved in the regulation of siderophores synthesis (Kojic et al. 1999; Saleh and Glick 2001). In addition to the genetic regulators, several environmental factors such as pH, elemental concentration, and nutrient availability also regulate the synthesis of siderophores in bacterial system (Duffy and Defago 1999).

1.4 Antibiotic-Mediated Suppression of Plant Diseases

Antibiosis is another major mechanism in biological control of plant diseases. A variety of antimicrobial agents have been isolated, identified, and characterized from several PGPR including the species of *Pseudomonas*, *Bacillus*, *Streptomyces*, *Trichoderma* sp., and *Lysobacter* sp. (Table 1.1). The most commonly reported antimicrobial agents are 2,4-diacetylphloroglucinol (Phl or DAPG), kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluterin, viscosinamide, xanthobaccin, zwittermycin A, tensin, tropolone, and cyclic lipopeptides (Thomashow et al. 1990; Defago 1993; Milner et al. 1995, 1996; Kim et al. 1999; Raaijmakers et al. 2002; Nielsen and Sorensen 2003; Islam et al. 2005). Some of these compounds have broad spectrum activity and also inhibit the growth of different groups of micro- and macroorganisms. As an example, DAPG produced by the fluorescent pseudomonads exhibits antifungal, antibacterial, and antihelmenthic activities (Thomashow and Weller 1996). Consequently, several biocontrol strains are able to produce multiple antimicrobial agents which can suppress several phytopathogens. For example, the biological control agent *Bacillus cereus* UW85 produces both zwittermycin A and kanosamine and inhibits several fungal

Table 1.1 Antibiotics produced by biological control agents

Antibiotics	Biological control agent	Target pathogen	References
2,4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium* sp.	Shanahan et al. (1992)
Phenazines	*Pseudomonas fluorescens* 2-79	*Gaeumannomyces graminis* var. *tritici*	Thomashow et al. (1990)
Mycosubtilin	*Bacillus subtilis* BBG100	*Pythium aphanidermatum*	Leclere et al. (2005)
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*	Moyne et al. (2001)
Zwittermycin A	*Bacillus cereus* UW85	*Phytophthora* sp.	Smith et al. (1993)
Bacillomycin and fengycin	*Bacillus amyloliquefaciens*	*Fusarium oxysporum*	Koumoutsi et al. (2004)
Iturin A	*Bacillus subtilis* QST713	*Botrytis cinerea* and *Rhizoctonia solani*	Paulitz an Belanger (2001)
Xanthobaccin A	*Lysobacter* sp.	*Aphanomyces cochlioides*	Islam et al. (2005)
Gliotoxin	*Trichoderma virens*	*Rhizoctonia solani*	Wilhite et al. (2001)

phytopathogens (Silo-Suh et al. 1994). The ability to produce multiple classes of antimicrobial agents is likely to enhance biological control activity.

Synthesis of antimicrobial agents in bacterial system is associated with the metabolic activity of the organism, which in turn depends upon the nutrient availability and environmental conditions such as pH, temperature, and other physicochemical conditions (Milner et al. 1995, 1996; Duffy and Defago 1999). The availability of trace elements, type of the carbon source, and genetic stability of the organism also affect the biosynthesis rate of secondary metabolites (Duffy and Defago 2000). Duffy and Defago (1999) also reported that the environmental conditions favoring the synthesis of one antimicrobial compound may not favor another. Thus, the varied arsenal of biological control agent may enable antagonists to perform their ultimate objective of pathogen suppression under the widest range of environmental conditions. As an example, in *P. fluorescens* CHA0 the presence of glucose (carbon source) stimulates the biosynthesis of DAPG and represses the biosynthesis of pyoluteorin. However, if the glucose level is depleted, pyoluteorin becomes the more abundant antimicrobial compound produced by this strain. This ensures the degree of flexibility for the antagonist when confronted with a different or changeable environment (Duffy and Defago 1999).

In addition to abiotic factors, several biotic conditions also affect the biosynthesis of secondary metabolites in bacterial system. The presence of certain metabolic by-products in the environment can affect the biosynthesis of antimicrobial compounds. As an example, the presence of salicylate and pyoluteorin can affect DAPG production in *P. fluorescens* CHA0 (Schnider-Keel et al. 2000). Furthermore, the antimicrobial synthesis is also influenced by the plant root exudates. Picard et al. (2000) reported that root exudates from the older plants induce the synthesis of DAPG, whereas exudates from the younger plants do not induce DAPG synthesis. Several studies have reported that the global regulators such as GacS and GacA,

sigma factors such as RpoS and RpoD, and quorum sensing molecule such as *N*-acyl homoserine lactone regulate the biosynthesis of antimicrobial compounds (Pierson et al. 1998; Bloemberg and Lugtenberg 2001; Haas and Keel 2003).

1.5 Role of Lytic Enzymes and Other By-Products in Direct Antagonism

Plant growth promoting bacteria secrete and excrete several lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, glucan, and DNA. Presence of these enzymes can suppress the growth and activities of the phytopathogens either directly or indirectly. For example, chitinase produced by *Serratia plymuthica* C48 inhibited the spore germination and germ tube elongation in *Botrytis cinerea* (Frankowski et al. 2001). The ability of the *Paenibacillus* sp. to produce extracellular chitinase is considered crucial to act as antagonist against *F. oxysporum* f. sp. *cucumernium.* β-1,3-glucanase produced by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 lyses the cell walls of *F. oxysporum* f. sp. *cucumerinum* (Singh et al. 1999). Laminarinase synthesized by the *Pseudomonas stutzeri* lyse the mycelia of *Fusarium solani* (Lim et al. 1991). Besides chitin and glucan, the skeleton of filamentous fungal cell wall contains lipids and proteins. Thus, proteases produced by the biocontrol agent may lyse the cell wall of fungal phytopathogens. Sivan and Chet (1989) reported that pretreatment of *F. oxysporum* with proteolytic enzymes increases their susceptibility to lysis by chitinase and 1,3-glucanase of *T. harzianum*. Furthermore, several studies reported that the products of lytic enzyme activity may also contribute to indirect disease suppression. For example, oligosaccharides derived from the fungal cell walls are known to be potent inducers of plant host defenses. Kilic-Ekici and Yuen (2003) reported that *Lysobacter enzymogenes* strain C3 has been shown to induce plant host resistance to disease through precise activities. However, the quantitative contribution of any and all of the aforesaid enzymes/compounds to disease suppression is likely to be dependent on the composition of the rhizosphere soil. As an example, in post-harvest disease control, addition of chitosan can stimulate microbial degradation of pathogens similar to that of an applied hyper parasite (Benhamou 2004). Lafontaine and Benhamou (1996) reported that amendment of plant growth substratum with chitosan suppressed the root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* in tomato. Similar to siderophores and antibiotics, the biosynthesis of lytic enzymes is also regulated by global regulators such as GacS/GacA or GrrA/Grrs and colony phase variation.

Several bacterial by-products also inhibit the growth and activities of phytopathogens. Hydrogen cyanide (HCN) is one of the most commonly reported by-products in most of the PGPR. Hydrogen cyanide effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by PGPR is believed to be involved in the suppression of phytopathogens.

Voisard et al. (1989) reported that HCN compounds produced by *P. fluorescens* contribute a significant role in biological control of black rot of tobacco caused by *Thielaviopsis basicola*. In addition to HCN compounds, volatile organic compounds emitted from the biological control agents also have a significant role in biological control of plant diseases. Howell et al. (1988) reported that volatile compound ammonia produced by *Enterobacter cloacae* is involved in the suppression *Pythium ultimum* induced damping-off of cotton. Chaurasia et al. (2005) reported that volatile compounds produced by *Bacillus subtilis* cause structural deformations in several phytopathogenic fungi. Kai et al. (2007) reported that the small organic volatile compounds emitted from the bacteria inhibited the mycelia growth of *Rhizoctonia solani*. In addition to antagonistic activity, volatile components emitted by PGPR also promote the growth of plants (Ryu et al. 2003). While it is clear that PGPR can release a wide range of volatile components into their surrounding environment, the type and the amount of volatile components produced in the presence of the pathosystem and the influence of the environmental factors have not been well documented. So this part of the study remains a frontier for discovery.

1.6 Degradation of Pathogen Virulence Factors

Another important mechanism of biological control is the degradation of pathogen virulence factors. For example, certain bacteria are able to detoxify albicidins, a phytotoxin which plays an important role in the pathogenesis of sugarcane leaf scald disease. The detoxification mechanism includes production of protein which inactivates albicidin by heat-reversible binding (Zhang and Birch 1996). In contrast to the aforesaid detoxification mechanism, an esterase produced by *Pantoes dispersa* is able to degrade the albicidins, rendering them inactive (Zhang and Birch 1997). Moreover, strains of *B. cepacia* and *R. solanacearum* are able to detoxify fusaric acid produced by various *Fusarium* sp. (Toyoda et al. 1988; Toyoda and Utsumi 1991).

Recent studies have reported that certain bacteria reduce or inhibit pathogen quorum-sensing capacity by degrading the autoinducer signal molecules, thereby blocking expression of virulence genes. Since most, if not all, bacterial plant pathogens rely upon quorum sensing signal to turn on virulence gene cascades, this approach has tremendous potential in biological control of plant disease. However, the role of PGPR and the influence of environmental factors in the degradation of the auto-inducer molecules and virulence factors have not been well documented. Hence, this area of research remains a hotspot for the researchers.

1.7 Induction of Resistance in Host Plants

Plants actively respond to a variety of environmental stimuli, including physical, chemical, and biological stress. In addition, plants also respond to a variety of chemical stimuli produced by different groups of microorganisms present in the rhizosphere region (Table 1.2). Such chemical stimuli can either induce or condition

Table 1.2 Bacterial determinants involved in induced systemic resistance in plants

Bacterial strain	Bacterial determinants	Plant species	References
Pseudomonas fluorescens CHA0	Antibiotics (DAPG)	*Arabidopsis*	Iavicoli et al. (2003)
Pseudomonas fluorescens WCS417	Lippopolysaccharide	*Arabidopsis*	Van Wees et al. (1997)
*Pseudomonas fluorescens*WCS374	Lippopolysaccharide	Radish	Leeman et al. (1995)
Pseudomonas putida WCS358	Lipopolysaccharide	*Arabidopsis*	Meziane et al. (2005)
Bacillus mycoides strain Bac J	Chitinase and β-1, 3 glucanase	Sugar beet	Bargabus et al. (2002)
Bacillus subtilis	2,3-butanediol	*Arabidopsis*	Ryu et al. (2004)
Bacillus pumilus	Chitinase and β-1, 3 glucanase	Sugar beet	Bargabus et al. (2002)
Serratia marcescens 90-166	Siderophore	Cucumber	Press et al. (2001)

plant host defenses through several biochemical changes that enhance resistant against subsequent infection by a variety of pathogens. Induction of the host defenses can be local or systemic, depending on the type, source, and amount of the microbial stimuli present in the rhizosphere region.

Peroxidase, chitinase, β-1,3-glucanase, 2,3-butanediol, lipopolysaccharide, Z,3-hexenal, and DAPG are the most common elicitors emitted by plant growth promoting bacteria (Iavicoli et al. 2003; Bargabus et al. 2004; Ryu et al. 2004; Ongena et al. 2004; Meziane et al. 2005). Recently, phytopathologists characterized these determinants and the pathways of induced resistance stimulated by PGPR. The first of these pathways, termed systemic acquired resistance (SAR), is mediated by salicylic acid (SA), a compound which is frequently produced following infection and typically leads to the expression of pathogenesis-related (PR) proteins. The PR proteins include a variety of enzymes some of which may act directly to lyse invading cells, reinforce cell wall boundaries to resist infection, or induce localized death.

The second pathway, termed induced systemic resistance (ISR), is mediated by jasmonic acid (JA) and ethylene, which are produced following application of either PGPR or nonpathogenic bacteria. As SAR, ISR is effective against different types of pathogens, but differs from SAR in that the inducing microorganism does not cause visible symptoms on the host plants. Thus far, *Pseudomonas*, *Burkholderia*, and *Bacillus* sp. have been shown to elicit ISR in different plants (Brooks et al. 1994; Barka et al. 2000; Ryu et al. 2004). Root treatment of *Phaseolus vulgaris* with *Pseudomonas putida* BTP1 leads to significant reduction of the disease caused by *Botrytis cinerea* on leaves. Ongena et al. (2005) isolated and characterized the molecular determinant of *P. putida* responsible for the ISR in bean plants. It has recently been reported that volatile organic compounds may play a key role in ISR process. For example, exposure to butanediol decreases the incidence of *E. carotovora* infection in *Arabidopsis* plants (Ryu et al. 2004). The volatile organic compounds secreted by *B. subtilis* GBO3 and *B. amyloquefaciens* IN937a were able to activate an ISR pathway in *Arabidopsis* and reduced the

incidence of *E. carotovora* infection (Ryan et al. 2001). However manifestation of ISR is depend on the combination of host plant and bacterial strain.

Biochemical or physiological changes in plants include induced accumulation of PR proteins such as PR-1, PR-2, chitinases, peroxidases, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and chalcone synthase (Park and Kloepper 2000; Ramamoorthy et al. 2001; Jeun et al. 2004). Recent studies have reported that some of the plant defense compounds (chalcone synthase) may trigger by *N*-acyl homoserine lactones that bacteria use for intraspecific signaling.

1.8 Conclusion and Future Prospects

Research into the mechanisms of biological control by PGPR has provided greater understanding of the multiple facets of disease suppression by these biological control agents. Still, most of the results are completely based on the in vitro studies, many remain to be learned from in vivo studies. Moreover, some of the questions need to be explained, such as response of introduced population to different management practices, factors that determine the successful colonization and expression of biocontrol traits, optimum physicochemical conditions for biological control agent to exert their suppressive capacities, and distribution of the antagonist in the environment. Answering these will advance the understanding of biological control.

Revelations about the mechanisms of biological control open new doors to design strategies for improving the efficiency of biological control agent. Identification of plant and microbial signal molecules that regulate the expression of biological control traits can be exploited for streamlining strain discovery that carry relevant biosynthetic gene. Characterization of all other genes and encoded proteins involved in the disease suppression, particularly those that stimulate antibiotic production and activity, can be exploited for screening the potential biological control agent. Along with this, molecular biology and biotechnology can be applied to further improve the strains that have prized qualities by creating transgenic strains that combine multiple mechanisms of action.

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Chapter 2
Bacteria for Plant Growth Promotion and Disease Management

Brahim Bouizgarne

2.1 Introduction

Soil is an excellent niche of growth of many microorganisms: protozoa, fungi, viruses, and bacteria. Some microorganisms are able to colonize soil surrounding plant roots, the rhizosphere, making them come under the influence of plant roots (Hiltner 1904; Kennedy 2005). These bacteria are named rhizobacteria. Rhizobacteria are rhizosphere competent bacteria able to multiply and colonize plant roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper 2001) where they are in contact with other microorganisms. This condition is wildly encountered in natural, non-autoclaved soils.

Generally, interactions between plants and microorganisms can be classified as pathogenic, saprophytic, and beneficial (Lynch 1990). Beneficial interactions involve plant growth promoting rhizobacteria (PGPR), generally refers to a group of soil and rhizosphere free-living bacteria colonizing roots in a competitive environment and exerting a beneficial effect on plant growth (Kloepper and Schroth 1978; Lazarovits and Nowak 1997; Kloepper et al. 1989; Kloepper 2003; Bakker et al. 2007). However, numerous researchers tend to enlarge this restrictive definition of rhizobacteria as any root-colonizing bacteria and consider endophytic bacteria in symbiotic association: Rhizobia with legumes and the actinomycete *Frankia* associated with some phanerogams as PGPR genera. Among PGPRs are representatives of the following genera: *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Thiobacillus*. Some of these genera such as *Azoarcus* spp., *Herbaspirillum*, and *Burkholderia* include endophytic species.

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However, *Pseudomonas* and *Bacillus* species constitute, together with *Streptomyces* species, the most bacteria often found in the rhizosphere of many crop plants.

In recent years, interest in the use of PGPR to promote plant growth has increased. Beneficial effect of PGPR on plant growth involves abilities to act as phytostimulators; biofertilizers. PGPR could enhance crop yield through nutrient uptake and plant growth regulators. PGPR could also act as biocontrol agents by production of antibiotics, triggering induced local or systemic resistance, or preventing the deleterious effects of xenobiotics by degradation (rhizoremediators) by acting as rhizoremediators (Jacobsen 1997; Somers et al. 2004; Aseri et al. 2008; Glick et al. 2007; Van Loon 2007). Their application as crop inoculants for biofertilization would be an attractive option to reduce the use of chemical fertilizers (Bloemberg and Lugtenberg 2001; Vessey 2003). In addition, PGPR have great adaptation to harsh environments including drought stress (Arshad et al. 2008; Arzanesh et al. 2011), salt stress (Mayak et al. 2004), high temperatures, dryness or heavy rainfalls in tropical countries (Da Mota et al. 2008), and contaminated environments (Burd et al. 2000; Gupta et al. 2002; Dell'Amico et al. 2008), indicating that they could contribute to ameliorate plant crops in areas with poor agricultural potential.

Biocontrol by rhizobacteria could involve PGPR and non-PGPR bacteria in the way that suppression of plant diseases could result in no enhancement of plant growth but only in protection against plant pathogens. Action of bacteria in the rhizosphere is also restricted by their ability to colonize the rhizosphere. Indeed, in practice, we cannot conclude that a bacterium is a PGPR only after its isolation from rhizobacteria and its reintroduction by plant inoculation followed by assessment of its ability to colonize rhizosphere and beneficial effect. This corresponds to only 2–5 % of rhizobacteria (Kloepper and Schroth 1978).

2.2 Root Colonization by Rhizobacteria

Schmidt (1979) proposed the term rhizosphere competence as related to soil microorganisms that show enhanced growth in response to developing plant roots. According to Weller (1988), root colonization is related to bacteria which can colonize the whole root system and survive during several weeks in the presence of the natural microflora. Later, Baker (1991) considered that root colonization is the ability of a microorganism, applied by seed treatment, to colonize the rhizosphere of developing roots. Both terms are used by authors to design the same process, that of the ability of microbial strains to grow and inhabit in the vicinity of roots (rhizosphere) or on the root system. The term rhizosphere effect designs the fact that bacterial density is higher in the rhizosphere in comparison to non-rhizosphere soil (Foster and Rovira 1978). Rhizosphere competence of rhizobacteria is strongly correlated with their ability to use organic acids as carbon sources, and the composition and quantity of root exudates influence also the nature of bacterial activity (Loper and Schroth 1986; Goddard et al. 2001). Abundance and

diversity of microorganisms in the rhizosphere are likely to be related to plant species due to differences in root exudation and rhizodeposition (Marschner et al. 2004; McSpadden Gardener 2004; Lemanceau et al. 1995). The root competence plays a major role in antagonistic activities of some bacteria. Root colonization is so important since poor colonization could cause decreased biocontrol activity. Indeed, population size was reported in many works as correlated to the efficiency of biocontrol activity against plant pathogens (Bull et al. 1991).

The ability to suppress disease by introduced *Pseudomonas* strains relies mainly on their ability to colonize the roots (Chin-A-Woeng et al. 2000) and their rhizosphere population density (Raaijmakers and Weller 1998). For example, the threshold population density required for significant suppression of take-all of wheat by two *Pseudomonas* spp strains; Q2-87 and Q8r1-96 is on average 1.2×10^5 CFU g^{-1} and 4.6×10^5 CFU g^{-1} root respectively (Raaijmakers and Weller 1998). Also, due to their lack of motility and consequently rhizosphere colonization, some *Pseudomonas* strains, producing the antibiotic phenazine, failed to suppress soil-borne pathogens (Chin-A-Woeng et al. 2003). Approaches aiming to enhance PGPR root colonization have focused on the effect of abiotic factors (Howie et al. 1987) and biotic factors (Notz et al. 2001): host genotype (Smith and Goodman 1999) and microbial genotypes (Landa et al. 2002, 2003). For instance, it was reported that plant growth promotion observed in tomato was more pronounced with two rhizosphere-competent streptomycetes *S. filipinensis* and *S. atrovirens* isolates than a non-rhizosphere-competent isolate. These two strains produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase and/or indole acetic acid (IAA) (El-Tarabily 2008).

2.2.1 Distribution and Localization of Root Competent Rhizobacteria

For the effective establishment of PGPR beneficial effects, the ability to colonize plant roots by introduced bacteria is an important trait. Attempts to measure external or internal amount of bacteria that colonize root are generally performed after root washing and disinfecting. In these studies, whole root systems or root segments are used. Generally, to determine colonization rate of the bacteria, enumeration of root colonizing bacteria, especially fluorescent pseudomonads, is classically performed by dilution plating (Ongena et al. 2000; Gamalero et al. 2004). Enumeration is performed on nutrient media generally supplemented with antifungal antibiotics such as yeast mannitol agar (YMA) with spectinomycin and kasugamycin (Chebotar et al. 2001) for the isolation of *Bradyrhizobium japonicum*. For the isolation of *Pseudomonads* and fluorescent *Pseudomonas* sp., TSA (Barnett et al. 1999) and King's medium B agar (Raaijmakers and Weller 1998) are used. The most used antibiotics are cycloheximide (which prevents the growth of fungi) and antibacterial compounds such as chloramphenicol and ampicillin (Simon and Ridge 1974;

van Wees et al. 1997). Media like actinomycete isolation agar or Olson medium (Olson 1968) or the rhizospherical soil extract medium (Bouizgarne et al. 2006) supplemented with nalidixic acid and cycloheximide are largely used for the isolation of Streptomycetes. Using a selective medium containing cycloheximide and carbenicillin, *Streptomyces lydicus* WYEC 108 were recovered from non-sterile soils. Monitored population of WYEC 108 in both roots and non-rhizospheric soils of pea, cotton and sweet corn planted in amended sterile and non-sterile soils revealed that over 30 days, the population remains stable at 10^5 CFU g^{-1} root, whereas in non-rhizosphere soil it decreases by 100-fold at least (Yuan and Crawford 1995).

Spontaneous chromosomal mutants or engineered strains with antibiotic resistance are widely used in dilution plating method. The most common resistance used is for rifampicin which is selective for rifampicin-resistant *Pseudomonas* spp. (Geels and Schippers 1983; Raaijmakers et al. 1999; Fließbach et al. 2009) and streptomycin (Asaka and Shoda 1996). Visualization of root colonization was assessed by various techniques: Immunofluorescence microscopy (Troxler et al. 1997; Gamalero et al. 2004), immunofluorescence colony (IFC) staining technique (Schobe and vanVuurde 1997; Raaijmakers et al. 1995) and scanning microscopy (Chin-A-Woeng et al. 1997; Tokala et al. 2002; Gamalero et al. 2004), and confocal laser scanning electron microscopy (Bloemberg et al. 2000; Bolwerk et al. 2003; Gamalero et al. 2004, 2005). In addition to its ability to quantify soil bacteria, bioluminescence genes method (lux gene) also allows to detect genetically engineered soil bacteria (de Weger et al. 1997). All these techniques enabled easier study of PGPR in their natural environment.

Work by Gamalero et al. (2004) concluded that the population dynamics showed spatiotemporal density variation according to the root zone. While *Pseudomonas fluorescens* A6RI density decreased with time in the apex, the elongation, and the young hairy zones, no variation with time was recorded in the hairy zone and the old hairy and the collar zones, and concluded that these variations could be due to patterns of exudates composition and concentrations along the root. *P. polymyxa* was found to be capable of colonizing the root tip and the intercellular spaces outside the vascular cylinder of *Arabidopsis thaliana* and barley (*Hordeum vulgare*) (Timmusk et al. 2005).

Visualization of cellular rhizosphere interactions between antagonistic strains (*Pseudomonas* and *Bacillus*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causal agent of tomato shoot and root rot, was performed using epifluorescence and confocal laser scanning microscopy (CLSM). By labeling these microorganisms differently with autofluorescent proteins, simultaneous detection enabled deep studies of these interactions in the tomato rhizosphere. According to these researches, biocontrol bacteria were able not only to colonize the tomato roots (Chin-A-Woeng et al. 1997; Bloemberg 2007), but also to colonize fungal hyphae, causing different stress effects to its growth (Bolwerk and Lugtenberg 2005) and actively attacking the pathogen, by producing antibiotic phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al. 1998; Bolwerk et al. 2003).

Generally, population density of actinomycetes is largely higher in rhizosphere in comparison with non-rhizosphere soils (Miller et al. 1989, 1990). SEM studies of the root colonization of *Streptomyces griseoviridis* showed a higher density in the rhizosphere of lettuce than in non-rhizosphere soil (Kortemaa et al. 1994). Also SEM study of *Streptomyces lydicus* WYEC 108 showed a particular interaction between *S. lydicus* strain WYEC 108 and nodules of *Pea*. It appeared to colonize nodulation sites, and then the vegetative hyphae moved onto root hairs and from the external surface of the root cells into the interior of the root cells, intermittently (Tokala et al. 2002). Moreover, PCR-DGGE analysis of DNA from colonized nodules revealed the presence of a *Streptomyces* band in addition to other bands corresponding to the plant and *Rhizobium*. The discovery of a native actinomycete colonizing the surface of a root nodule of a pea plant from an agricultural field in north Idaho demonstrated that this phenomenon could be frequent in nature (Tokala et al. 2002).

2.2.2 Molecular and Biochemical Basis of Root Colonization

However, classical cultivation-based analysis has the disadvantage that only a small proportion of the bacterial populations can be recovered (Amann et al. 1995). More accurate techniques are actually used to quantify bacteria: measuring bacterial activity by thymidine and leucine incorporating techniques (Söderberg and Bååt 1998), immunological techniques such as ELISA (REF) and IFC staining technique (Mahaffee et al. 1997), flow cytometry (Tombolini et al. 1997; Gamalero et al. 2004) and bioluminescent *lux* gene tagged bacteria (Mahaffee et al. 1997; Kragelund et al. 1997), and fluorochrome-labeled RNA-directed probes (Assmus et al. 1995). *Pseudomonas* colonies isolated from the roots of wheat on King B medium and harboring the genes for Phl (2,4-diacetylphloroglucinol) were subsequently quantified by colony hybridization with a Phl-probe followed by polymerase chain reaction (PCR) analysis using Phl-specific primers (Raaijmakers and Weller 1998).

Some of these works showed that higher colonization patterns were found near the collar zone (Kragelund et al. 1997; Gamalero et al. 2004) where root exudation activity is higher (Grayston et al. 1996) in comparison with the apical zone. In addition, these researches agreed that the preferential location of bacteria is situated at the junction between epidermal cells (Chin-A-Woeng et al. 1997; Gamalero et al. 2004, 2005; Lagopodi et al. 2002; Bolwerk et al. 2003) or between and inside the epidermal and cortical cells (Troxler et al. 1997).

In order to study bacterial distribution and organization in the root zones, molecular fingerprinting techniques such as amplified rDNA restriction analysis (ARDRA), whole-cell repetitive sequence-based polymerase chain reaction (rep-PCR), random amplified polymorphic DNA (RAPD) analysis, and restriction fragment length polymorphism (RFLP) allowing to detect bacteria were performed by several authors. Other methods such as the use of genetically engineered bacteria by introduced marker gene or a reporter gene to detect innate activity of the bacterium are used. An example is the use of LacZ which encodes for the β-galactosidases (Kluepfel et al. 1991).

PCR could also detect bacteria by analyzing total rhizosphere DNA or rhizosphere 16S rDNA fragments (Smalla et al. 2001), or amplification of specific genes such us those encoding antibiotics. An example is the use of two oligonucleotide primers Phl2a and Phl2b that targeted the gene for 2,4-diacetylphloroglucinol (Phl) (Raaijmakers and Weller 1998). PCR amplification of target *phlD* genes from 2,4-diacetylphloroglucinol (2,4-DAPG) producers provides a technique sensitive enough to detect log 2.4 cells per sample (McSpadden-Gardener et al. 2001). Colony hybridization followed by PCR analysis was used to determine the frequency of 2,4-DAPG producing wheat root-associated fluorescent *Pseudomonas* in take-all disease suppressive and conductive soils, and showed that in conductive soils these strains were not detected or were detected at densities at least 40-fold lower than those in the suppressive soils. Moreover, in suppressive soils, 2,4-DAPG producing *Pseudomonas* spp. were present on roots of wheat at densities above the threshold required for significant suppression of take-all of wheat (Raaijmakers et al. 1997). Genetic profiles of over a dozen distinct genotypes within a worldwide collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. isolated from soils suppressive to *Fusarium* wilt or take-all were analyzed, and isolates belonging to two BOX-PCR genotypes (D-genotype strains and P-genotype strains) were found to be more aggressive colonists of the rhizosphere of pea plants than isolates of other genotypes, suggesting that biosynthetic gene phlD profiles were predictive of their rhizosphere competence (Landa et al. 2002). Furthermore, *P. fluorescens* Q8r1-96, a representative D-genotype strain, was a potent competitor toward representatives of the less competent A, B, and L genotypes when coinoculated in a 1:1 ratio in the wheat rhizosphere over several successive cycles (Landa et al. 2003).

Using DGGE fingerprints of PCR-amplified 16S rDNA of whole bacterial communities in three plants: strawberry, potato, and oilseed rape, smalla et al. (2001) found significant differences in microbial community abundance between soil and rhizosphere communities. Similar results were found for *Bacillus* and *Paenibacillus* which are more abundant in bulk soil than in plant tissues (McSpadden Gardener 2004) using a ribotyping method.

The use of genetically engineered strains defected in some characteristics important in the attachment to plant roots could affect dramatically their effectiveness. It was reported in *Pseudomonas* that flagella (de Weger et al. 1987), pilli (Vesper 1987), O-antigens of lipopolysaccharide (LPS) (de Weger et al. 1989), an agglutinin present in the root (Anderson et al. 1988; Glandorf et al. 1994), and the outer membrane protein OprF (de Mot et al. 1992) are involved in root colonization, particularly in the bacterial attachment to roots. Mutants defective in the synthesis of the O-antigen of LPS are impaired in rhizosphere competence (de Weger et al. 1989; Dekkers et al. 1998). However, genetic determinants involved in the biochemical interactions between root plants and bacteria are poorly understood. On the other hand, flagella and LPS are also reported as bacterial determinants recognized by plants in the process of triggering systemic resistance (Gómez-Gómez et al. 1999; Leeman et al. 1995a, 1995b; Van Peer and Schippers 1992; Duijff et al. 1997; Bakker and Schippers 1995).

Plant–bacteria communication involves diverse signaling molecules. Strains belonging to *Bacillus* were reported as producers of volatile organic compounds

such as acetoin and 2,3-butanediol involved in plant–bacteria communication (Ryu et al. 2003). Aldehydes, ketones, and alcohols had been found to be involved in *A. thaliana* recognition of *Bacillus* strains by triggering morphogenetic changes in root system, consisting of stimulation of primary root growth and/or lateral root development. A good correlation between biomass production and lateral root growth was shown, suggesting that this kind of chemical communication might be of ecological relevance toward enhancing root colonization and reinforcing symbiotic interactions between plants and their associated bacterial populations. However, molecular mechanisms and signaling pathways involved in *A. thaliana* responses to volatile organic compounds remain poorly understood (Gutiérrez-Luna et al. 2010). Despite the well-studied dynamics of root colonization ability of Actinomycetes (Merzaeva and Shirokikh 2006; Franco et al. 2007), little is known about biochemical and molecular traits involved in this interaction as it has been mostly studied on pathogenic actinomycetes, particularly *Clavibacter michiganensis* where bacterial exopolysaccharides have been shown to bind to receptor proteins present on the plasma membrane of potato cells (Shafikova et al. 2003; Bermpohl et al. 1996).

2.3 Rhizobacteria Antagonistic to Plant Disease Agents

2.3.1 Gram Negative Bacteria

The most important group of PGPR among Gram negative bacteria are the genera *Pseudomonas*.

2.3.1.1 Pseudomonas

PGPR effect of *Pseudomonas* was largely reviewed (Lemanceau 1992). Bacteria belonging to *Pseudomonas* were reported as PGPR for many crops of potato (*Solanum tuberosum* L.) (Burr et al. 1978; Schippers et al. 1987), radish (*Raphanus sativus* L.) (Kloepper and Schroth 1978), sugar beet (*Beta vulgaris* L.) (Suslow and Schroth 1982), and lettuce (Chabot et al. 1993).

Strains of fluorescent pseudomonads used in biocontrol have contributed greatly to the understanding of the mechanisms involved in disease suppression. Many of these bacteria could prevent plant diseases by various mechanisms: antibiosis, competition, or parasitism. Within the genus *Pseudomonas*, *Pseudomonas fluorescens* which are ubiquitous rhizosphere inhabitant bacteria are the most studied group (Weller 1988). They were shown to have a higher density and activity in the rhizosphere than in bulk soil. When introduced on seed or planting material, they promote plant growth or control plant diseases by suppressing deleterious rhizosphere microorganisms. They are able to compete aggressively for sites in

the rhizosphere and prevent proliferation of phytopathogens by niche exclusion, production of antibiotics and siderophores, or inducing systemic resistance (De Weger et al. 1986; Haas and Défago 2005; Krishnamurthy and Gnanamanickam 1998); by stimulating plant growth by facilitating either uptake of nutrients from soil (De Weger et al. 1986); or by producing certain plant growth promoting substances (Ryu et al. 2005; Spaepen et al. 2007). Fluorescent pseudomonads have been applied to suppress *Fusarium* wilts of various plant pathogens (Lemanceau and Alabouvette 1993), *Clavibacter michiganenis* subsp. *michiganesis*, causal agent of tomato bacterial canker (Amkraz et al. 2010). In addition, their presence is correlated with disease suppression in some suppressive soils (Kloepper et al. 1980a; Lemanceau et al. 2006). Examples of commercially available biocontrol products from *Pseudomonas* are Bio-save (*P. syringae*) and Spot-Less (*P. aureofaciens* Tx-1).

Van Peer et al. (1991) reported protection of carnation from fusarosis due to phytoalexin accumulation upon treatment with *Pseudomonas* strain WCS417. Other works followed including the use of *P. fluorescens* as an inducing agent to prevent the spread of various plant pathogens (Maurhofer et al. 1994; Duijff et al. 1994a; Leeman et al. 1995b; Pieterse et al. 2000). *P. fluorescen*s CHA0 showed ability to protect tobacco against the tobacco necrosis virus concomitant with a systemic accumulation of salicylic acid and associated with the induction of multiple acidic pathogenesis-related proteins, including PR-1a, -1b, and -1c (Maurhofer et al. 1994). Inoculation of *A. thaliana* by *P. fluorescens* WCS417r and of rice by WCS374r conducted to induced systemic resistance (ISR) respectively to *Pseudomonas syringae* pv. *tomato* (Pieterse et al. 2000) and to the leaf blast pathogen *Magnaporthe oryzae* (De Vleesschauwer et al. 2008).

2.3.2 Gram Positive Bacteria

The most important group of PGPR among Gram positive bacteria are *Bacillus*, *Paenibacillus*, and *Actinomycetes*.

2.3.2.1 *Bacillus* and *Paenibacillus*

Different species of *Paenibacillus* can induce plant growth by fixing atmospheric nitrogen (Von Der Weid et al. 2002), and producing auxins (Lebuhn et al. 1997; Bent et al. 2001; Da Mota et al. 2008) and cytokinin (Timmusk et al. 1999). Benificial effects were reported in lodgepole pine (*Pinus contorta*) (Bent et al. 2001) and spruce (*Picea* sp.) (Shishido et al. 1995) after inoculation of *P. polymyxa* strain. *Bacillus* strains could also repress soil-borne pathogens (Von Der Weid et al. 2005) and induce plant resistance to diseases following root colonization (Timmusk and Wagner 1999).

In the opposite to *Pseudomonas* and other nonspore-forming bacteria, *Bacillus* spp. are able to form endospores that allow them to survive for extended periods

under unfavorable environmental conditions. This trait is relevant in their relative durable viability when stored for a relatively long period (shelf-life). *Bacillus* species have been reported as plant promoting bacteria in a wide range of plants (Deepa et al. 2010; Bai et al. 2003; Kokalis-Burelle et al. 2002; Kloepper et al. 2004). Different *Bacillus* species were reported to be effective biocontrol agents in greenhouse or field trials (Stabb et al. 1994; Kloepper et al. 2004). Isolates of *Bacillus subtilis* inhibited *S. cepivorum* in vitro and were able to suppress the incidence of onion white rot, leading to an increased onion emergence and yield (Utkhede and Rahe 1980). The suppression of onion white rot could be due to a possible antibiotic production and probably also metabolization of onion produced stimulants of sclerotial germination (Utkhede and Rahe 1980). Members of *Bacillus* were reported as producers of antibiotics inhibiting various phytopathogens including *F. oxysporum* f. sp. *ciceri* (Kumar 1999) and *Rhizoctonia solani* (Asaka and Shoda 1996).

Mechanisms involved in *Bacillus* eliciting plant growth promotion include auxin production (Idris et al. 2004; Deepa et al. 2010), increased uptake availability of phosphorus (Idris et al. 2002; Deepa et al. 2010), biocontrol abilities (Asaka and Shoda 1996; Jacobsen et al. 2004), and induction of systemic resistance (Zehnder et al. 2000; Jetiyanon et al. 2003; Bargabus et al. 2003; Kloepper et al. 2004). An example of the use of *Bacillus* as biocontrol PGPR agents include the use of two *B. subtilis* strains (G1 and B3), and two *Bacillus amyloliquefaciens* strains (FZB24 and FZB42) in tobacco, either in the presence or absence of tobacco mosaic virus (TMV). In these experiments, they significantly reduced disease severity. Commercial available biocontrol products include Kodiak (*B. subtilis* strain GB03), Serenade (*B. subtilis* QST 713), YieldShield (*Bacillus pumilus* strain GB34), Companion (*Bacillus subtilisformis*, *B. megaterium*), and EcoGuard (*Bacillus licheniformis* strain SB3086).

Bacillus strains were also reported to be potent inducers of systemic resistance (ISR). Jetiyanon et al. (2003) observed that one PGPR mixture, *B. amyloliquefaciens* strain IN937a and *B. pumilus* strain IN937b, protected plants by inducing systemic resistance against southern blight of tomato caused by *Sclerotium rolfsii*, anthracnose of long cayenne pepper caused by *Colletotrichum gloeosporioides*, and mosaic disease of cucumber caused by cucumber mosaic virus (CMV). In a greenhouse experiment, induced resistance to CMV resulted in 32 % of diseased tomato plants in the most effective PGPR treatments with *B. subtilus* IN937b compared with 88% in the nonbacterized plants (Zehnder et al. 2000). *Bacillus* spp. have been tested in field trials for their capacity to reduce the incidence and severity of the tomato mottle virus (ToMoV) that is transmitted by whiteflies (Murphy et al. 2000; Zehnder et al. 2001) and against CMV (Zehnder et al. 2001).

The ISR displayed against CMV on tomato can be obtained under field conditions albeit at variable extents than that reported from greenhouse experiments (Raupach et al. 1996). *Bacillus thuringiensis* induced accumulation of PR proteins in coffee against *Hemileia vastatrix* (Guzzo and Martins 1996). *B. amyloliquefaciens* strain EXTN-1 induced pathogenesis-related genes including PR-1a against anthracnose disease caused by *Colletotrichum orbiculare* in cucumber (Park et al. 2001; Jeun et al. 2001). In addition to induction of phenylalanine ammonia-lyase (PAL), and

3-hydroxy-3-methylglutaryl CoA reductase (HMGR) genes, EXTN-1 induced transcript accumulation of defense-related genes of PR, particularly PR-1a mRNA, upon challenge inoculation with the Pepper mild mottle virus in tobacco, while EXTN-1 treatment of *Arabidopsis* wild type Col-0 resulted in the activation of PR-1 and the ethylene encoding gene PDF1.2 (Ahn et al. 2002).

2.3.2.2 Actinomycetes

Actinomycetes are Gram-positive bacteria characterized by a genome with high G+C ratio. They are for the most aerobic, but some of them can grow anaerobically. Several Actinomycetes form branching filaments and possess mycelial growth and some species produce external spores.

Despite the fact that actinomycetes are largely spread in the nature especially in telluric ecosystems and that they were strongly studied since they are the origin of numerous antibacterial and antifungal compounds and some are used in biocontrol, only few works are interested in their usefulness as PGPR for plants like wheat (Aldesuquy et al. 1998; Hamdali et al. 2008a) and broccoli (Hasegawa et al. 2008). Recent works demonstrated that plant promotion relies on the ability of the Actinomycetes to solubilize phosphate (El-Tarabily et al. 2008; Hamdali et al. 2008b) or to produce phytohormones (El-Tarabily 2008; Hamdali et al. 2008a), showing the great interest of actinomycetes solubilizing phosphate in soils deficient in available soluble phosphorus (P). In greenhouse experiments, rhizosphere-competent *Micromonospora endolithica* induced increase in available P in the soil, promoted the growth of roots and shoots of bean plants in comparison with a non-phosphate-solubilizing, non-rhizosphere-competent isolate (*M. olivasterospora*) (El-Tarabily et al. 2008). El-Tarabily (2008) reported that the plant growth promotion was most pronounced with one actinomycete strain *S. filipinensis* than with another isolate *S. atrovirens* in greenhouse experiment, probably due to the ability of *S. filipinensis* to produce both ACC deaminase and IAA while *S. atrovirens* produce only ACC deaminase.

It is likely that more interest was addressed to the antibiotic production by actinomycetes or their biopesticide capacities since almost all works were initially interested in these topics. Thus, most studied PGPR actinomycetes possess antibacterial or antifungal activity as they were initially screened for works aiming to suppress a plant disease (de Vasconcellos and Cardoso 2009; El-Tarabily and Sivasithamparam 2006; Hamby and Crawford 2000). Examples of commercial biocontrol products from actinomycetes are Mycostop (*Streptomyces griseoviridis* K61), Actinovate (*Streptomyces lydicus*), and Nogall (*Agrobacterium radiobacter* Strain K1026).

Merriman et al. (1974) reported the use of the PGP *Streptomyces griseus* isolate with biocontrol abilities toward *R. solani* in carrot. Antagonistic Streptomycetes were also used to promote the growth of coniferous plants. In Brazil, one *Streptomyces* isolate genetically close to *Streptomyces kasugaensis* able to inhibit the

growth of *Fusarium* and *Armillaria* pine rot showed also plant promotion in growth of *Pinus taeda* seedlings under greenhouse experiment (de Vasconcellos and Cardoso 2009). El-Abyad et al. (1993) described the use of three *Streptomyces* spp., *S. pulcher*, *S. canescens*, and *S. citreofluorescens*, effective in the control of some tomato diseases including those caused by *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum*, *Alternaria solani*, *Pseudomonas solanacearum*, and *Clavibacter michiganensis* subsp. *michiganensis* in tomato. As seed-coating, tomato growth was significantly improved with the tree antagonistic.

S. violaceusniger YCED9, an antifungal producer (Hamby and Crawford 2000), showed also carrot growth promotion under gnotobiotic conditions. From eight strains shown to be strong *P. ultimum* antagonists, only one (strain WYEC 107) significantly enhanced lettuce growth in the absence of *Pythium ultimum* in glass-house pot studies over a 20-day experiment (Crawford et al. 1993). However another isolated antagonistic strain to *P. ultimum*, *Streptomyces lydicus* strain WYEC 108, with demonstrated PGPR effect in carrots and beets in the absence of fungal pathogen stress (Hamby 2001) had shown in another work an increase in average plant stand, plant length, and plant weight of pea and cotton seedlings grown in either *Pytium ultimum*-infested sterile or non-sterile soils (Yuan and Crawford 1995). In addition to increasing shoots length and plant and root wet weights in pea seedlings in both growth chamber and greenhouse experiments, *Streptomyces lydicus* strain WYEC 108 was also found to increase root nodulation frequency by *Rhizobium* spp. and nodule size and number as shown by the more numerous and vigorous nodules found in *Streptomyces*-colonized plants than in control. Also, an increase in the number of bacteroids per nodule, nitrogenase activity, and nodular assimilation of iron was reported (Tokala et al. 2002). Due to its high potential as fungal antagonist, its good establishment in the rhizosphere of various plants at significant levels (10^4 CFU g^{-1} of soil), and as it can easily be reisolated for 26 months after inoculation (Crawford et al. 1993). *S. lydicus* strain WYEC 108 led to the formulation and the commercialization of Actinovate® and Actino-Iron®, a well-known biocontrol product (Crawford et al. 2005).

2.4 Bacterial Antagonism: Protection Against Phytopathogens

Generally plant diseases cause 10–20 % loss in production (James 1981). The use of antibacterial and antifungal chemicals is deprecated in view of sustainable agricultural practices. Hence, an alternative to chemical control of plant diseases by the use of bacteria able to antagonize phytopathogenic is considered as a more environmentally friendly process. Biological control of soil-borne pathogens with antagonistic bacteria has been intensively investigated. In this mode of action, direct interaction between PGPR and the endogenous microflora is necessary. PGPR can promote plant growth by suppressing diseases caused by soil-borne pathogens (Van Loon and Glick 2004).

Rhizobacteria can antagonize pathogens through competition, production of antibiotics, or secretion of lytic enzymes (Van Loon and Bakker 2003) that make them a potent tool for reducing damages through preventing deleterious effects of phytopathogens. The main bacteria are representatives of the genera *Pseudomonas*, *Bacillus*, and *Streptomyces*. Numerous studies on bacteria antagonistic to phytopathogens include bacteria such as fluorescent *Pseudomonas* and *Bacillus subtilis* (Kloepper et al. 1989).

2.4.1 Antagonism by Production of Lytic Enzymes

Lytic enzymes are glucanases, proteases (Dunne et al. 1997), cellulases, and chitinases. Bacteria could parasitize disease-causing fungi by the production of these enzymes. Some enzyme producing bacteria are able to destroy oospores of phytopathogenic fungi (El-Tarabily 2006) and affect the spore germination and germ-tube elongation of phytopathogenic fungi (Sneh et al. 1984; Frankowski Lorito et al. 2001). A positive relationship was observed between chitinase production and the antifungal activity of chitinolytic *P. fluorescens* isolates (Velazhahan et al. 1999). Production of extracellular cell wall degrading enzymes has been associated with biocontrol abilities of the producing bacteria (Fridlender et al. 1993; Valois et al. 1996; Singh et al. 1999; El-Tarabily 2006). Tn5 mutants of one of the *Enterobacter* which were deficient in chitinolytic activity were unable to protect plants against the disease (Chernin et al. 1995). In addition, enzyme producing bacteria were successfully used in combination with other biocontrol agents, leading to a synergistic inhibitory effect against pathogen (Dunne et al. 1998; Someya et al. 2007). Table 2.1 gives examples of enzymes produced by biocontrol bacteria.

2.4.2 Antagonism by Antibiosis

Antibiotics produced by bacteria include volatile antibiotics (hydrogen cyanide, aldehydes, alcohols, ketones, and sulfides) and nonvolatile antibiotics: polyketides (diacetyl phloroglucinol; DAPG and mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives: pyocyanin, phenazine-1-carboxylic acid; PCA, PCN, and hydroxy phenazines) (de Souza et al. 2003), and phenylpyrrole antibiotic (pyrrolnitrin) (Ahmad et al. 2008). *Bacillus strains* produce a variety of lipopeptide antibiotics (iturins, bacillomycin, surfactin, and Zwittermicin A).

Introduction of selected antagonistic fluorescent pseudomonads into the rhizosphere can effectively suppress soil-borne plant diseases. *B. subtilis* strain RB14 produces the cyclic lipopeptide antibiotics iturin A and surfactin active against several phytopathogens. *B. subtilis* strains were able to control damping-off of

Table 2.1 Examples of lytic enzymes produced by biocontrol bacteria

Enzyme	Producing bacteria	Target phytopathogen and host plant	References
Chitinases	*Aeromonas caviae*	*Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *vasinfectum* (cotton) and *Sclerotium rolfsii* (beans)	Inbar and Chet (1991)
	Arthrobacter sp.	*Fusarium* (carnation)	Koths and Gunner (1967), Sneh (1981)
	Arthrobacter	*Fusarium moniliforme* var *subglutinans* (southern pines)	Barrows-Broaddus and Kerr (1981)
	Enterobacter agglomerans, *Bacillus cereus*	*R. solani* (cotton)	Chernin et al. (1995, 1997), Pleban et al. (1997)
	Bacillus circulans and *Serratia marcescens*	*Phaeoisariopsis personata* (peanut)	Kishore et al. (2005)
	Enterobacter agglomerans, *Bacillus cereus*	*R. solani* (cotton)	Chernin et al. (1995, 1997), Pleban et al. (1997)
	Paenibacillus illinoisensis	*R. solani* (cucumber)	Jung et al. (2003)
	Pseudomonas	*Fusarium oxysporum* f. sp. *cucumerinum* (cucumber)	Sneh et al. (1984)
	Serratia plymuthica	*Bothrytis cinerea* and *Sclerotinia sclerotiorum* (cucumber)	Kamensky et al. (2003)
	Serratia marcescens	*Sclerotium rolfsii* (beans) and *R. solani* (cotton)	Chet et al. (1990)
	Streptomyces lydicus	*Pythium* and *Aphanomyces*	Mahadevan and Crawford (1997)
Glucanases	*Streptomyces* sp.	*Phytophthora fragariae* (raspberry)	Valois et al. (1996)
	Pseudomonas cepacia	*R. solani*, *Sclerotium rolfsii* and *Pythium ultimun*	Fridlender et al. (1993)
	Actinoplanes philipinensis and *Micromonospora chalcea*	*Pythium aphanidermatum* (cucumber)	El-Tarabily (2006)
	Lysobacter enzymogenes	*Pythium* (sugar beet)	Palumbo et al. (2005)
Chitinases and glucanases	*Serratia marcescens*, *Streptomyces viridodiasticus*, *Micromonospora carbonacea*	*Sclerotinia minor* (lettuce)	El-Tarabily et al. (2000)
	Streptomyces sp. and *Paenibacillus* sp.	*Fusarium oxysporum* f. sp. *cucumerinum* (cucumber)	Singh et al. (1999)
Chitinases, proteases, and cellulases	*Bacillus subtilis*, *Erwinia herbicola*, *Serratia plymuthica*, *Actinomycete*	*Eutypa lata* (grapevine)	Schmidt et al. (2001)
Proteases	*Stenotrophomonas maltophilia*	*Pythium ultimum* (sugar beet)	Dunne et al. (1997, 1998)

tomato caused by *Rhizoctonia solani* and was found to produce an antifungal antibiotic inhibiting *F. oxysporum* f. sp. *ciceris*, causal agent of wilt disease in chickpea (Asaka and Shoda 1996; Kumar 1999). Table 2.2 gives some examples of antibiotics produced by biocontrol bacteria.

Most strains of *Pseudomonas* spp. used in biocontrol produce one or several antibiotic compounds with antifungal abilities in vitro (Raaijmakers et al. 2002). Phenazine derivatives produced by fluorescent pseudomonads were reported as implicated in biocontrol of take-all disease (Weller and Cook 1983). *P. fluorescens* 2-79, an in vitro producer of phenazine-l-carboxylate, was reported to suppress *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller 1988). 2,4-DAPG producers play a key role in the natural suppression of take-all disease of wheat (Raaijmakers et al. 1997, 1999; Raaijmakers and Weller 1998). Suppressive soils to take-all lost its suppressiveness when indigenous DAPG-producing fluorescent *Pseudomonas* spp. was eliminated by pasteurization. Moreover, suppressiveness was able to be transferred to conductive soils when DAPG-producing *Pseudomonas* strains were introduced (Raaijmakers and Weller 1998).

Numerous investigations demonstrate that *Streptomyces* produce numerous secondary metabolites with antibiotic properties. Currently, 42 % of the 23,000 known microbial secondary metabolites are produced by actinobacteria. Actinomycetes and particularly Streptomycetes produce 70–80 % of known bioactive natural products (Berdy 2005). A large number of these antibiotics were exploited in experimental works in laboratories or greenhouses. However, few of these antibiotics are commercialized.

The genus *Streptomyces* is the largest producer of secondary metabolites. The antagonistic properties of *Streptomyces* against numerous phytopathogens including *Alternaria brassicicola*, *Collectotrichum gloeosporioides*, *F. oxysporum*, *Penicillium digitatum,* and *Sclerotium rolfsii* (Khamna et al. 2009) and *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f.sp *lycopersici*, and *F. oxysporum* f. sp. *asparagi*, the causal agents of wilt diseases, are well established. Geldanomycin produced by *Streptomyces hygroscopicus* var. *geldonus* was applied to suppress *Rhizoctonia solani* in soil (Rothrock and Gottlieb 1984). Furthermore, compounds responsible for the antifungal activity of some *Streptomyces* species have been identified: e.g., cyclohex-imide from *S. griseus*, kasugamycine from *S. kasugaensis*, Blastcidin-S from *S. griseochromogenes*, and Rhizovit from *S. rimosus* etc.

Bacterial strains may protect plants from phytopathogenic fungi due to the volatile antibiotic HCN production (Ahmad et al. 2008). *P. fluorescens* CHAO enhanced root growth and could suppress black root rot of tobacco caused by *Thielaviopsis basicola* under gnotobiotic conditions. CHAO excretes several metabolites with antifungal properties including pyoverdine, DAP, pyoluteorin, and HCN (Ahl et al. 1986; Maurhofer et al. 1995). It was also suggested that HCN might constitute a stress in the plants, provoking an enhancement of their resistance to fungal diseases (Défago et al. 1990). Suppressive effect on black root rot was found to be related to hydrogen cyanide production as demonstrated by the less protective effect of hcn mutant defective in HCN biosynthesis and effective

Table 2.2 Selected examples of antibiotics produced by biocontrol bacteria

Antibiotics	Producing organism	Target organism	References
Bacillomycin	*Bacillus*	*Aspergillus flavus*	Moyne et al. (2001)
Kanosamine	*Bacillus cereus*	*Phytophthora medicaginis*	Milner et al. (1996)
Zwittermycin A	*B. cereus* and *B. thuringiensis*	*Phytophthora*	Silo-Suh et al. (1998)
	Bacillus spp.	*Sclerotinia sclerotiorum*	Zhang and Fernando (2004)
	B. cereus	*Phytophthora parasitica* var. *nicotianae*	He et al. (1994)
Iturin	*B. subtilis*	*Pythium ultimum*, *R. solani*, *F. oxysporum*, *S. sclerotiorum* and *M. phaseoli*	Constantinescu (2001)
Iturin A and Surfactin	*B. subtilis*	*R. solani*	Asaka and Shoda (1996)
Pyrrolnitrin	*Burkholderia cepacia*	*R. solani*	El-Banna and Winkelmann (1988)
	Pseudomonas fluorescens	*Gaummanomyces graminis* var *tritici*	Tazawa et al. (2000)
		R. solani	Howell and Stipanovic (1979)
	Enterobacter agglomerans	*Agrobacterium tumefaciens*, *Clavibacterium michiganense*, *Xanthomonas campestris*, *Pseudomonas syringae* pv. *syringae*	Chernin et al. (1996)
2,4-DAPG	*Pseudomonas*	*Xanthomonas oryzae* pv. *oryzae*	Velusamy et al. (2006)
Pyoluteorin	*P. fluorescens*	*Py. ultimum*	Howell and Stipanovic (1980)
Phenazines	*P. fluorescens*	*Gauemannomyces graminis* var. *tritici*	Weller and Cook (1983), Brisbane and Rovira (1988)
Phenazine-1-caboxylate	*P. fluorescens*	*Gauemannomyces graminis* var. *tritici*	Thomashow et al. (1990)
	P. aureofaciens	*Sclerotinia homeocarpa*	Powell et al. (2000)
Phenazine-1-carboxamide	*P. chlororaphis*	*Fusarium oxysporum f. sp.radicis-lycopersici*	Chin-A-Woeng et al. (1998), Bolwerk et al. (2003)
Viscosinamide	*P. fluorescens*	*R. solani*	Nielsen et al. (2002)
	P. fluorescens	*Py. ultimum*	Thrane et al. (2000)
Amphisin	*P. fluorescens*	*Py. ultimum* and *R. solani*	Andersen et al. (2003)
Geldanomycin	*Streptomyces hygroscopicus*var. *geldonus*	*R. solani*	Rothrock and Gottlieb (1984)
Oligomycin A	*Streptomyces libani*	*Botrytis cinerea*	Kim et al. (1999)
Polyoxin D	*Streptomyces cacaoi* var.	*R. solani*	Isono et al. (1965)
Kasugamycin	*Streptomyces kasugaensis*	*P. oryzae*	Umezawa et al. (1965)

disease suppression when hcn+ genes were re-introduced into the mutant genome or inserted into the genome of an initially nonactive strain (Voisard et al. 1989).

The role of antibiosis in the protection against phytopathogens was widely reviewed (Raaijmakers et al. 2002). Although there are numerous reports on the lack of correlation between in vitro antibiosis and effectiveness in field pointing out the limitations of using in vitro assays, many others concluded that at least a proportion of laboratory-discovered active isolates could be effective in soil. In general among a group of actinomycetes isolated from soil, while a number of in vitro antagonist actinomycetes may be active in soil, those with no in vitro activity are also inactive in soil (Broadbent et al. 1971). At least, in vitro assays have the advantage of selecting only antibiotic producers and excluding bacteria acting by other mechanisms.

Attempts to establish a causal relationship between antibiotic production revealed in vitro and biocontrol activity had been investigated, three main methods are used: (1) Direct detection and quantification of the antibiotics in the rhizosphere after inoculation through extraction and HPLC purification (Haas and keel 2003). Detection in some cases may be difficult due to the fact that in vitro culture conditions generally differ from those of rhizosphere. Indeed, biotic and abiotic factors including chemical instability of the antibiotic, irreversible binding to soil colloids or organic matter, or microbial decomposition could hamper direct detection of the antibiotic (Thomashow et al. 1997). (2) By use of reporter genes which could "report" the expression of antibiotic biosynthetic genes in the rhizosphere (Haas and keel 2003). This was reported in *Pseudomonas* for the expression of various antibiotics; 2,4-diacetylphloroglucinol (Notz et al. 2001), pyoluteorin (Kraus and Loper 1995), phenazine (Georgakopoulos et al. 1994), and lipopeptide antibiotics (Koch et al. 2002), and (3) genetic manipulation of bacterial antibiotic producers which is a powerful tool to ascertain their role in disease suppression. First work on this topic was performed by Thomashow and Weller (1988) by constructing pseudomonad mutants that lacked phenazine production and showing, thereafter, that these mutants were defective to control a plant disease. Tn5 insertion-derived mutants defective in phenazine synthesis (Phz-) were less effective in biocontrol of take-all disease in comparison with the parental strain of *P. fluorescens*. Moreover, effectiveness of some of these mutants was restored with cloned DNA from the effective parental strain. Mutations in the biosynthetic gene cluster of DAPG reduced biocontrol activity of fluorescent pseudomonads (Keel et al. 1992). Using ARDRA, Sharifi-Tehrani et al. (1998) compared the biocontrol activity of a collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. and found that strains producing only 2,4-DAPG were more effective than pyoluteorin- and 2,4-DAPG-producing strains against *Fusarium* crown and root rot of tomato and *Pythium* damping-off of cucumber.

In addition, some works showed that the in vitro activities could not be related to the in situ activity. Spontaneous mutants of two scab-suppressive S*treptomycetes* that were defective in in vitro pathogen inhibition activity against *Streptomyces scabies* demonstrated significant scab biocontrol activity, suggesting that the

pathogen inhibition activity detected in vitro may not be an accurate predictor of scab biocontrol (Schottel et al. 2001).

2.4.3 Antagonism by Competition: Siderophore Production

Iron is important for plant health and metabolism. It is found in proteins such as nitrogenase, ferredoxins, cytochromes, and leghemoglobin. PGPR bacteria could perform uptake of iron from soil and provide plant with this element. The most widely studied rhizospheric bacteria with respect to the production of siderophores are fluorescent pseudomonads. Siderophores are low-molecular-mass microbial compounds with high affinity for iron. They possess an iron uptake system (iron-binding ligand) able to chelate Fe^{3+} molecules. They are often induced under limiting Fe^{3+} concentrations to allow bacteria to partially fulfill their iron requirement.

Siderophores represent a large biochemically diverse group produced by either plants or plant associated microorganisms (Loper and Buyer 1991). They include pyoveridins produced by *Pseudomonas*; catechols produced by *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, and enterobacteriacea; hydroxamates produced by *Erwinia carotovora*, *Enterobacter cloacae*, and various fungi; and rhizobactin produced by *Rhizobium meliloti*.

Pyoverdine which is a yellow-green, water-soluble fluorescent pigment is the major class of siderophores produced by fluorescent pseudomonad. However, strains of *P. aeruginosa*, *P. syringae*, and *P. putida* could also produce pyoverdine. The chemical structure of pyoverdine has been elucidated and the presence of a chromophore consisting of a 2,3-diamino-6,7-dihydroxyquinoline derivative which is responsible for the fluorescence was reported (Wendenbaum et al. 1983; Leong 1986). Siderophores can be used not only by their producing bacteria (Ongena et al. 1999), but also by other microorganisms. Fluorescent *Pseudomonas* exclusively recognizes the ferric complex of its own PVD. Thus differences in PVD structure affect the biological activity of the siderophores (Hohnadel and Meyer 1988). More than 30 structures of PVDs, differing mainly in their peptide chain, have been described (Budzikiewicz 1997).

Siderophore-producing microbes could contribute to various alterations in plants via the action of siderophores on ferric nutrition (Bar-Ness et al. 1991). Siderophores produced by PGPR could contribute to enhanced growth (Kloepper et al. 1980b). When various plants growing on soil or nutrient solution were supplemented by pyoverdin or ferriopyoveridne, they showed, with few exceptions, enhanced chlorophyll content, and enhanced iron content in the roots and ferric reductase activity (Duijff et al. 1994b, c). In other cases, they have reverse action (Becker et al. 1985)

Siderophores are produced by *Pseudomonas* bacteria to compete for iron and consequently impairing growth of soil-borne phytopathogens, and thus are considered as a control mechanism for many pathogens (Duijff et al. 1994a; Schippers

et al. 1987; Bakker 1989). In addition, in vitro assays showed that the inhibition of pathogens based on competition for iron tends to decrease with increasing iron content of the medium (Duijff et al. 1993).

Suppressive soils to fusarium wilts are known to have a very low solubility of ferric iron (Alabouvette et al. 1996). Consequently a strong iron competition occurs in these soils. In addition, the ability to produce siderophores is likely to contribute to the root-colonizing ability of *Pseudomonas* strains, their antagonistic properties, and their usefulness in biocontrol (Leong 1986; Ran et al. 2005).

The role of these microorganisms in disease-suppressive soils particularly to fusarium wilts was shown to be related to siderophore-mediated iron competition. Addition of *Pseudomonas* pyoverdine to soils conducive to fusarium wilts and to *G. graminis* var. *tritici* confer them suppressiveness (Kloepper et al. 1980a). In addition, when soil was treated either by *Pseudomonas* or its pyoverdine, reduced chlamydospore germination of pathogenic *F. oxysporum* was observed (Elad and Baker 1985a), suggesting a possible role of pyoverdines in soil fungistasis and suppressiveness. In addition, some siderophores like pyocyanin and pyoveridin are essential for the induction of systemic resistance (Audenaert et al. 2002; Leeman et al. 1996; Meziane et al. 2005).

Actinomycetes are also reported as siderophore producers (Khamna et al. 2009). Endogenous siderophore (ferrioxamine) and exogenous siderophore (ferrichrome) have been studied in *Streptomyces pilosus* (Muller et al. 1984; Muller and Raymond 1984). *S. lydicus* WYEC108 was found to produce hydroxamate-type siderophores (Tokala et al. 2002). *Streptomyces violaceusniger* strain YCED9 was reported as able to chelate iron under limiting conditions (Buyer et al. 1989).

Evidences for the in situ production of siderophores and their involvement in biocontrol include the following: (1) *Variation in iron availability of the soil:* increasing the iron amount in soil by lowering soil pH through amendment of H_2SO_4 or iron synthetic chelator resulted in loss of suppression of *Fusarium* wilt by *Pseudomonas* (Elad and Baker 1985b). (2) *Addition of siderophores or synthetic chelators to soil*: introduction of pyoverdin in soil resulted in reducing chlamydospore germination of *Fusarium* (Elad and Baker 1985a) and reducing Fusariosis and take-all disease (Kloepper et al. 1980a). Addition of a synthetic chelator; the ferrated form of ethylenediaminodi (*o*-hydroxyphenyl) acetic acid Fe-EDDHA to the nutrient solution for the plants diminished the disease-suppressive effect of *Pseudomonas putida* WCS358 to suppress *Fusarium* wilt of radish caused by *F. oxysporum* f. sp. *raphani* (de Boer et al. 2003), while addition of EDDHA or its ferrated form to conductive soil rendered it suppressive to *Fusarium* wilt of cucumber, flax, and radish (Scher and Baker 1982). (3) *Genetic evidences*: Expression of siderophore biosynthesis genes in the rhizosphere by *P. fluorescens* in which a promoter from a siderophore biosynthesis gene was cloned (reporter gene) (Loper and Lindow 1991) and comparison of the biocontrol abilities of wild-type producing strains and their mutants defective in siderophore production. Bakker et al. (1988) demonstrated that mutants defective in the synthesis of pyoverdine (Pvd-) and able to use pyoverdine produced by a coinoculated wild-type strain showed a great establishment in potato compared with a mutant not able to use pyoverdine.

Wild-types of *Pseudomonas putida* WCS358 which relatively suppress *F. oxysporum* f. sp. *dianthi* in carnation roots were found to depend only on siderophore-mediated competition for iron. Subsequently, its mutant defective in siderophore biosynthesis was ineffective. This fact provides the proof that siderophores were implicated in the suppressivness of *Fusarium* wilt by this strain (Duijff et al. 1993). Similar results were found in *P. putida* WCS358 for *Fusarium* wilt in radish caused by *F. oxysporum* f. sp. *raphani* (de Boer et al. 2003). However, as disease suppression does not rely only on siderophore production, lack of evidence in the use of mutant-derived strains has been reported. Mutants from *Pseudomonas* sp. WCS417r or *P. putida* strain RE8 defective in siderophore biosynthesis are still able to ensure comparable or a less effective disease suppression in carnation and radish in comparison to wild types due to resistance induction and probably also antibiosis (Duijff et al. 1993; de Boer et al. 2003). In Addition, iron-regulated molecules but non-siderophores could be implicated in disease suppression. In the case of *G. graminis* var. *tritici* (Kloepper et al. 1980a), partial contribution of an iron-regulated nonsiderophore to this suppressiveness is not excluded (Thomashow et al. 1990). Also, Gill and Warren (1988) reported a negatively iron-regulated fungistatic agent to *Pythium ultimum* in iron-limited cultures of *Pseudomonas* sp. NZ130.

2.5 Conclusion

Sustainable agriculture, based on environmentally friendly methods, tends to use bacteria as tools that could by the way reduce the use of chemicals. It is advantageous for sustainable agriculture as introduced bacteria could act as biofertilizers and as biopesticides. In this way, PGPR could constitute a group of bacteria of great importance. Recently, biopesticides are receiving worldwide attention for the sustainability of the agricultural system. Researches had interested the selection of bacteria able to antagonize most deleterious phytopathogens. Unfortunately, most of the works on the biocontrol effect of rhizobacteria were conducted in axenic conditions at laboratory-scale and greenhouse-controlled conditions. Few works were conducted under field conditions. However, the effectiveness of a biocontrol agent depends mainly on its interaction with other microorganisms, the controlled phytopathogen, the plant, and the rhizosphere environment.

Root competence of bacteria with in vitro antagonistic effects toward phytopathogens is one of the most important traits to be considered when bacteria are introduced in native soils where they are subjected to interaction with both roots and other microorganisms. Efficient competition for colonization sites is an important prerequisite for effective biocontrol. In some cases, relatively long time is needed for checking the effectiveness in disease suppression. Also, host crop could affect rhizosphere colonization and competitiveness of antagonistic bacteria as plant response to rhizosphere colonization seems to be bacterium specific. In addition, the sensitivity of phytopathogenic fungi in some cases depends on its

life cycle stage and propagules. For instance, mycelia, zoosporangia, zoospore cysts, and zoospores of *Pythium ultimum* showed difference in sensitivity to 2,4-DAPG (de Souza et al. 2003). For all these reasons, only few examples of biocontrol agents-based products succeeded in field trials and thus have been commercialized for use in agriculture.

A remarkable diversity of metabolites with antibiotic activity is produced by *Pseudomonas*, *Bacillus*, and *Streptomycetes* strains. Some of these microorganisms could produce simultaneously more than one compound (for example, *P. fluorescens* strains CHAO and Pf-5) and/or act by more than one mechanism (e.g., antibiosis and competition for nutrients). Rhizobacteria that could also induce systemic resistance confer protection against phytopathogens to plants. DAPG and siderophores such as pyoverdine have been described as inducers of systemic plant resistance. In contrast to antagonism by antibiosis or siderophore production where the population size should be maintained during the biocontrol process, it is sufficient that the plant and the inducing agent be in contact for a limited period and once induced, systemic resistance is expressed systemically throughout the plant and maintained for prolonged periods.

A better understanding of the major mechanism displayed in field soils will suggest what conditions are to be provided in order to optimize the antagonistic activities of inoculant strains. In this optic, controlled root exudation or nutritional amendment could lead to more successful disease management. Bacteria with more than one beneficial effect are of great interest in biocontrol. By combining strains with different disease-suppressive mechanisms, the impact of filed fluctuating biotic and abiotic conditions could be minimized as some biocontrol mechanism could be effective even if others are unfunctional. In addition, such combinations could be effective against multiple phytopathogens. However, the use of bioinoculants must be taken with some precautions. Measures must be taken to avoid nontarget effect of the introduced bacteria, to stabilize them in soil systems, and thus to guarantee durability of their beneficial effect and their good performance.

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Chapter 3
Bacterial Endophytes of Perennial Crops for Management of Plant Disease

Rachel L. Melnick, Bryan A. Bailey, and Paul A. Backman

3.1 Endophytes

Internal plant tissues are far from a sterile environment, as nearly all tested plant species have been found to be colonized by microbes persisting as epiphytes and endophytes. Endophytes are microorganisms that inhabit the internal tissues of a plant without causing disease (Surette et al. 2003). The term "endophyte" was developed from "endotroph," used to describe the endomycorrhizal association (Frank 1885) and also defines ferns colonized with endophytic algae (Campbell 1908). Endophytes are known to inhabit seeds, ovules, root, stems, leaves, and branches. Endophytes form a unique association with their plant host that can be neutral or beneficial to the plant. Endophytes reduce herbivory (Koh and Hik 2007), promote plant growth (Taghavi et al. 2009), increase mineral uptake (Malinowski et al. 2000), biologically fix nitrogen (Doty et al. 2009; Stoltzfus et al. 1997), suppress disease (Bae et al. 2011; Melnick et al. 2008; Kloepper et al. 2004), and induce plant defense cascades (Bae et al. 2011; Bailey et al. 2006; Kloepper et al. 2004; Melnick et al. 2011).

The occurrence of endophytic bacteria has been known for over 60 years (Hollis 1951). Bacterial endophytes colonizing the intercellular space of plants have been isolated from nearly every plant species sampled (Ryan et al. 2008). Endophytes are associated with perennials such as cacao (Melnick et al. 2011), spruce (Cankar et al. 2005), and diverse Atlantic timber species (Lambais et al. 2006); biennials such as sugar beets (Bargabus et al. 2002) and carrots (Surette et al. 2003); and annuals such

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as corn (McInroy and Kloepper 1995) and potatoes (Sessitsch et al. 2002), among many other plant species. Bacterial endophytes have been isolated from nearly all plant tissues, including pollen (Madmony et al. 2005) and ovaries (Sugawara et al. 2004). There tends to be higher total endophyte populations in roots than in aerial plant tissues (Rosenblueth and Martínez-Romero 2006; Hallman et al. 1997). Additionally, due to the availability of free water in the phyllosphere (leaf surface), epiphytic leaf populations are typically more numerous than endophytic populations (Beattie and Lindow 1999).

Microbes, in addition to colonizing plants, can also colonize the internal tissues of other microorganisms without causing disease. Bacteria and viruses have been isolated inhabiting the hyphae of fungal endophytes, indicating the potential for beneficial tritrophic endophytic interactions (Hoffman and Arnold 2010; Roossinck 2011). An additional tritrophic interaction is the endophytic colonization of bacterium-induced root nodules by a second distinct bacterium (Bai et al. 2002). Bacterial endophytes that lack the ability to cause nodules were found to inhabit root nodules of red clover (Sturz et al. 1997) and soybean (Bai et al. 2002). *Bacillus cereus* UW85 is not a root nodulating bacterium, but seed treatment of soybeans with this isolate increased root nodules formed by another bacterium, suggesting that there may be synergistic effects of a diverse bacterial community in root nodules (Halverson and Handelsman 1991). Turner and Backman (1991) found similar results for *B. subtilis* (GB03) treated peanut plants. For a more extensive review of bacterial endophytes and their associated hosts, see Hallman et al. (1997), Berg and Hallmann (2006), and Rosenblueth and Martínez-Romero (2006).

Gram-negative endophytic bacteria are easier to isolate, culture, and study, and have therefore been more intensively studied than Gram-positive bacteria. Endospores are formed by some Gram-positive bacteria such as *Bacillus* spp. and allow the organisms to survive in extreme environments (Driks 1999). The spore coat of the endospore is composed predominately of proteins rich in tyrosine and cystine (Driks 1999). Additionally, the DNA is saturated by small acid-soluble proteins protecting the genetic material (Driks 2004). Endospores allow gram-positive bacteria to survive extreme conditions, such as the high radiation zone in the area immediately surrounding the Chernobyl, Ukraine, disaster (Zavilgelsky et al. 1998). Agricultural activities cause stress to native soil microbial communities, and these stresses can also select for endospore-forming isolates. As a result, the dominance of endospore-forming bacteria can be a bioindicator of agricultural history (Nilsson and Renberg 1990). Due to the resistant nature of endospores, they are often sought after for commercial products. Endospore-forming bacteria are easy to formulate, can be combined in formulated products with agrochemicals, surfactants, etc., or during application, and have a long shelf life (Francis et al. 2010; Fravel 2005). Species such as *Bacillus* and *Streptomyces* are readily culturable using traditional microbiological techniques, resulting in the skewed isolation and overrepresentation of this group of bacteria from environmental samples.

3.1.1 Diversity of Bacterial Endophytes

Several other factors can impact the diversity of bacterial endophytes including plant genotype, plant tissue, and plant age. According to Adams and Kloepper (2002), different cotton genotypes had large differences in bacterial population levels and the diversity of the bacterial communities present in seeds, stems, and roots. The radicles of germinating cotton varieties Auburn 56 and Rowden were more highly colonized by endophytic bacteria than were other tested genotypes. Genotype Deltapine 50 had a higher population density of endophytic bacteria than all other tested genotypes when grown in the field, suggesting that the genetic, morphological, or physiological differences are important in determining endophytic bacteria populations (Adams and Kloepper 2002). The community of stem-colonizing bacterial endophytes differed between two thermophilic sweet pepper cultivars. The predominant bacterial genera in cultivar Milder Spiral were *Microbacterium*, *Micrococcus*, *Rhodococcus*, and *Staphylococcus*, while the predominant species in cultivar Ziegenhorn Bello were *Staphylococcus aureus* and *Bacillus* spp. (Rasche et al. 2006).

Plant tissues offer different environments to endophytes due to differences in nutrient availability, environmental fluctuations, etc. The distinct environments in these spatially and physiologically different tissues result in qualitative and quantitative differences in bacterial community membership when niches are compared throughout a single plant. In isolations of bacterial endophytes from Chardonnay grapes, *Curtobacterium* spp., *Pseudomonas* spp., and *Streptomyces* spp. were isolated from cane and leaf tissue, but were absent from the trunk and roots. The trunk and roots were home to a totally different collection of species within the bacterial community (West et al. 2010). Endophytic bacterial communities of *Betula pendula*, *Pinus silvestris*, and *Sorbus aucuparia* differed between roots and leaves/stems (Izumi et al. 2008) of the trees. In addition to differences in composition of communities between plant tissues, some tissues are not readily inhabited by endophytes. Endophytic bacteria and fungi were isolated from leaves of several different cultivars of citrus, but all sampled seeds lacked endophytes (Araújo et al. 2001). The lack of bacterial endophytes in citrus seed is an exception. Bacterial endophytes have been detected in both coffee and cacao seeds (Posada and Vega 2005; Vega et al. 2005). In the coffee study, they also found that of all the coffee berry tissues sampled, seeds had the highest populations of endophytic bacteria, including both gram-negative and gram-positive species. Mundt and Hinkle (1976) isolated endophytic bacteria from the seeds and ovules of 27 different plant species. These seeds did not appear to be home to a diverse range of culturable bacteria, as 93 % of seeds samples were inhabited solely by one culturable species, although this species was variable between seeds of the same and different plants.

3.1.2 Classification of Endophytes

Endophytes can be classified into several categories: pathogens of other hosts, avirulent pathogens of the same host, nonpathogens of other hosts, and nonpathogens of the same host. Nonpathogens of other hosts are endophytes isolated from one asymptomatic plant species that have the ability to colonize another species, while nonpathogens of the same host can be reintroduced into the species they were originally isolated from. Commercial products containing nonpathogenic endophytes isolated from another host combined with nonpathogens from the same host have been developed (Bargabus et al. 2002; Brannen and Kenney 1997; Bacon et al. 2001; Zeriouh et al. 2011). Endophytic relationships involving pathogens of other hosts can be beneficial to plants, as the nonhost may still recognize the microbe as a potential pathogen and activate its defenses even though the endophyte is incapable of causing disease in this nonhost. Endophytic colonization of cabbage with the cotton pathogen *Xanthomonas campestris* pv. *malvacearum* reduced the severity of black rot, caused by the pathogen *Xanthomonas campestris* pv. *campestris* throughout the growing season (Backman and Tuzun 1999). This same strain of *X. campestris* pv. *malvacearum* was capable of endophytically colonizing cacao leaves for a month and induced the expression of cacao defense-related transcripts (de Mayolo 2003). Endophytic relationships involving avirulent pathogens often have similar effects as endophytic relationships involving pathogens of other hosts. Inoculation of broccoli with an avirulent strain of *Pereonospora parasitica* reduced disease caused by pathogenic strains of *P. parasitica* (Monot et al. 2002). Inoculation of canola with an avirulent race of the blackleg pathogen *Leptosphaeria masculans* activated the hypersensitive response, while remaining incapable of eliciting disease symptoms (Li et al. 2006). Although not capable of causing disease, avirulent pathogens can often rapidly activate the same plant defense pathways as pathogenic organisms that cause disease (Pieterse et al. 1998).

The capacity to colonize and protect plants in a range of pathosystems is often considered essential for the success of a commercial bioproduct in the agricultural market. The ability to apply a product for management of multiple diseases means a wide range of growers are able to buy and utilize the product. A nonpathogenic endophytic *Bacillus* spp. from tomato was capable of endophytic colonization of cacao leaves and reduced disease in a detached leaf assay (Melnick et al. 2008). *Burkholderia* sp. strain PJN, originally isolated from onion, promoted grapevine growth and inhibited the growth of the noble rot pathogen *Botrytis cinerea* through endophytic colonization (Barka et al. 2002; Compant et al. 2005). Additionally, *Bacillus cereus* UW85, originally isolated from alfalfa seedlings (Handelsman et al. 1990), colonized the roots and rhizosphere of soybeans (Halverson et al. 1993) and increased soybean yield in field trials (Osburn et al. 1995).

It is generally accepted that nonpathogenic endophytes isolated from the same host are highly suited for biological control since they are adapted to the environment from which they were isolated. Plant-associated *Bacillus* spp. from apple were able to colonize apple leaves and fruit preharvest as well as apple fruits in the

postharvest environment (Poleatewich et al. 2011). Melnick et al. (2011) isolated 69 endophytic Gram-positive bacteria from cacao tissues. Of these isolates, inundative application of chitinolytic *Bacillus pumilus* ET to cacao leaves utilizing a Silwet adjuvant for stomatal penetration reduced lesion diameter of *Phytophthora capsici* in growth chamber studies (Melnick et al. 2011). Preliminary field experiments in young plants indicated *B. pumilus* ET also reduced witches' broom, caused by *Moniliophthora perniciosa* (Melnick et al. 2009). *Bacillus subtilis* stain L25 suppressed the growth of chestnut blight pathogen *Cryphonectria parasitica* (Wilhelm et al. 1998). Similar research isolating native endophytes and testing them as biological control agents has been conducted in citrus (Lacava et al. 2004), poplar (Taghavi et al. 2009), carrots (Surette et al. 2003), sugar beets (Bargabus et al. 2003), and a wide range of other plant species.

3.1.3 Endophyte Responses to the Environment

Endophytes are better protected from stress caused by changes in the environment than are epiphytes due to their position within plant tissues. Research by Melnick et al. (2008) demonstrated dynamic changes over time in the relative levels of inoculated *Bacillus* sp. populations in the epiphytic and endophytic environments over a 2-month period. It was suggested that there was a correlation between endophytic population levels and level of ISR as demonstrated by lesion expansion caused by *P. capsici*. In this study, they observed that endophytes were increasingly suppressed as disease suppression approached maximum levels. Later, as endophytic populations were at minimal levels (approaching undetectable), disease suppression also began to recede. These data point to the strong probability that falling endophyte populations were probably a result of suppression by plant defense products. It was further observed that when ISR was at its highest levels, the majority of CFUs of the inducing *Bacillus* were endospores, while when defenses were lowest, a large majority were found as vegetative cells. The ratio of vegetative cells to spores in the epiphytic environment was very stable throughout the experiment, but interestingly, these epiphytic populations may well have provided the reservoir population for restocking the endophytic population as the level of ISR receded and the internal leaf environment became more conducive to the growth of the *Bacillus* sp. (Melnick et al. 2008).

One likely major component influencing the fluctuation of population levels is the movement of bacteria between epiphytic and endophytic environments. In bean plants, internal populations of pathogenic *P. syringae* pv. *syringae* B782a increased at 15 days after bacterization due to inward movement through stomates (Sabaratnam and Beattie 2003), leaving open the possibility that endophytic bacteria may also move in a similar manner. Additionally, endophytic rhizobium species were shown to exit the stomates of tobacco leaves to colonize the epiphytic phyllosphere, indicating that the movement can occur in both directions (Ji et al. 2010). Work with grapevines demonstrated that epiphytic bacteria could enter the internal plant tissues by natural

or human-based means, such as pruning (West et al. 2010). Endophytic *Burkholderia* sp. PsJN readily colonized the epiphytic and endophytic portions of *Vitis vinifera* roots in tissue culture, using endoglucanase to degrade cell walls to internally colonize roots (Compant et al. 2005). Similarly, aerial tissue was colonized through the vascular transport of the root endophyte *Pseudomonas aureofaciens* of corn (Lamb et al. 1996). The bacteria spread through the transpiration stream to colonize stems and leaves driven by water movement (Compant et al. 2005). Endophytes can potentially be vertically transmitted in tissue culture. Propagation of endophyte colonized material can lead to colonization of the derived plantlets.

Work with endophytic *B. cereus*, originally isolated from tomatoes and applied to young cacao plants, demonstrated that populations of these bacteria in the endophytic environment fluctuated between log 2.5 and log 5.5 CFU/cm^3 leaf tissue, while epiphytic populations remained relatively stable during the 68-day sampling period (Melnick et al. 2008). Additionally, endophytic population levels of *B. cereus* BP24 were below the detection threshold 25 days after inoculation, yet recovered 33 days after inoculation. The epiphytic population may have been the source for endophytic colonists (Melnick et al. 2008). Despite the suggested movement of bacteria between the epiphytic and endophytic environments, little research has focused on the fluctuations and interactions of these communities.

Seasonal temperature changes altered the species diversity and abundance of the bacterial community of *Ulmus* spp. (Mocali et al. 2003). Chilling of heat tolerant sweet peppers resulted in an altered bacterial community as assessed by the diversity, complexity, and/or abundance of stem endophytes (Rasche et al. 2006). Despite the endophytic habitat protecting bacterial endophytes from environmental stresses, both short- and long-term changes in endophytic communities can result from environmental fluctuations. Populations of native endophytic *Bacillus* spp. in cacao leaves from field grown trees were variable, fluctuating by approximately 1.0 log CFU/cm^3 (Melnick et al. 2011). Populations of isolates derived from cacao were more stable than endophytes from other hosts. The native bacterial and *Bacillus* communities of cacao leaves sampled after 3 months following inundative application of endophytic *Bacillus* spp. had similar species abundance and diversity to those before application of endophytes. Despite having observed fluctuations of singular species in growth chamber studies, bacterial communities in field grown cacao trees returned to pre-application diversity levels from an endophytic population shift (Melnick et al. 2011) when given enough time.

3.2 Biological Control

In agriculture, biological control is the use of beneficial microorganisms to reduce plant pests, such as disease and insects. The term "biological control" itself can be misleading, as microbes only suppress plant disease and are rarely capable of controlling disease. Russian scientists started work on "bacterial fertilizers" to enhance plant growth in the 1950s (Backman et al. 1998). During this period, it is

estimated that famers were treating millions of hectares of crops with bacterial fertilizers (Cooper 1959). Dunleavy (1955) utilized *B. subtilis* to suppress damping off of sugar beets. Early work by Broadbent et al. (1971) found that 40 % of 3,500 isolated soil-inhabiting bacteria were antagonistic to nine pathogens on agar growth medium, with only 4 % being antagonistic to the pathogens in the soil. This work set forth the precedent of screening large numbers of isolates as well as the notion that only a small portion of environmental isolates may be effective in biological control. In 1974, Baker and Cook wrote the first book solely about biological control of plant pathogens, which summarized the early work in the field (Baker and Cook 1974).

3.2.1 Modes of Action

The mode of action of biological control agents can be divided into direct, indirect, and mixed-path antagonism. Direct modes of action include parasitism such as hyperparasitism and mycoparasitism. Indirect modes of action include competition and induction of plant defenses. Mixed-path modes of action include antibiosis, antagonism, and other less characterized mechanisms. Mycoparasites are parasites of fungi, and have been utilized in biological control to suppress fungal pathogens. In terms of commercial success, *Trichoderma* spp. are the most researched and commercialized mycoparasites (Harman et al. 2004). *Trichoderma* spp. form intimate contact during mycoparasitism and can be found coiling around plant pathogenic fungi (Inbar et al. 1996). Once contact is established, the *Trichoderma* spp. can directly penetrate the hyphae of plant pathogens through combined appersorium formation and production of cell wall degrading enzymes, such as chitinase and glucanase (Harman et al. 2004). The key to the success of mycoparasitism in biological control is that the biological control agent (BCA) must come in direct contact with the targeted pathogen and must persist in the same environment as the pathogen. In terms of bacteria, bacterial BCAs are not known to be mycoparasites of fungi. However, bacterial species have been found to be parasites of nematodes. Gram-positive *Pasteuria penetrans* effectively reduces damage of the root-knot nematodes through direct colonization and parasitism of nematodes resulting in lysis and death of the pest (Davies et al. 1988). Commercial production has been difficult since *P. penetrans* in an obligate symbiont and the bacterium must be produced on nematode-infected tomato roots.

Competition in terms of biological control occurs when a BCA obtains resources faster than a pathogen within a shared habitat. Several commercial BCAs operate primarily through the mechanism of niche displacement. *Pseudomonas fluorescens* A506 (BlightBan A506) colonizes apple and pear blossoms and prevents *E. amylovora* from reaching adequate populations for quorum sensing by excluding resources required for the pathogen (Wilson et al. 1992; Wilson and Lindow 1993). Additionally, colonization of trees with *P. fluorescens* A506 prevents frost damage, as the BCA outcompetes ice nucleating bacteria for nutrients, reducing their

population by nearly 100-fold (Lindow et al. 1996). BlightBan A506 is registered to protect almond, cherry, pome fruits, potato, strawberry, and tomato from frost damage (Stockwell and Stack 2007). Colonization of several crops and protection against multiple diseases have resulted in the continued success of BlightBan.

Another mixed-path mode of action is antagonism. Antagonism can be broken down into production of lytic enzymes or antimicrobials by the BCA to make the shared environment inhospitable for pathogens (McSpadden-Gardener 2002). Antagonistic compounds impair pathogen growth, reproduction, sporulation, and infection processes to reduce disease. Traditionally dual-culture *in vitro* assays conducted on Petri dishes have been a key initial screen to identify organisms producing antagonistic compounds. Increased knowledge of the multiple modes of action used by BCAs has reduced the use of this method as a primary selection step since an *in vitro* screen on agar does little to mimic the natural environment and readily eliminates potential BCAs that utilize other modes of action. NoGall is an example of commercial success using antibiosis as a primary mode of action. *Agrobacterium radiobacter* K1026, the active microbe in NoGall, produces the bacteriocin agrosin 84 which kills the crown gall pathogen *Agrobacterium tumefaciens* (Reader et al. 2005). The close genetic relationship of these two species allows them to colonize the same niche. Similarly, *Bacillus* spp. found in several commercial products produce antibiotics and secrete them in the environment shared with plant pathogens (Gupta and Utkhede 1986; Toharisman et al. 2005; Stein 2005). An advantage of *Bacillus* produced antibiotics is that they are often effective against a range of plant pathogens (Kloepper et al. 2004). Additionally, bacteria including *Bacillus* spp. are known to produce lytic enzymes such a chitinase and glucanases which degrade the cell walls of fungal pathogens (Chernin et al. 1995; Frändberg and Schrnürer 1998; Kishore and Podile 2005; Kobayashi et al. 2002; Kokalis-Burelle et al. 1992; Pleban et al. 1997). Some bacteria can produce phytohormones and nutrient solubilizing enzymes that produce plant-growth promoting rhizobacteria (PGPR) effects. These traits coupled with the ability to effectively colonize and dominate the rhizosphere are largely responsible for the beneficial effects of *Bacillus subtilis* GB03, commercially sold as Kodiak (Brannen and Kenney 1997).

The last and most recently recognized mode of action is induction of host defenses, more commonly known as induced resistance. When understanding plant-associated microbes, it should be recognized that plant disease is a rare circumstance in which the pathogen avoids early recognition by the host and early induction of host defenses or does not elicit a timely host response during the initial interaction with the plant (Zehnder et al. 2001). Some beneficial microbes can activate plant defense cascades, resulting in disease suppression (Pieterse et al. 1996). Although effective in reducing disease, activation of plant defense can be costly to the plant, due to the energy required for the production of proteins and plant metabolites (Heil 2001). Some beneficial microbes overcome this high cost by priming the plant for defense. Priming occurs when BCAs do not fully activate plant defense cascades, but instead stimulate slight changes in gene expression and/or metabolism, preparing the plant to rapidly hyperactivate specific defense

response in the presence of the pathogen (van Hulten et al. 2006). In other words, primed plants have a slightly increased expression of key defense genes that allows them to have a faster and stronger defense response upon infection by a pathogen (van Hulten et al. 2006). The advantage of priming over full induction of plant defense is that there is a reduced biological cost to the host plant in the absence of the pathogen (van Hulten et al. 2006).

There are several advantages to induced resistance. Induced resistance is often effective again a broad range of pathogens since it utilizes plant defenses (van Wees et al. 1999) evolved for broad-spectrum activity. Induced resistance can also act systemically, impacting disease in an area spatially separated from the BCA. Rhizosphere colonizing bacteria have been shown to reduce foliar diseases through production of systemic defense signals (Kloepper et al. 2004; Cartieaux et al. 2003; Heil and Bostock 2002; Zehnder et al. 2001). Colonization of cucumber roots with combinations of plantgrowth promoting rhizobateria (PGPR) reduced the severity of cucumber mosaic virus on the foliage, despite lack of colonization in leaves (Jetiyanon and Kloepper 2002). Colonization of tobacco roots with *Bacillus* spp. reduced the severity of cucumber mosaic virus (Kloepper et al. 2004). Overall, the key to understanding the modes of action utilized by BCAs is to understand that BCAs can readily utilize multiple modes of action to reduce disease. Developing BCAs with multiple modes of action by having one robust organism or multiple organisms may provide more success in disease management.

3.2.2 Biological Control of Perennial Plant Diseases with Bacterial Endophytes

Biological control in woody perennial crops offers many challenges that are not encountered with annual crops. One of the largest issues to overcome with perennial crops is the absence of crop rotations to reduce levels of pathogen inoculum. For example, an apple tree may be in an orchard for 30 years. Pathogens of perennial crops can overwinter on debris, but also overwinter/off season on the plants themselves. Cleistothecia of powdery mildew of grapevine (*Uncinula necator*) overwinter in leaf scars and crevices of bark while the mycelium can also overwinter in dormant buds (Pearson and Gadoury 1987). During spring, ascospores are discharged from the cleistothecia to infect developing leaves and perpetuate the disease into the next growing season (Pearson and Gadoury 1987). Similarly, the conidia of *Venturia inaequalis*, causal agent of apple scab, overwinter in inner bud tissue (Holb et al. 2004). Mummified non-abscised fruit serve as a refuge for overwintering conidia of *Monilinia fructicola*, causal agent of peach brown rot (Landgraf and Zehr 1982).

Tropical crops are under constant disease pressure, and managing inoculum sources in tropical woody perennials is even more difficult than for temperate perennials. Vascular wilt of the perennial tropical crop Naranjilla (*Solanum quitoense* L.), caused by *Fusarium oxysporum*, has decimated production in

Ecuador (Ochoa et al. 2001). Farmers faced with this pathogen by simply abandoning plantations or replanting elsewhere every few years in order to continue producing the crop (Ochoa et al. 2001). Guava is widely grown throughout the tropics, but succumbs to many fruit rots, such as *Pestalotiopsis* species (Keith et al. 2006) and *Phomopsis destructum* (Rao et al. 1976). Since both of these crops are actively growing year round, there is always a potential for disease to occur. Despite the perennial nature of their hosts, inoculum for most tropical diseases is not consistent through the year. For example, the mushrooms of *Moniliophthora perniciosa*, causal agent of witches' broom of cacao, require an alternating wet/dry cycle typically found during the rainy season (Meinhardt et al. 2008). While far more mushrooms form during the rainy season, infections still occur during the dry season, forcing farmers to scout for disease and conduct phytosanitatry practices year round.

3.2.2.1 Delivery and Entrance of Endophytes

A key step to use of bacterial endophytes in biological control is the development of methods to facilitate colonization of the plant. Most commercial BCAs are formulated so that they can be applied in a manner similar to fungicides through aerial sprays, seed treatments, roots dips, and incorporation into soil mixes (Fravel 2005). One challenge to use of bacterial endophytes with woody plant species is physically getting the endophytes into the plant. In work with cotton, endophytes were introduced into the stems by puncturing the stem with a needle (Chen et al. 1995). This method allowed the bacteria to survive in the stems for nearly a month, although the bacteria did not move further than 5.0 cm from the inoculation point (Chen et al. 1995). Another method is using surfactants, such as Silwet, which reduce the surface tension of the solution, allowing for substomatal infiltration (Melnick et al. 2008). Additionally, timing of application can be essential for effective management of disease. The key success with the use of BlightBan A-506 (*Pseudomonas fluorescens*) on tree fruit is application timing. Since the bacterium colonizes internal flower parts (stigma, nectaries, etc.), farmers must time the application of BlightBan with flower opening, allowing the bacterium to colonize the flowers before the pathogenic *Erwinia amylovora* bacterium. The shortcoming of this strategy is that flowers are opening every day for 2 weeks, and all flowers are susceptible to *Erwinia* when they open (Bubán et al. 2003). Timing sprays to coordinate with flower opening, including prediction of rain events that deliver the bacterium, is critical to success (Wilson et al. 1992; Wilson and Lindow 1993).

For some tree species, endophytes naturally spread through seeds, grafting, or bud wood. These types of vertical transmissions are most commonly seen with fungal endophytes of grasses (Afkhami and Rudgers 2008). Vertical transmission has been suggested for bacterial endophytes, but this is an under-researched topic. Although not as well studied as in perennial grasses, vertical transmission has been found in woody perennial plants. Natural bacterial endophytes were detected in

Eucalyptus seeds and the same species were detected in developing seedlings (Ferreira et al. 2008). Ferreira et al. (2008) created gfp-labeled strains of the endophytic bacterium, *Pantoea agglomerans*, and tracked the movement of the species from seeds into the roots of developing *Eucalyptus* seedlings to confirm that vertical transmission occurred. We need a better understanding of how widespread and effective vertical transmission of bacterial endophytes is in plants.

3.3 Case Study: Biological Control of Cacao Pod Diseases

Theobroma cacao L. is an economically significant crops, as the beans (seeds) produced in pods (fruits) are processed into chocolate and cocoa products. Cacao is a tropical crop grown in countries located between the tropics of Capricorn and Cancer. Approximately 70 % of cacao is grown by smallholder farmers. Traditionally, cacao trees have been intercropped with timber and fruit trees, which provide shade for the cacao trees and additional income for the farmers. Additionally trees were often planted from seed, leading to significant genetic diversity within a field. In recent years, improved clones have been introduced and are being planted in monoculture under full sun and in large hectarage farms, changing disease management needs (Hebbar 2007).

Many pods are lost early during development to a physiological condition known as cherelle wilt. The physiological initiation of cherelle wilt is unknown, but the trigger causes xylem occlusion by a mucilage-like substance which results in wilting and pod death (Nichols 1961). Even if developing pods survive past the critical period for cherelle wilt (<70 days), they can still be lost from the destructive effects of three major pod rotting diseases. Black pod is caused by several *Phytophthora* spp. (Guest 2007). Symptoms of pod infection are dark necrotic lesions and rotten beans. Frosty pod rot and witches' broom are caused by two distinctive agaric *Moniliophthora* spp. Symptoms of frosty pod rot, caused by *Moniliophthora roreri*, are pod malformation and the development of necrotic lesions which rapidly become covered with mycelium bearing powdery white meiospores (2*N* spores) (Evans 1981). Infections of maturing pods by *M. perniciosa*, causal agent of witches' broom disease, may not be seen until the pod is opened to expose the rotten beans. Additionally, infection of young pods and flower cushions leads to the development of parthenocarpic fruit known as cherimoyas, which later become necrotic and remain on the tree as a source of basidiocarps. Management of these cacao diseases consists of phytosanitation to remove disease branches and pods in order to reduce inoculum and planting of tolerant clones. Fungicides can improve yield, but are often too costly for small-holder farmers as well as pose risks to the health of the applicator and the environment. For these reasons, there has been increased research on biological control as a sustainable disease management option.

Previous work by Melnick et al. (2008) found that an endophytic *Bacillus cereus* from tomatoes could colonize cacao foliage and suppress *P. capsici* in detached leaf assays. Although *Bacillus* sp. BT8 was capable of reducing disease in detached leaf assays, the isolate was not native to cacao growing regions, making release of these

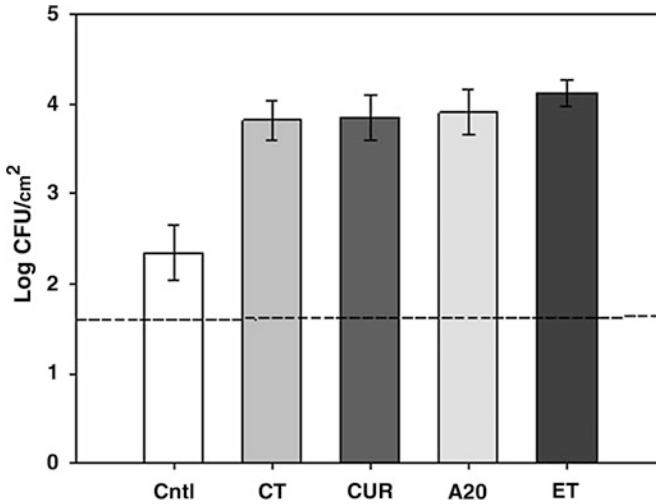

Fig. 3.1 Mean initial epiphytic colonization of immature pods (log CFU/cm^2) found on "Nacional" cacao pods at 24 h after treatment when sprayed with either 0.2 % Silwet control or 0.2 % Silwet + log 8.0. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET Control pods had low levels of naturally occurring endospore-forming bacterial endophytes. The *dotted line* indicates the minimum detectable level of log 1.8 CFU/cm^2. *Bars* extending from means indicated the standard error of that mean

isolates in cacao production areas difficult. Melnick et al. (2011) expanded on this work by isolating native endophytic endospore-forming bacteria from cacao flower cushions, pods, leaves and branches from trees grown in Ecuador. Isolates were screened to determine whether they possessed attributes of BCA, such as chitinase production, antagonism toward the three cacao pods diseases, ability to colonize cacao seedlings, and ability to reduce *Phytophthora* lesion expansion in a detached leaf assay by 34–44 % (Melnick et al. 2011).

Field trials were conducted to test how elite bacterial isolates (*Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET) affected cacao disease when applied to immature trees grown under two cacao farming strategies (monoculture and intercropped) typical of what is found in Ecuador. Intercropped cacao trees (cacao grown under shade of forest trees) were "Nacional" trees having been derived from several cacao clones. We found that application of bacteria with the organo-silicon surfactant Silwet (0.2 %) did not alter normal pod development. Twenty-four hours after application, applied bacteria were detected in both the epiphytic (Fig. 3.1) and endophytic environments (Table 3.1).

Endophytic bacteria survived in both the epiphytic and endophytic environments. There were no detectable endophytic colonists in control pods immediately after application. Despite control pods initially lacking endophytic endospore-forming bacteria, they were detected in some control pods pieces 3 months after bacterization. Endophytic colonists were not equally dispersed throughout the pod, as endospore-forming bacteria were not isolated from all pod sections.

Table 3.1 Mean percentage of sampled pods with endophytic endospore-forming bacterial colonists

Treatment	Initial	3 mai
Control	0	33.3
CT	100	100
CR	100	66.7
A20	100	66.7
ET	100	33.3

Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET. Initial indicates the percentage of pods with endophytic colonization 24 h after inoculation with bacteria, while 3 mai indicates the percentage of pods with any heat stable endophytic colonists tested 3 months after inoculation (mai)

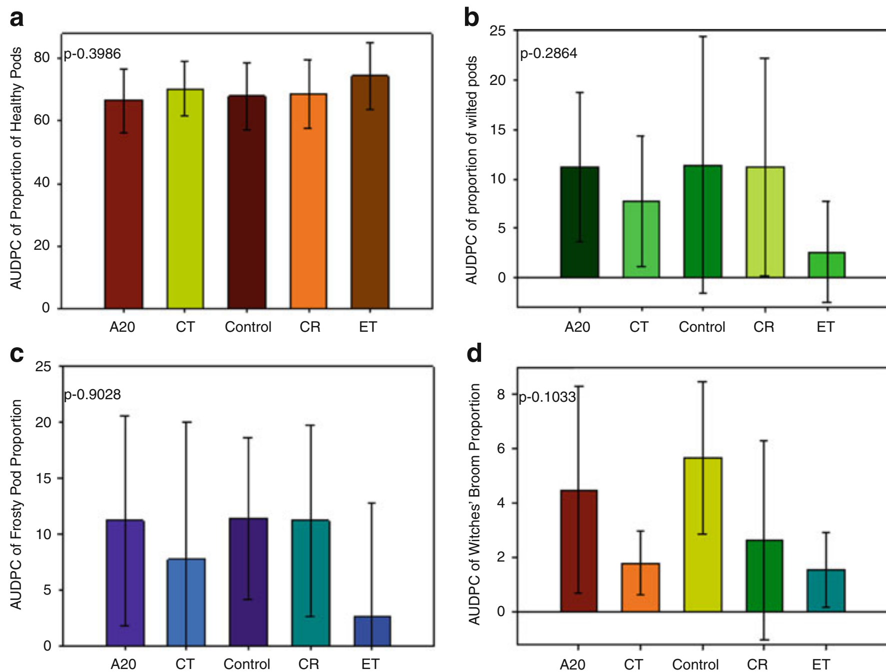

Fig. 3.2 Mean AUDPC of incidences of (**a**) healthy pods, (**b**) cherelle wilt, (**c**) frosty pod, and (**d**) witches' broom out of 78 "Nacional" pods per treatment blocked by location at the INIAP-EET. Control pods were sprayed with 0.20 % Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU.cm^2 solution of bacterial isolate + 0.20 % Silwet L-77. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET. Pods were sprayed in January and evaluated monthly in February, March, April, and May for the presence of cherelle wilt, frosty pod, and witches' broom. *Bars* extending from means indicated the standard error of that mean

Pods were counted and disease was assessed monthly. In the intercropped "Nacional" trees, *B. pumilis* ET significantly increased the number of healthy pods 1 month after bacterization ($p = 0.0262$) (Fig. 3.2a), but not at later time

points. Pods treated with *B. pumilis* ET also had less pods lost to cherelle wilt at 1 month after bacterization ($p = 0.044$), after which, pods were physiologically resistant to cherelle wilt. Therefore, the incidence of cherelle wilt did not increase beyond that point. Despite an increase in the number of healthy pods early in the season, no isolate reduced losses to frosty pod rot or witches' broom.

Despite the successes of the bacteria in reducing cherelle wilt in Nacional pods, no treatment caused season-long disease suppression as seen in a reduction in the AUDPC (Fig. 3.2). Fewer pods overall were lost to cherelle wilt or witches' broom. Despite the 83 % reduction in cherelle wilt in *B. pumilis* ET-treated pods, the high levels of variability in the control treatment precluded a significant effect.

3.3.1 Results from a Large Farm Producing Sun-Grown Cacao

All plants on the commercial farm were clonally propagated "CCN-51" clones, known for their high yield and reduced levels of disease. *B. pumilis* ET significantly decreased cherelle wilt on CCN-51 trees throughout the study when compared to both control pods and pods treated with *Lysinibacillus sphaericus* A20. This bacterium appears to have increased resistance to cherelle wilt during the susceptible period. Despite the wilt reduction, the number of healthy pods throughout the season did not increase.

Bacterial treatments did not reduce overall black pod (Fig. 3.3c) or frosty pod (Fig. 3.3d) incidence, but *B. pumilis* ET decreased overall cherelle wilt (Fig. 3.3b) when compared to both controls and *L. sphaericus* A20.

In both "Nacional" and "CCN-51" plots, *B. pumilis* ET reduced cherelle wilt, but did not reduce cacao disease. One confoundment in these experiments was the fact that a higher frequency of pod-set from cherelle wilt suppression provided more pods that might be infected with fungal pod diseases, causing a statistical skew in the data.

3.3.2 Conclusions

These finding are one of the first studies in which application of a BCA suppressed cherelle wilt which had previously been ascribed to "self-thinning." Fungal mycoparasites used against frosty pod in Peru (Krauss and Soberanis 2001) and black pod rot in Africa (Deberdt et al. 2008) had no impact on cherelle wilt in field trials. In the key cacao production zones of central and south America, it is common to see 50–60 % of young pods lost in the first 6 weeks following flower fertilization. The reduction in losses to cherelle wilt by *B. pumilis* ET followed the same trend at both replicated field sites, despite differences in genotype, environment, and field management strategies between the two fields. Reducing the number of pods lost to cherelle wilt could potentially increase the number of healthy pods, thus potentially increasing yield. Loss of "CCN-51" pods to cherelle wilt was reduced by 52 % by

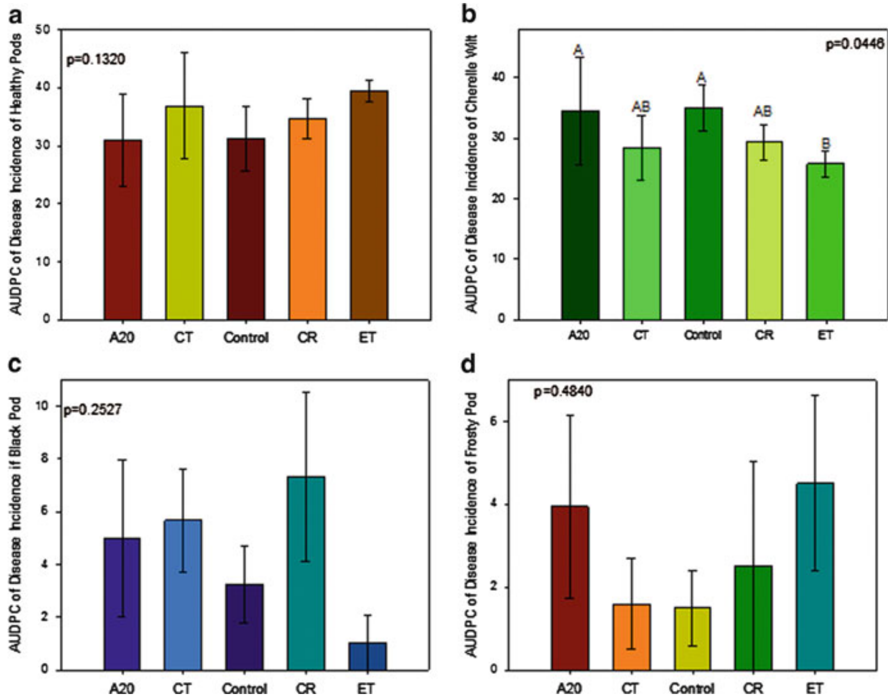

Fig. 3.3 Mean AUDPCs for incidence of (**a**) healthy pods, (**b**) cherelle wilt, (**c**) frosty pod, and (**d**) witches' broom out of 82 "CCN-51" pods per treatment blocked by tree location at the Rio Lindo farm. Control pods were sprayed with 0.20 % Silwet L-77, while the remaining treatments were sprayed with log 8.0 CFU/cm^2 solutions of *Bacillus* isolate + 0.20 % Silwet L-77. Treatments were control, *Bacillus cereus* CT, *Bacillus subtilis* CR, *Lysinibacillus sphaericus* A20, and *Bacillus pumilis* ET. Pods were sprayed in January and evaluated monthly in February, March, April, and May for the presence of cherelle wilt, frosty pod, and witches' broom. *Bars* extending from means indicate the standard error of that mean

B. pumilis ET, while loss of Nacional pods was reduced by 38 %. If cherelle wilt is simply physiological wilting, then hormone imbalances might initiate xylem occlusion. *Bacillus* spp. are known to have the ability to produce phytohormones (Idris et al. 2007) and also affect plasticity of plant cells (Kerff et al. 2008). If *B. pumilis* ET is found to produce phytohormones in future experiments while colonizing pod tissue, they could potentially prevent cherelle wilt initiation.

The most consistent observation at both sites was that one application of bacterial endophytes was not enough to reduce disease throughout the 4–5-month period from early pod set to harvest. The protection against cherelle wilt required a much shorter term of protection, lasting only until pods were physiologically resistant to cherelle wilt, which was roughly March in these experiments. Although *B. pumilis* ET decreased pods lost to cherelle wilt, it also increased the number of healthy pods which could have become infected, adding a complicating factor to the experiment. The increased numbers of healthy pods warrant additional measures to

protect the pods until harvest. Based upon previous research and the need to protect pods throughout the season, experiments are ongoing to assess the effect of monthly application of bacteria endophytes for control of cacao diseases.

3.4 Case Study: Endophytic *Curtobacterium flaccumfaciens* and Reduction of Citrus Variegated Chlorosis

Xylella fastidiosa is a xylem-limited Gram-negative bacterium which causes diseases in at least 10 woody species including grapevine and citrus (Hopkins 1989). The bacterium is transmitted between plants by xylem feeding insects (Hopkins 1989). *X. fastidiosa* sbsp. *pauca* (*Xfp*) infection of sweet oranges (*Citrus sinensis* L) results in citrus variegated chlorosis (CVC) disease (Lacava et al. 2007a). As the name suggests, the bacterium induces leaf chlorosis as well as gummy lesions on the abaxial surface of leaves, which become necrotic. CVC infection results in smaller fruit with hard rinds, making them unmarketable (Amorim et al. 1987). Additionally, tree growth is stunted and twig and branch dieback occurs (Roberto et al. 1996). All sweet orange cultivars are susceptible to the disease (Lacava et al. 2007a). Since its arrival in Brazil in 1987 (Amorim et al. 1987), production of sweet oranges has been drastically reduced. A difficulty in management of the disease is related to transmission of the pathogen by xylem feeding sharpshooter leafhoppers (Roberto et al. 1996) and the xylem limited nature of the pathogen. Current recommendations are purchasing disease-free nursery stock, insecticidal treatments to manage vectors, and phytosanitation (Almeida et al. 2001). Due to the inability to directly attack the pathogen in the plant, scientists decided to assess the potential of utilizing bacterial endophytes to manage CVC.

Araújo et al. (2001) assessed the diversity of endophytic fungi and bacteria isolated from citrus rootstock. Thirty-six distinct bacterial isolates were isolated from the endophytic portion of the roots, with several of these isolates being inhibitory to the citrus pathogen *Guignardia citricarpa*, causal agent of black spot of citrus (Araújo et al. 2001). This work was then expanded to assess the diversity of endophytes inhabiting the branches. Araújo et al. (2002) compared differences between the bacterial communities and their relationship to *Xfp*. The bacterial communities of healthy, CVC infected and symptomatic, and CVC infected yet asymptomatic plants were compared using traditional isolation techniques and culture-independent technologies. There were no differences in the number of endophytic bacterial strains isolated from these trees, but asymptomatic plants had a higher frequency of *Curtobacterium flaccumfaciens* (Araújo et al. 2002). Additionally, there was a positive correlation between disease intensity and abundance of *Methylobacterium* spp. (Araújo et al. 2002). This observation may be related to the fact that a *M. extorquens* isolate stimulated *Xfp* growth on Petri dishes (Lacava et al. 2004). Other endophytic isolates, in particular, *Methylobacterium mesophilicum* and *C. flaccumfaciens*, were found to be inhibitory to *Xfp* and were further screened for their potential in biological control of CVC (Lacava et al. 2004).

Madagascar periwinkle (*Catharanthus roseus*) has been used as a model plant to study *Xfp* in the greenhouse (Lacava et al. 2007a). This system was utilized to study the interaction of inhibitory bacterial endophytes with *Xfp in planta* (Lacava et al. 2007b). Inoculation of *C. roseus* with citrus *Xfp* reduced flower number, stunted growth, stunted leaf size, and caused wilting (Lacava et al. 2007a), similar to the reaction in citrus. Once the model system was developed, scientist screened *M. mesophilicum* and *C. flaccumfaciens* for their ability to reduce CVC. *M. mesophilicum* was inoculated into *C. roseus* via seed treatment, causing a shift in the native bacterial communities of the plant, particularly in root tissue (Andreote et al. 2006). In its interaction with *Xfp*, some endophytic *Methylobacterium* spp. produce hydroxamate-type siderophores which stimulated *Xfp* growth (Lacava et al. 2008), supporting results from earlier studies in which there was increased *Methylobacterium* colonization with CVC disease (Araújo et al. 2002). Additionally, *M. mesophilicum* preferentially colonized xylem vessels (Gai et al. 2009). The sharpshooter insects *Bucephalogonia xanthophylls*, CVC vectors, were also shown to be able to vector *M. mesophilicum*, suggesting that the insect may also vector endophytic bacteria (Gai et al. 2009). When plants inoculated with *C. flaccumfaciens* were challenged with the pathogen, no disease symptoms developed as *C flaccumfaciens* colonized the same niche and the pathogen and produced bacteriocins effective against *Xfp* (Lacava et al. 2007a). Further work on this bacterium may provide citrus farmers with another management option for CVC.

3.5 Biological Control in the -Omics Age

Traditional microbiology methods often underestimate the diversity of species present in endophytic communities, as approximately 90–99 % of microbes cannot be cultured (Curtis et al. 2002; Pace 1997). Combining culture-based and culture-independent technologies can provide a better picture of endophytic communities present in a host. Additionally, they can be used to determine how inundative application of a BCA affects the community of beneficial and pathogenic microbes. Work by Gilbert et al. (1993) illustrated that inundative application of *Bacillus cereus* UW85 to soybean roots drastically altered rhizosphere bacterial communities. Berg et al. (2005) used terminal restriction length polymorphism analysis to estimate the endophytic and ectophytic bacterial (combined rhizosphere and phyllosphere) community inhabiting different potato tissues and the soil. They found that the rhizosphere and endorhizosphere communities were home to more species of antagonistic bacteria than aboveground plant parts (Berg et al. 2005). Melnick et al. (2011) analyzed community diversity of cacao leaves at three months using automated ribosomal intergenic spacer analysis (ARISA) after application of endophytic *Bacillus* spp. to determine whether inundative application of bacteria could incite long-term shifts in the native microbial communities. Despite application with Silwet adjuvant, followed by robust endophytic colonization immediately following application, the bacterial community had fully recovered from inundative application by the 3-month

sampling date by returning to a similar microbial community as found in nontreated leaves (Melnick et al. 2011).

A range of molecular technologies have been developed to estimate the numbers of individual species in an interaction as well as to determine the diversity of endophytic bacterial communities present in plants. One technique with a diverse range of applications is real-time PCR. Real-time PCR simultaneously amplifies and quantifies gene fragments. Amplification-specific fluorescent dyes are detected by the machine to quantify the amount of DNA amplicons. The detection step can be performed using two methods. One methodology utilizes primers which have a fluorescence reporter label which is only detected once it has hybridized with the target (Arikawa et al. 2008). The other methodology utilizes nonspecific fluorescent dyes that intercalate into the double-stranded DNA amplified from targets (Arikawa et al. 2008).

Real-time PCR is an invaluable technique for rapid diagnosis of plant diseases, especially when the pathogenic organism cannot be cultured, such as viruses (Schaad and Frederick 2002). Knowledge of changes in the genes of different pathovars of a species can help determine the pathovar of isolated organisms without the need to conduct Koch's postulate. With the increasing ease of extracting DNA using pre-assembled kits, diagnosticians can potentially determine causal agents in hours, as they will not have to wait for pathogen sporulation or for the microorganism to grow in media. Additionally, since 96-well plates are typically used, analyses can be simultaneously performed using multiple primers targeting multiple pathogens and utilizing multiple samples.

Quantitative real-time PCR (QPCR) is an invaluable tool for estimating the population levels of endophytes. Tellenbach et al. (2010) developed QPCR primers to estimate the biomass of endophytic fungus *Phialocephala fortinii* inhabiting plant roots. Lacava et al. (2006) used QPCR to access population levels of the bacterial endophyte *Methylobacterium mesophilicum* in the presence of xylem-limited pathogen *Xylella fastidiosa* in the model plant *Catharanthus rosea*. They found the endophytic population increased by nearly 200-fold in the presence of the pathogen compared to disease-free plants (Lacava et al. 2006). Studies using bacteria often combini serial dilution plating with QPCR to confirm findings.

QPCR has been used to assess the effects of colonization by endophytes on plant gene expression. Bailey et al. (2006) found that endophytic colonization of cacao seedlings with *Trichoderma* spp. induced the expression of plant expressed sequence tags (ESTs) related to osmotic stress response and defense. The ESTs' induction patterns were *Trichoderma* isolate specific. Additionally, *Trichoderma* had altered expression of ESTs related to nutrient acquisition in a low nutrient environment (Bailey et al. 2006). Pavlo et al. (2011) assessed whether colonization of Arabidopsis with potato endophytes *Pseudomonas* sp. or *Methylobacterium* sp. induced expression of defense genes via QPCR. In the absence of the pathogen *P. syringae* pv. *tomato* DC3000, there was a slight change in gene expression (Pavlo et al. 2011). Plants colonized with endophytes and then challenged with the pathogen had higher expression levels for marker genes for ISR and SAR than plants simply colonized with the endophyte or challenged with the pathogen, suggesting that the endophytes primed the plants for defense (Pavlo et al. 2011).

Estimation of gene expression is not only important in the understanding of plant–microbe interactions, but also in understanding the induction of resistance by endophytes. This technology is not only useful in detecting host gene expression in response to endophytic colonization, but also to estimate gene expression of the plant-associated microbes. Bailey et al. (2006) used QPCR to determine how endophytic colonization of cacao with *Trichoderma* spp. altered gene expression in the plant, indentifying seven cacao ESTs which were induced by the fungus. Additionally, QPCR was used to estimate expression of *Trichoderma* ESTs *in planta* to gain a better understanding of the genes utilized during the endophytic interaction. Similar work was conducted on the interaction of the plant root nodulating bacteria and its legume host using a specialized dual genome microarray (Barnett et al. 2004). Microarrays, often known as gene chips, are specialized slides in which DNA spots of specific sequences are attached or printed on the surface. The target cDNA is then hybridized to the chip. If a gene is expressed by the plant, then the cDNA will form a probe–target hybrid which can be detected with chemiluminescence to estimate the expression levels of the genes in the sample. The advantage of microarrays over QPCR is that tens of thousands of gene are on the chips, allowing scientist to measure shifts in expression of many genes with just one chip as opposed to thousands of QPCR reactions. Verhagen et al. (2004) used the Arabidopsis GeneChip to determine which genes were induced during rhizobacteria-induced systemic resistance in response to colonization of the rhizosphere with *P. fluorescens* WC471r. This work provided researchers with a list of potential genes involved in the ISR pathway. While a very useful technology, scientists are limited by the genes present on the chip and the availability of a microarray for a specific species. Microarrays have been used and are available commercially for perennial *Vitis vinifera* (Waters et al. 2005), citrus (Martinez-Godoy et al. 2008), and poplar (Azaiez et al. 2009).

Another use for microarray technology is the Phylochip. Instead of the microarray having genes of one organism, the DNA bound to the chip is 16S RNA genes from thousands of bacterial species. The Phylochip was used by Weinert et al. (2011) to demonstrate that potato cultivar impacted the abundance of specific plant-associated bacterial genera. Phylochip technology has also been applied to communities of endophytic bacteria. Sagaram et al. (2009) used this technology to study the diversity of endophytic bacteria associated with citrus leaf midribs. Through this study, an increased abundance of nine taxa of bacteria was observed in leaves having symptoms of Huanglongbing disease, caused by "*Candidatus* Liberibacter asiaticus." Pathogen "*Canididatus* Liberibacter asiaticus", which cannot be cultured using current methodologies, was present at 200 times higher population levels in symptomatic leaves than asymptomatic leaves. These results confirmed that Phylochip technology could potentially be used to detect specific pathogens in addition to estimating total diversity of microbes (Sagaram et al. 2009). Although it is a powerful technology, it is only as good as the genes on the chip. Currently, there is no PhyloChip for fungal species.

There are other less costly molecular technologies based upon whole-community fingerprinting that have been utilized to assess microbial communities. One technique is automated ribosomal RNA intergenic spacer analysis (ARISA). PCR is conducted to amplify the rRNA spacer region (Jensen et al. 1993). Automation involves using a DNA analyzer to measure the length of the rRNA spacer region and the fluorescence of the fragment to estimate the diversity and abundance of the microbial community (Fisher and Triplett 1999). ARISA cannot estimate exact counts or identify the organisms present, but can provide useful estimates of diversity and abundance. Bacterial identification can be accomplished only if the amplicons are run on a gel and the individual bands are excised and sequenced. ARISA can be used to assess total bacterial or fungal communities or specific populations of microorganisms, depending on primer design. ARISA has most often been utilized to assess the diversity of bacterial communities in the soil and rhizosphere, but some researchers have utilized this technology for assessment of endophytic communities. Manter et al. (2010) used ARISA to demonstrate difference in the endophytic bacterial community associated with the roots of different potato cultivars. ARISA has also been used to assess endophytic diversity in perennial plants such as cacao (Melnick et al. 2011) and several Brazilian Atlantic forest tree species (Lambais et al. 2006).

Another similar technology is the use of terminal restriction fragment length polymorphism (T-RFLP) analysis. While ARISA primers amplify the intergenic spacer region, T-RFLP primer amplicons amplify a region containing a restriction site. The amplicons are exposed to a restriction enzyme and the resulting fragments are separated via DGGE or capillary electrophoresis. Benítez et al. (2007) used T-RFLP to assess not only the bacterial soil communities that developed under different soil management strategies, but also to assess differences in pathogenic communities causing damping-off. T-RFLP has been used to assess endophytic bacterial community structure in sweet pepper (Rasche et al. 2006), potato (Sessitsch et al. 2002), wheat (Conn and Franco 2004), poplar (Ulrich et al. 2008), and many other plants.

A further technology is the use of pyrosequencing to assess microbial communities. The concept of pyrosequencing is best described as sequencing by synthesis. The DNA sequence is obtained from the complementary strand as it is sequenced from the target strand. Pyrosequencing is an evolving field with new technologies bring continually released. The technology generates a plethora of data in a fraction of the time required for Sanger sequencing methodologies. Manter et al. (2010) assessed the bacterial endophytes of potato roots using 454 sequencing technology in which primers amplified regions of the 16S rRNA gene. These data were compared to those generated from bacterial ARISA (B-ARISA). Both analyses demonstrated that the bacterial communities differed between cultivars (Manter et al. 2010). The advantage of pyrosequencing is the ease at which species identification can be determined, since the 16S rRNA genes are directly sequenced. In B-ARISA, all individual bands would have to be removed from DGGE gels and then sequenced to determine the exact identification of the species within the microbial community, a process obviously not required by direct sequencing. Relative abundances can be estimated based on the number of amplicons from a specific species.

3.6 Conclusions

Despite years of research on bacterial endophytes as tools to manage plant diseases, new technologies and new crops still leave many areas open for research that will lead to more optimal disease control. While previous research focused on annual crops, utilizing bacterial endophytes for management of diseases in perennial crops is an ever-increasing area of research. Molecular tools have expanded our knowledge on the role of endophytes in managing diseases. Whether it is measuring something as small as expression of a host gene or something as large as determining the abundance and diversity of species in a bacterial community, knowledge of the interaction of bacterial endophytes with their host and other microorganisms in the endosphere will continue to expand our knowledge of the role of bacterial endophytes in nature. High-throughput sequencing can lead to discovery of new groups of microorganisms which may play an important role in biological control, yet cannot be cultured using current methodologies. Understanding gene expression in perennial crops can provide a better understanding of induced resistance in plants. Perennial crops are more complicated than model annual plants. Perennial crops grown in the field support large and diverse microbial communities, certainly more diverse and competitive than those found in plants grown in a laboratory situation. Molecular methodologies have allowed researchers to gain a better understand of the complex nature of the interaction between endophytic microbial communities and their perennial hosts.

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Chapter 4
Biological Control of Pathogens and Plant Growth Promotion Potential of Fluorescent Pseudomonads

R. Subashri, G. Raman, and Natarajan Sakthivel

4.1 Introduction

Modern agricultural practises have augmented the use of chemicals to enhance crop productivity. However, indiscriminate use of hazardous chemicals has resulted in soil pollution. Incorporation of harmful pesticides and insecticides as residues in our agricultural products has reached an alarming limit. Consequently, there has been a profound upward trend in the incidence of diseases associated with exposure to such toxic chemicals. Hence, recently the focus has shifted towards environment-friendly strategies to control devastating pathogens using inexpensive biocontrol microbes. These natural practises can preserve environment quality and conserve natural resources (Rigby and Caceres 2001; Lee and Song 2007). Fluorescent pseudomonads are promising microbial agents that offer dual benefits of enhancing the crop growth and productivity while suppressing plant pathogens. Among the diverse range of fluorescent pseudomonad bacteria, specific strains that belong to *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa* and *P. chlororapis* have the immense potential to be exploited for biological control because of their inherent capacity for the production of an array of metabolites (Thomashow et al. 1990; Sunish Kumar et al. 2005; Pathma et al. 2011) and enzymes (Salisbury 1994; Ayyadurai et al. 2006, 2007; Ravindra Naik et al. 2008) which mediate both plant growth-promotion (Sakthivel and Gnanamanickam 1987) and biological control of pathogens (Raaijmakers and Weller 1998; Rosales et al. 1995) in a wide variety of economically important agricultural crops.

Fluorescent pseudomonads possess many traits that make them well suited as biological control and growth-promoting agents. The beneficial attributes of fluorescent pseudomonads include (1) the ability to grow rapidly *in vitro* and *in vivo*,

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(2) the ability to utilise seed and root exudates, (3) the potential to colonise and multiply in the rhizosphere, (4) the capability to produce a wide spectrum of bioactive metabolites, (5) the competence with other microorganisms for rhizosphere niche and availability of nutrients and (6) the adaptability efficiently to environmental stresses. This article reviews the biological control and growth-promoting potential of specific beneficial group of fluorescent pseudomonad bacteria for the enhancement of crop production.

4.2 Biological Diversity of Pseudomonads

Pseudomonas is an enormously diverse genus of γ-*Proteobacteria* (Galli et al. 1992). This genus consists of ubiquitous saprophytic members of plant, animal and human pathogens. They are typically Gram-negative, chemoheterotrophic motile rods with polar flagella. Members of the genus *Pseudomonas* have very simple nutritional requirements and grow well under normal conditions in mixed populations with other types of microorganisms (Foster 1988). Den Dooren deJong (1926) first characterised *Pseudomonas* strains phenotypically on the basis of their nutritional features. The fundamental study on *Pseudomonas* resulted into an extensive phenotypic characterisation in which the genus was subdivided into species and species groups (Stanier et al. 1966). These characterisation studies were supported by numerical analysis (Sneath et al. 1981) and DNA–DNA hybridisation (Palleroni and Doudoroff 1972) and rRNA–DNA hybridisations (Palleroni et al. 1973) and pseudomonad bacteria were grouped into five groups based on the relatedness of their rRNA genes.

4.2.1 rRNA Groups of Pseudomonads

4.2.1.1 rRNA Group I

The largest rRNA group consists mostly of saprophytic bacteria (*P. fluorescens*, *P. putida*, *P. chlororaphis*) or pathogenic bacteria for humans (*P. aeruginosa*), plants (*P. cichorii*, *P. marginalis*, *P. syringae*, *P. savastanoi*) and mushrooms (*P. agarici*, *P. tolaasii*) and *P. stutzeri*, *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes*. Taxonomically the fluorescent pseudomonad bacteria such as *P. aeruginosa* and *P. fluorescens* are remarkably heterogeneous species (Doudoroff and Palleroni 1974; Palleroni 1992).

4.2.1.2 rRNA Group II

The second RNA group is called the *Pseudomallei-cepacia* group. It contains a group of pathogenic species, with an exception of *P. pichettii* (Ralston et al. 1973). The most remarkable species is *P. cepacia*, which is a plant pathogen and also a

significant human opportunistic pathogen (Ederer and Matsen 1972). This group also contains *P. marginata* (*P. glaioli*), *P. caryophylli*, *P. pseudomallei*, *P. mallei* and *P. solanacearum*.

4.2.1.3 rRNA Group III

The third rRNA group is represented by five species. Two of the species *P. acidovorans* (*Comamonas acidovorans*) and *P. testosteroni* (*Comamonas testosteroni*) have been shown to be so distantly related to other *Pseudomonas* sp. that a new genus, *Comamonas*, has been proposed (De Vos et al. 1985). The other three phytopathogenic species are *P. avenae*, *P. rubrilineans* and *P. konjaci*. These groups are phenotypically different from one another.

4.2.1.4 rRNA Group IV

Group IV comprises *P. diminuta* and *P. vesicularis*. These two strains stand as an out group and do not show affinity with any other *Pseudomonas* (Ballard et al. 1968).

4.2.1.5 rRNA Group V

The fifth rRNA homology group constitutes *P. maltophilia* (now S*tenotrophomonas maltophilia*) (Palleroni and Bradbury 1993) together with X*anthomonas* species. *P. maltophilia*, the saprophytic bacterium, can be found in many natural habitats and it is also frequently present in clinical specimens (Palleroni et al. 1973). A number of *Pseudomonas* species have not yet been assigned to RNA homology groups. The marine species, facultative autotrophs, the poly-β-hydroxyl butyrate utilising pseudomonads are among them.

In common with the other species of the genus *Pseudomonas*, the fluorescent pseudomonad bacteria are Gram-negative, strictly aerobic, polar flagellated rods. All fluorescent pseudomonad bacteria fall into one of the five rRNA groups (Palleroni et al. 1973) and the Guanine-plus-Cytosine (G + C) content of their DNA ranges from 58 to 68 mol% (Palleroni 1975).

4.3 Plant Growth-Promoting and Disease Management Mechanisms of Fluorescent Pseudomonads

There are various direct and indirect ways of plant growth-promotion and disease management by fluorescent Pseudomonads.

4.3.1 Phosphate Solubilisation

Phosphorus is an important macronutrient essential for plant growth and development. Soil contains a wide range of organic phosphorus substrates, but to make this form available for plant nutrition, it must be hydrolysed to inorganic phosphorus (Glass 1989). Also a large portion of soluble inorganic phosphate applied to soil as chemical fertiliser is rapidly immobilised soon after application and becomes unavailable to plants (Dey 1998). The principal mechanism for the mineralization of organic phosphorus is the production of organic acids and acid phosphatases. Most of the strains belonging to fluorescent pseudomonad species such as *P. chlororaphis*, *P. putida*, *P. aeruginosa P. monteilli*, *P. plecoglossicida*, *P. fluorescens P. fulva*, and *P. moselli* are among the most powerful phosphate solubilisers (Cattelan et al. 1999; Bano and Musarrat 2003; Sunish Kumar et al. 2005; Ravindra Naik et al. 2008; Jha et al. 2009). It has been reported that 41 % of fluorescent pseudomonad bacteria isolated from banana rhizosphere were found to be phosphate solubilising bacteria (Ravindra Naik et al. 2008).

4.3.2 Phytohormones

Phytohormones are involved in several stages of plant development like cell division, cell elongation, tissue differentiation and apical dominance. Fluorescent pseudomonads produce various phytohormones such as auxins, gibberellins, cytokinins and abscisic acid (Streit et al. 1996; Patten and Glick 2002). Auxin, indole-3-acetic acid (IAA), is an important phytohormone. Fluorescent pseudomonads are found to produce significant amount of IAA (Salisbury 1994; Sunish kumar et al. 2005) which stimulates the density and length of root hairs which improve plant uptake potential for water and other nutrients, thereby stimulating plant growth. In addition to IAA, several pseudomonad species also produce gibberellins and cytokinins. Cytokinins are believed to be the signals involved in mediating environmental stress from roots to shoots (Jackson 1993). Ethylene is a gaseous phytohormone commonly induced by wounding in plants (Salisbury 1994) which causes inhibition of root growth. Various strains of pseudomonads produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase (an enzyme which cleaves ACC), the immediate precursor to ethylene, thereby inhibiting ethylene production which in turn leads to an increase of root growth in plants. ACC deaminase gene of pseudomonads stimulates plant growth even in heavy metal (cadmium) contaminated soils (Belimov et al. 2001). List of phytohormones produced by fluorescent pseudomonad bacteria is given in Table 4.1.

Table 4.1 Phytohormones produced by fluorescent pseudomonads

Phytohormone	Producer strain	References
Auxin	*P. putida* GR12-2	Xie et al. (1996)
	P. monteilli	Ravindra Naik et al. (2008)
Cytokinins	*P. fluorescens*	Garcia de Salamone et al. (2001), Vessey (2003)
ACC deaminase	*P. fluorescens*	Wang et al. (2000)
	P. aeruginosa Pw60, 61	Ravindra Naik et al. (2008)
Gibberellins	*Pseudomonas* spp.	Gutierrez-Manero et al. (2001)

4.3.3 Iron Absorption

Iron is an essential nutrient of plants, but it is relatively insoluble in soil solutions. Plant roots can readily absorb ferrous (Fe^{2+}) ion, but ferric ion (Fe^{3+}) is the most prevalent form in soil (Salisbury and Ross 1992). Siderophores are small high affinity iron chelating compounds that facilitate the reduction of iron and hence favours easy absorption by plants. Under iron limiting conditions, several species of pseudomonads produce many fluorescent yellow siderophores such as pyoverdins (Budzikiewicz 1993, 1997), pyochelin (Cox et al. 1981), pseudomonine (Lewis et al. 2000; Mossialos et al. 2000; Mercado-Blanco et al. 2001), quinolobactin (Matthijs et al. 2007) and ornicorrugatin (Matthijs et al. 2008). The production of siderophores has been linked to the disease-suppressing ability of fluorescent pseudomonads (Loper 1988). Siderophore production by fluorescent pseudomonads is influenced by an array of factors, such as concentration of iron (Kloepper et al. 1980a, b); nature and concentration of carbon and nitrogen sources (Park et al. 1988); level of phosphates (Barbhaiya and Rao 1985); degree of aeration (Lenhoff 1963); presence of trace elements such as magnesium (Georgia and Poe 1931), zinc (Chakrabarty and Roy 1964) or molybdenum (Lenhoff et al. 1956) and temperature (Weisbeek et al. 1986). Different types of siderophores produced by fluorescent pseudomonad bacteria are presented in Table 4.2.

4.3.4 Nitrogen Fixation

Several species of pseudomonads are involved in the process of nitrogen fixation, thereby enhancing plant growth and productivity (Nicole et al. 2003). *Pseudomonas stutzeri* A1501 is found to be involved in denitrification under anaerobic conditions, nitrification under aerobic conditions and nitrogen fixation under microaerophilic conditions (Yan et al. 2010). Strains of *Pseudomonas* sp. (Raverkar and Konde 1988; Li and Alexander 1988) and *P. flluorescens* were reported for their ability to stimulate rhizobia–legume symbiosis in pea (Andrade et al. 1998), red clover (Marek-Kozaczuk and Skorupska 2001) and soybean (Li and Alexander 1988). Many reports indicate that various strains of pseudomonads possess *nif* genes, signifying their role in nitrogen fixation. Several species of pseudomonads are involved in the denitrification process (Gamble et al. 1977).

Table 4.2 Siderophores produced by fuorescent pseudomonads

Siderophore	Producer strain	References
Pyoverdins	*Pseudomonas* sp. B10	Budzikiewicz (1997), Kloepper et al. (1980a, 1980b)
	P. aeruginosa	Meyer (2000), Lamont and Martin (2003)
	P. fluoresens 3551	Loper et al. (2008)
	P. putida WCS358	Van Wees et al. (1997)
	P. fluorescens WCS374	Mohammad et al. (2009)
Pyochelin	*P. aeruginosa* PAO1	Cox et al. (1981), Buysens et al. (1996)
	P. fluorescens CHAO	Lewis et al. (2000)
	P. stutzeri KC	
	P. aeruginosa	Audenaert et al. (2002), Sun et al. (2006)
Pseudomonine	*P. stuzeri*, *P. putida*	Lewis et al. (2000), Mossialos et al. (2000)
	P. fluorescens	Mercado-Blanco et al. (2001)
	P. aeruginosa PUPa3	Sunish Kumar et al. (2005)
	P. aeruginosa FP10	Ayyadurai et al. (2006)
	P. fulva FP23	Ravindra Naik et al. (2008)
	*P. fluorescens*WCS374	Mohammad et al. (2009)
Yersiniabactin	*P. syringae*	Jones et al. (2007), Petermann et al. (2008)
	P. syringae DC300	Bultreys et al. (2001), Youard et al. (2007)
Quinolobactin	*P. fluorescens* 1740	Matthijs et al. (2007)
Achromobactin	*P. syringae* B728a	Franza et al. (2005), Berti and Thomas (2009)
Corrugatin	*P. fluorescens*	Matthijs et al. (2007)
Ornicorrugatin	*P. fluorescens* AF76	Matthijs et al. (2008)

4.3.5 Antimicrobial Compounds

Antibiotics are organic low molecular weight compounds produced by microorganisms, which even at low concentrations are deleterious to the growth and metabolism of other microorganisms. Antibiotic production of fluorescent pseudomonads is recognised as an important factor in the disease-suppressing ability of this group of bacteria (James and Gutterson 1986; Gutterson et al. 1988; Thomashow et al. 1990). Antibiotics produced by fluorescent pseudomonads include phenazines (Gurusiddaiah et al. 1986; Thomashow and Weller 1988; Pierson and Thomashow 1992; Chin-A-Woeng et al. 1998), phenolics (Keel et al. 1990, 1992; Vincent et al. 1991; Shanahan et al. 1992), pyrrole-type compounds (Homma and Suzui 1989; Pfender et al. 1993), polyketides (Nowak-Thompson et al. 1997; Kraus and Loper 1995) and peptides (Nielsen et al. 1999, 2000; Sorensen et al. 2001; de Bruijn et al. 2008; Loper et al. 2008). Different types of antibiotics produced by fluorescent pseudomonad bacteria are presented in Table 4.3.

4.3.6 Lytic Enzymes

Apart from the production of antibiotics and other secondary metabolites, pseudomonads are found to produce an array of lytic enzymes by which they exert their ability to suppress phytopahogenic fungi (Martin and Loper 1999;

Table 4.3 Antibiotics produced by fluorescent pseudomonads

Antibiotics	Producer strain	References
Phenazine-1-carboxylic acid	*P. fluorescens* 2-79	Gurusiddaiah et al. (1986)
	P. fluorescens 2-79RN10	Weller and Cook (1983)
	P. aureofaciens 30-84	Thomashow et al. (1990)
	P. chlororaphis	Pierson and Thomashow (1992)
	P. putida P15	Pathma et al. (2011)
Dimer of phenazine-1-carboxylic acid	*P. fluorescens* Pf23	Sakthivel and Sunish Kumar (2008)
Phenazine-1-carboxamide	*P. aeruginosa* PUPa3	Sunish Kumar et al. (2005)
2-Hydroxyphenazine	*P. chlororaphis* PCL1391	Chin-A-Woeng et al. (1998)
Pyocyanin	*P. aeruginosa* PAO1	Baron et al. (1997)
Phloroglucinols		
2,4-diacetylphloroglucinol	*P. fluorescens* Pf-5, Q2-87, CHAO, PFM2, Q8r1-96	Howell and Stipanovic (1979), Vincent et al. (1991)
	P. fluorescens F113	Shanahan et al. (1992), Keel et al. (1992), Levy et al. (1992), Flaishman et al. (1990), Raaijmakers and Weller (1998)
Pyrrolnitrin	*P. fluorescens* BL914, 915	Kirner et al. (1998), Ligon et al. (2000)
	P. aureofaciens A10338.7	Elander et al. (1968)
	P. cepacia 5.5B	Cartwright et al. (1995)
Isopyrrolnitrin	*Pseudomonads* spp.	Hashimoto and Hattori (1966a)
Oxypyrrolnitrin	*Pseudomonads* spp.	Hashimoto and Hattori (1966b)
Monodechloro-pyrrolnitrin	*P. pyrrolnitrica*	Hashimoto and Hattori (1968)
Polyketides		
Pyoluteorin	*P. fluorescens* Pf-5, CHAO	Howell and Stipanovic (1979), Keel et al. (1992)
Mupirocin	*P. fluorescens* NCIMB10586	El-Sayed et al. (2003)
2,3-Deepoxy-2,3-didehydro rhizoxin	*P. borealis* MA342	Tombolini et al. (1999)
Rhizoxin analogues	*P. fluorescens* Pf-5	Loper et al. (2008)
Peptides		
Viscosinamide	*P. fluorescens* DR54	Nielsen et al. (1998)
Tensin	*P. fluorescens* 96.578	Nielsen et al. (2000)
Amphisin	*Pseudomonas* sp. DSS73	Sorensen et al. (2001)
Masstolides A	*P. fluorescens* SS101	de Bruijn et al. (2008)

Neilsen and Sorensen 1999; Picard et al. 2000). Chitinase, cellulase and glucanase enzymes hydrolyse chitin, cellulose and β-1,3-glucan which are major cell wall components of various phytopathogenic fungi. *P. cepacia* producing glucanase is found to inhibit the proliferation of pathogenic fungi such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Friedlender et al. 1993). Pseudomonads are also found to produce various enzymes such as protease, pectinase and xylanase which also contribute to disease suppression. A reduction in the activity of these enzymes correlates with a reduction in virulence (Beraha et al. 1983). Role of chitinase and glucanase in biological control has been well documented (Shapira et al. 1989; Nielsen et al. 1998; Lim et al. 1991). Pseudomonads are also found to produce enzymes such as peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase which arrested the pathogen colonisation in crops such as sugarcane (Viswanathan et al. 2003).

4.3.7 Volatiles such as Hydrogen Cyanide

Hydrogen cyanide (HCN), a volatile compound produced by fluorescent pseudomonads, exerts biocontrol activity against plant pathogens (Sacherer et al. 1994; Bagnasco et al. 1998; Rodriguez and Fraga 1999; Siddiqui 2006). Production of HCN is found to be a common trait of *Pseudomonas* (88.89 %) (Ahmad et al. 2008). HCN inhibits the enzyme cytochrome oxidase and other metalloenzymes (Voisard et al. 1989) of the pathogens and hence assists the plants in the control of soil-borne diseases (Blumer and Haas 2000).

4.3.8 Induced Systemic Resistance

Induced systemic resistance (ISR) is a phenomenon wherein plant growth-promoting rhizobacteria activate an array of biochemical pathways to trigger the plant's defence mechanisms against a broad spectrum of phytopathogens (Van Loon et al. 1998). Signalling pathways involved are salicylic acid pathway and the pathway of ethylene and jasmonic acid (Pieterse et al. 2001). Seed treatment with *P. aeruginosa* resulted in the rapid accumulation of pathogenesis-related (PR) enzymes such as chitinase, β-1,3-glucanase, peroxidases and lyases. Bacterial determinants such as outer membrane lipopolysaccharides (LPS), flagella, iron-regulated metabolites, volatile compounds, antibiotics and cyclic lipopeptides are reported to activate ISR (Bakker et al. 2007; Iavicoli et al. 2003; Meziane et al. 2005; Ongena et al. 2008; Ryu et al. 2004; Tran et al. 2007). Fluorescent pseudomonad strains that show ISR against various plant pathogens are presented in Table 4.4.

Table 4.4 Induced systemic resistance by PGPR pseudomonads

Pseudomonad strain	Target pathogen	Host plant	References
Pseudomonas WCS417	*F. oxysporum*	Carnation	Van Peer et al. (1991)
Pseudomonas WCS374	*F. oxysporum*	Radish	Hoffland et al. (1996)
Pseudomonas WCS374	Turnip crinkle virus (TCV)	Arabidopsis	Mohammad et al. (2009)
P. putida 89B-27	*C. orbiculare*	Cucumber	Wie et al. (1991)
P. fluorescens strain S97	*P. syringae*	Bean	Alstrom (1991)
P. aeruginosa 7NSK2	*B. cinerea*	Grapevine	Verhagen et al. (2010)

4.4 Bacterisation Techniques

Bacterisation is the process of inoculating plant seeds, seed pieces or roots with inoculant bacteria to enhance plant growth and to suppress phytopathogens. Treatments with bacterial inoculants include drench application (Babalola et al. 2007b; Vleesschauwer and Hofte 2005), seed bacterisation (Babalola et al. 2007a; Kumar et al. 2009), seedling treatment (Babalola et al. 2007a), bioformulation, biopreparation, dual treatment (Lavania et al. 2006) and multiple delivery (Nakkeeran et al. 2005). The choice of the method depends on the crop, soil type and the nature of the bacterial inoculum. Many commercial preparations such as "Ecomonas" and "Florezen P" are available in the market which can be directly used. The efficacy of combined application method was comparable with fungicide treatments (Rabindran and Vidhyasekaran 1996). Beneficial microbial agents and biocontrol bacteria may be applied to the crops to be treated by one of the method.

4.4.1 Seed Bacterisation

In this method, bacterial suspension was prepared using the log phase cultures (10^{10} cells/ml) and the seeds were exposed in the above suspension for 30 min and then dried for 3 h before sowing (Babalola et al. 2007a; Kumar et al. 2009). Aqueous methyl cellulose is added to the bacterial suspension as an adhesive and preservative in commercial formulations. Seed treatment of maize with *Pseudomonas* spp. GRP3A, PRS9 and *P. chlororaphis* ATCC 9446 increased seed germination, shoot and root lengths and dry weight of seedlings (Sharma and Johri 2003). ISR, plant growth-promotion and sheath blight control were observed when rice seeds were treated with strains of *P. aeruginosa* (Saikia et al. 2006).

4.4.2 Direct Inoculation of Liquid Culture into Soil

In this method, the microorganism is multiplied in large quantity as liquid culture. These liquid cultures are either first mixed in the soil before sowing or applied in the furrows. In the case of rice, these are applied by sprinkling in the water-logged beds

(Wang et al. 2009). The in-furrow inoculants provide a larger amount of bacteria to the crop plant than seed inoculation. Mixing the potting soil with suspensions of *P. aeruginosa* mutants along with soil drench conferred resistance against blast and sheath blight diseases of rice by eliciting ISR (Vleesschauwer and Hofte 2005).

4.4.3 Seedling Treatment

Liquid culture containing the biocontrol bacteria in log phase is prepared. The seedlings and saplings are dipped in the above culture for about 30 min followed by immediate sowing (Babalola et al. 2007a; Kloepper et al. 1980a, b). This method is highly suited for rice, vegetable and fruit saplings (Niknam and Dawan 2003; Al-Taweil et al. 2009).

4.4.4 Foliar Application

This method is based on the fact that bacterial cells and exudates can be absorbed into plant via epidermal cells and stomatal pores from where they are transported to the growing zones of the plants, causing the desired effects. Application is more or less uniform when the bacterial cultures are spread on leaves as foliar spray. But the survival rates and the application efficiency are dependent on the microenvironment (Nakkeeran et al. 2005) and the time of application. Commercial formulation of *P. fluorescens* applied by foliar method conferred disease resistance and hence increased total grain yield (Vijay Krishna Kumar et al. 2009).

4.4.5 Carrier-Based Inoculation

In this method, bacterial cells mixed with carrier materials such as peat lignite, charcoal and farmyard manure (Rabindran and Vidhyasekaran 1996). Carrier material provides a conducive environment for the microorganisms to remain viable for a longer period. This method is mostly accepted and widely used because it supports transportation and retains cell viability. Microencapsulation is an advanced technology in which there is a controlled release of microbes from formulations (Fages 1992; Smith 1995; Rojan et al. 2011). In contrast, conventionally used solid and liquid formulations encompass several problems with respect to the low viability of microorganisms during storage and field applications.

4.5 Biocontrol of Plant Diseases and Yield Enhancement by Fluorescent Pseudomonads

Fluorescent pseudomonad bacteria are the most promising group of beneficial bacteria due to their multiple attributes for crop productivity. This specific group of bacteria could be used as prospective agents due to their ability to maintain soil health, promote plant growth and suppress phytopathogens (Table 4.5 and 4.6).

4.5.1 Wheat Diseases

Take-all disease of wheat is caused by the fungus *Gaeumannomyces graminis* var. *tritici*. Take-all is controlled by crop rotation, but it is also suppressed by continuous monoculture following an outbreak of the disease which is known as “Take-all decline” (TAD) (Andrade et al. 1995). Strains of *P. fluorescens* (2-79 and 13-79) from the USA were reported as biological control agents against *G. graminis* var. *tritici* (Capper and Higgins 1993). Take-all caused by the soil-borne fungal pathogen *G. graminis* var. *tritici* is one of the most destructive root diseases in wheat and other cereal grain crops. Take-all decline is strongly associated with the development of antagonistic microorganisms in the wheat rhizosphere. The most prominent antagonistic microorganisms are bacteria of the genus *Pseudomonas* which are able to suppress *G. graminis* var. *tritici* in both saprophytic and parasitic stages. Phenazine-1-carboxylic acid as a biocontrol determinant is produced by *P. fluorescens* 2-79 that controls take-all disease. The strain *P. fluorescens* 2-79, originally isolated from the rhizosphere of wheat, was found to suppress take-all disease (Thomashow and Weller 1988). *Fusarium culmorum* causes seedling blight, foot rot and head blight diseases of cereals, resulting in yield loss. *P. fluorescens* strains MKB 100 and MKB 249, *P. frederiksbergensis* strain MKB 202 and *Pseudomonas* sp. strain MKB 158 were effective in ameliorating the negative effects of *F. culmorum* on seedling germination of six wheat cultivars and on stem base infection of wheat cv. GK-Othalom. Chitosan has been shown to reduce Fusarium seedling blight disease of wheat caused by seed-borne *F. graminearum* (Reddy et al. 1999). It was found that even lower doses (1,000 versus 2,000–8,000 ppm) were effective in reducing *F. culmorum* seedling blight of wheat, both as a stem base treatment and as a soil amendment. Chitosan exhibited direct antifungal activity against *Candida albicans*, *F. oxysporum*, *Aspergillus fumigatus* and *Aspergillus parasiticus*. Chitosan induced systemic host resistance to *F. culmorum* against seedling blight. There are reports on chitosan-induced systemic resistance against different plant pathogens, including fungi, in a plant species-dependent manner. Soil amendment with chitosan or with culture filtrate of either *Pseudomonas* sp. strain MKB 158 or *P. fluorescens* strain MKB 249 was reported to reduce *Fusarium* seedling blight of wheat (Johansson et al. 2003; Khan et al. 2004).

4.5.2 Rice Diseases

Treatments with *P. fluorescens* 7-14 have been reported to control rice blast caused by *Magnaporthe grisea* (Chatterjee et al. 1996; Gnanamanickam and Mew 1992). Production of an antifungal antibiotic by *P. fluorescens* is the mechanism known to mediate the biological disease suppression. Biological suppression of blast disease of rice was afforded by bacteria applied either as seed treatment or as root-dip infiltration. Among these, Pf 7-14 applied as infiltration gave a maximum of 28.7 and 25.2 % blast disease control in the two separate field experiments. Root-dip applications also performed better than seed treatments. The blast lesions did not appear on the resistant rice cultivars (C101LAC and C101PKT). Rice stem and leaf but not root tissues which received Pf 7-14 and *P. putida* V14i showed increases in salicylic acid (SA) levels over the native SA levels found in the untreated controls. It has been proved that bacteria which are spatially separated from the pathogen are involved in the induction of ISR against the rice blast pathogen. SA levels which increase during bacteria-induced systemic resistance contribute to the suppression of rice blast by about 25 %. The results on disease suppression when taken together with limited bacterial migration would support the suggestion that the blast reductions are caused by the nonmigratory bacteria which remain on the roots or inside the rice stem. These data suggest that the benefits of bacterial treatments could only be realised through properly timed foliar spray applications of bacteria. Such applications will sustain adequate bacterial populations on the rice foliage and achieve maximum (70 $\pm$ 80 %) disease control (Chatterjee et al. 1996). SA-mediated ISR is caused by *P. fluorescens* 7-14 and *P. putida* V14i that are spatially separated from the rice blast pathogen *M. grisea* and plays a significant role in the biological control of rice blast disease (Krishnamurthy and Gnanamanickam 1997).

Rice sheath blight, caused by *Rhizoctonia solani* Kuhn (Sexual stage: *Thanetophorus cucumeris*), is one of the major production constraints in rice-growing countries and ranks next to blast in causing economical loss. Effective biological control of soil-borne diseases can be achieved by applying *P. fluorescens* that are insensitive to toxic metabolites produced by plant pathogens because of their ability to detoxify toxins. Several rhizobacteria are known to detoxify the toxins produced by fungal pathogens and they have been developed as biocontrol agents to control fungal diseases of crop plants. The rice sheath blight fungus, produces oxalic acid (OA). An OA-detoxifying strain of *P. fluorescens*, PfMDU2, was isolated from the rhizosphere of rice and its efficacy in controlling sheath blight of rice was demonstrated under greenhouse conditions. Strain of *P. fluorescens* PfMDU2 was isolated from the rhizosphere soil of rice amended with OA. This bacterium was tested for its ability to inhibit the mycelial growth of *R. solani in vitro* by the dual culture technique. PfMDU2 was highly effective in inhibiting the mycelial growth of *R. solani* and further, it was demonstrated that seed treatment followed by soil application with talc-based powder formulation of *P. fluorescens* PfMDU2 significantly reduced the severity of sheath blight by 75 % compared to untreated control plants. The mode of actions of *P. fluorescens* that inhibit various soil-borne plant

pathogenic fungi include biosynthesis of antibiotics, production of HCN, production of hydrolytic enzymes, production of siderophores and competition for substrates. Successful bacterial antagonists often show a combination of synergistic mechanisms (Nagrajkumar et al. 2005).

P. fluorescens strains PF1 and FP7 were reported as antagonistic bacteria for sheath blight of rice (Nandkumar et al. 2000). Suspension culture or a talc-based formulation of biocontrol bacteria was used to control disease severity and promote plant growth under glasshouse or field conditions. Upon challenge inoculation of the pathogen, the treated plants had smaller lesions compared to the untreated control plants. Furthermore, the plants grew faster and greener with longer roots and shoots than the untreated plants. *Pseudomonas*-treatment played a dual role by reducing disease severity and promoting the growth of the plant, resulting in increased biomass and yield. It has been established that fluorescent pseudomonad bacteria enhance plant growth in several ways by producing plant growth regulators, such as gibberellins, cytokinins and indole acetic acid, which can either directly or indirectly modulate the plant growth and development. These bacteria were also reported to produce chitinase in the culture medium which gets further accelerated when the medium was supplemented with chitin (Velazhahan et al. 1999; Viswanathan and Samiyappan 2000). The increased chitinase activity in chitin medium implies that *Pseudomonas* strains are able to degrade the complex chitin polymer, which is the major component of fungal cell walls. More chitinolytic activity in culture medium inoculated with FP7 suggested that the strain FP7 performed well with the addition of chitin as a substrate. Since the fungal cell wall contains chitin, the FP7 bacteria in the plant rhizosphere might have produced more chitinase, and the enhanced chitinase activity might be one of the reasons for the increased disease reduction indicated that induced systemic resistance in rice may be due to the elicitation of defence mechanisms involving peroxidase and chitinases. Fluorescent pseudomonads are also known to produce salicylic acid which acts as local and systemic signal molecules in inducing resistance in plants. PF1 strain had typical PGPR activity and induced both peroxidase and chitinase enzymes, while FP7-mediated ISR appears to be associated with the involvement of induced plant chitinase as well as its own chitinase to suppress the pathogen. Hence, the addition of chitin to the talc-based formulation may enhance the effect of ISR. Antibiotic production by *Pseudomonas* strains also revealed that FP7 and PF1 have the capacity to produce 2,4-diacetyl phloroglucinol (DAPG) and phenazine (Nandkumar et al. 2000).

4.5.3 Cotton Diseases

Damping-off is a disease of cotton incited by *P. ultimum*. Many rhizobacteria which are found in cotton rhizosphere exhibit potent inhibition against this pathogenic fungus by different mechanisms. These beneficial bacteria are mainly *P. fluorescens* type. Lopper (1988) reported a pseudomonad fluorescent strain 3551 which showed

antagonism against *Pythium* sp. It has been proved that strain 3551 inhibits the growth of *P. ultimum* due to its potential to produce siderophore. Mutant strain of this bacterium did not show the suppression of *Pythium*. Several studies showed the production of siderophores and secondary metabolites by fluorescent pseudomonad bacterium. Therefore, antagonistic effect of this strain also may be contributed by secondary metabolites. This biocontrol strain also induces the host resistance against this pathogen. Numerous studies have demonstrated the role of 2,4-DAPG-producing *Pseudomonas* spp. in the suppression of a wide variety of plant pathogens, including fungi, bacteria and nematodes. The sensitivity of various infectious propagules of *P. ultimum* var. *sporangiiferum* to 2,4-DAPG produced by *P. fluorescens* strain CHAO was studied in detail. The effects of pH and level of acetylation on activity of phloroglucinols against mycelial growth of *P. ultimum* were also assessed. It is shown that lower pH has a significant effect on the activity of 2,4-DAPG against mycelial growth of *P. ultimum*. Changes in pH in the rhizosphere of plants, growing in agricultural soils, by as much as 2 pH units may occur. In general, the pH changes induced by roots lead to acidification, with more pronounced effects observed for dicot plants. Also microorganisms, including pathogenic fungi, can alter the pH to make nutrients or trace elements more readily available. As a result of these changes in pH, the activity of antimicrobial compounds produced by competing microorganisms may also change. It has been observed that *P. ultimum* acidifies unbuffered, liquid medium (potato dextrose broth) in a 7-day period from pH 6.5 to 4.5. This reduction in pH and coordinate increase in toxicity of 2,4-DAPG may explain some of the discrepancies in inhibitory concentrations of 2,4-DAPG reported in the different experiments. Based on TEM observations, it appears that 2,4-DAPG does not affect the cell wall structure and composition of hyphal tips of *P. ultimum* (de Souza and Raaijmakers 2003).

4.5.4 Tomato Diseases

The root-knot nematode *Meloidogyne javanica* is one of the most economically important pest causing severe damages to a wide variety of crops, particularly to tomato. Certain root-associated strains of fluorescent pseudomonad bacteria produce and excrete metabolites that are inhibitory to soil-borne plant pathogens. Siddiqui and Shahid Shaukat (2003) showed that 2,4-DAPG-producing *P. fluorescens* CHAO could affect egg hatch and induce mortality in juveniles of *M. javanica*. Since natural soil with a large number of soil microorganisms including deleterious soil-borne plant pathogens was used in this study, it is not sure whether observed suppression of the root-knot nematode was solely due to the application of DAPG-producing *P. fluorescens* in such soil. It is clearly demonstrated that tomato plants treated with *P. fluorescens* strain CHAO reduced nematode penetration rates in roots. Results suggest that *P. fluorescens strain* CHAO reduces nematode infection by inducing systemic resistance in tomato plants against *M. javanica* because the bacteria and nematode were spatially

separated. Leeman et al. (1996) demonstrated that antibiotics and siderophores may function as stress factors or signals inducing local and systemic host resistance. These results suggest that CHAO releases 2,4-DAPG during early growth stages which elicit systemic resistance in tomato against nematode.

P. syringae pv. tomato causes bacterial speck disease of tomato and has been demonstrated to be virulent on *Arabidopsis* as well (Dong et al. 1991; Whalen et al. 1991). *P. fluorescens* strain WCS417 shows antagonistic activity against *P. syringae* by inducing systemic response in tomato. To demonstrate ISR activity of this antagonistic bacteria, *Arabidopsis*-based model system using *P. syringae* pv tomato as challenging pathogen and for induction, a rifampicin-resistant mutant of the PGPR strain WCS417 of *P. fluorescens* (*P. fluorescens* WCS417r; Van Peer et al. 1991) was used.

It was documented that *P. fluorescens* WCS417r effectively protects *Arabidopsis* against infection by *P. syringae* pv. tomato. Root colonisation by *P. fluorescens* WCS417r resulted in a marked delay in symptom development and reduction in disease severity. Challenge inoculation with *P. fluorescens* WCS417r reduced both the visible symptoms caused by *P. syringae* infection and the growth of this pathogen in the leaves. Because inducing bacteria and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that *P. fluorescens* WCS417r-induced protection is plant mediated. Among the bacterial determinants implicated in eliciting metabolic events in plants is the outer membrane LPS (Graham et al. 1977; Mazzucchi et al. 1979; Dazzo et al. 1991; Newman et al. 1995). Earlier, it was demonstrated that the LPS of *P. fluorescens* WCS417r is involved in eliciting systemically enhanced resistance in carnation (Van Peer and Schippers 1992) and radish (Leeman et al. 1995), indicating that PGPR-mediated protection is accomplished by induction of ISR in the plant. In *Arabidopsis*, this resistance response is effective against bacterial leaf pathogen.

The plant growth-promoting rhizobacterium *P. aeruginosa* 7NSK2 produces three siderophores when iron is limited: the yellow-green fluorescent pyoverdin, the salicylate derivative pyochelin and salicylic acid. This *Pseudomonas* strain was shown to be an efficient antagonist against *P. splendens*, the causative agent of tomato damping-off. The role of pyoverdin and pyochelin in the suppression of *P. splendens* was demonstrated by using various siderophore-deficient mutants derived from *P. aeruginosa* 7NSK2. Mutant KMPCH inhibited *P. splendens* but was less active than the parental strain. This residual protection could be due to the production of salicylic acid. Salicylic acid is known to induce systemic acquired resistance in plants. A rise in the level of salicylic acid increases systemic acquired resistance. Salicylic acid produced by rhizobacteria might be taken up by plants, thereby inducing resistance systemically. Superoxide, hydrogen peroxide and hydroxyl free radicals represent reactive oxygen species (ROS) that are thought to be involved in induction of disease resistance in plants. It is attractive to hypothesise that active oxygen species generated by the pyochelin–pyocyanin interaction induce resistance in tomato plants, which results in an enhanced protection against *Pythium*-induced damping-off. It remains to be shown whether or not this phenomenon can occur on plant roots. Only pyochelin is reported to be

involved in free-radical formation. In fact, most iron chelators, including pyoverdin, appeared to have free-radical scavenging properties. The observed antagonism of *P. aeruginosa* 7NSK2 towards *P. splendens* could be explained by pyoverdin-mediated iron competition and induction of resistance by pyochelin (Saskia et al. 1995).

4.5.5 Sugarcane Diseases

Red rot of sugarcane caused by the fungus *Colletotrichum falcatum* Went (Perfect state: *Glomerella tucumanensis*) is one of the oldest recorded diseases and has caused significant losses both to the cane growers and to sugar factories in India and other countries. Various fungicides have been used to control the disease, but limited success was achieved under field conditions (Singh and Singh 1989). Hence, plant protection chemicals are not useful for managing the red rot disease. In this context, management of red rot disease through biocontrol agents is increasingly capturing the attention of scientists as an alternative, environment-friendly strategy for the disease management.

The PGPR strains SS1, SS2 and SS3 that belong to *P. fluorescens* native to sugarcane rhizospheric soil have been isolated and their efficacy against the pathogen was demonstrated under laboratory, greenhouse and field conditions. Application of fluorescent pseudomonads to rhizosphere region had induced several defence-related enzymes such as chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase in sugarcane stalks which arrested the colonisation and spread of pathogen in the stalk (Viswanathan et al. 2003). The red-rot pathogen *C. falcatum* Went is known to produce a phytotoxic metabolite, anthroquinone. It has been established that this toxic metabolite is host specific and produces part of the disease symptoms. Recently, specific strains of *Pseudomonas* spp. effective against the pathogen have been identified. Samples treated with *P. fluorescens* strains FP 7 and VPT 4 along with phytotoxic metabolites did not produce any symptom on the leaves. It suggests that the reduction of symptoms may be possibly due to inactivation of the toxic metabolite by bacterial inoculants. The PGPR formulation was applied three times, seed treatment while planting and soil application twice in the field. Talc formulation of PGPR strains significantly reduced red rot disease incidence when the treated canes were challenge inoculated with pathogen. When PGPR strains were evaluated for their efficacy against the disease in endemic locations, strains of *P. fluorescens* such as EP1, Pfl and CHAO and *P. putida* KKM1 strongly suppressed the red rot disease development in field experiments. In addition to their efficacy against red rot disease in sugarcane, the strains significantly improved seed germination, number of millable canes (NMC) and cane yield (Malathi et al. 2002).

Strains of *Pseudomonas* spp. have also been found to induce systemic resistance against *C. falcatum* (Viswanathan and Samiyappan 1999a). Suppression of C. *falcatum* by the bacterial strains may be due to the production of antifungal secondary metabolites or by the bacterial strains-induced chitinases in sugarcane (Viswanathan and Samiyappan 1999b).

4.5.6 Banana Diseases

Bunchy top caused by Banana bunchy top virus (BBTV) is one of the most destructive diseases of banana. BBTV infects almost all banana cultivars, retarding the growth of infected plants and causing substantial economic losses. Two strains of the PGPR *P. flourescens* (Pf1 and CHAO) formulated with the carrier chitin for the ability to promote the growth of banana plants were investigated for their efficacy in controlling BBTV. Banana plants treated at planting and at the third, fifth and seventh month after planting had significantly reduced bunchy top incidence under field conditions, compared with the control treatment. The reduction in disease incidence was more pronounced with the chitin-amended CHAO strain. The chitin-amended CHAO strain also increased the leaf nutrient status and enhanced growth, bunch yield and the quality of the fruits compared to untreated plants. *P. fluorescens* strains CHAO and Pf1 and in combination with chitin were investigated for their biocontrol efficacy against BBTV. Bioformulation of *P. fluorescens* CHAO with chitin was effective in reducing the BBTV incidence under glasshouse and field conditions. *P. fluorescens* strains might stimulate the production of biochemical compounds associated with the host defence. The presence of phenolic compounds in plants or their synthesis in response to infection has often been associated with resistance (Ingham 1972). It is well known that resistant plants contain more phenols or produce polyphenols more rapidly than susceptible ones. Multifold increase in phenol content was observed in *P. fluorescens*-treated plants along with pathogen inoculation compared with the infected control plants. *P. fluorescens* strains are capable of inducing high levels of defence enzymes in banana, and it could be speculated that induced enzyme activities by *P. fluorescens* may be associated with the biosynthesis of phenolic compounds that have been considered as major determinants in inducing systemic resistance against BBTV disease, besides increasing the bunch yield. Banana plants treated with *P. fluorescens* CHAO alone or in combination with chitin showed an increase in PR-2 (β-1,3-glucanase) and PR-3 (chitinase) proteins. Thus, the induction of PR-proteins corresponding to a reduction in BBTV infection in banana supports the hypothesis that the resistance induced by fluorescent pseudomonad strains are systemic (Kavino et al. 2007; Mathiyazhagan et al. 2008).

4.5.7 Sugar Beet Diseases

The important root pathogenic fungi of sugar beet are the oomycetes, *Aphanomyces cochleoides* and *P. ultimum* and the basidiomycete *R. solani*. Antagonistic fluorescent pseudomonad bacteria producing antifungal substances have shown a potential for biological control of the pathogen *P. ultimum* in sugar beet. *P. fluorescens* DR54 was isolated as an antifungal agent towards plant pathogens causing damping-off in sugar beet. The antifungal activity of *P. fluorescens* DR54 towards both *P. ultimum*

and *R. solani* was primarily determined by production of the cyclic lipopeptide, viscosinamide. This cyclic lipopeptide was also produced in the spermosphere and rhizosphere of sugar beet when the seedlings were treated with *P. fluorescens* DR54 and grown in soil microcosms. Pot and microcosm experiments supported the field observations, demonstrating a clearly improved emergence of healthy sugar beet seedlings in the presence of the *P. fluorescens* DR54 inoculant, which established in the rhizosphere surrounding the seedling roots. The observation that low disease level in presence of *P. fluorescens* DR54 concurred with reduced mycelial biomass and sclerotia formation by *R. solani*. *R. solani* growth may be exerted by antagonistic mechanisms such as production of antibiotic (viscosinamide) and hydrolytic, cell wall-degrading enzyme (chitinase). Direct surface attachment and microcolony growth of *P. fluorescens* DR54 cells on the surface of *R. solani* hyphae were observed using Gfp-labelling of bacteria (Thrane et al. 1999, 2000; Nielsen et al. 2002).

4.5.8 Tobacco Diseases

Strain CHAO of *P. fluorescens* suppressed black root rot of tobacco, caused by *Thielaviopsis basicola*, under gnotobiotic conditions in an artificial soil containing vermiculite as clay mineral. Many facts indicate that competition for iron is not the mechanism of suppression of tobacco black root rot, caused by *T. basicola*, in the gnotobiotic system. The siderophore-negative mutant CHA400 suppressed disease as effectively as the wild-type strain CHAO in the soils containing vermiculite, and strain CHAO suppressed disease more effectively in the iron-rich soil (vermiculite) than in the iron-poor soil (illite); the addition of $FeCl_3$ to vermiculite did not reduce the capacity of the bacteria to suppress disease; the addition of $FeCl_3$ to illite increased it; iron-free siderophores did not inhibit the growth of *T. basicola* in vitro. The endoconidia of *T. basicola* may contain enough endogenous iron to initiate germination and infection. The addition of $FeCl_3$ to illite increased the capacity of strain CHAO and its siderophore-negative mutant CHA400 to suppress disease. This indicates that the bacteria need sufficient iron to suppress disease (Keel et al. 1989). Maurhofer et al. (1994) indicated that induced protection of tobacco against tobacco necrosis virus by *P. fluorescens* CHAO was associated with the production of pyoverdine. Thus, systemic resistance by bacteria appears to involve multiple mechanisms.

4.6 Concluding Remarks

Overuse of chemicals is reported to affect plant nutrition and subsequently reduce the total yield. Phytopathogens are becoming increasingly tolerant to chemicals and emerged as a major threat for crop productivity. Modern agricultural practises helped to increase the food production, but still 10–16 % of the harvest is lost

Table 4.5 Biological control of phytopathogens by fluorescent pseudomonads

Biocontrol strain	Crop	Disease	Pathogen	References
P. fluorescens BL915	Cotton	Seedling damping-off	*Rhizoctonia solani*	Ligon et al. (2000)
P. cepacia 5.5B	Cotton	Damping-off	*R. solani*	Cartwright et al. (1995)
P. fluorescens Pf-5	Tobacco	Black root rot	*Pythium ultimum*	Howell and Stipanovic (1979)
	Cotton	Damping-off	*R. solani*	Howell and Stipanovic (1979)
P. fluorescens CHAO	Tobacco	Black root rot	*P. ultimum*	Keel et al. (1992)
	Tomato	Damping-off	*P. splendens*	Buysens et al. (1994)
	Wheat	Take-all	*Gaeumannomyces graminis gramini* var. *tritici*	Keel et al. (1992)
P. fluorescens 3551	Cotton	Damping-off	*P. ultimum*	Lopper (1988)
Pseudomonas spp.	Cucumber	Damping-off	*P. aphanidermatum*	Elad and Chet (1987)
P. putida NIR	Soyabean	Damping-off	*P. ultimum*	Paulitz (1991)
P. aeruginosa 7NSK2	Tomato	Damping-off	*P. splendens*	Buysens et al. (1994)
P. fluorescens Hv37a	Barley	Damping-off	*P. ultimum*	Gutterson et al. (1986)
P. fluorescens DR54	Sugar Beet	Damping-off	*R. solani*	Nielsen et al. (1999)
P. fluorescens 2-79,13-79	Wheat	Take-all	*G. gramnis var. tritici*	Thomashow and Weller (1988)
P. fluorescens PfMDU	Rice	Sheath blight	*R. solani*	Nagrajkumar et al. (2005)
P. putida KKM1	Sugarcane	Red rot	*Colletotrichum. falcatum*	Malathi et al. (2002)
P. fluorescens PGS12	Corn	Damping off	*F. oxysporum*	Georgakopoulos et al. (1994)
P. chlororaphis 30-84	Wheat	Take-all	*G. gramini* var. *tritici*	Pierson and Thomashow (1992)
P. putida	Cucumber	Fusarium wilt	*Fusarium oxysporum*	Park et al. (1988)
Pseudomonas spp.	Cucumber	Fusarium wilt	*F. oxysporum* f. sp. *cumuerinum*	Sneh et al. (1984)
P. aeruginosa PNA1	Chickpea	Damping-off	*F. oxysporum*	Anjaiah et al. (1998, 2003)
P. chlororaphis PCL1391	Tomato	Root rot	*F. oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng et al. (1998)
P. fluorescens	Rice	Sheath rot	*Sarocladium oryzae*	Sakthivel and Gnanamanickam (1987)
P. aeruginosa PUPa3	Rice	Sheath rot	*S. oryzae*	Sunish kumar et al. (2005)
		Sheath blight	*R. solani*	
P. aeruginosa	Wheat	Foliar disease	*Septoria tritici*	Baron et al. (1997), Flaishman et al. (1990)
P. fluorescens Q8r1-96	Wheat	Take-all	*G. gramini* var. *tritici*	Raaijmakers and Weller (1998)
F113	Sugar beet	Damping-off	*P. ultimum*	Shanahan et al. (1992)
PFM2	Wheat	Foliar disease	*S. tritici*	Levy et al. (1992)
P. putida	Radish	Fusarium wilt	*F. oxysporum*	Scher and Baker (1982)

Table 4.6 Enhancement of crop growth and productivity by fluorescent pseudomonad bacteria

Bacterial strain	Crop	Enhancement of plant growth and yield	References
Pseudomonas sp.	Potato	Increase in root dry weight (44–201 %)	Frommel et al. (1991)
		Stem length increase (26–28 %)	
		Increase in lignin up to 43 %	
		Enhancement of stem hair formation (55–110 %)	
Pseudomonas sp.	Wheat	Increase in yield up to 27 % and control of Take-all	De Freitas and Germida (1990)
P. cepacia	Wheat	Stimulation of plant growth	De Freitas and Germida (1990)
P. fluorescens		Biocontrol against pathogen, *Rhizoctonia solani*	
P. putida		Suppression of the growth of pathogen, *Leptosphaera maculans*	
P. cepacia MR85, R85 *P. putida* MR111, R105	Wheat	Increase in grain yield	De Freitas and Germida (1992b)
P. cepacia R55, R85	Wheat	Increase in dry weight (62–78 %), root weight (92–128 %)	De Freitas and Germida (1991)
P. putida R104		Increase in dry shoot weight (28–48 %)	
		Antagonism against pathogen, *Rhizoctonia solani*	
P. cepacia R85 *P. fluorescens* R104, 105 *P. putida* R111	Wheat	Increase in grain yield (46–75 %)	De Freitas and Germida (1992a)
P. chlororaphis 2E3, O6	Wheat	Increase in growth (8–6 %)	Kropp et al. (1996)
		Inhibition against pathogen, *Fusarium culmorum*	
P. corrugate	*Amaranthus paniculatus*	Increase in plant growth and nitrogen content	Pandey et al. (1999)
P. fluorescens *P. putida* TL3, BK1	Potato	Increase in tuber yield (14–33 %)	Burr et al. (1978)
P. fluorescens	Wheat	Increase in seedling height and number of heads and yield in *Pythium*-contaminated sites	Weller and Cook (1986)
*P. fluorescens*63-28 R17-FP2, QP5, R15-A4	Tomato	Increase in yield up to 18.2 %	Gagne et al. (1993)

P. corrugate 13 *P. fluorescens* 63-28	Cucumber	Increase in yield	McCullagh et al. (1996)
P. fluorescens 63-49	Cucumber	Increase in fruit number (18 %)	McCullagh et al. (1996)
P. fluorescens Pf5	Blueberry	Leaf area and stem diameter increase	de Silva et al. (2000)
P. putida *P. putida biovar* B	Canola	Increase in yield up to 57 %	Kloepper et al. (1988)
P. fluorescens	Canola	Increase in yield (6–13 %)	Kloepper et al. (1988)
P. putida	Cucumber	Increase in root length	Uthede et al. (1999)
P. putida GR12-2	Canola	Increase in root elongation Greater phosphate uptake and growth	Lifshitz et al. (1987)
P. putida GR12-2	Canola Lettuce Tomato Barley Wheat Oat	Increase in root elongation Reduction in ethylene synthesis (ACC deaminase)	Hall et al. (1996)
P. putida W4P63	Potato	Increase in yield (10.2–11.7 %) Suppression of potato soft rot	Xu and Gross (1986)
Pseudomonas sp.	Canola	Increase in root dry weight (11–52 %)	Bertrand et al. (2001)
Pseudomonas sp. PsJN	Potato	Increase in plant dry weight	Frommel et al. (1993)
Fluorescent pseudomonad strains A1, B10, TL3,BK1, E6	Potato	Significant yield increase	Kloepper et al. (1980a, 1980b)
Fluorescent pseudomonad strains A1, B2, B4, E6.RV3, SH5	Sugar beet	Increase in seedling mass	Suslow and Schroth (1982)
Pseudomonas sp.	Potato	Increase in yield (10–37 %)	Howie and Echandi (1983)
Pseudomonas sp.	Potato	Increase in yield (9–20 %)	Geels et al. (1986)
Pseudomonas sp.	Potato	Increase in yield (14–33 %)	Kloepper et al. (1989)

(continued)

Table 4.6 (continued)

Bacterial strain	Crop	Enhancement of plant growth and yield	References
P. fluorescens	Rice	Increase in plant height, tiller number and grain yield (3–160 %)	Sakthivel and Gnanamanickam (1987)
Pseudomonas sp.	Canola	Increase in growth	Kloepper et al. (1988)
Pseudomonas sp.	Lettuce Canola Cucumber Tomato	Increase of root and shoot weights	Van Peer and Schippers (1988)
Pseudomonas sp. 7NSK2	Maize Barley Wheat	Increase in yield (15–25 %)	Iswandi et al. (1987)
Pseudomonas W34	Lettuce Tomato	Increase in seedling biomass in soils infested with *Meloidogyne incognita*	Hoffmann-Hergarten et al. (1998)
Pseudomonas sp.	Maize	Increase in yield (8–14 %)	Lalande et al. (1989)
P. syringae pv. *phaseolicola*	Bean	Increase in protein	Alstrom (1995)

due to plant diseases. As the communities of bacteria on the plants are complex, in-depth understanding of the dynamics of the plant–bacterial interaction is required to exploit them for biological control. Strategies that would lead to the development of more consistent and reliable methodology for the selection and application of fluorescent pseudomonad bacteria to inhibit crop pathogens and subsequent enhancement of crop productivity may be envisaged.

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Chapter 5
Disease Management in Staple Crops: A Bacteriological Approach

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5.1 Introduction

Plant diseases cause losses and damages to crop yield and quality and also reduce resource-use efficiency. Diseases account for at least 10% of crop losses globally and are, in part, responsible for the suffering of 800 million people who do not get adequate food to eat (Strange and Scott 2005). Among the most notable historical diseases were the potato late blight epidemics in Western Europe and rice blast in China and India (Savary et al. 2006). Similarly, direct economic loss such as the estimated one billion dollars loss in one year occurred to American corn growers from southern corn blight (C/O *Cochliobolus maydis*). Many plant diseases even though cause less loss annually but collectively represent sizable loss to farmers and can reduce esthetic values of landscape plants and harm gardens. Diseases are the most difficult type of plant injury to diagnose and manage. A plant disease is a condition that does not allow the plant to function normally. Plant diseases are the result of complex interaction of host, pathogen, and environment, which form a disease triangle (Fig. 5.1). They normally are of two types: noninfectious and infectious. Noninfectious diseases caused by nonliving agents do not spread from plant to plant, whereas infectious diseases caused by living agents are reproducible and spread from plant to plant. The main casual agents for infectious diseases of plants are fungi, bacteria, and virus. In developing countries, agriculture is the driving force for economic growth where food demand is critical because of the slow rate of net production in relation to population growth (Dubey et al. 2010). However, countries of tropical and subtropical regions suffer more from diseases due to congenial climatic conditions and environment (Roy 2003).

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Staple food constitutes a dominant portion of a diet and supplies a high proportion of energy and nutrient, which varies from place to place. There are more than 5,000 edible plant species in the world. Among them, only 15 plant species provide 90% of the world's food energy intake. However, rice, maize, and wheat comprise two-third of the human food consumption. These three are alone staples of over four billion people against the seven billion population of the world. India is one of the largest producers of rice, accounting for 20% of all world rice production. Rice is India's prominent crop and is the staple food of the eastern and southern parts of the country. The demand for rice production is projected to 128 million tons for the year 2012 and will require a production level of 3,000 kg/ha, significantly greater than the present average yield of 1,930 kg/ha. Rice continues to hold the key to sustained food security in the country, so even if rice production area stabilizes or registers negative growth, future rice production must be achieved exclusively through yield improvement (Tiwari 2002) and disease management.

Maize ranks as third most important cereal in India. Its cultivation area has slowly expanded over the past few years from 6.6 to 8.6 mha (2002–2011). The productivity has also increased from 1.6 to 2.6 t/ha in the same period. Maize demand will continue to increase in view of increasing demand in poultry and livestock sectors (Paroda and Kumar 2000). Maize is a promising option for diversifying agriculture due to its resilience to changing climate in upland areas of India. The growing demands of the staple food due to increase in the population and the yield loss due to various diseases should be given utmost importance. Although the applications of many strategies for the protection of crops from diseases were taken up, approximately one third of the global food production is estimated to be destroyed by pests and pathogens (Arthur and Thorne 2003). The hurdles faced by the crop protection scientists in the twenty-first century are the evolution of resistant strains of pests and pathogens and lack of appropriate crop protection technologies in lower input farming systems (Beddington 2010; Godfray et al. 2010). The challenges for staple food production are now intensifying. Even in industrialized crops, there is an increasing pressure to optimize inputs, reduce environmental impact, but at the same time minimize the risk of widespread crop failure. Emerging infectious diseases (EIDs) caused by plant pathogens can develop into unpredicted and very serious epidemics, owing to the influence of various characteristics of the pathogen, host, and environment. Devastating epidemics, having social implications by increasing the rate of urbanization, occurred in the past in Europe, and many other EIDs still occur with high frequency in developing countries.

The disease-causing agents need to be controlled quantitatively and qualitatively by improved crop protection strategies to prevent such damage to ensure a substantial contribution to the food security and economy of the people. Different strategies were taken until to date to manage and control plant diseases (Bargabus et al. 2004; Benhamou 2004; Chisholm et al. 2006; Heydari et al. 2007; Kloepper et al. 2004; Islam et al. 2005). The use of chemical fertilizers and pesticides in agriculture had contributed significantly to the spectacular improvements in crop productivity over the past decades. However, the widespread use of chemicals in agriculture causes

degradation of soil, undesirable effects on nontarget organisms, possible carcinogenicity of some chemicals, environmental pollution, development of resistant races of pathogens, etc. The effects of chemicals used in disease management had motivated people to think for an alternative approach in the twenty-first century to prevent, mitigate, or control plant diseases (Jamalizadeh et al. 2008; Pimenta et al. 2010). There are also strict regulations and political pressure to remove the hazardous chemicals from the market. Researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases (Pal and Gardener 2006). The decreasing efficacy of the fungicides and the risks associated with their fungicide residues on leaves and fruits have highlighted the need for a more effective and safer alternative control measures.

Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world and consumer demand for pesticide-free food, has led to search for substitutes for these agricultural inputs. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent (Compant et al. 2005). Therefore, there is a need for a new solution to plant disease problems that provide effective control while minimizing negative consequences for human health and the environment (Sahaf et al. 2007). More effective, efficient, and durable crop protection measures are, therefore, a priority. Successful disease management programs taken into account the environment, the pathogen, and the host.

Biological control is likely to be more effective than chemical-based disease control. The rich microbial diversity provides a seemingly endless resource for this purpose. Plant growth-promoting rhizobacteria are most widely studied group of plant growth-promoting bacteria (PGPB), are associated to almost all plant species, and are commonly present in many environments (Kloepper and Schroth 1978), which can also establish endophytic populations (Gray and Smith 2005). Antagonistic bacteria are considered ideal biological control agents because of the rapid growth, easy handling, and aggressive colonization of the rhizosphere (Weller 1988; Velusamy et al. 2006). The complexity of the interactions, the involvement of the numerous mechanisms of disease suppression by a single microorganism, and the adaptedness of most biocontrol agents to the alien environment in which they are used all contribute to the belief that biocontrol will be more durable than synthetic chemicals. Two decades ago, Schroth and Hancock (1982) stated that the biological control of plant diseases is an attractive, challenging, but illusive and frustrating area of study for plant scientists and microbiologists. Biological control agents occur naturally and are the principal reason why the diseases are not catastrophic naturally. In many instances, sufficient knowledge is not available to explain how biological control operates naturally or how various biotic and abiotic factors manipulated. Bacteria are excellent source of antagonists due to their multiple mechanism of disease control. Apart from their direct role in disease suppression, they also produce metabolites which enhance plant growth or trigger the induction of systemic resistance which immunizes the plant, thereby preventing plant disease (Van Loon et al. 1998). The signal molecules elicit defense mechanisms in plants by activating quiescent defense genes which are present in healthy plants (Vidhyasekaran 1988a, b).

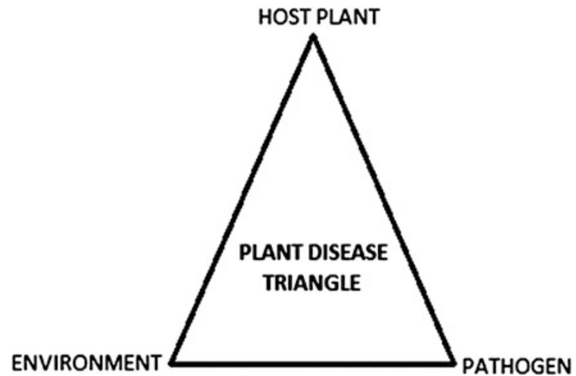

Fig. 5.1 Schematic diagram of plant disease triangle

Biological control of plant disease is defined as "The involvement of the use of beneficial microorganisms, such as specialized fungi or yeast or bacteria to attack and control the plant pathogens and the diseases they are causing" (Fravel 2005). Biocontrol methods and strategies involve appropriate and timely manipulation of antagonist population to suppress pathogens in various inoculum sources or on host plants (Saxena et al. 2000). Biological control of plant pathogens though gaining popularity in majority of crops, its utilization in rice and maize ecosystem is still in its infancy.

The major diseases of rice (*Oryza sativa*) and maize (*Zea mays*) prevalent in India are as follows: rice (1) blast (*Magnaporthe oryzae*), (2) sheath blight (*Rhizoctonia solani*), (3) sheath rot (*Sarocladium oryzae*), (4) bacterial blight (*Xanthomonas oryzae* pv. *oryzae*), (5) rice tungro disease (rice tungro bacilliform virus and rice tungro spherical virus), and (6) brown leaf spot (*Bipolaris oryzae*); maize (1) maydis leaf blight (*Helminthosporium maydis*), (2) sorghum downy mildew (*Peronosclerospora sorghi*), (3) brown stripe downy mildew (*Sclerophthora rayssiae*), (4) crazy top downy mildew (*Sclerophthora macrospora*), (5) sugarcane downy mildew (*Peronosclerospora sacchari*), (6) pythium stalk rot (*Pythium aphanidermatum*), (7) bacterial stalk rot (*Erwinia carotovora*), (8) common rust (*Puccinia sorghi*), (9) charcoal rot (*Macrophomina phaseolina*), (10) brown spot (*Physoderma maydis*), and (11) turcicum leaf blight (*Exserohilum turcicum*). This review, therefore, aims to discuss the developments in research taken place in the field of disease control in these two crops using different bacterial genera.

5.2 Bacterial Species as a Measure of Disease Control

To combat high incidence of fungal, bacterial, and viral diseases, biocontrol agent seems to play a tremendous role in agriculture in the coming days. The present review aims only on the bacterial agent to control plant diseases. During the research of last decades, several bacterial genera like *Arthrobacter* (Mitchell and Hurwitz 1965), *Agrobacterium* (Thomson 1987), *Alcaligenes* (Martinetti and

Loper 1992), *Azotobacter* (Meshram and Jager 1983), *Bacillus* (Fiddaman and Rossall 1995; Sharga 1997), *Pseudomonas* (Dowling and O'Gara 1994; Gomes et al. 1996), *Escherichia coli* (Roberts et al. 1997a, b), *Enterobacter* (Roberts et al. 1997b), *Burkholderia* (King and Parke 1993), *Rhizobium* and *Bradyrhizobium* (Chakraborty and Purkayastha 1984), *Serratia* (Berg and Behl 1997), etc. were found to have the potentiality to control plant diseases caused by fungal pathogens both under in vitro and in vivo conditions.

Agrobacterium radiobacter strain 84 was used for the first time as commercial biocontrol agent against crown gall disease (Kerr 1980). *Alcaligenes* sp. strain MFAI inhibited the growth of *Fusarium oxysporum* f. sp. *dianthi* and minimized the impact of disease presumably as a result of iron chelation by producing siderophore (Martinetti and Loper 1992). Campbell (1989) reported the role of *Bacillus* as a source of antagonist for many plant pathogens. *Bacillus* endowed with added ecological advantage due to their endospores which are resistant to extreme environments. The most interesting is *Bacillus subtilis* A 13, isolated by Broadbent et al. (1971) which was found to be antagonistic to several plant pathogens. Since 1983, it has been sold as bioinoculant under the trade name QUANTUM−4000 (Turner Jr 1987). *Bacillus* spp. and actinomycetes became very attractive biological control agents due to their abundance in soil and the production of various biologically active metabolites against range of pathogens (Silo-Suh et al. 1994).

Bacillus cereus UM85 protects alfalfa seedlings from damping-off disease caused by *Phytophthora medicaginis* (Handelsman et al. 1990), tobacco seedlings from *Phytophthora nicotianae* (Handelsman et al. 1991), peanut from *Sclerotinia minor* (Phipps 1992), and cucumber fruits from *Pythium aphanidermatum* (Smith et al. 1993). *Bacillus subtilis* RB 14 and *Bacillus* BS 153 could suppress the *Rhizoctonia solani* (cause damping-off disease of tomato) and *Botrytis cinerea* (cause chocolate spot symptoms on faba beans), respectively (Asaka and Shoda 1996; Sharga 1997). *Bacillus subtilis* and other *Bacillus* sp. were also reported to control postharvest diseases (Sholberg et al. 1995). Howell et al. (1988) applied *Enterobacter cloacae* strain ECH-1, which controls damping-off of cotton caused by *Pythium ultimum* by producing ammonia. Costa and Loper (1994) evaluated and characterized that siderophore of *E. cloacae* strain EcCT 501 is responsible for disease suppression. Fluorescent pseudomonads emerged as the most potential and promising group of PGPB, which has revolutionized in the field of biological control (Gomes et al. 1996; Saxena et al. 2000). The ability of fluorescent pseudomonads to readily colonize plant roots, to use diverse carbon sources that exudates from roots and their simple nutritional requirements have made them the most potential agent for disease management (Mazzola and Cook 1991).

5.3 Mechanism of Biological Control

Biological control of plant diseases is a result of many different types of ecological interactions and is the basis of the mechanism of biological control agents (Saravanakumar et al. 2009; Zhu et al. 2010). A system of action may be explained

as the strategy against a pathogen by beneficial microorganisms (Liu et al. 2010). The mechanisms attributed to disease-suppressive microorganisms for biocontrol of plant diseases mainly include antibiosis, competition, cell wall-degrading enzymes, biosurfactants, and induced systemic resistance (ISR) (Lo et al. 1998). The different mechanisms are probably never mutually exclusive. However, a successful biocontrol agent is by and large equipped with several attributes which generally work synergistically; for example, pseudomonads produce 2,4-diacetylphloroglucinol (DAPG), an antibiotic, and may also induce host defenses (Kloepper et al. 1980; Lafontaine and Benhamou 1996; Leeman et al. 1995; Maurhofer et al. 1994; Silva et al. 2004; Castoria and Wright 2009; Jamalizadeh et al. 2011). DAPG producers also have the ability to aggressively colonize roots, a trait that might further contribute to their biocontrol property. The mechanism and potentiality of biocontrol agents with special emphasis on bacteria for stimulating plant growth and managing soil and plant health have been discussed and reviewed in several papers and books (Sturz et al. 2000; Pandey et al. 2011; Aeron et al. 2011a). Proper understanding of the mechanisms of the biological control of plant disease may permit us to deliberately manipulate the rhizosphere to develop more conducive biocontrol strategies (Fravel 1988; Handelsman and Parke 1989).

The mode of action of beneficial or disease-suppressing microorganisms is either a direct or an indirect antagonism. Usually pathogens are antagonized by the presence and activities of other microorganisms they encounter (Haas and Défago 2005; Pal and Gardener 2006; De Weert and Bloemberg 2007). Direct antagonism is the outcome of physical contact and/or from high degree of selectivity of the mechanism(s) expressed by the biocontrol-active microorganisms in relation to pathogen, i.e., degradative enzymes, production of antibiotics, and signal interference. In contrast, indirect antagonism results from the activities that do not involve targeting a pathogen by biocontrol-active microorganism(s), i.e., competition for space and nutrition, production for siderophores and biosurfactants, and ISR (Kloepper et al. 1980; Leeman et al. 1995; Lafontaine and Benhamou 1996; Silva et al. 2004).

5.3.1 Enzymatic Control

Extracellular hydrolytic enzymes, viz., chitinases, β-1,3-glucanases, lipases, cellulases, and proteases produced by microbial biocontrol agents, play an important role in suppressing the growth and development of plant pathogens (Chernin and Chet 2002). The lytic enzymes produced by biocontrol agents interfere with the growth and activities of pathogen by hydrolyzing the polymeric compounds, including chitin, proteins, celluloses, hemicelluloses, etc., and consequently killing or suppressing the growth of pathogens (Markowich and Kononova 2003; Arora et al. 2007). Direct parasitism by the antagonists on the pathogen propagules has been reported to play a role in biological control of foliar plant diseases

(Droby and Chalutz 1994). The occurrence of in vitro lysis of fungal cell wall either by chitinase or β-1,3-glucanases alone or in combination has also been established (Lam and Gaffney 1993). The ability of *Serratia marcescens* to produce the chitinase enzyme is considered crucial for its antagonistic activity against *Sclerotium rolfsii* (Ordentlich et al. 1988). *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 also suppress *Fusarium oxysporum* f. sp. *cucumerinum* by the same mechanism. Lim et al. (1991) showed that chitinase and glucanase synthesized by *Pseudomonas stutzeri* digest and lyse mycelia of *Fusarium solani*.

The β-1,3-glucanases synthesized by *Streptomyces* sp. strain 385 and *Paenibacillus* sp. strain 300 lyse cell wall of *Fusarium oxysporum* f. sp. *cucumerinum*. In fact, *Burkholderia cepacia* also synthesizes β-1,3-glucanases and consequently destroys the cell walls of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *P. ultimum* (Fridlender et al. 1993). Palumbo et al. (2005) observed the biocontrol activity of *Lysobacter enzymogenes* strain C3 against Bipolaris leaf spot disease caused by *Pythium* spp. and attributed it to its ability to produce β-1,3-glucanase. Sometimes, these hydrolytic enzymes may act synergistically with antibiotics and thus enhance the antagonistic effects of biocontrol bacteria (Schirmboock et al. 1994; Fogliano et al. 2002). The role of chitin-degrading enzymes in biological control and plant defense mechanism has now been extensively studied (Broglie et al. 1991; Jach et al. 1995; Gupta et al. 2006). Recently, genetic basis for the role of these enzymes in biocontrol has been revealed. A chitinase (ChiA)-deficient mutant of *S. marcescens* was shown to have reduced inhibition of fungal germ tube elongation and reduced biocontrol of *Fusarium* wilt of pea seedling in a greenhouse assay. Furthermore, when ChiA gene from *S. marcescens* was inserted into the non-biocontrol *Escherichia coli*, the transgenic bacterium reduced disease incidence of southern blight of bean caused by *S. rolfsii* (Lo 1998).

5.3.2 Antibiotic-Mediated Suppression

Antibiotics, low molecular weight organic compounds, produced as secondary metabolites by some microorganisms, inhibit the growth or other metabolic activities of other microorganisms at low concentrations, i.e., less than 10 ppm (George 2002). Several microbes produce and secrete compounds with antibiotic activity (Islam et al. 2005; Leclere et al. 2005; Shahraki et al. 2009). Antibiotics may have either a cidal effect or a static effect on certain microorganisms (Leclere et al. 2005). Different antibiotics have different specific modes of action, viz., inhibition of cell wall formation, interference with protein synthesis and membrane integrity, inhibition or interference with DNA synthesis, and inhibition of synthesis of essential small molecules of miscellaneous effects (Walker et al. 2001).

A variety of volatiles/nonvolatiles are produced by microbes, viz., amphisin, 2,4-diacetylphloroglucinol (DAPG), ammonia, butyrolactones, hydrogen cyanide, oomycin A, phenazine (Phz), pyoluteorin, pyrrolnitrin, tensin, tropolone, phloroglucinols (Phl), oligomycin A, kanosamine, zwittermicin A, xanthobaccin

(Nielsen et al. 2002; Raaijmakers et al. 2002; de Souza et al. 2003), and the most recently discovered 2-hydroxymethyl-chroman-4-one (Kang et al. 2004), D-gluconic acid (Kaur et al. 2006), and 2-hexyl-5-propyl resorcinol (HPR) (Cazorla et al. 2006). However, DAPG is the most extensively studied antibiotic (Raaijmakers et al. 2002) and the first clearly implicated antibiotics involved in biocontrol. The phenazine derivatives produced by fluorescent pseudomonads contribute to disease suppression by *P. fluorescens* strain 2-79 and *P. fluorescens* strain 31-84, which control take-all of wheat (Weller and Cook 1983; Brisbane and Rovira 1988). Recent findings stated that some antibiotics may offer untapped resources of compounds to deal with the alarming ascent of multidrug-resistant pathogenic bacteria (Isnansetyo et al. 2003; Lodewyckx et al. 2002). Productions of allelochemicals are highly influenced by the quality and quantity of nutrients and are also subjected to quorum sensing (Haas and Keel 2003). Plant growth and development also influence antibiotic production. Biological activities of antibiotic producers are induced by the activities of the roots of older plants more than roots of young plants, which subsequently results in selective pressure against other rhizosphere microorganisms (Picard et al. 2000).

A significant role is also played by host genotype in disease-suppressive interactions of plants with microbial biocontrol agents (Smith et al. 1999). Regulatory cascades of these antibiotics involve GacA/GacS or GrrA/GrrS, RpoD and RpoS, *N*-acyl-homoserine lactone derivatives (Bloemberg and Lugtenberg 2001; Haas and Keel 2003), and autoregulation (Brodhagen et al. 2004). Antibiotic synthesis of the cell is tightly linked to the overall metabolic status of the cell (Thomashow 1996). Elements, particularly zinc and carbon source, influence the genetic stability/instability of bacteria, consequently affecting their ability to produce secondary metabolite production (Duffy and Defago 2000). The conditions favoring the production/release of one secondary antimicrobial metabolite may not favor the production of other by the same strain. Thus, a biocontrol strain may produce a variety of compounds under different environmental conditions, which enable them to carry out pathogen suppression under a broad range of conditions; for example, in the presence of glucose as carbon source, DAPG synthesis is induced and pyoluteorin is repressed in *P. fluorescens* CHAO. Pyoluteorin is, however, abundantly produced when glucose gets depleted (Duffy and Defago 1999). To be effective, antibiotic must be produced in situ in sufficient quantities at the precise time of interaction with the pathogen (El-Ghaouth et al. 2002). Recent evidences suggest that planned manipulation of microbial communities could be a highly effective form of biocontrol agent (English and Mitchells 1988; Boehm and Hoitink 1992).

5.3.3 Competition for Nutrients and Niches

Microbial competition for nutrients and niches is a fundamental mechanism by which PGPR bacteria protect plants from phytopathogens (Duffy 2001). Competition for nutrients and niches (CNN) among disease-suppressing microorganisms

and pathogen has been observed as an important limiting factor for the development of plant diseases (Kamilova et al. 2005). The rhizosphere regions are significant carbon sinks, and the photosynthetic allocation to this zone is as high as 40% (Degenhardt et al. 2003). Competition is defined as niche overlap, resulting from a situation where there is a simultaneous demand for the same scarce resource by two or more groups of microbial population (Droby and Chalutz 1994). This process is considered to be a potential and indirect interaction whereby pathogens are excluded by depletion of a food base or by physical occupation of site (Lorito et al. 1994).

The chemical attractants present in root exudates include organic acids, amino acids, specific sugars, etc. from diverse nutrient-rich niches attracting the diversity of microorganisms including phytopathogens (Welbaum et al. 2004). Microbial competition for nutrients and niches is a fundamental mechanism by which plant growth-promoting bacteria protect plants from phytopathogens (Duffy 2001). Exudates with antimicrobial agents provide ecological niche to microorganisms having adequate enzymatic machinery to detoxify them (Bais et al. 2004). The extracellular capsule present in some bacteria helps them in the adhesion process (Spadaro and Gullino 2003). The available nutrient sources in the soil and rhizosphere are generally not sufficient for microorganisms, and thus, microbes must effectively compete for available nutrients of phytosphere and rhizosphere (Keel et al. 1989; Loper and Buyer 1991).

On plant surfaces, host-released nutrients include exudates, leachates, or senesced tissues. Additionally, nutrients can also be obtained from waste products of other organisms (Keel et al. 1989). Nonpathogenic microorganisms generally protect the plant by rapid colonization, thereby exhausting the available substrates so that none are available for pathogens to grow. Earlier, Anderson et al. (1988) observed "agglutinin," a plant glycoprotein, and correlated its potentiality with reference to *Pseudomonas putida* to colonize the root system. The quantity and quality of chemoattractants present in plant root exudates are under genetic and environmental control (Bais et al. 2004). The potentiality of plant growth-promoting bacteria (PGPB) depends either on the abilities or to take advantage of that environment. For example, in *Azospirillum* spp., chemotaxis is induced by amino acids, sugars, and organic acids, but the degree of chemotactic response differs to each of these compounds among strains (Reinhold et al. 1985).

PGPB may also be uniquely equipped to sense these chemoattractants, as, for example, rice exudates induce stronger chemotactic responses of endophytic bacteria than to PGPB present in the rice rhizosphere (Bacilio-Jime'nez et al. 2003). In some instances, bacterial lipopolysaccharides (LPS), in particular the O-antigen chain, can also contribute to root colonization (Dekkers et al. 1998). Recently, it is demonstrated that fast bacterial growth rate and their ability to synthesize vitamin B1 exude NADH dehydrogenases that contribute to plant colonization by PGPB (Simmons et al. 1996). Root colonization is also influenced by the ability of PGPB to secrete a site-specific recombinase. Transfer of recombinase gene from competent *P. fluorescens* into noncompetent *P. fluorescens* strain enhances the ability of the latter to colonize root tips

(Dekkers et al. 2000). Simmons et al. (1997) reported that amino acid synthesis is required for root colonization by *P. fluorescens* WCS365, indicating the importance of amino acid prototrophy in rhizosphere competence. Although amino acids are present in root exudates, it is not sufficient for auxotrophic *P. fluorescens* WCS365 to support root tip colonization (Knee et al. 2001).

5.3.4 Siderophore-Mediated Competition for Nutrients

Biological control can involve inhibition of the pathogen by depriving it of nutrients. Iron competition is the best studied example in this context. Most of the iron of the earth's crust is found in highly insoluble form of ferric hydroxide, and consequently, only a small concentration at or below 10^{-18} M is available to organisms in soil solution at neutral pH. This low concentration cannot support the microbial growth, which generally requires a minimum of 10^{-6} M concentrations of iron. Iron is an essential element for all living organisms, and the scarcity of its bioavailable form in rhizosphere leads to intense competition among the microbial communities (Loper and Henkels 1997). They are produced by almost all aerobic and facultative anaerobic microorganisms that play an important role in disease suppression (Haggag Wafaa and Abo Sadera 2000). Siderophore biosynthesis is generally regulated by a consortium of molecules like iron-sensitive Fur proteins; the global regulators GacS and GacA; the sigma factors RpoS, PvdS, and Fpvl; and quorum-sensing autoinducers such as *N*-acetyl homoserine lactone and site-specific recombinases (Cornelis and Matthijs 2002; Ravel and Cornelis 2003). However, evidence is also there for the noninvolvement of the global regulators in siderophore biosynthesis. Saleh and Glick (2001) and Compant et al. (2005), however, observed that siderophore synthesis was not significantly regulated by GacS and RpoS in *Enterobacter cloacae* CAL2 and UW$ and RpoS in *P. putida* strain WCS 358. In *Serratia plymuthica* strain IC1270, GrrA/GrrS but not GacS/GacA is involved in siderophore biosynthesis (Ovadis et al. 2004).

Nevertheless, the siderophore biosynthesis is influenced by factors like pH, level of iron, the presence of other trace elements, and an adequate supply of carbon, nitrogen, and phosphorus (Duffy and Defago 1999). Kloepper et al. (1980) were the first to demonstrate the importance of siderophore production as a mechanism of biological control of *Erwinia carotovora* by *P. fluorescens* strains A1, BK1, TL3B1, and B10. A correlation was established between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *F. oxysporum* (Elad and Baker 1985; Sneh et al. 1984). Mutants that failed to produce some siderophores such as pyoverdine were poor in their ability to suppress different phytopathogens (Keel et al. 1989; Loper and Buyer 1991). Infection of plants by pathogen occurs only after seed dormancy is broken in presence of stimulants from host plants. Thus, both the beneficial and pathogenic microbes compete for the specific stimulants released from the germinating seeds or growing roots.

The stimulants may be fatty acids or their peroxidation products or volatile compounds like ethanol and acetaldehyde (Lo et al. 1998). Nelson (1987) reported the evidence of sporangia germination of *Pythium* sp. within 24-h exposure to stimulants from germinating seeds of plants. Norton and Harman (1985) reported the 60-fold increase in populations of *Pythium ultimum* after exposure to volatiles from aged pea seeds in soil. Fatty acids of C16–C18 also stimulated spore germination of *Thielaviopsis basicola* in vitro (Papavizas and Kovacs 1972). The volatile peroxidation products of unsaturated fatty acids may be potent stimulants for fungal germination, since as little as 200 mg/l of 2,4-hexadienal in aerial solution stimulated conidia germination in *Alternaria alternata* (Harman and Hayes 1994). Thus, biocontrol agent can provide protections to plant from disease landing by efficient interception of these host-released stimulants before pathogen can use them (Lo et al. 1998).

5.3.5 Role of Biosurfactants

Biosurfactants are structurally diverse group of surface-active molecule, synthesized extracellularly, or as part of the cell membrane by microorganisms (Chen et al. 2007; Anandaraj and Thivakaran 2010). The ability to reduce surface tension is a major characteristic of surfactant. Stanghellini and Miller (1997) for the first time explored the potentiality of biosurfactant in biological control of plant diseases. Biosurfactant-producing microorganisms are abundant in every imaginable environment worldwide, including soil (Bodour et al. 2003), marine environments (Gerard et al. 1997), wastewater (Haddad et al. 2008), hydroponic systems (Hultberg et al. 2008), human lung tissue (Tingpej et al. 2007), and, last but not least, on plant surfaces (de Souza et al. 2003; Perneel et al. 2007).

Biosurfactants supposedly act through modification of surface properties, alteration of compound bioavailability, and interaction with membranes. The principal action of surfactant depends on its specific structure and production characteristics. The little changes in structure can cause major alteration in biological activity, which can either be beneficial for the bacteria itself or for the plant, or cause deleterious effects on competing microorganisms or on host plants, as in case of bacterial pathogens (D'aes et al. 2010). Rhamnolipids can cause lyses of the zoospores of many oomycete plant pathogens. Several reports (Kim et al. 2000; Nielsen et al. 2000; De Jonghe et al. 2006) have been published about the possible use of rhamnolipid-producing bacteria to suppress zoospore-producing pathogens. *Pseudomonas aeruginosa,* a pathogenic bacterium, causing serious infections in animals, is also a common rhizosphere inhabitant. In some cases, it exhibits PGP effects and production of rhamnolipid-type biosurfactants (Soberon-Chavez et al. 2005; Perneel et al. 2008).

Regulation of rhamnolipid production by *P. aeruginosa* has been very well studied. A network involving two quorum-sensing systems (RhlR/RhlI and LasR/LasI) as well as the Pseudomonas quinolone signal (PQS), several sigma factors,

the GacS/GacA two-component system, and multiple other transcriptional regulators are responsible for rhamnolipid biosynthesis regulation, suggesting that many environmental and internal signals can be integrated to ensure timely production of these compounds (Venturi 2006). Biosurfactants, including rhamnolipids and cyclic lipopeptides (CLPs), disrupt the membrane integrity of *Phytophthora* zoospores, leading to complete elimination of infectious zoospore populations within minutes (Tran et al. 2007). Rhamnolipids and CLP also inhibit growth of *Phytophthora capsici* and other oomycete pathogen (Kim et al. 2000; De Jonghe et al. 2006). Biosurfactants contribute in different ways to ecological fitness of the producing bacteria. They reduce the surface tension of hydrophobic plant surfaces such as leaf cuticle or act as wetting agents, thereby changing the viscosity of the surface (Lindow and Brandl 2003; Harshey 2003). Morikawa et al. (2000) reported that the activity of two very powerful surfactants, surfactin and arthrofactin, is strongly affected by specific amino acid modification, protonation of amino acid side chains, linearization of the peptide activity, and length of the fatty acid chain. Some CLP essential for biofilm formation like viscosin and massetolide produced by *P. fluorescens* strains SBW25 and SS101, while mutants in putisolvin and arthrofactin production of *P. putida* and *Pseudomonas* sp. MIS38, form thicker and unstable biofilms (Roongsawang et al. 2003; D'aes et al. 2010).

Biofilm formation is important for colonization process. Rhamnolipids play a role in initial formation of microcolonies on a surface, development and maintenance of a mature biofilm consisting of mushroom-shaped structures and water channels, and in dispersion of bacteria from biofilms. Thus, rhamnolipids may have concentration-dependent effects (Davey et al. 2003; Pamp and Tolkier-Nielsen 2007). *Bacillus subtilis* 6051 strain needs surfactin for biofilm formation on the roots of *Arabidopsis* (Turnbull et al. 2001; Bais et al. 2004). Root colonization is important for plant pathogens by providing as the delivery system for antifungal metabolites and increases their epiphytic fitness on leaf surface (Chin-A-Woeng et al. 2000; Morris and Monier 2003).

Biosurfactants also help strains to get access to nutrients. By increasing the wettability, allow solubilization and diffusion of substrates and emulsification of water-soluble compounds, making them more available to bacteria. Alternatively, biosurfactant may increase uptake of hydrophobic substrates by altering cell surface properties. Rhamnolipids increase cell surface hydrophobicity of *P. aeruginosa* by causing release of lipopolysaccharides from the outer membrane, thereby enhancing the contact between the cell and the hydrophobic substrate. In contrast to making compounds more available, biosurfactants may protect their producers against toxic compounds such as heavy metals or hydrocarbons, by binding them or encapsulating them in micelles (D'aes et al. 2010). Perneel et al. (2008) observed that rhamnolipids produced by *P. aeruginosa* PNA1 increase the efficacy of phenazine-1-carboxamide (PCN) in suppression of disease caused by *Pythium* sp. Similarly, Debode et al. (2007) showed that interference between the modes of action of phenazines and biosurfactants is responsible for suppression of *Verticillium* microsclerotia viability in vitro.

Biosurfactants in particular CLPs can act as cytolytic agents leading/causing the destruction of membranes and subsequent death of the pathogens. Usually, CLPs are usually toxic towards specific groups of organisms, showing less significant activity against others. These differences in activity are due to the structural properties of both surfactant and membrane. *P. fluorescens* DR54 produces viscosinamide, one of the best CLPs with antifungal properties to control *P. ultimum* and *R. solani* damping-off on sugar beet (Nielsen et al. 1998; Thrane et al. 2001). Some lipopeptide-producing bacteria induce defense responses in plants. *B. subtilis* S499 produces biosurfactant, viz., fengycins and surfactins, which in turn provide an ISR-mediated protective effect on tomato plant against *Botrytis cinerea* (Ongena et al. 2007). Surfactin induces the early defense responses in tobacco cell cultures. The most potent elicitors are surfactin homologues with longer fatty acid chains (C14 and C15), while surfactins with C12 and C13 fatty acid chains have markedly less active. Ramification of fatty acid chains improves eliciting capacity of surfactin, while linearization or methylation of the peptide part leads to decrease in its activity (Jourdan et al. 2009). Tran et al. (2007) observed that rhamnolipids and CLPs produced by *Pseudomonas* are able to induce resistance. Massetolide A produced by *P. fluorescens* SS101 induces resistance against tomato late blight caused by *Phytophthora infestans*. Varnier et al. (2009) showed that rhamnolipids trigger defense responses in grapevine cell culture against *Botrytis cinerea*.

5.3.6 Detoxification and Degradation of Virulence Factors

Microorganisms can adopt the mechanism of disease suppression by detoxification of the pathogenic virulence factor; for example, albicidin toxin produced by *Xanthomonas albilineans* is easily detoxified by certain microorganisms (Basnayake and Birch 1995; Zhang and Birch 1997). The proteins released by the beneficial microorganisms can bind reversibly or irreversibly with the toxin for detoxification. Bacteria like *Burkholderia cepacia* and *Ralstonia solanacearum* hydrolyze fusaric acid, a phytotoxin produced by various *Fusarium* sp. (Compant et al. 2005). However, pathogen toxin showed broad-spectrum activity and suppresses growth of microbial competitors or detoxifies antibiotics as a defense mechanism of pathogenic microorganisms against biocontrol agents (Schouten et al. 2004).

Most bacterial plant pathogens depend upon autoinducer-mediated quorum sensing to turn on gene cascades for virulence factors. Certain PGPB break the potentiality by degrading autoinducer signals, thereby blocking the expression of various virulence genes (Newton and Fray 2004). Endophytic bacteria also synthesize compounds with antagonistic property towards the pathogens (Compant et al. 2005). For example, munumbicins, an antibiotic produced by endophytic *Streptomyces* sp. strain NRRI 30562, inhibit the in vitro growth of *P. ultimum* and *F. oxysporum*. Interestingly, it is revealed from certain evidences that the

antagonistic property of endophytic bacteria decreases as the bacteria colonize the host plant interiors, suggesting their antagonistic property as tissue type specific or site specific (Sturz et al. 1999).

5.3.7 Induced Systemic Resistance

Induced resistance is defined as an enhancement of the plant defense activity against a broad spectrum of pathogens that is acquired after appropriate stimulation. The plants respond to a variety of environmental stimulating factors, including gravity, light, temperature, physical stress, water, nutrient availability, and chemicals produced by microorganisms (Audenaert et al. 2002). ISR is elicited by some bacteria and is phenotypically similar to systemic acquired resistance (SAR) (Van Loon et al. 1998).

SAR is induced in plants when they respond to primary infection by a pathogen, resulting in hypersensitive reaction which leads to local necrotic lesion of brown, desiccated tissue (Compant et al. 2005). ISR is mediated by a jasmonate-/ethylene-sensitive pathway. However, some PGPB trigger salicylic acid-dependent pathway by producing nanogram of salicylic acid in the rhizosphere (De Meyer et al. 1999). ISR is effective against various types of pathogens but differs from SAR because the inducing PGPB does not cause any visible symptoms on the host plant. ISR was first recorded in *Dianthus caryophyllus* with reduced susceptibility of *Fusarium* wilt (Van Peer et al. 1991) and on *Cucumis sativus* with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare* (Wei et al. 1991). However, the combination of host plant and bacterial strains determines the expression of ISR (Kilic-Ekici and Yuen 2004). The endophytic bacteria also observed to exhibit the ISR activity. For example, *Burkholderia phytofirmans* PsJN triggers ISR against *Botrytis cinerea* on grapevine (Ait Barka et al. 2002) and *Verticillium dahlia* on tomato (Compant et al. 2005). Several *Pseudomonas* spp. are able to induce ISR in a wide range of plants against different pathogens (Van Loon 2007).

The activation ISR of the plant defense system increases the plant cell wall strength, alters host physiology, and enhances synthesis of plant defense chemicals (Ramamoorthy et al. 2001; Nowak and Shulaev 2003). In several fluorescent Pseudomonads, a combination of ISR elicitors, viz., siderophores, O-antigen, and flagella, works in concert to induce the ISR effect (Bakker et al. 2003). ISR is associated with an increase in sensitivity to the related hormones rather than an increase in their production, which might lead to the activation of a partially different set of defense genes (Hase et al. 2003). In *Burkholderia phytofirmans* PsJN–grapevine interactions, ISR is associated with the accumulation of phenolic compounds, resulting in strengthening of cell walls in exodermis and cortical cells during endophytic colonization of bacterium. Biochemical and physiological changes in plants during ISR include accumulation of pathogenesis-related proteins such as PR-1, PR-2, chitinases, and some peroxidases. However, certain bacteria

do not induce production of PR proteins but rather increase accumulation of peroxidases, phenylalanine, ammonialyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase (Compant et al. 2005).

5.4 Control of Different Diseases of Rice by Using Bacteria

Rice is one of the staple crops and is attacked by various pathogens. Here we discuss some of the salient features of biological control of selected diseases of rice.

5.4.1 Bacterial Blight

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. o*ryzae* is one of the most important and oldest known diseases of rice. It is the most devastating disease of rice in tropical countries and is associated with many epidemics. The symptoms include small, green, water-soaked spots at the tips and margins of fully developed leaves. The spots may further expand along the veins, merge, and become chlorotic then necrotic forming opaque, white- to gray-colored lesions. The disease was first observed by the farmers of Japan in 1884 (Tagami and Mizukami 1962) and occurs mainly in China, Korea, India, the Philippines, Sri Lanka, Myanmar, Laos, Taiwan, Thailand, and Vietnam. In India, it has been observed in rice-growing states like Andhra Pradesh, Bihar, Haryana, Kerala, Orissa, Punjab, and Uttar Pradesh. The disease occurred in an epidemic form during 1998 in the Palakkad district of Kerala (Venkatesan and Gnanamanickam 1999) and, since then, has been observed in severe proportions almost every year (Velusamy et al. 2006). New diseases appear with a change of rice ecology, when agriculture moves towards higher productivity. However, bacterial blight is not a new disease, and its importance to rice production was recognized only after the introduction of new cultivars.

The studies pertaining to the use of bacterial agents to control bacterial blight are meager. An antibacterial metabolite, kanosamine, produced at the rate of 1.6 mg/ml by a *Bacillus* strain ALP 18 amended with peptone sucrose agar (PSA) inhibited the growth of *X. oryzae* pv. *oryzae* (Vasudevan 2002). Kanosamine production by other *Bacillus* spp. has also been reported earlier (Cron et al. 1958; Milner et al. 1996; Umezawa et al. 1967). The rate of disease suppression by four Bacillus species, viz., *Bacillus lentus*, *B. cereus*, *B. circutans*, and *Bacillus* sp. (CAL 9) and *Bacillus* sp. (MON 2-17), was studied. They were applied @10^8 cfu/ml in 1% carboxymethy-cellulose as seed treatment of rice variety IR-24 before sowing besides the two foliar sprays on 35th and 45th day after planting. The disease suppression ranged from 36 to 59% and 52 to 56% in net house and field experiments, respectively, while in case of variety Jyothi, it was 21 to 57% and 18 to 54%, respectively, in net house and field experiments (Gnanamanickam 2009). A yield of 40 μg 2,4-diacetylphloroglucinol

(DAPG)/ml was extracted from the 72-h grown culture of *P. fluorescens* PTB9 and was tested for its antibiosis activity against *X. oryzae* pv. *oryzae* which inhibited the growth of pathogen in vitro (Gnanamanickam 2009). Ji et al. (2008) used a novel strain of *Lysobacter antibioticus* 13-1 for biological suppression of BB blight. In net house experiments, whole bacterial broth culture (WBC) of this novel strain reduced disease intensity better than zhongshengmycin (1%) (Ji et al. 2008). However, disease suppression by *L. antibioticus* 13-1 varies significantly among different rice cultivars and pathogen strains involved. *Lysobacter enzymogenes* strain C3 is a well-known biocontrol agent for many fungal diseases (Giesler and Yuen 1998; Jochum et al. 2006). In another experiment, *Erwinia herbicola* present on rice leaf surfaces lowers the pH of rice leaves by producing an acid and consequently suppresses the growth of BB pathogen (Babu and Thind 2005).

5.4.2 Rice Blast

Rice blast caused by *Magnaporthe oryzae* is responsible for spotting and blasting of the foliage and blighting of the panicles. The disease is destructive under conditions of high monsoon rainfall and high atmospheric humidity and becomes an obstruction for rice cultivation in tropical areas of the world. Blast disease suppression in upland rice cultivar UPLRi-5 was carried out using *Pseudomonas* strains. *Bacillus* strains 4-03 and 33 also reduced leaf blast. For controlling rice blast, seeds of rice cultivar UPLRi-5 were coated with strain of *Pseudomonas* and *Bacillus* followed by their spray. Valasubramanian (1994) reported that *P. fluorescens* 7-14-mediated blast suppression was due to the production of an antibiotic. On the other hand, *Serratia marcescens* strain B2 contains a set of chitinase genes that enabled them to suppress the growth of pathogenic fungi including *Magnaporthe oryzae* (Someya et al. 2002). The cloned chitinase gene of *S. marcescens* transferred to *Erwinia ananas* NR-1 strain inhibited the mycelial growth and conidial germination of rice blast fungus and also caused bursts in the mycelial tips (Someya et al. 2003). Similarly, *Streptomyces sindeneusis* 263 strain isolated from rice fields effectively suppressed the growth of *M. oryzae* (Zarandi et al. 2009).

One of the most elegant developments in blast control is the discovery and commercial use of antibiotics. *Streptomyces griseochromogenes* produce an antibiotic blasticidin-5, which is considered as the first antibiotic commercially introduced for blast control. It is effective even when applied at a very low concentration (10–20 ppm) as spray. However, higher concentration of blasticidin-5 is phytotoxic, producing chlorotic and necrotic spots on the foliage of rice plants. The blasticidin-5-mediated disease suppression is due to inhibitory action on protein synthesis of fungus (Misato 1961). Ashizawa et al. (2005) controlled rice leaf blast by preinoculating rice leaves by avirulent isolates of *Pyricularia oryzae*. Non-rice pathogen *Bipolaris sorokiniana* caused rice blast disease suppression. It can be applied either as preinoculation or foliar spray. This indicates the involvement of induced resistance in blast suppression. Smith and Metraux (1991) realized the role of

ISR when they used *Pseudomonas syringae* pv. *syringae* as the inducer of systemic and induced resistance (ISR) in rice. ISR as a mechanism of disease suppression operates in every host–pathogen interaction and widens the efficacy of disease resistance and protection of crop plants (Walters et al. 2005).

5.4.3 Sheath Blight

The causal organism of sheath blight disease of rice (*Oryza sativa* L.) is *Rhizoctonia solani* Kuhn and is one of the most widespread and severe diseases of rice. It causes heavy losses of the yield under favorable conditions (Ou 1985). The initial symptoms consist of lesions on the sheath of lower leaves at late tillering or early internode elongation stages. Under the favorable conditions of low sunlight, high humidity, and warm temperature, the infection spreads rapidly by means of runner hyphae to upper plant parts, including leaf blades and adjacent plants. Lesion may completely encompass the entire leaf sheath and stem. The disease is currently managed only through excessive application of chemical fungicides which are toxic, non-environment friendly, and expensive for resource-poor farmers. Therefore, greater emphasis is now given on biological control to develop effective alternative strategy (Table 5.1).

Several strains of *P. fluorescens* have been successfully used as biological control agent of rice sheath blight (Gnanamanickam 2009). *Pseudomonas fluorescens* 7-14 strain controls leaf blast up to 79% and sheath blight up to 85% in IR50 rice variety (Valasubramanian 1994). Phenazine like antifungal antibiotic appeared to be the primary contributor of its disease suppression ability (Gnanamanickam et al. 1994; Chatterjee et al. 1996). *Pseudomonas putida* V14-I, a chitinase producer, suppressed sheath blight (ShB) disease in IR50 rice cultivar by 60–80% (Thara and Gnanamanickam 1994). *P. putida* suppressed this disease up to 60% when used as seed treatment, root dip, and as sprays. Disease control efficiency, however, was very low (8%) when used only as seed treatment (Krishnamurthy and Gnanamanickam 1998). *Bacillus polymyxa* VLB16 produced a heat-tolerant protein that suppresses ShB disease up to 67% in IR24 when applied as seed treatment with an additional root dip and foliar sprays (Kavitha et al. 2005). A group of fluorescent pseudomonads present in Indian soil produce antibiotic 2,4-diacetylphloroglucinol (DAPG) which is found to suppress ShB disease (Immanuel 2006). The highest level of reduction of ShB was recorded by the treatments with *P. fluorescens* strain W4 (Immanuel 2006). Prolonged exposure of pathogenic fungi to DAPG causes bursting of mycelia tips. In plate assay, DAPG concentration >100 μM completely inhibited the growth of *R. solani*. Talc-based formulations of *P. fluorescens* strains Pf1 and PfALR2 after seed treatments or sprays cause suppression of sheath blight disease of rice (Rabindran and Vidhyasekaran 1996). The rice field soils of slightly acidic nature (pH 5.0) and soils of boron toxicity are more suitable soil conditions for bacterial treatments to suppress ShB (Gnanamanickam 2009).

The water-soluble formulation of *Bacillus megaterium* (1 g formulation/100 g seed) is reported to be effective in suppressing sheath blight in greenhouse and

Table 5.1 Major rice diseases and their biocontrol agents

Sl. No.	Disease	Causal agent	Biocontrol agent	References
1	Bacterial blight	*Xanthomonas oryzae* pv. *oryzae*	*Bacillus lentus* *B. cereus* *B. circulans* *Pseudomonas fluorescens* *Lysobacter antibioticus*	Vasudevan (2002) Velusamy and Gnanamanickam (2003) Velusamy et al. (2006) Ji et al. (2008)
2	Blast	*Magnaporthe oryzae*	*Bacillus polymyxa* *B. pumilus* *B. coagulans* *P. fluorescens* *Enterobacter agglomerans*	Gnanamanickam and Mew (1992) Valasubramanian (1994) Kavitha (2002)
3	Sheath blight	*Rhizoctonia solani*	*B. megaterium* *B. polymyxa* *B. pumilus* *B. coagulans* *B. laterosporus* *P. fluorescens* *P. putida* *P. aeruginosa* *Enterobacter agglomerans* *Serratia marcescens*	Thara and Gnanamanickam (1994) Krishnamurthy and Gnanamanickam (1998) Sakthivel (2002) Kavitha (2002) Vasudevan (2002)
4	Sheath rot	*Sarocladium oryzae*	*P. fluorescens* *P. aeruginosa* *B. subtilis* *B. pumilus*	Sakthivel (1987) Sakthivel and Gnanamanickam (1987) Vasudevan (2002)

small field trials after seed treatment, followed by three sprays at the seedling, tillering, and flowering stages (Kanjanamaneesathian et al. 2009). A novel formulation of *B. megaterium* endospores is developed and can be used either as broadcast or spray application. The formulation is effervescent fast-disintegrating granules composed of lactose, polyvinylpyrrolidone K-30, and effervescent base (citric acid, tartaric acid, and sodium bicarbonate). Floating pellets composed of hydrogenated vegetable oil (HVO), lactose, microcrystalline cellulose (Avicel® PH101), and a disintegrant; cross-linked sodium carboxymethylcellulose also contain spores of *B. megaterium* are prepared by extrusion–spheronization process. The bacterial pellets showed promising result in suppression of the development of sheath blight lesions in greenhouse experiment (Wiwattanapatapee et al. 2004).

Siderophore-producing *P. fluorescens* biovar 3 inhibited the mycelial growth of *R. solani* and also reduced the germination and caused lysis of the sclerotia of the pathogen (Kazempour 2004). Another siderophore-producing rhizobacterial strains of *B. cereus* Z 2-7 and *Enterobacter* sp. B41 strain SPR7 cause ShB disease suppression and increase in yield due to improved uptake of nutrients and induction of defense enzymes (Naureen et al. 2011). Recently, *Pseudomonas fluorescens* MDU2 is also effective in inhibiting the mycelial growth of *R. solani*, which can grow even at 250 mM concentration of oxalic acid (Nagarajkumar et al. 2005).

Rice seed treatment followed by root-dipping and a foliar spray with *P. fluorescens* strains Pf1 and FP7 showed higher induction of ISR against *R. solani* (Vidhyasekaran and Muthamilan 1999). Production of secondary metabolites such as salicylic acid, antibiotics, Fe-chelating siderophores, and cyanide are most often associated with fungal suppression by fluorescent Pseudomonads in the rhizosphere of several crops (Rabindran and Vidhyasekaran 1996; Singh et al. 2010; Khare et al. 2011; Aeron et al. 2011b). *Pseudomonas fluorescens* strain Pf 003 is found to be highly effective in controlling ShB pathogen of rice with inhibition ranging from 50 to 58% (Reddy et al. 2010). Seed bacterization of cv. IR58 and cv. IR64 increased germination from 78 to 93% and 89 to 97%, respectively. Moreover, seed bacterization by *B. subtilis*, *B. laterosporus*, *B. pumilus*, *P. aeruginosa*, *S. marcescens*, and *Erwinia herbicola* provides sheath blight protection in the glass house trial (Rosales et al. 2008).

5.4.4 Sheath Rot

The causal organism of sheath rot disease is *Sarocladium oryzae*. The alternative host of the pathogen includes maize, pearl millet, sorghum, *Echinochloa colona* (L.) Link (jungle grass), *Eleusine indica* (L.) Gaertn. (goose grass), *Leptochloa chinensis* (L.) Nees (red sprangletop), *Oryza rufipogon* (red rice), *Zizania aquatica* (annual wild rice), and *Zizaniopsis miliacea* (giant cutgrass). The fungus grows best at 20–28 °C. High amount of nitrogen, high relative humidity, and dense crop growth favor disease development. The symptoms are most severe on the upper most leaf sheath that encloses the young panicle during the boot stage. Lesions are oblong or irregular oval spots with gray or light brown centers and a dark reddish brown diffuse margin. Early or severe infection may affect the panicle so that it only partially emerges. The emerged portion of the panicle rots, with florets turning red brown to dark brown consequently not allowing grain setting. A powdery white growth consisting of spores and hyphae of the pathogen is usually observed on the inside of the affected leaves. The disease appears late during the growing season of the rice crop. The disease is important when the crop proceeds towards maturity stages. The disease causes discoloration of the sheath and affects the marketable quality of the grains.

There are very few studies on the biocontrol of sheath rot disease of rice plant. *Pseudomonas fluorescens* strain suppresses the sheath rot disease (Sakthivel 2002). This Pfcp strain causes suppression of 54% Sh-R incidence in IR20 rice in greenhouse test. Bacterization of rice cultivars with *P. fluorescens* enhanced plant height, number of tillers, and grain yields 3–160% (Sakthivel and Gnanamanickam 1987). At IRRI, bacterial strains were isolated from rice rhizosphere and showed antagonistic activity towards *Sarocladium oryzae* (Mew et al. 1993). Sakthivel and others have been doing research on the toxins produced by sheath rot pathogen.

5.5 Control of Different Diseases of Maize by Using Bacteria

Maize is another crop that is attacked by a variety of pathogens, biological control of which is discussed below.

5.5.1 Downy Mildew

The causal organism of downy mildew of maize is *Peronosclerospora sorghi* (Weston and Uppal) C.G. Shaw. The disease is considered as extremely destructive in maize (*Zea mays* L.), grain sorghum (*Sorghum bicolor* L.), and forage sorghum (*Sorghum vulgar* L.). The pathogen is known to cause both local and systemic symptoms (Shaarawy et al. 2002) with the latter usually producing higher economic loses. The disease is spread by different ways including air and seed and can also be soilborne (Kutama et al. 2008).

Bacillus subtilis inhibited germination of oospores and conidia of *P. sorgi*. Disease infection percentages were different according to the treatments, i.e., spray, seed soaking, and spray plus seed soaking. Seed treatment with the biocontrol agents was more effective than individual treatment of seed soaking or spray. Dual treatment using *Bacillus subtilis* and *Trichoderma viridae* showed effective inhibitory effect on the oospores and conidial germination (Sadoma et al. 2011). Downey mildew of maize was significantly controlled by seed treatment and foliar sprays of a talc-based formulation of a *Pseudomonas fluorescens* strain under greenhouse and field conditions (Kamalakannan and Shanmugam 2009).

5.5.2 Southern Leaf Corn Blight

The causal organism for southern corn leaf blight (SCLB) disease of maize is *Cochliobolus heterostrophus* (Drechsler). Drechsler [anamorph = Bipolaris maydis (Nisikado) Shoemaker; synonym = *Helminthosporium maydis* Nisikado] is a widespread disease throughout most of hot humid corn-growing areas of the world. Current practices for controlling plant diseases are based largely on application of synthetic pesticides. The role of biocontrol in controlling this disease is being explored now because of development of resistant varieties (Huang et al. 2010).

Bacillus subtilis B47, an endophytic bacterium, inhibited the growth of *Bipolaris maydis*. The inhibitory effect that was significantly higher than chlorothalonil, a fungicide, is considered to be due to the cyclic lipopeptide antibiotic iturin A2 (Yun-feng et al. 2012). Lu et al. (2006) also reported the ability of *B. subtilis* ST-87-14 to suppress SCLB in the greenhouse and fields. *Bacillus cereus* C1L has also been observed to suppress SCLB effectively in greenhouse as well as field conditions

(Huang et al. 2010). *Bacillus* sp. CHMI inhibits southern corn leaf blight (Wang et al. 2009). Earlier, Muhammad and Amusa (2003) reported the suppression of the growth *H. maydis* by *B. subtilis* and *B. cereus*. A lytic factor has been reported to be located in the walls of strains of *B. subtilis* (Young et al. 1974), suggesting that this might have diffused out in the surrounding medium causing zones of inhibition and consequently preventing seedling blight. Antagonistic microorganisms mediated antibiotics played an important role in biological control of plant diseases (Yun-feng et al. 2012).

Antibiotics, BTL and verlamelin, produced by *Bacillus* strain B-TL2 and *Acremonium strictum* BCP showed strong inhibitory effect against mycelial growth of *B. maydis* in vitro (Zhang et al. 2008). Members of the *B. subtilis* family produce a wide variety of antimicrobial substances which can be exploited for controlling plant diseases (Yun-feng et al. 2012). *Bacillus* spp. also produce ISR elicitors, viz., lipopeptides, volatile compounds, and bacteriocin, responsible for biocontrol (Choudhury and Johri 2009). *Streptomyces* spp. strains (DAUFPE 11470, DAUFPE, 14632) decrease the incidence of *Bipolaris maydis* after maize seed treatments. Treatments with strain cells and antibiotics together exhibited greater efficacy in reducing the incidence of disease as compared to the treatments only with antibiotics produced by the *Streptomyces* strains (Bressan 2003).

5.5.3 Charcoal-Rot Disease

The causal organism of charcoal-rot disease is *Macrophomina phaseolina*. The disease is favored by warm, dry growing conditions and is often associated with drought stress although prevalent under humid tropical conditions. The pathogen can infect a broad array of major crops including maize, bean, sorghum, soybean, and cotton (Mayek-Perez et al. 2002). Cultural and chemical strategies for disease management are not adequate to control the disease effectively and economically. The disease is controlled by soil fumigation using methyl bromide chloropicrin (Smith and Krugman 1967). The largest disadvantage of the soil fumigation is the simultaneous killing of beneficial microorganisms (Singh et al. 2008). Recently, interest in biological control (Table 5.2) has been increased due to public concerns over the use of chemicals in the environment in general and the need to find alternatives to the use of chemicals for disease controls in particular (Whipps 2001).

Bacillus spp. has been identified as potent antagonists against *M. phaseolina* (Muhammad and Amusa 2003). *Bacillus subtilis* BN1 exhibited strong antagonistic activity against *M. phaseolina*. The strain causes vacuolation, hyphal squeezing, swelling, abnormal branching, and lysis of mycelia. The cell-free culture filtrate (CFCF) of *B. subtilis* BN1 was found to be concentration dependent and completely inhibited the fungal growth (Singh et al. 2008). Singh et al. (2008) used sawdust-based formulation of *B. subtilis* BN1 for consistently delivering critical number of viable cells, i.e., $\geq 10^6$ cfu g^{-1}, for successful suppression of disease.

Table 5.2 Major maize diseases and their biocontrol agents

Sl. No.	Disease	Causal agent	Biocontrol agent	References
1	Downey mildew	*Peronosclerospora sorghi*	*Bacillus subtilis* *Pseudomonas fluorescens*	Sadoma et al. (2011)
2	Southern leaf corn blight	*Helminthosporium maydis*	*B. subtilis* B47 *B. subtilis* ST-87-14 *Bacillus cereus* C1L *Bacillus* strain B-TL2 *Acremonium strictum* BCP *Streptomyces* spp.	Yun-feng et al. (2012) Lu et al. (2006) Huang et al. (2010) Zhang et al. (2008) Bressan (2003)
3	Charcoal rot	*Macrophomina phaseolina*	*Bacillus* spp. *P. fluorescens*	Muhammad and Amusa (2003) Pal (1996)
4	Bacterial stalk rot	*Erwinia carotovora*	*B. thuringiensis*	Dong et al. (2004)
5	Ear rot	*Stenocarpella maydis*, *S. macrospora*	*Bacillus subtilis* *Pseudomonas fluorescens* *Pantoea agglomerans*	Petatan-Sagahon et al. (2011)

Pseudomonas sp. strain EM 85 isolated from rhizosphere of maize plant reported to have inhibitory effect against charcoal-rot disease (Pal 1996). Pal et al. (2001) also reported two bacilli isolates MR-11(2) and MRF isolated from maize rhizosphere having antagonistic property against three plant diseases including charcoal-rot disease of maize. Combined application of two bacilli significantly reduced *Macrophomina*-induced charcoal rots of maize by 56.04%. The possible mechanisms by which fluorescent pseudomonads and bacilli exhibit biocontrol have been reported by various workers (Emmert and Handelsman 1999; Gupta et al. 2002; Bhatia et al. 2003; Deshwal et al. 2003; Joshi et al. 2006). Antifungal antibiotics and fluorescent pigments produced by *Pseudomonas* sp. EM85, antibiotics and antifungal volatiles produced by *Bacillus* sp. MR-11(2), and antibiotic of *Bacillus* sp. MRF might be involved in biocontrol of charcoal-rot disease of maize (Pal et al. 2001).

5.5.4 Bacterial Stalk Rot

The casual organism of bacterial stalk rot disease of maize is *Erwinia carotovora*. Dong et al. (2004) reported *Bacillus thuringiensis* as an antagonistic agent against *E. carotovora*. *Bacillus thuringiensis* suppresses the quorum-sensing-mediated virulence of plant pathogen *Erwinia carotovora*, through a new form of microbial antagonism, signal interference. *E. carotovora* produces and responds to acyl-homoserine lactone (AHL) quorum-sensing signals to regulate antibiotic production

and expression of virulence genes, whereas *B. thuringiensis* strains possess AHL-lactonase, which is a potent AHL-degrading enzyme. *B. thuringiensis* did not seem to interfere with the normal growth of *E. carotovora*; rather, it abolished the accumulation of AHL signal when they were cocultured. The biocontrol efficiency is correlated with the ability of bacterial strains to produce AHL-lactonase.

5.5.5 Ear Rot

The casual organisms of ear rot of corn are *Stenocarpella maydis* and *Stenocarpella macrospora*. The disease is one of the most destructive diseases of maize crop worldwide. These fungi are important mycotoxin producers that also cause different diseases in farmed animals and represent an important risk for humans (Petatan-Sagahon et al. 2011). Petatan-Sagahon et al. (2011) reported bacteria like *Bacillus subtilis*, *Pseudomonas* spp., *P. fluorescens*, and *Pantoea agglomerans* isolated from maize rhizosphere with inhibitory activity against these phytopathogens.

5.6 Commercial Formulations

The availability of the commercial formulation of biocontrol agents (BCAs) in the market for control of plant diseases is still in its infancy due to different degrees of limitations (Fravel 2005; Mercier and Lindow 2001). The problems can be overcome by better understanding of the environmental factors limiting biological control agents, mechanism of interaction among different components of biocontrol system, extensive research, investment in both research and production of low cost commercial formulations, public awareness, etc. (Mercier and Lindow 2001; Heydari et al. 2007; Ardakani et al. 2009). However, the development of commercial formulation of biocontrol agent (BCA) is an important area of microbiological research, concerning mainly on the development of low cost, most effective, and suitable for all types of farming systems and preservation of microbial activity for a period long enough to enable delivery of an effective product for field application (Ramamoorthy et al. 2001).

The most successful and effective product would be the one that can be used for disease management with existing machinery or methods. The biological control agents, therefore, are generally formulated as wettable powders, dusts, granules, and aqueous or oil-based liquid products, with various additives to attain all the desirable attributes along with their biocontrol activity. Nowadays, a number of biocontrol agents are available for the control of fungal plant diseases (Ardakani et al. 2009). Even though many small, privately owned companies with limited product line are developing and are in the process of registration, there still are large companies with diverse product lines that include a variety of agrochemicals, and biotechnological products playing an important role in the development and marketing of products. These products are standalone or formulated as mixture of microorganisms.

The biopesticide industry alliance is establishing a certification process to ensure standards for efficacy, quality, and consistency for lifting the global marketing perception. In this process, it is also important to concentrate on training of growers, formulation of biocontrol microorganisms, and the role of environmental factors (Ardakani et al. 2009). Despite the limitations of commercial formulation, many of the bacterial formulation products are available for control of rice and maize diseases (Table 5.3).

5.7 Limitations of Bacterial Control of Diseases

Biocontrol involves sustained manifestation of interactions among the plant, pathogen, the biocontrol agent, the microbial community, and the physical environment. Due to complex nature of interaction among the components of biocontrol system, it is poorly understood area of research despite of its tremendous unexplored potential in disease management (Handelsman and Stabb 1996). Due to the complexity of the system, variations in the environmental conditions, and influence of many seen/unseen factors, the practical results have been variable.

Thus, despite some spectacular results in biocontrol research, there remains a general skepticism born of past failures (Fravel 2005). The time taken to understand the complete system is slow as compared to the other research areas. Despite the presence of tremendous unexplored knowledge and potential in biological control research, it is one of the slowly advanced areas of research. In addition, there has been relatively little investment in the development of low cost commercially viable products for biological control of diseases, partly due to the high cost of developing, testing efficacy and risk, registering, and marketing of such products (Ardakani et al. 2009). Though extreme research was lacking in this field but in recent years throughout the world, significant work has started, owing to increased consciousness and realization of huge potential of biocontrol agents. The funding agencies coming up for research in biological disease management to know better about biological control agents and their feasibility in disease management (Fig. 5.2).

5.8 Monitoring Biological Control Agents in the Environment

Introduction of biological control agents (BCAs) causes adverse effect on the nontarget native species. Conservationists from different parts of the world worry that nonindigenous species used for disease control can devastate native ecosystems. Howarth (1991) for the first time raised the problem of native species destruction on application of BCA. This gives an alarm for probable extinctions of the micro- and macroflora and hidden potential of biological control agents. The use

Table 5.3 Bacterial biopesticides commercialized for the control of rice and maize plant pathogens (Nakkeeran et al. 2005; Cawoy et al. 2011)

Sl. No.	Product	Bioagent	Diseases/pathogen	Crop	Company	Country
1	Ballad®	*Bacillus subtilis*	Rice blast (*Pyricularia oryzae*); root rot (*Rhizoctonia oryzae*); leaf spot (*Bipolaris maydis*); bacterial spot (*Xanthomonas* spp.)	Rice and others	AgraQuest Inc., USA	USA
2	Biosafe®	*B. pumilus*	Foliar blight	Several crops	Lab. Biocontrole Farroupilha, Brazil	Brazil (not sold, only for use in the company)
3	Biosubtilin	*Bacillus subtilis*	*Fusarium*, *Pythium*	Several crops	Biotech International Ltd.	India
4	Cease®	*Bacillus subtilis*	Stillborn and foliar pathogens	Rice, maize and others	Bio Works Inc., USA	USA, Mexico
5	Companion®	*Bacillus subtilis*	Leaf spot (*Erwinia carotovora*);Root rots (*Fusarium oxysporum*, *Pythium); Seedling blight* (*Rhizoctonia solani*)	Maize, rice, and others	Growth Products Ltd., USA	USA
6	Ecoshot	*Bacillus subtilis*	Ear rots minor (*Botrytis cinerea*)	Maize and others	Kumiai Chemical Industry, Japan	Japan
7	FZB24®WG, FZB24®Ii, FZB24®TB	*Bacillus subtilis*	Root rot (*Rhizoctonia solani*)	Maize, rice, and others	ABiTEP GmbH, Germany	Germany
8	HiSticK N/T®/ Subtilex®/ Pro-Mix®	*Bacillus subtilis*	Rot (*Rhizoctonia solani*, *Pythium*, *Fusarium*)	Maize, rice, and others	Becker Underwood, USA Premier Horticulture Inc., Canada	USA, Canada
9	System 3-*Bacillus subtilis* GB03 and chemical pesticides	*Bacillus subtilis*	Seedling pathogen	Rice and others	Helena Chemical Co., Memphis, USA	USA
10	RhizoVital®42Ii and RhizoVital 42 TB	*B. amyloliquefaciens*	Soilborne pathogens	Maize and others	ABiTEP GmbH, Germany	Germany

(continued)

Table 5.3 (continued)

Sl. No.	Product	Bioagent	Diseases/pathogen	Crop	Company	Country
11	Botrycid®	*B. cepacia*	*Soilborne pathogens*	Maize, rice, and others	Safer Agrobiologicos, Colombia	Colombia
12	Sublic®	*Bacillus* sp.	Root rot	Maize, rice, and others	ELEP Biotechnologies, Italy	Italy
13	Mycostop	*Streptomyces lydicus*	Root rot	Maize, rice, and others	Verdera Oy, Finland	USA, Germany, Canada, Finland
14	Spot-less Biofungicide®	*Pseudomonas aureofaciens*	Rot	Maize and others	Turf Science Laboratories, USA	USA

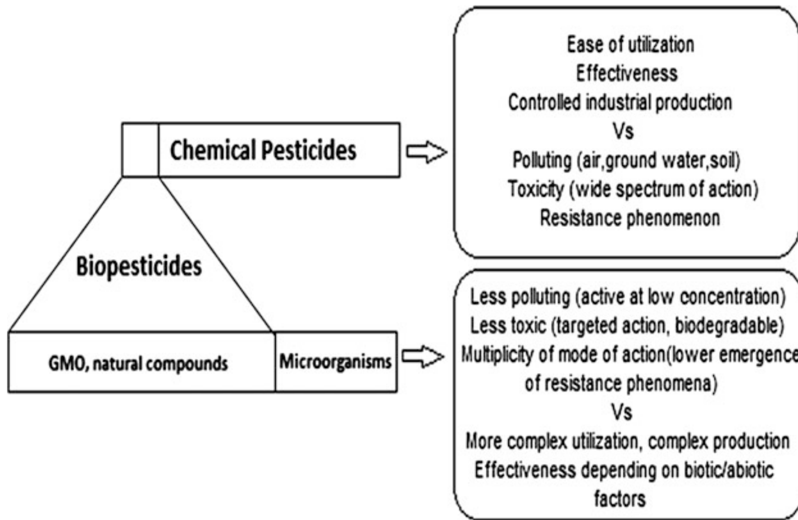

Fig. 5.2 Market share and (dis)advantages of microbial biopesticides versus chemical phytosanitary products (Cawoy et al. 2011)

of practice of biocontrol should be regulated and guided by state laws and specific protocols that are all designed to ensure the safety and effectiveness of the biocontrol program. Australia is the only nation with a specific law for classical biological control, the Australian Biological Control Act of 1984. Its primary task is to bring public awareness and approves upon a finding of no significant harm to any person or to the environment (Delfosse 1985).

The degree of success of a biocontrol-mediated disease management depends upon the biotic, abiotic, and procedural factors. There should be rigorous testing of biocontrol agents (BCAs) for target specificity to ensure that the biocontrol organism attacks only the target organism. An adequate protocol should be followed to prevent future damage.

Specific sets of guidelines for testing, quarantine, release, and target specificity should be followed, and there should be a technical advisory group for monitoring the biocontrol agents. Movement of exotic biocontrol agents across the states must be authorized and approved. The guidelines emphasize predicting the effectiveness of agents, monitoring the consequences of releases (including redistribution of releases), and farming partnerships to ensure adequate monitoring.

To monitor the biological control, the following points need to be clarified: select the biocontrol agents with the potential to control the target pathogen; release only the safe and approved agents; strict legislation should be made to regulate the BCAs; appropriate protocols should be used for release and documentation; monitor the impact on target pathogen; avoid further application of biocontrol agent when effective or control is achieved; and proper assessment of changes in plant and animal communities along with the complete ecosystem should be done.

5.9 Future Prospects and Conclusion

The bacterial control agent will completely replace chemical in foreseeable future and is very doubtful. But we can at least expect some reduction in the use of chemicals. Thus far, most approaches include the single antagonist concept; however, a biological system approach that involves integration of various factors for disease suppression might provide a better alternative. The biological control agents could be used as one component of the integrated management program to achieve the best possible results. Biological control really developed as an academic discipline during the 1970s and is now a mature science supported both in the public and private sectors. Research related to biological control is published in scientific journals. Additionally, some academic journals are specifically devoted to this discipline (Heydari and Pessarakli 2010). In India, several research schemes, institutions, awareness programs, monitoring techniques, funds for boosting agriculture, and small business innovation are taken in hand.

Such ventures are intended to be conduits for academic research that can be used to develop new products of commercial importance (Spadaro and Gullino 2005). Much has been learned from the biological control research conducted over the past 40 years. But, in addition to learning the lessons from the past, biocontrol researchers need to look forward to define new and different questions and the answers to which will facilitate new biocontrol strategies, technologies, and applications. In recent times, advances in computing, molecular biology, analytical chemistry, and statistics have added new dimensions to research that aimed at characterizing the structure and functions of biological control agents, pathogens, and host plants at molecular, cellular, physiological, and ecological levels. Ecological factors play very important roles in the performance and potentiality of biological control agents (BCAs). To develop efficient and effective biological control system, the following factors need to be clarified and studied, viz., (1) distribution of various pathogens and their natural antagonists in the environment; (2) optimum field conditions which influence suppressive capacities of control agents; (3) response of native and introduced populations to different management practices; (4) studies of factors responsible for successful colonization and expression of biocontrol traits in fields like signal molecules of plant and microbial origin that regulate expression of biocontrol traits, mechanism of defense induction in plants; (5) search for more effective strain variants for broad base field applications; (6) use of rDNA technology for manipulation of plants and biological control agents; (7) development of proper formulations to enhance field activities of known biocontrol agents; (8) role of other gene products involved in pathogen suppression; (9) efficacy of using consortium of novel strains in place of individual agents; and (10) development of effective biocontrol–cultivar combinations by plant breeders. In recent times, farmers have become more interested to use biological control rather than chemical control. The biological controls can be expected to play an important role in integrated pest management (IPM). A good agricultural practice, however, includes appropriate site selection, crop rotation,

tillage, fertility, and water management as first line of defense for successful disease management. The use of disease-resistant varieties backs as the next line of defense. The biological control agents should be deployed as the next line of defense (Jacobsen et al. 2004).

However, if all three lines of defense are not sufficient to ensure plant health and production, then less specific and less harmful chemical toxins can also be used. In general, regulatory and cultural concerns about the human health and safety are the primary economic drivers promoting the adoption of biological control strategies in urban and rural landscapes (Heydari and Pessarakli 2010). Keeping in view of the unexplored potentiality of biological control, research in this area is meager. The biological control system for disease management is a complex one; thus, rigorous research and investment are needed to make a safe and effective biocontrol agent. There should be collaboration between all branches of science instead of a standalone research. Researchers get set back due to certain limitation and complexity related to biological control of disease management.

The inhibitory behavior of certain bacteria does not confirm its effective and ideal characteristics. The researchers of different branches from microbiology, pathology, ecology, soil biology, chemistry, molecular biology, chemistry, environmental science, etc. should put their effort and mind together to make revolution in the field of disease management. In the near future, integrated researches in biological control surely take a new era in disease management.

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Chapter 6
Beneficial Bacteria for Biological Control of Fungal Pathogens of Cereals

Mojibur R. Khan

6.1 Introduction

Fourteen crop plants provide the bulk of human food, of which eight are cereals (Strange and Scott 2005). According to an estimate, 10 % of global food production is lost due to plant disease (Strange and Scott 2005). Reducing disease-associated cereal crop losses is key to both increasing yields and providing a steady and healthy food supply to a burgeoning human population. Common practices for controlling plant disease include plant disease resistance breeding, manipulation of plant culture practices and, to a greater extent, the use of synthetic chemicals (Strange 1993). The persistence and long-term toxicity of fungicides to nontarget organisms, including humans, has generated worldwide concern, both societal and scientific, regarding their future use. This has necessitated the re-evaluation of synthetic chemicals as a final solution to pest disease management (Saxena and Pandey 2001). Many of the synthetic chemicals may lose their usefulness due to revised safety regulations, concern over nontarget effects, or development of resistance in pathogen populations (Emmert and Handelsman 1999). Thus there is a need for new solutions to plant disease problems that provide effective control, while minimizing the negative consequences for human health and the environment (Emmert and Handelsman 1999).

Biological control (i.e., using microorganisms to suppress plant disease) offers a powerful alternative to the use of synthetic chemicals. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Increasing the abundance of a particular strain in the vicinity of a plant can suppress disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem (Gilbert et al. 1993). The basic prerequisite for the

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success of a biological control program is good adaptation of the biocontrol agent to the local environmental conditions in which it is to be used (Romero et al. 2004). Idealistically, biological control would be more robust and durable than chemical disease control in cases where the "biocontrol" agent employs numerous mechanisms of disease suppression (Cook 1993).

Until recently, research on biological control of fungal plant pathogens had been confined within the members of the fungal genera *Trichoderma*. However, growing evidence suggests that bacteria have great potential to control fungal plant pathogens. Growing interest among scientists for bacterial biocontrol agents resulted in identification of a range of bacterial species having great potential against plant pathogens. We present here some highlights of global research activities on bacterial biological control of major fungal cereal diseases, commercial biocontrol products based on bacteria, mode of action of antifungal bacteria, screening methods used for selecting the potential biocontrol bacteria, and future challenges and prospects.

6.2 Antifungal Bacteria Against Major Cereal Diseases

Researches around the world have reported antifungal activity of a range of bacterial species against various cereal diseases (Table 6.1). Although members of various bacterial genera have been found to possess cereal disease suppression capability, it seems that those belonging to fluorescent pseudomonads and *Bacillus* spp. are more effective.

6.2.1 Wheat and Barley Diseases

The major fungal wheat diseases are Fusarium head/seedling blight, Septoria tritici leaf blotch, take-all and net blotch disease caused by *Fusarium* species, *Mycosphaerella graminicola*, *Gaeumannomyces graminis*, and *Pyrenophora teres,* respectively. The bacteria *Pseudomonas fluorescens* (strains MKB 100, MKB 158, and MKB 249), *P. frederiksbergensis* strain MKB 202, and *Chryseobacterium* sp. strain MKB 277 were found to be very effective biocontrol bacteria in reducing Fusarium seedling blight disease symptoms in both wheat and barley seedlings under controlled environmental conditions (Khan et al. 2006). *Pseudomonas fluorescens* strain MKB 100 is also very effective against net blotch disease of barley (Khan et al. 2010). In another study, the bacteria *P. fluorescens* (strains MKB 158 and MKB 249) and *P. frederiksbergensis* strain MKB 202 were found to be effective in reducing Fusarium head blight disease symptoms in both wheat and barley plants under both glasshouse and field conditions (Khan and Doohan 2009). They were also effective in restoration of yield of wheat and barley under field conditions. In the same study *P. fluorescens* MKB 158 and MKB 249 were able to reduce the

Table 6.1 Examples of bacteria having biocontrol ability against major cereal diseases

Crop	Plant disease	Pathogen	Biocontrol bacteria	References
Wheat	Fusarium seedling blight	*Fusarium culmorum*	*Pseudomonas fluorescens* (strains MKB 100, MKB 158, and MKB 249); *P. frederiksbergensis* strain 202; *Chryseobacterium* sp. strain MKB 277	Khan et al. (2006)
	Fusarium head blight	*Fusarium culmorum*	*Pseudomonas fluorescens* (strains MKB 158 and MKB 249); *P. frederiksbergensis* strain 202	Khan and Doohan (2009)
	Fusarium head blight	*Fusarium graminearum*	*Lysobacter enzymogenes* strain C3	Jochum et al. (2006)
	Septoria tritici leaf blotch	*Mycosphaerella graminicola*	*Bacillus megaterium* strain MKB135	Kildea et al. (2008)
Barley	Fusarium seedling blight	*Fusarium culmorum*	*Pseudomonas fluorescens* (strains MKB 100, MKB 158, and MKB 249); *P. frederiksbergensis* strain 202	Khan et al. (2006)
	Fusarium head blight	*Fusarium culmorum*	*Pseudomonas fluorescens* (strains MKB 158 and MKB 249); *P. frederiksbergensis* strain 202	Khan and Doohan (2009)
	Net blotch	*Pyrenophora teres*	*Pseudomonas fluorescens* strain MKB 100	Khan et al. (2010)
Rice	Rice blast	*Pyricularia oryzae*	*Streptomyces sindeneusis* isolate 263	Zarandi et al. (2009)
	Rice blast	*Pyricularia oryzae*	*P. fluorescens* strain 7-14	Gnanamanickam and Mew (1992)
	Rice sheath-blight	*Rhizoctonia solani*	*Bacillus megaterium* strain 16	Kanjanamaneesathian et al. (2007)
Maize	Banded leaf and sheath blight	*Rhizoctonia solani*	*Bacillus subtilis* strain br23	Muis and Quimiob (2006)
	Foot rots and wilting	*Fusarium moniliforme*	*Pseudomonas* sp. strain EM85; *Bacillus* spp. strains MRF and MR-11(2)	Pal et al. (2001)
	Collar rots/stalk rots and root rots and wilting	*Fusarium graminearum*	*Pseudomonas* sp. strain EM85; *Bacillus* spp. strains MRF and MR-11(2)	Pal et al. (2001)
	Charcoal rots	*Macrophomina phaseolina*	*Pseudomonas* sp. strain EM85; *Bacillus* spp. strains MRF and MR-11(2)	Pal et al. (2001)
	Preemergence damping-off	*Pythium ultimum*	*Pseudomonas fluorescens* strain AB254	Callan et al. (1990)

mycotoxin contamination of wheat and barley flour. Jochum et al. (2006) observed that treatment of wheat spikelets with the bacterium *Lysobacter enzymogenes* strain C3 significantly reduced Fusarium head blight disease symptoms in greenhouse tests. Kildea et al. (2008) observed that the bacterium *Bacillus megaterium* strain MKB 135 could significantly inhibit the Septoria tritici leaf blotch disease of wheat under field conditions.

6.2.2 Rice Diseases

Blast disease of rice caused by the fungus *Pyricularia oryzae* is considered to be one of the major diseases of rice. Zarandi et al. (2009) observed that spraying of rice seedlings with *Streptomyces sindeneusis* isolate 263 resulted in the reduction of blast disease symptoms under glasshouse conditions. Earlier, Gnanamanickam and Mew (1992) observed that spraying of rice seedlings with a cell suspension of the bacterium *P. fluorescens* strain 7-14 reduced the blast disease symptoms under field conditions. Another major rice disease is sheath blight caused by the fungus *Rhizoctonia solani*. Kanjanamaneesathian et al. (2007) reported that spraying of *Bacillus megaterium* strain 16 was as effective as the fungicide “Iprodione” in reducing the percentage of rice seedling with sheath blight disease symptoms.

6.2.3 Maize Diseases

Biocontrol activity of bacteria has been found against several maize diseases. Muis and Quimio (2006) observed that treatment of maize seeds with *Bacillus subtilis* strain br23 could significantly restore grain yield of maize in banded leaf and sheath blight infested field plots and its effect was better than the fungicide “captan” used for seed treatment. Pal et al. (2001) reported that bacterial genera, namely, *Pseudomonas* sp. EM85 and *Bacillus* spp. MRF and MR-11(2) could significantly reduce foot rots and wilting, collar rots/stalk rots and root rots and wilting, and charcoal rots of maize under field conditions. Callan et al. (1990) found that *P. fluorescens* AB254 could provide protection against preemergence damping-off in naturally infested soil.

6.3 Mode of Action

For a successful biological control program against fungal cereal diseases, it is very important to understand the mode of action of potential biocontrol bacteria. A biocontrol bacterium may employ different mechanism(s) to antagonize a pathogen such as by competition, antibiosis, and elicitation of induced systemic

resistance (ISR) in the host plant. A biocontrol bacterium may compete against a pathogen for space and/or nutrients. A fast growing bacterium may outgrow the pathogen and thus restrict the growth of a pathogen on or around the plant. Moreover, bacteria may compete for essential nutrients with the pathogen. Members of *Pseudomonas* species and *Bacillus* species produce a range of different antibiotics against plant pathogens which might contribute to their disease suppression effect (reviewed by Shoda 2000; Haas and Defago 2005). Members of *Pseudomonas* species produce several types of antibiotics including phloroglucinols, pyrolnitrin, pyoluteorin, cyclic peptides, phenazines besides siderophore and production of volatile antibiotic hydrogen cyanide, which may contribute to disease suppression effect as well. Members of *Bacillus* species produce several types of antibiotics including siderophores, lipopeptides, bacillomycin, iturin, mycosubtilin, bacilysin, fengymycin, and mycobacillin. A biocontrol bacterium may suppress the plant disease by eliciting ISR mechanism in the plant, in which due to colonization of the biocontrol bacteria the plant develops resistance against invading pathogens. Members of *Pseudomonas* species and *Bacillus* species have been shown to elicit ISR mechanism in different plant species against various fungal pathogens.

6.4 Screening Methods

Research on biological control starts with the generation of a bacterial culture collection and screening of the isolates against the pathogen. Conventionally, the bacterial isolates are co-cultivated on agar plates along with the pathogen (Fig. 6.1). If the growth of the pathogen is inhibited due to presence of any bacterium, then it is thought to have antagonistic activity and further tested in planta. However, this method has severe drawback as it can detect only direct antagonism mediated by secretion of antibiotic(s) in the agar plate. Moreover, these in vitro tests cannot mimic exactly the environmental conditions on the plants in the field where the biocontrol agent will encounter the pathogen. On the contrary, a potential biocontrol bacterium may not show in vitro inhibitory activity against a pathogen. Furthermore, in vitro dual culture tests cannot detect the other two modes of bacterial antagonism, i.e., competition for space and nutrients and elicitation of ISR. Ideally any screening tests for potential biocontrol bacteria should include the four elements, viz. plant, pathogen, bacterial isolate, and the proper environment where the disease occurs (i.e., field tests). This is practically impossible while screening a large collection of bacteria. The next option will be glasshouse tests in pots including all the four elements. If the second option is also not possible, then at least an in vitro test should include at least plant (tissue such as seed or leaf segment), pathogen, and bacterial isolate (Fig. 6.2). This test will still exclude the “environment” element. It will save time and space for screening a large bacterial collection. However, the bacteria which show disease suppressing effect in such test should then be tested in the glasshouse and field. For example, in our in vitro studies *P. fluorescens* MKB 156

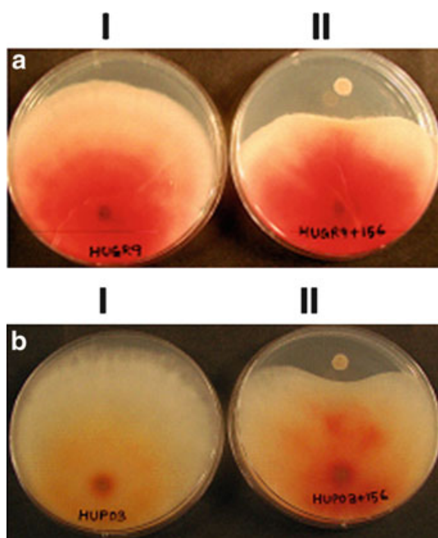

Fig. 6.1 Growth inhibition of *Fusarium* spp. by *Pseudomonas flourescens* (strain MKB 156) in dual cultures tests. *F. graminearum* (strain HUGR9) (**a**) and *F. poae* (strain HUPO3) (**b**) grown in the absence (**I**) and presence (**II**) of *Pseudomonas fluorescens* (strain MKB 156) for 7 days on potato dextrose agar plates

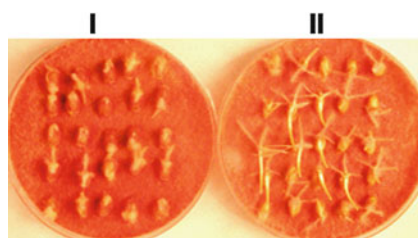

Fig. 6.2 Bacterial inhibition of in vitro coleoptile retardation of germinating wheat (cv. GK-Othalom) seeds caused by *Fusarium culmorum* (strain FCF 200). Effect of seed treatment with *Pseudomonas flourescens* (strain MKB 158) on *F. culmorum*-induced coleoptile growth retardation. Nontreated (**I**) and bacterial-treated (**II**) seeds were germinated on *F. culmorum*-inoculated potato dextrose agar plates and photographed 4 days postincubation

has shown growth inhibitory activity against *Fusarium* species, the pathogens of Fusarium seedling blight disease (Fig. 6.1) (Khan et al. 2006). However, this bacterium is unable to control Fusarium seedling blight disease (Khan et al. 2006) in glasshouse conditions. On the other hand, *P. fluorescens* MKB 158 can inhibit both *Fusarium* seedling and head blight diseases of cereals, but did not show any inhibitory activity in dual culture tests against the same pathogens (Khan et al. 2006; Khan and Doohan 2009).

6.5 Commercial Biocontrol Products

Research on biological control of plant diseases is relatively new compared to pest control. Despite this, a few commercial products are already available in the market against cereal diseases. We have presented some examples of the commercially available bacterial biocontrol products which can be used against fungal cereal diseases in Table 6.2. The bacterium *Burkholderia cepacia* is in the market under the trade name "Deny" (Helena Chemical Company, TN, USA) which can be used to control wheat and barley diseases caused by the fungal genera *Rhizoctonia, Pythium*, *Fusarium*. Pratibha Biotech (Hyderabad, India) has been marketing a *P. fluorescens* under the trade name "Flick" which can be used to control blast, sheath blight, sheath rot, brown spot, and seedling rot diseases of rice. Scientific Agriculture Laboratory (Madurai, India) has a product based on a *P. fluorescens* under the trade name "Fluroissal" which can be used to control root rot, stem rot, and wilt diseases of rice, wheat, and maize. Biotech International Ltd. (Greater Noida, India) has a product based on a *P. fluorescens* under the trade name "Biomonas" which can be used to control cereal diseases caused by *Rhizoctonia*, *Pythium*, *Fusarium*. The same company markets another bacterium *Bacillus subtilis* under the trade name "Biosubtilin" which can be used to control general fungal diseases of cereals. Jay Bio Tech (Pune, India) has a product based on a *P. fluorescens* under the trade name "Jay-Pseudo" which can be used to control blast, sheath blight of rice. BioAgri AB (Stockholm, Sweden) markets a *Pseudomonas chlororaphis* under the trade names "Cedomon" and "Cerall" which can be used to control diseases caused by *Fusarium* spp. in wheat, rye, and triticale.

6.6 A Case Study: Biological Control of Fusarium Diseases of Cereals

6.6.1 Diseases Caused by Fusarium *species*

Fusarium fungi can cause diseases on most cultivated plants, including all members of the Gramineae (Parry et al. 1995). *Fusarium* spp. cause seedling blight, foot rot, and head blight [Fusarium head blight (FHB)] diseases of cereals (Parry et al. 1995). Seedling blight and foot rot diseases cause extensive damage to growing seedlings (Wiese 1977) and lead to a reduction in plant establishment, number of heads, and grain yield (Wong et al. 1992; Humphreys et al. 1998). FHB is a major cereal disease worldwide (McMullen et al. 1997). *Fusarium* infection of cereal heads leads to a reduction in the yield and quality of the cereal grains (Pirgozliev et al. 2003). The infected grains carry over inoculum that can cause Fusarium seedling blight when such seeds are sown (Winson et al. 2001). *Fusarium* spp. can produce a wide range of toxins [i.e., deoxynivalenol (DON)] in the infected heads

Table 6.2 Examples of commercial bacterial biocontrol agents available against cereal diseases

Product name	Active ingredient	Active against cereal diseases	Company
Deny	*Burkholderia cepacia*	Barley and wheat diseases caused by *Rhizoctonia, Pythium, Fusarium*	Helena Chemical Company 225 Schilling Blvd., Collierville, TN 38017 USA
Flick	*Pseudomonas fluorescens*	Blast, sheath blight, sheath rot, brown spot, and seedling rot diseases of rice	Prathibha Biotech 5-5-35/75, Kukat Pally, Hyderabad, India
Fluroissal	*Pseudomonas fluorescens*	Root rot, stem rot, wilt diseases of rice, wheat, and maize	Scientific Agriculture Laboratory 3/321, Kavimani Street, Indian Bank Colony, Narayanapuram, Madurai-625014, Tamil Nadu, India
Biomonas	*Pseudomonas fluorescens*	Cereal diseases caused by *Rhizoctonia*, *Pythium*, *Fusarium*	Biotech International Ltd. No. B-2160-C, Surajpur, Greater Noida-301306, Uttar Pradesh, India
Biosubtilin	*Bacillus subtilis*	Fungal diseases of cereals	Biotech International Ltd. No. B-2160-C, Surajpur, Greater Noida-301306, Uttar Pradesh, India
Jay-Pseudo	*Pseudomonas fluorescens*	Blast, sheath blight of rice	Jay Bio Tech 32, Market Yard, Gultekdi, Pune-411037, Maharashtra, India
Cedomon and Cerall	*Pseudomonas chlororaphis*	Diseases caused by *Fusarium* spp. in wheat, rye, and triticale	BioAgri AB Uppsala Stockholm, SE-75109, Sweden

which are hazardous to animal and human health (Placinta et al. 1999). There has been limited success in controlling *Fusarium* diseases of cereals by cultural, genetical, and chemical measures (Pirgozliev et al. 2003). Recently, biological control has shown some promise against *Fusarium* diseases of cereals (Johansson et al. 2003; Khan et al. 2006; Khan and Doohan 2009).

6.6.2 Global Scenario

During 1942, a severe FHB outbreak in Ireland decreased wheat yield by up to 55 % (McKay 1957). A second outbreak during 1954 was responsible for wheat and oat yield reductions up to 50 % (McKay 1957). Severe infestations were widespread in wheat crops in England in 1982, 1992, and 1993 (Parry et al. 1984; Jennings and Turner 1996). In Romania, Tusa et al. (1981) and Munteanu et al. (1972) reported

that, in epidemic years, FHB of wheat caused losses of approximately 40 % in some regions of the country, with up to 70% yield loss recorded in some fields. In Hungary, according to Kukedi (1972), wheat yields were depressed by 40–50 % in some areas following a severe attack of FHB in 1970. A survey carried out between 1951 and 1985 in Yangtze river valley of China recorded 19 FHB outbreaks, with grain yields of wheat reduced by 5–15 % in years when moderate epidemics of FHB were recorded and up to 40 % in years when disease epidemics were severe (Zhuping 1994). During 1980, in the Atlantic Provinces of Canada, FHB was responsible for between 30 % and 70 % wheat yield loss (Martin and Johnston 1982). FHB epidemics in wheat and barley occurred in southern Idaho in 1982 and 1984 and resulted in estimated yield losses as high as 50 % (Michuta-Grimm and Foster 1989). In the USA, FHB has reached epidemic levels in several years during the last decade, causing yield losses and discounted prices were paid for the reduced quality seed (Windels 2000). From 1998 to 2000 direct and secondary economic losses due to FHB for all crops in the Northern Great plains and Central USA were estimated to be worth $2.7 billion (Nganje et al. 2002).

6.6.3 Biological Control

It is surprising that there are so few reports of biocontrol of FHB, given the importance of the disease. It could be presumed that the short time period during which cereal heads are sensitive to the disease could offer an ideal opportunity for a biological solution to the FHB problem and would avoid the hazards associated with late fungicide application (Parry et al. 1995). Although biocontrol using either microorganisms or biochemicals offers a positive alternative to chemical pesticides, the overall contribution of biocontrol represents about 1 % of agricultural chemical sales, whereas fungicides represent approximately 15 % of pesticide sales (Lidert 2001; Fravel 2005). No commercial biocontrol product has yet been released for the control of FHB disease of cereals, but there is experimental evidence that indicates that this is a feasible disease control strategy.

There has been very limited research on biological control Fusarium seedling blight disease of cereals. In a screen for potential disease control organisms and agents, the bacteria *P. fluorescens* (strains MKB 100, MKB 158, and MKB 249), *P. frederiksbergensis* strain MKB 202, and *Chryseobacterium* sp. strain MKB 277 significantly reduced the extent of wheat and barley seedling blight disease symptoms caused by *F. culmorum* (up to 91 % reduction) (Khan et al. 2006). Strains of *Bacillus cereus* and *Stenotrophomonas maltophilia* have also been shown to reduce Fusarium seedling blight disease caused by *F. graminearum* under glasshouse conditions (Bello et al. 2002). In Sweden, Johansson et al. (2003) reported that treatment of winter and spring wheat with selected isolates of fluorescent pseudomonads and *Pantoea* sp. suppressed seedling blight of wheat caused by *F. culmorum* and *M. nivale* as effectively as did the fungicide guazatine in repeated glasshouse and field trials (by >85 %, relative to control treatments).

The bacteria, *P. fluorescens* strains MKB 158 and MKB 249 and *P. frederiksbergensis* strain 202 were capable of reduced both the severity of FHB disease symptoms caused by *F. culmorum* on wheat and barley ($\geq$23 %; $P \leq 0.050$) and the disease-associated loss in 1,000-grain weight ($\geq$16 %; $P \leq 0.050$) under both glasshouse and field conditions when applied 24 h prepathogen inoculation (Khan and Doohan 2009). Glasshouse studies showed that these bacteria were more effective in controlling disease when applied 24 h pre- as opposed to 24 h postpathogen inoculation. The most striking finding was that, in the *F. culmorum*-inoculated field trials, treatment with either of the two *P. fluorescens* strains (MKB 158 or MKB 249) also significantly reduced the DON levels in wheat and barley grain (74–78 %; $P \leq 0.050$). This was the first report detailing the ability of fluorescent pseudomonad bacteria to control FHB disease and simultaneously reduce mycotoxin contamination of wheat and barley under field conditions.

Interestingly, the bacterium *P. fluorescens* strain MKB 158 caused a suppression of expression of key *Fusarium* gene (Trichodiene synthase) involved in trichothecene mycotoxin biosynthesis in the infected stem base tissue of wheat and augmented expression of a wheat class III plant peroxidase gene (a pathogenesis-related plant defense gene). A soil inoculation test showed that this bacterium can control wheat and barley seedling blight disease symptoms when spatially separated from the pathogen which indicated that it can elicit ISR mechanism in the seedling against the disease. Subsequent functional genomics analysis in our laboratory revealed that MKB 158-mediated ISR against *Fusarium* in the barley seedling takes place involving novel plant hormone-mediated pathways (Khan et al. unpublished data). Further research is underway in this line to understand the exact role played by these hormones in barley defense against *Fusarium*. We have not confirmed yet whether the strain MKB 158 can elicit similar ISR against FHB disease as well. Our preliminary functional genomic studies indicate that at least it can elicit a local resistance mechanism with upregulation of many wheat genes in the heads (Petti et al. 2010). The bacterium significantly affected the accumulation of 1,203 barley transcripts associated with diverse functions, including detoxification, cell wall biosynthesis, and the amplification of host defense responses. The transcriptome studies also revealed new insights into bacterium-mediated priming of host defenses against necrotrophs, including the positive effects on grain filling, lignin deposition, oxidative stress responses, and the inhibition of protease inhibitors and proteins that play a key role in programmed cell death.

6.7 Future Challenges and Prospects

Intensification of crop cultivation to feed the burgeoning human population demands use of chemicals for controlling cereal diseases. Growing concern about the effects of chemicals has led to increase in demands for organic products throughout the globe. Although research on biological control of plant diseases

involving fungi is quite old, there is no commercial biocontrol product available that can substitute chemicals. Growing evidence suggests the potential of bacteria to control fungal cereal diseases. However, more research is necessary to find suitable candidate for each fungal disease which should be able to control diseases in variable environmental conditions as per with chemical agents. Research should target the diverse bacterial populations throughout the globe to find suitable agents, acknowledging the fact that conventional techniques have targeted only the culturable bacteria which constitute only less than 1 % in any given habitat. Therefore, advance molecular tools such as metagenomic research should be employed to explore for potential antifungal genes among the unculturable bacteria.

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Chapter 7
Biological Control of Peronosporomycete Phytopathogen by Bacterial Antagonist

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7.1 Introduction

The peronosporomycetes are phylogenetic relatives of brown algae and diatoms under the kingdom of Straminipila (Dick 2001). They are devastating pathogens of plants, animals, fishes, crustaceans, and microorganisms (Margulis and Schwartz 2000). Several species of this group of microorganisms such as *Phytophthora infestans* and *Plasmopara viticola* are listed among the top ten economically most important plant pathogens, resulting in multibillion-dollar crop losses worldwide (Agrios 1997; Haverkort et al. 2008). *Phytophthora* spp. have long been recognized worldwide as destroyer of plants. About 80 identified species of *Phytophthora* are causing some of the world's most economically important and devastating diseases in over 2,000 plant species (Abad et al. 2008). Among them, *Ph. infestans*, the cause of potato and tomato late blight disease was responsible for the Great Irish Potato Famine in the mid-nineteenth century. This disease is still widespread throughout potato-growing regions of the world and is virtually impossible to grow potato without some form of late blight disease control. Several other species such as *Ph. cinnamomi*, *Ph. capsici*, *Ph. megasperma*, *Ph. parasitica*, *Ph. erythroseptica*, *Ph. fragariae*, and *Ph. palmivora* are also extremely destructive on their hosts causing primarily root and lower stem rots, but also some cankers, twig blights, and fruit rots (Yang et al. 1994; Carruthers et al. 1995; Valois et al. 1996; Ko et al. 2009; Schisler et al. 2009; Timmusk et al. 2009). One of the dangerous aspects of *Phytophthora* pathogens is emergence of new and more virulent species. Record of new species of *Phytophthora* has become customary from new hosts as well as from new countries or even continents.

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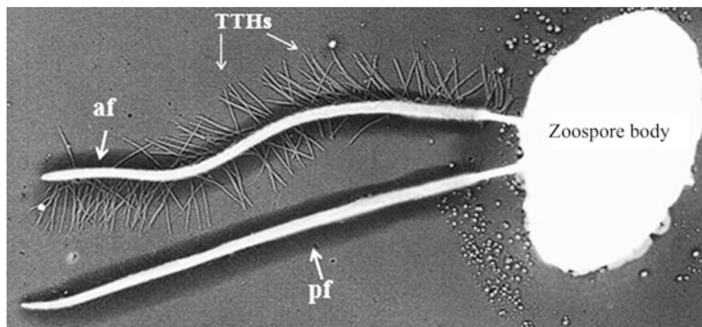

Fig. 7.1 Transmission electron micrograph (TEM) of a glutaraldehyde-fixed *Aphanomyces cochlioides* zoospore with flagella. *af* Anterior flagellum ornamented with two rows of tubular hairs, *pf* Posterior flagellum covered with very fine hairs, *TTHs* Tripartite tubular hairs on an anterior flagellum

The soilborne phytopathogenic peronosporomycetes such as *Pythium* spp. and *Aphanomyces* spp. cause devastating damping-off as well as root rot of seedlings in many crop and forest plants (Whipps and Lumsden 1991). They attack seeds after planting and rot them before they germinate. Moreover, root and crown rot caused by *Pythium* spp. and *Aphanomyces* spp. has become an increasing problem in different crops (Deora et al. 2006; Timmusk et al. 2009) and resulting inadequate plant stand in both nursery and field. Another historically infamous peronosporomycete is *Plasmopara viticola*, a causal agent of downy mildew disease of grapevine, which almost inundated the European wine industry in late nineteenth century. This obligate biotrophic pathogen was introduced into Europe from North America in 1976 (Gobbin et al. 2006). Downy mildews caused by various species of biotrophic peronosporomycete genera such as *Plasmopara*, *Basidiophora*, *Hyaloperonospora*, *Sclerospora*, etc. are serious pathogens of many crops and responsible for substantial amount of annual yield losses (Shetty et al. 1995). Because of the obligate parasitic mode, they are difficult to study at molecular level. Hence, our knowledge on their biology is very limited.

The peronosporomycetes infect their host plants through asexually generated characteristic biflagellate motile zoospores (Fig. 7.1). The zoospores have powerful sensory transduction system to locate and aggregate potential infection sites of the host and then rapidly undergo necessary morphological alterations for invading host tissues (Islam et al. 2001, 2002). The infection cycles of the peronosporomycetes are extremely rapid, which result epidemic for large area of crops within a few days under favorable environment. Despite the economical and environmental importance of the peronosporomycete diseases, they are difficult to control due to their unique cellular features, such as cell wall composition and/or lack of sterol metabolism (Nes 1987). Rapid development of resistance against agrochemical is also complicated in traditional agrochemical-based plant protection approaches against the peronosporomycetes. Moreover, the deleterious effects and consequences of the use of synthetic chemicals to the environment and nontargeted organisms are also discouraging their

use against the peronosporomycetes. Therefore, development of effective biologically rational management strategies against the peronosporomycete phytopathogens are badly needed.

Biological control involves the use of organisms, their products, genes, or gene products to control undesirable organisms (pests) and favor desirable organisms, such as crops, trees, beneficial organisms, and insects (National Academy of Sciences 1987). The organism that suppresses the disease or pathogen is referred to as the biological control agent (BCA). In last three decades, we have witnessed a dramatic development in research on biological control of plant diseases including those caused by the peronosporomycetes. Plant pathologists have been fascinated by the perception that disease suppressing soil or antagonistic plant-associated microorganisms could be used as environment-friendly biocontrol agents (Haas and Defago 2005). The concept of biological control is becoming popular not only because of increasing public concern about the use of hazardous chemical pesticides, but also uncertainty or inefficiency of current disease control strategies against the peronosporomycetes (Cook 1993; Islam et al. 2005a, b). Biological control strategies attempt to enhance the activities of BCA either by introducing high populations of a specific BCA or by enhancing the conditions that enable a BCA in their natural habitat to suppress the diseases (Nelson 2004). BCAs are easy to deliver, increase biomass production, and yield and improve soil and plant health (Burr et al. 1978; Kloepper et al. 1980b; Stockwell and Stack 2007). Isolation and characterization of new potential BCAs and understanding their ecology, behavior, and mode of action are considered as major foci in current biocontrol research (Islam et al. 2005b, 2011). The rapid development of convenient techniques in molecular biology has revolutionized this field by facilitating the identification of the underlying molecular mechanism of pathogen suppression (Islam et al. 2005b, 2011; Islam 2008) and by providing means for construction of "superior" BCAs through genetic engineering (Fenton et al. 1992; Bainton et al. 2004).

A large body of literature indicates that biological control agents such as bacterial antagonists can significantly suppress the disease caused by peronosporomycete phytopathogens and increase the yield of crops. Bacterial antagonists commonly studied and deployed for the control of peronosporomycete diseases include *Pseudomonas*, *Bacillus*, *Burkholderia*, *Lysobacter*, *Actinobacter*, *Enterobacter*, *Paenibacillus*, and *Streptomyces* (Anjaiah et al. 1998; Handelsman et al. 1990; Liu et al. 2007a; Islam et al. 2004, 2005b, 2011). Suppression of pathogens or diseases by the biocontrol agents is accomplished by several ways, such as production of antibiotics or lytic enzymes (Osburn et al. 1995; Palumbo et al. 2005; Perneel et al. 2008; Islam et al. 2011), competition for specific nutrient (e.g., iron or carbon) (van Dijk and Nelson 1998; Heungens and Parke 2000; Lee et al. 2008), induction of systemic resistance in the host plants (Yan et al. 2002; Zhang et al. 2010), and parasitizing pathogen's hyphae (Tu 1978) and/or reproductive structures (Khan et al. 1997).

Although several good reviews on biocontrol of plant diseases have been published (McSpadden Gardener and Fravel 2002; Compant et al. 2005; Haas and Defago 2005; Weller 2007), however, there is no review so far been published

specifically on biocontrol of peronosporomycete phytopathogens by bacterial antagonists. Recently, potentials for biological control of plant diseases by *Lysobacter* spp. (Islam 2011) and *Bacillus* spp. (Borriss 2011) have been reviewed. In this chapter, we attempt to review current knowledge on biocontrol of peronosporomycete phytopathogen by the bacterial antagonists. This review covers activities of BCAs against pathogens in both *in vitro* and *in vivo* conditions. The mode of action and application of BCAs in the practical field are also discussed.

7.2 Bioassay Methods to Screen Antagonistic Bacteria, Detect and Identify Antiperonosporomycetal Compounds

Selection of a potential isolate is the first and foremost important step in biological control by bacterial antagonist. Isolation of bacteria in a suitable culture medium from soil, plant, and water generates huge number of taxonomically diverse population. It is always challenging for researchers to find a convenient method to screen these vast population of isolated bacteria and identify the potentially active strain based on *in vitro* antagonism against a target pathogen. Over the years, some convenient *in vitro* bioassay methods have been developed in different laboratories. Some widely used bioassay methods are briefly discussed in this section.

Among the various bioassay methods, dual culture assay is a relatively simple and rapid screening method, which involves the cocultivation of two organisms on a soft agar medium in a Petri dish (Fig. 7.2). The area of the inhibition zone is taken as a measure of antagonistic potential of the isolates. However, microscopic observation on growth inhibited hyphal tips reveals that antagonistic bacteria exert diverse morphological alterations on the approaching hyphae and thus inhibit normal polar growth of the peronosporomycetes which can be seen in naked eye (Deora et al. 2006). The diversity of morphological alterations in the affected hyphae appears to be associated with the mode of action of antagonism by the bacteria (Fig. 7.2).

Other bioassay methods for assessment of activities of BCAs include (1) double layer agar method (Kraus and Loper 1992); (2) homogeneous solution method for motility and viability assay of zoospores (Islam et al. 2005b, 2011); (3) detached leaf assay (Islam et al. 2011); (4) inverted lid method (Ongena et al. 1999); (5) hyphal column assay (Yang et al. 1994); (6) bioassay to detect antiperonosporomycetal substances such as thin layer chromatography (TLC) (Islam et al. 2005b, 2011), high performance liquid chromatography (HPLC) (Nakayama et al. 1999), gas chromatography (GC) (Ko et al. 2009); (7) test with negative mutants (Becker and Cook 1988); (8) molecular detection of antibiotic biosynthesis genes (Chung et al. 2008); (9) use of antibiotic gene transcription in situ (Meyer et al. 2010); (10) lytic enzyme assay (Ko et al. 2009); (11) antiperonosporomycetal protein assay (Woo et al. 2002); (12) detection of siderophores (Schwyn and Neilands 1987); and (13) advanced microscopic study to understand mode of action (Islam 2008, 2010).

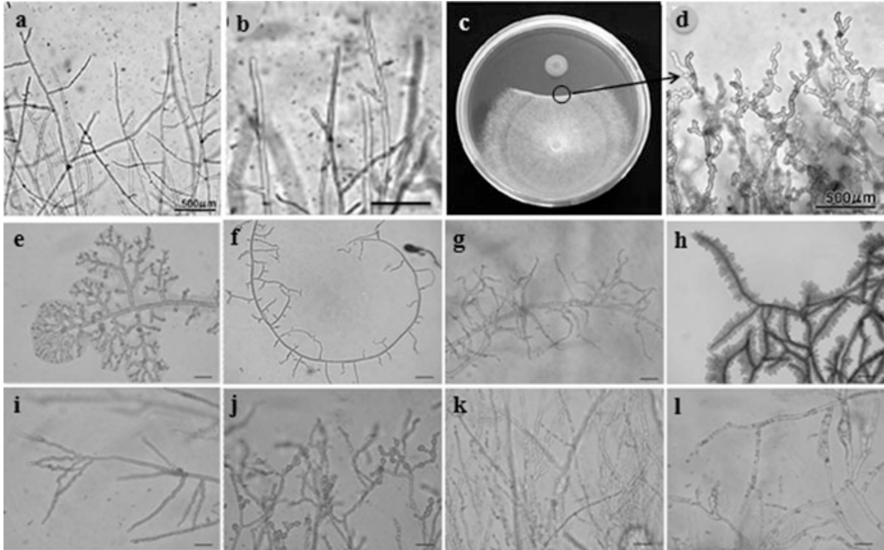

Fig. 7.2 Morphological alterations due to *in vitro* interactions between antagonistic bacteria and peronosporomycete phytopathogens in a dual culture on agar plate (adapted from Islam et al. 2005b and Deora et al. 2006). (**a**), (**c**)–(**h**) *Aphanomyces cochlioides* AC-5, and (**b**), (**i**)–(**l**) *Pythium aphanidermatum* PA-5. (**a**) Normal hyphal growth of AC-5; (**b**) normal hyphal growth of PA-5; (**c**) inhibition of AC-5 mycelial growth in the presence of *Lysobacter* sp. SB-K88 (Islam et al. 2005b); (**d**) curly growth of AC-5 hyphae approaching an SB-K88; (**e**) *Pseudomonas jessenii* strain EC-S101: excessive hyphal branching or hyperbranching; (**f**) *Stenotrophomonas maltophilia* EC-S105: curling; (**g**) *Delftia* sp. EC-107: longer and pointed tip with irregular growth; (**h**) *Bacillus subtilis* EC-S108: colonization of bacteria on hyphae; (**i**) *P. jessenii* EC-S101: apical branching; (**j**) *Pseudomonas* sp. EC-S102: swelling; (**k**) *Pseudomonas* sp. EC-S102: extensive vacuolation; (**l**) *Bacillus subtilis* EC-S108: decrease in normal branching, swollen hyphae and necrosis. Scale bars, (**e**)–(**l**) 50 μm

7.3 *In Vivo* Disease Suppression by Various Biocontrol Bacteria

Bacteria from diverse origins and taxonomic genera such as *Bacillus*, *Pseudomonas*, *Streptomyces*, *Burkholderia*, *Lysobacter*, and *Enterobacter* have been shown high potentials to suppress various plant diseases caused by the peronosporomycete phytopathogens (Table 7.1). Literature of some notable groups or genera of biocontrol bacteria are reviewed in this section.

7.3.1 Pseudomonas spp.

Pseudomonas spp. are most researched BCAs against peronosporomycete phytopathogens. Members of this bacterial genus are ubiquitous in soils and rhizosphere of plants. They have many traits that make them one of the best

Table 7.1 List of biocontrol bacteria, source of isolation, target pathogens, and mode of application on suppression of plant diseases caused by the peronosporomycete phytopathogens

Species/strain	Source	Mode of application	Target pathogen	Disease	Reference
Acinetobacter sp. LCH001	*Cinnamomum camphora* stems	–	*Ph. capsici*	–	Liu et al. (2007a)
Actinoplanes spp.	–	Sporangia on clay granules or as a root dip	*Py. ultimum*	Damping-off and root rot of Geraniums and poinsettias	Filonow and Dole (1999)
		Sporangia on clay granules	*Py. ultimum*	Damping-off of table beet and bush bean	Khan et al. (1997)
Ac. campanulatus, *Micromonospora chalcea*, *Streptomyces spirali*	Cucumber roots	Pruned root-dip method	*Py. aphanidermatum*	Damping-off and crown root rot of cucumber	El-Tarabily et al. (2010)
Bacillus cereus UW85	Roots of alfalfa	Seeds coated with UW85 grown in TSB, CB, or Min 3C and dried. Methyl cellulose is used as adhesive	*Ph. megasperma* f. sp. *medicaginis*	Damping-off of alfalfa	Handelsman et al. (1990), Silo-Suh et al. (1994)
		Spore-based formulation	*Ph. sojae*	Damping-off and root rot of soybean	Osburn et al. (1995)
Ba. megaterium	Rhizosphere of pepper plants	Seedlings drench with bacterial inoculum	*Ph. capsici*	Blight or crown blight disease on pepper plants	Akgül and Mirik (2008)
Ba. mycoides MW27	Pea rhizosphere soil	Prill, granule, and seed coat formulations	*A. euteiches*	Root rot of pea	Wakelin et al. (2002)
Ba. pumilus SE34 *Ps. fluorescens* 89B61	–	Bacterial suspensions to soilless growth media	*Ph. infestans*	Late blight of tomato	Yan et al. (2002)
Ba. pumilus INR7	–	Seed treatment with INR7	*Pl. halstedii*	Downy mildew of sunflower	Nandeeshkumar et al. (2008)
Ba. subtilis BBG100 (transformant)	*Ba. subtilis* ATCC 6633	Seed treatment with BBG100	*Py. aphanidermatum*	Damping-off of tomato	Leclère et al. (2005)

Ba. subtilis HS93	Roots of pepper plants	Seeds soaked in saccharose suspension containing bacteria	*Ph. capsici*	*Phytophthora* root rot of sweet pepper	Sid Ahmed et al. (2003a)
		Seed treatment and root drenching with bacteria or its chitin formulation	*Ph. capsici*	*Phytophthora* root rot of pepper plant	Sid Ahmed et al. (2003b)
Ba. subtilis strain M4	Soil	Seeds treated with vegetative cells of bacteria	*Py. aphanidermatum*	Damping-off of tomato	Ongena et al. (2005)
Ba. subtillis	Rhizosphere soil of healthy red pepper	Seeds soaked in bacterial suspension	*Ph. capsici*	*Phytophthora* blight of red pepper	Lee et al. (2008)
Ba. subtilis cot1	Contaminated *Continus*-tissue culture	Dipping microplants and seedlings into bacterial suspension	*Py. ultimum* *Phytophthora* spp.	Damping-off of *Astilbe*, *Photinia*, *Hemerocallis*, and *Brassica*	Berger et al. (1996)
Ba. subtilis ME488	Soil	Seed treated with bacteria in methyl cellulose	*Ph. capsici*	*Phytophthora* blight of red pepper	Chung et al. (2008)
Ba. subtilis KS1	Grape berry skin	–	*Pl. viticola*	Downy mildew of grapevine	Furuya et al. (2011)
Ba. subtilis (commercial formulation serenade)	Commercial formulation	Spray bacterial suspension	*Ph. infestans*	Potato late blight	Stephan et al. (2005)
Ba. safensis, *Lysinibacillus boronitolerans*, *Ba. subtilis* subsp. *subtilis*, *Ba. pumilus*, *Ba. macauensis*	Roots of soybean seedling	Soil drench for all strains. Seed treatment for strain 1PC-11 (one or more strain separately or jointly)	*Ph. capsici*	*Phytopthora* blight of squash plant	Zhang et al. (2010)
Bu. cepacia AMMD, *Ps. fluorescens* PRA25	Rhizosphere of pea	Seed mixed with bacteria and then air-dried	*Pythium* spp.	Preemergence damping-off	Parke et al. (1991), Bowers and Parke (1993), King and Parke (1993)

(continued)

Table 7.1 (continued)

Species/strain	Source	Mode of application	Target pathogen	Disease	Reference
Bu. cepacia AMMDR1	Pea rhizosphere	Seed treatment or root dip inoculation	*Py. aphanidermatum*	Preemergence damping off of pea	Heungens and Parke (2000)
			Py. aphanidermatum *A. euteiches*	Postinfection stage of preemergence damping-off and root rot of pea	Heungens and Parke (2001)
Bu. cepacia BC1	Soybean root	Preinfiltrated seed coated with bacterial antagonist	*Py. ultimum* *Py. arrhenomanes* *Fusarium graminearum*	Damping-off of corn	Mao et al. (1998)
E. clocae	Cucumber seeds and cotton hypocotyls	Seed soaked in bacterial suspension with methocel adhesive	*Pythium* spp.	Seed rot and preemergence damping-off of cotton	Nelson (1988)
E. clocae, *Acinetobacter calcoaceticus*, *Pseudomonas* spp., *Pantoea agglomerans*	Seeds of cotton, perennial ryegrass, zucchini, tomato, onion, and cotton hypocotyls	Sand containing cylinders drenched with bacterial suspension	*Py. ultimum*	Seed rot and damping-off of cotton	van Dijk and Nelson (1998)
E. clocae EcCT-501R3	Cotton hypocotyls	Seed placed on soil and drenched with bacteria	*Py. ultimum*	Damping-off of cotton	van Dijk and Nelson (2000)
		Seed treatment	*Py. ultimum*	Seed infection of cucumber	Windstam and Nelson (2008a,b)
E. clocae	–	Bacterial suspension poured on seed placed in soil	*Py. ultimum*	Damping-off of carrot, cotton, cucumber, lettuce, radish, tomato, and wheat	Kageyama and Nelson (2003)

Lysobacter enzymogenes 3.1T8	Root tips of cucumber grown on used rock wool	Addition of unwashed bacterial suspension grown on R2A medium or its filtrate to seed and/or nutrient solution	*Py. aphanidermatum*	Root and crown rot of cucumber	Folman et al. (2003, 2004)
		Washed bacterial cells plus chitosan on top of each rock wool block	*Py. aphanidermatum*	Root and crown root rot of cucumber	Postma et al. (2009)
L. enzymogenes strain C3	Turf grass leaf surface	Bacterial seed coating using methyl cellulose as adhesive	*Py. ultimum* var. *ultimum*	Damping-off of sugar beet	Palumbo et al. (2005)
L. (*Stenotrophomonas*) sp. strain SB-K88	Fibrous roots of sugar beet	Seeds are mixed with bacterial pellet and air dried	*Pythium* spp.	Damping-off of sugar beet	Nakayama et al. (1999)
			Ap. cochlioides	Damping-off of sugar beet and spinach	Islam et al. (2004, 2005b), Islam (2010)
L. antibioticus HS124	Rhizosphere soil	Seedling pot is amended with bacterial culture	*Ph. capsici*	*Phytophthora* blight of pepper plant	Ko et al. (2009)
Paenibacillus sp., *St. sioyaensis*	Soil and peat moss	Unknown	*Py. aphanidermatum*	Unknown	Hong and Meng (2003)
Paenibacillus sp. strain B2	Mycorrhizosphere of *Sorghum bicolor*	Strain B2 alone or with *Glomus mosseae*	*Ph. parasitica*	Root rot of tomato plants	Budi et al. (1999)
Pae. polymyxa	Wheat (B2), and peanut (B5 and B6) rhizosphere	Roots of 2 week seedlings soaked in cultures of *P. polymyxa*	*Py. aphanidermatum*	Damping-off of *Arabidopsis*	Timmusk et al. (2009)
Ps. fluorescens Pf5	Cotton rhizosphere	Seeds treatment with bacteria	*Py. ultimum*	Damping-off of cotton seedlings	Howell and Stipanovic (1980)
		Seed treatment with bacteria suspended in methyl cellulose	*Py. ultimum*	Damping-off of cucumber	Kraus and Loper (1992)

(continued)

Table 7.1 (continued)

Species/strain	Source	Mode of application	Target pathogen	Disease	Reference
Ps. fluorescens Pf1	Crop rhizosphere	Seed treatment with bacteria suspended in methyl cellulose or with talc formulation	*Py. aphanidermatum*	Damping-off of tomato and hot pepper	Ramamoorthy et al. (2002)
Pseudomonas sp. DSS73	Rhizoplane of sugar beet seedlings	Bacterized seed	*Py. ultimum*	*Pythium*-root disease	Koch et al. (2002), Andersen et al. (2003)
Ps. fluorescens DR54	Rhizosphere of sugar beet	Bacterial suspension applied on seed surface	*Py. ultimum*	Damping-off of sugar beet	Nielsen et al. (1998, 1999)
Ps. aeruginosa PNA1	Rhizosphere of chickpea	Seeds are soaked in bacterial suspension.	*Py. splendens*	Damping-off of bean	Anjaiah et al. (1998)
		Bacteria applied to soil	*Py. splendens* *Py. myriotylum*	Damping-off of bean root rot of cocoyam	Tambong and Höfte (2001), Perneel et al. (2008)
		–	*Ph. capsici*	*Phytophthora* blight of pepper	Kim et al. (2000)
Ps. fluorescens strain F113 and M114 (pCU2031)	Sugarbeet rhizosphere	Seed dipping into bacterial suspension	*Py. ultimum*	Damping-off sugar beet	Fenton et al. (1992)
Ps. fluorescens	Sugar beet rhizosphere	Seeds soaking in bacterial suspension	*Py. ultimum*	Damping-off of sugar beet	Delany et al. (2001)
Ps. fluorescens	Rhizosphere of sugar beet	Seed soaking in bacterial suspension	*Py. ultimum*	Damping-off of pea	Bainton et al. (2004)
Pseudomonas spp. strain B324 and A708	Roots of wheat	Seed treated with bacteria suspended in methyl cellulose	*Pythium* spp.	Preemergence seedling blight of wheat	Becker and Cook (1988)
Ps. aeruginosa 7NSK2	Rhizosphere of barley	Seeds soaked in bacterial suspension	*Py. splendens*	Damping-off of tomato	Buysens et al. (1996)
Ps. fluorescens AB254	Rhizosphere soil	Seed coated with bacteria (methyl cellulose) and dried	*Py. ultimum*	Damping-off of sweet corn (*Zea mays*)	Callan et al. (1990)

Ps. chlororaphis Tx-1	–	Addition to hydroponic nutrient solution	*Py. aphanidermatum* *Py. dissotocum*	Root rot peppers	Chatterton et al. (2004) Khan et al. (2003)
		–	*Ph. megasperma*	Root rot asparagus	Carruthers et al. (1995)
Ps. fluorescens, *Ba. cereus* HY06, *Ps. chlororaphis*, *Ps. corrugata*, *Bu. gladioli*, *Comamonas acidovorans*	–	Bacterial application to hydroponic nutrient solution	*Py. phanidermatum*, *Py. dissotocum*	*Pythium* root rot of *Chrysanthemum*	Liu et al. (2007b)
Ps. fluorescens CHA0 *Ps. fluorescens* strain CHAO and CHAO/pME3090	Tobacco rhizosphere	Unknown	*Py. ultimum*	Damping-off of cucumber, cress, and wheat	Maurhofer et al. (1994, 1995)
Ps. fluorescens CHA0r	Tobacco rhizosphere	Soaking pots of 1-week-old seedlings with bacterial suspension	*Hyaloperonospora parasitica*	Arabidopsis downy mildew	Iavicoli et al. (2003)
Ps. fluorescens CHA0, *Ps. fluorescens* KD	Rhizosphere of tobacco (CHAO) and wheat (KD)	Soil inoculation	*Py. ultimum*	*Pythium* root rot of wheat	Meyer et al. (2010)
Ps. fluorescens strains B5 and X *Ps. corrugate* strain R117 *Bacillus subtilis* strains B2 and B6	–	Seed treatment (sugar beet) or drenching (cucumber) or seed coating	*Py. ultimum*	Damping-off of sugar beet and cucumber	Georgakopoulos et al. (2002)
Ps. corrugata, *Ps. fluorescens*, *Ps. marginalis*, *Ps. putida*, *Ps. syringae*, *Ps. viridiflava*	Peat, barks, and composts	bacterial application to rock wool	*Py. ultimum* *Py. aphanidermatum*	Damping-off of tomato	Gravel et al. (2005)

(continued)

Table 7.1 (continued)

Species/strain	Source	Mode of application	Target pathogen	Disease	Reference
Ps. fluorescens 2-79R, *Bu. cepacia*1-23, *Pseudomonas* sp. 1-30	Wheat roots	Seed soaking in bacterial suspension with methyl cellulose as adhesive	*Pythium* spp.	*Pythium* root rot of winter wheat	Milus and Rothrock (1997)
Pseudomonas sp. (fluorescent pseudomonads)	Rhizosphere and the rhizoplane of cotton	Bacterial suspension pipetted over seed	*Py. ultimum*	Root disease in cotton	Hagedorn et al. (1989)
Ps. fluorescens strain Hv37aR2	Barley roots	Seeds soaked in bacterial suspension and air dried	*Py. ultimum*	*Pythium* disease of cotton	Howie and Suslow (1991)
Ps. fluorescens strain (PfT-8)	Tomato rhizosphere	Bacterial suspension in lignite-based formulation	*Pythium* spp.	Damping-off of tomato	Jayaraj and Radhakrishnan (2008)
Ps. fluorescens 3551	Cotton rhizosphere	Seeds dipped in bacterial suspension	*Py. ultimum*	Preemergence damping-off of cotton	Loper (1988)
Ps. fluorescens (EBL 20-PF)	Chili leaf	Seed or soil treatment with talc-based formulation	*Py. aphanidermatum*	Damping-off of chili	Muthukumar et al. (2011)
Pseudomonas strains BTP1, BTP7	Barley and tomato roots	Addition of bacterial suspension to hydroponic nutrient	*Py. aphanidermatum*	*Pythium* root rot of cucumber	Ongena et al. (1999)
Ps. fluorescens-putida ML5 *Ps. putida* R20	ML5 from pericarp of sugar beet seed. R20 from rhizosphere of lima bean	Dry seed coating with bacteria in methyl cellulose and talc	*Py. ultimum*	Seed rot and damping-off of sugar beet	Osburn et al. (1989)
Ps. putida N1R	–	Seed soaked in bacterial suspension with pelgel as sticker	*Py. ultimum*	Damping-off of pea, cucumber, and soybean	Paulitz and Loper (1991)
Ps. fluorescens B5, *Ps. corrugata* 2140	–	Pelleted seeds were soaked in bacterial suspension and air-dried	*Py. ultimum*	Damping-off of sugar beet	Schmidt et al. (2004a, b, c)

Ps. fluorescens strain SS101	Rhizosphere of wheat	Bulb or soil treatment	*Py. intermedium*	Root rot hyacinth bulb	de Souza et al. (2003b)
		Leaves were immersed into bacterial suspension	*Ph. infestans*	Tomato late blight	Tran et al. (2007)
		Bacteria applied to soil as an atomized mist	*Pythium* spp.	*Pythium* disease of apple seedling	Mazzola et al. (2007)
Ps. fluorescens, *Serratia* spp., *Bacillus* spp.	Soil from pea field	Bacterial suspensions were applied to soil	*Py. ultimum*	Damping-off of field pea	Wang et al. (2003)
Pseudomonas spp., *Ba. megaterium*, *Arthrobacter histidinolovorans*, *Cytophaga johnsonae*	Roots of sugar beet	Washed and unwashed bacteria applied to pelleted seed and then partially dried	*Py. ultimum*, *A. cochlioides*	Sugar beet damping-off complex	Williams and Asher (1996)
Ps. fluorescens, *Pseudomonas* sp. *Enterobacter* sp., *E. cloacae*	–	Mixed microbial suspension was used to inoculate shallow puncture wounds on tubers.	*Ph. erythroseptica*	Pink rot of potato tubers	Schisler et al. (2009)
Pseudomonas sp. strain EC-S101, *Stenotrophomonas maltophilia* EC-S105	Rhizoplanes of spinach and sugar beet	Sterilized seeds coated with bacteria partially	*Ap. cochlioides*	Damping-off of sugar beet and spinach	Deora et al. (2005)
Pseudomonas jessenii strain EC-S101	Rhizoplane of spinach	Seed coating	*Ap. cochlioides*	Damping-off of sugar beet	Deora et al. (2006)
Ps. fluorescens ECO-001	Rhizoplane of a weed *Plantago asiatica*	–	*Ap. cochlioides AC-5*	Damping-off of sugar beet	Islam and Fukushi (2010)
Ps. fluorescens UOM SAR14	–	Seed treatment	*Sclerospora graminicola*	Downy mildew of pearl millet	Raj et al. (2003)
Ps. fluorescens WCS417r	–	Soil inoculation	*H. parasitica*	Downy mildew in *Arabidopsis thaliana*	Van der Ent et al. (2008)

(continued)

Table 7.1 (continued)

Species/strain	Source	Mode of application	Target pathogen	Disease	Reference
Ps. fluorescens	Rhizosphere of pearl millet	Seed treatment, and/or foliar application	*Sc. graminicola*	Downy mildew of pearl millet	Umesha et al. (1998)
Ps. fluorescens 0990, *Ps. putida* 06909	Citrus rhizosphere soil	Unknown	*Ph. parasitic*	Citrus root rot	Yang et al. (1994)
Rhizobium japonicum	–	Inocula of *Rhizobium* applied to potted soil	*Ph. megasperma*, *Ph. megasperma*, *Py. ultimum*	Root rot of soybean	Tu (1978)
Serratia plymuthica HRO-C48	Rhizosphere of oilseed rape	Seeds are soaked in bacterial suspension	*Py. apahnidermatum*	Damping-off of cucumber	Pang et al. (2009)
Se. marcescens 90-166, *Ps. Fluorescens* 89B-61, *Ba. pumilus* SE34, *Ba. pumilus* T4, and *Ba. pasteurii* C-9	–	Seed treatment or root drenches	*Peronopora. tabacina*	Blue mold of tobacco	Zhang et al. (2001)
Se. plymuthica C-1, *Chromobacterium* sp. C-61, *L. enzymogenes* C-3	Soils	Bacteria in chitin media applied as soil drench	*Ph. capsici*	*Phytophthora* blight	Kim et al. (2008)
Stenotrophomonas maltophilia strain W81	Rhizosphere of sugar beet	Seed dipping into bacterial suspension	*Py. ultimum*	Sugar beet	Dunne et al. (1997, 2000)
Streptomyces spp.	Soils	Potting mix inoculated with the spores	*Py. ultimum*	Root disease of lettuce	Crawford et al. (1993)
Streptomyces spp.	Raspberry roots, potato rhizosphere, and soil	Bacterial agar blocks set around the developing roots of each plantlet	*Ph. fragariae* var. *rubi*	Root rot of raspberry	Valois et al. (1996)
Streptomyces spp.	Soil	Spore suspension applied over each seed after sowing	*Ph. medicaginis* and *Ph. sojae*	Root rot of alfalfa and soybean	Xiao et al. (2002)

Streptomyces sp. AMG-P1	–	Culture extract sprayed on seedlings	*Phytophthora* and *Pythium* spp.	Late blight of red pepper and tomato	Lee et al. (2005)
Streptomyces spp. strain BSA25 and WRA1 and *Mesorhizobium ciceri* WSM1666 or Kaiuroo 3	Roots of lentil, chickpea, pea, faba bean, and wheat	Dry seed coating in *Streptomyces* with Arabic gum and *Rhizobium* applied over-head soil	*Ph. medicaginis*	Root rot of chick pea	Misk and Franco (2011)
Streptomyces sp. ANK313 *Streptomyces* sp. ANK302	Soil	Tested on motility of zoospore	*Pl. viticola*	Downy mildew of grapevine	Abdalla et al. (2011) Zinada et al. (2011)
Streptomyces sp. strain B5136	Sea water	Leaf disk assay	*Pl. viticola*	Downy mildew of grapevine	Islam et al. (2011)
Streptomyces sp. AP77	Seawater of a *Porphyra yezoensis* cultivation facility	–	*Py. porphyrae*	Red rot of red alga (*Porphyra* spp.)	Woo et al. (2002)
St. halstedii AJ-7	Soil	Red-pepper seeds were soaked in the culture broth	*Ph. capsici*	*Phytophthora* blight of red pepper	Joo (2005)

BCAs against various phytopathogens (Weller 2007). It is now over 40 years since *Pseudomonas* spp. were first recognized as a potential BCA. Within this period, intensive research has given rise to many well-characterized *Pseudomonas* BCAs (Table 7.1). Among them, the fluorescent *Pseudomonas* spp. received paramount importance due to their efficacy in biocontrol activity. A well-characterized fluorescent pseudomonad is *Ps. fluorescens* Pf5, suppresses several *Pythium* diseases, including damping-off disease in cotton (Howell and Stipanovic 1980) and cucumber caused by *Py. ultimum* (Kraus and Loper 1992). Another strain of *Ps. fluorescens*, Pf1 controls damping-off diseases in tomato and hot pepper caused by *Py. aphanidermatum* (Ramamoorthy et al. 2002). A strain of fluorescent *Pseudomonas*, DSS73 isolated from the rhizoplane of sugar beet seedlings, displayed suppression of root-pathogenic *Py. ultimum* by producing biosurfactant antibiotics (Sørensen et al. 2001; Nielsen et al. 2002; Andersen et al. 2003). *Ps. fluorescens* strain DR54 isolated from sugar beet rhizosphere showed high biocontrol activity against *Pythium* damping-off in sugar beet (Nielsen et al. 1998). Similarly, *Ps. fluorescens* strain F113 isolated from sugar beet in Ireland displayed damping-off disease suppression in sugar beet and pea caused by *Py. ultimum* (Fenton et al. 1992; Delany et al. 2001; Bainton et al. 2004).

An important strain CHA0 of *Ps. fluorescens* was isolated from roots of tobacco grown near Payern, Switzerland, in a soil naturally suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* (Stutz et al. 1986). This strain has shown one of the broadest range of potential biocontrol and growth-promoting mechanisms of any PGPR described so far. CHA0 suppresses *Pythium* damping-off of cucumber and wheat and infection of *Arabidopsis* by *Hyaloperonospora parasitica* (Maurhofer et al. 1995; Iavicoli et al. 2003; Meyer et al. 2010). It is also equally effective against nonperonosporomycete fungi, viruses, and nematodes (Keel et al. 1992; Maurhofer et al. 1994; Siddiqui and Shaukat 2003). Application of *Ps. fluorescens* SS101 to soil or bulbs effectively controls root rot of flower bulb crops caused by *Py. intermedium* in both laboratory and small-scale field experiments (de Souza et al. 2003b). Strain SS101 when applied to soil controls *Pythium*-root rot of apple seedlings (Mazzola et al. 2007) and when applied to leaves controls late blight of tomato caused by *Ph. infestans* (Tran et al. 2007).

Ps. aeruginosa PNA1, isolated from the rhizosphere of chickpea, has widely been shown biocontrol efficacy against a number of phytopathogenic fungi and peronosporomycetes (Anjaiah et al. 1998). This strain demonstrated *in vivo* biocontrol activity against various peronosporomycetes including *Py. splendens* on bean (Anjaiah et al. 1998), *Py. myriotylum* on cocoyam (Tambong and Höfte 2001; Perneel et al. 2008), and *Ph. capsici* on pepper (Kim et al. 2000). Another strain of *Ps. aeruginosa*, 7NSK2 isolated from the rhizosphere of barley, promotes the growth of several crops and suppresses *Py. splendens*-induced damping-off in tomato (Buysens et al. 1996). Isolates of *Ps. chlororaphis* (previously *Ps. aureofaciens*) strongly suppressed *Py. aphanidermatum* in roots of pepper, cucumber, and chrysanthemum (Chatterton et al. 2004; Khan et al. 2003; Liu et al. 2007b), and *Ph. megasperma* in roots of asparagus (Carruthers et al. 1995) and moderately suppressed *Py. dissotocum* in roots of hydroponic

chrysanthemums (Liu et al. 2007b). *Ps. putida* strain N1R provides biocontrol of *Ps. ultimum* on soybean, pea, and cucumber (Paulitz and Loper 1991). Similarly, *Ps. jessenii* strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 efficiently suppressed *in vivo* damping-off disease caused by *Ap. cochloides* (Deora et al. 2005). Some pseudomonads have been found to protect plants from various pathogens by inducing systemic resistance. For example, *Ps. fluorescens* strains UOM and SAR14Ps control *Sclerospora graminicola* on pearl millet (Raj et al. 2003) and *Ps. fluorescens* WCS417r controls *Hy. Parasitica* on *Arabidopsis* (van der Ent et al. 2008).

7.3.2 Bacillus spp.

The spore-forming bacteria, *Bacillus* spp. are considered one of the most effective biocontrol candidates for plant diseases caused by peronosporomycetes. A large body of literature is available concerning biological control of peronosporomycete phytopathogens by various strains of *Bacillus* spp., some important research findings are reviewed (Table 7.1). Bacterial strain antagonistic to a certain peronosporomycete on plate assay may not necessarily be effective in suppressing disease in in vivo or vice versa. For example, *Ba. cereus* UW85 is not inhibitory to *Ph. megasperma* f. sp. *medicaginis* on plates, but its application to the alfalfa seeds suppresses the damping-off disease caused by the same pathogen in the field conditions (Handelsman et al. 1990). In contrast, the same strain was inhibitory to *Ph. sojae* on plate assay, but increased yields only on the susceptible cultivar where *Phytophthora* root rot was a factor. At another site where *Phytophthora* root rot was not a factor, UW 85 increased plant stands significantly over untreated seeds regardless of *Phytophthora* root rot resistance (Osburn et al. 1995). Both additive and nonadditive responses were also observed when several strains were applied together for disease control. For example, a nonadditive response was demonstrated by Everts and Armentrout (2001) for powdery mildew of pumpkin. Several *Bacillus* spp. were also found effective in suppressing *Phytophthora* blight in pepper and squash caused by *Ph. capsici*. Preinoculation of pepper plants with three strains of *Ba. megaterium* alone or in combination, significantly reduced disease severity of *Phytophthora* blight or crown blight caused by *Ph. capsici* in field experiments (Akgül and Mirik 2008). Several strains of *Ba. subtilis* isolated from the rhizoplane and rhizosphere pepper have been found useful for suppression of *Phytophthora* blight of pepper when applied as seed coating (Sid Ahmed et al. 2003a, b; Lee et al. 2008; Chung et al. 2008). The mixture of *Ba. safensis* T4 + *Lysinibacillus boronitolerans* SE56 significantly improved control efficacy compared to the individual strain (Zhang et al. 2010).

Several lines of evidence suggest that inoculation of *Bacillus* spp. may induce systemic resistance in the host plant. Several strains of *Ba. safensis*, *Lysinibacillus boronitolerans*, *Ba. pumilus*, and *Ba. macauensis* have also been shown to induce protection squash against *Phytophthora* blight (Zhang et al. 2010). Similarly,

Ba. pumilus INR7 + T4 + SE56 and INR7 + *Ba. subtilis* IN937a + T4 + SE56 tended to induce higher levels of disease reduction compared to individual strains. *Ba. pumilus* strains INR7 and SE34 were also successful to elicit systemic protection against downy mildew caused by *Plasmopara halstedii* in sunflower (Nandeeshkumar et al. 2008) and late blight on tomato caused by *Ph. infestans* (Yan et al. 2002), respectively. Downy mildew, caused by *Pl. viticola*, was also reduced on grape berry skins and leaves by treatment with *Ba. subtilis* KS1 (Furuya et al. 2011).

A common soil bacterium, *Paenibacillus* spp., displayed biocontrol activity against several diseases caused by peronosporomycetes. *Paenibacillus* sp. strain B2 isolated from the mycorrhizosphere of *Sorghum bicolor* displayed antagonistic activities against some soilborne pathogens when applied with Glomus mosseae (Budi et al. 1999). Application of strain B2 alone or with *G. mosseae* to tomato plants significantly reduced root necrosis caused by *Ph. parasitica*. Besides, this bacterium also enhanced colonization of mycorrhizae in the rhizosphere of tomato. A strain of *Pae. illinoisensis* KJA-424 isolated from soil in the west coast of Korea reduced root mortality of pepper plants caused by *Ph. capsici* when it (in 0.2 % colloidal chitin) was applied into the pot soil. Timmusk et al. (2009) reported that pretreatment of *Arabidopsis* root with some strains of *Pae. polymyxa* showed significant protection against subsequent infection by *Py. aphanidermatum*. The survival rates of *Py. aphanidermatum* infected plants were higher when the seedlings were pretreated with B2 and B5 strains than that of B6 treatment.

7.3.3 Burkholderia spp.

Burkholderia spp. formerly erroneously identified as *Pseudomonas* spp. showed biocontrol activities against several peronosporomycetal diseases. For example, pea seeds treated with *Burkholderia* (*Pseudomonas*) *cepacia* strains AMMD and AMMDR1 (rifampicin resistant mutant) resulted in increased seedling stand and reduced preemergence damping-off caused by *Py. ultimum* and *Py. sylvaticum*. Seed treatment with *Bu. cepacia* (strain AMMD) and *Ps. fluorescens* (PRA25) alone or in combination with captan effectively suppressed damping-off disease of pea (Parke et al. 1991). Application of *Bu. cepacia* increased seedling emergence (40 %) and yield (48 %) compared with captan alone. Strains AMMD or PRA25 significantly suppressed early stage of diseases, while strain AMMD also suppressed the final incidence of disease at harvest over 2 successive years (Bowers and Parke 1993). *Bu. cepacia* AMMDR1 significantly reduced *Py. aphanidermatum* postinfection colonization and damping-off of pea seeds, even when the bacteria were applied 12 h after zoospore inoculation (Heungens and Parke 2001). The level of biocontrol of *Pythium* damping-off by AMMD depended on the host genotype, while the seed treatment with bacteria did not reduce the symptoms of *Aphanomyces* root rot (King and Parke 1993). *Bu. cepacia* AMMDR1 significantly reduced colonization

of taproots by *Ap. euteiches* mycelium, when roots were dip inoculated in a concentrated cell suspension (Heungens and Parke 2001). Both root-dip inoculation of bacterial suspension at lower concentrations and seed inoculation resulted in lower numbers of bacteria near the root tip early on in the infection process.

7.3.4 Enterobacter cloacae

Enterobacter cloacae is a common seed-associated bacterium (Hadar et al. 1983), which is an effective biological control agent that suppresses seed infections, protecting a number of plant species from *Py. ultimum*-induced damping-off (Table 7.1) (Nelson 1988; van Dijk and Nelson 1998, 2000). Seeds previously inoculated with *E. clocae* strains NRRL B-14095 and NRRL B-14096 suppressed preemergence *Pythium* damping-off of cucumber compared to control. The performance of biocontrol by *E. clocae* was varied in different plant species. *E. cloacae* was equally effective in controlling *Pythium* damping-off when placed on the seeds of various crops such as carrot, cotton, rye, lettuce, radish, tomato, and wheat (Nelson 1988; Kageyama and Nelson 2003). However, it was ineffective in biocontrol of diseases in corn (Kageyama and Nelson 2003); pea (Hadar et al. 1983; Kageyama and Nelson 2003); and soybean, snap bean, and lima bean.

7.3.5 Lysobacter spp.

The *Lysobacter* spp. are ubiquitous inhabitants in the diverse environment that have some unique features including gliding motility, high genomic G + C ratio (65–72 %), and brush-like polar fimbriae (Islam et al. 2005a, b). This genus have gained broad interest for several reasons such as (1) rich source for production of a variety of novel antibiotics, such as lysobactins or katanosins (Bonner et al. 1988), cephabacins (Lee et al. 2008), tripropeptins (Hashizume et al. 2001, 2004), and macrocylic lactams such as xanthobaccins (Hashidoko et al. 1999; Nakayama et al. 1999; Yu et al. 2007); (2) production of a wide variety of extracellular cell wall degrading enzymes such as β-lytic proteases (Sid Ahmed et al. 2003b), endopeptidase (Muranova et al. 2004), keratinases, β-1,3 glucanases (Palumbo et al. 2003), cellulase (Ogura et al. 2006), and lysoamidase (Riazanova et al. 2005); (3) ability to suppress plant diseases and colonize plant surfaces (Martin 2002; Islam et al. 2004, 2005b; Islam 2008, 2010); and (4) exhibition of wolf-pack-like micropredatory behavior (Martin 2002; Islam 2010). Some of these unique features of *Lysobacter* spp. are advantageous for using them as BCAs against phytopathogens (Zhang et al. 2001; Folman et al. 2004; Islam et al. 2005a; Kobayashi et al. 2005; Ji et al. 2008). Among 21 identified species, biocontrol activity of *L. enzymogenes* strains C3 and 3.1T8 and *Lysobacter* sp. SB-K8 has extensively been investigated. The biocontrol potentials of *Lysobacter* spp. including their mode of action have recently been reviewed (Islam 2011).

7.3.6 Actinomycetes

Numerous surveys of soil bacteria have identified considerable number of strains of actinomycetes as potential BCAs (Khan et al. 1997; Filonow and Dole 1999; Crawford et al. 1993; Misk and Franco 2011). One of the advantages of actinomycetes is their ability to produce spore. These spores are long lived and resistant to heat and desiccation, and maintain a stable population over the time. Actinomycetes such as *Actinoplanes* spp. have shown great promise for reducing *Pythium* root rot in horticultural plants in the greenhouse. Several strains of *Actinoplanes* spp. (W57, W257, or 25844) were applied on clay granules at 5 % or 0.5 % w/w to *Py. ultimum* oospores-infested soil-less potting mix 5 days prior to replanting geranium or poinsettia seedlings. Application of *Actinoplanes* spp. generally reduced root rot severity and increased plant stand compared to nontreated plants after 6 week grown in a greenhouse (Filonow and Dole 1999). When strain W257 was applied as granules or as a root dip, it was as effective as the fungicide metalaxyl in reducing the root rot. When strains 25844, W57, and W257 were applied as granules at 5 % (w/w) to field plots infested with oospores of *Py. ultimum*, only strain 25844 consistently increased emergence and reduced root rot of table beets compared to controls (Khan et al. 1997). In the same study, strain 25844 at 1 % (w/w) also increased the emergence of bush beans at 28 days after planting in *Py. ultimum*-infested plots, but lower rates were found ineffective. Similarly, El-Tarabily et al. (2010) showed that the endophytic actinomycetes such as *Ac. campanulatus*, *Micromonospora chalcea*, and *Streptomyces spiralis*, when applied individually or in combination significantly promoted plant growth and reduced damping-off and crown and root rot of cucumber caused by *Py. aphanidermatum* under green house conditions. These isolates when applied individually or in combination to cucumber seedlings, also promoted growth and yield and reduced seedling damping-off and root and crown rot of mature cucumber plant in the field (El-Tarabily et al. 2010).

The use of streptomycete actinomycetes as biological control agents against the peronosporomycete disease has received considerable attention (Crawford et al. 1993; Valois et al. 1996; Xiao et al. 2002; Joo 2005; Lee et al. 2005; Misk and Franco 2011; Abdalla et al. 2011; Islam et al. 2011) (Table 7.2). Eleven strains of actinomycetes belong to the genus *Streptomyces* significantly reduced the root rot index caused by *Ph. fragariae* var. *rubi* when inoculated on raspberry plantlets (Valois et al. 1996). In contrast, antiperonosporomycetal compounds originating from actinomycetes appear to be selectively active against *Phytophthora* and *Pythium*. Spraying tomato seedlings with culture broth of *Streptomyces* sp. AMG-P1 resulted highly inhibitory effect against late blight disease caused by *Ph. infestans* at 500 μg freeze-dried weight per milliliter, whereas its antibiotic paromomycin showed potent in vivo activity against red pepper and tomato late blight diseases with 80 and 99 % control value, respectively, at 100 μg/ml (Lee et al. 2005).

The use of *Streptomyces* spp. in biocontrol of plant diseases depends on their potential inhibitory effects on the pathogen. Eight isolates having potential pathogen-inhibitory capabilities were subsequently tested for their ability to control *Phytophthora* root rots on alfalfa and soybean in sterilized vermiculite and naturally infested field soil. The *Streptomyces* isolates significantly reduced root rot severity in alfalfa and soybean caused by *Ph. medicaginis* and *Ph. sojae*, respectively (Xiao et al. 2002). Similarly, in a greenhouse experiment, *Strepmyces* sp. BSA25 and WRA1 with the highest antagonistic capabilities against broad spectrum of pathogens were tested for their ability to control *Phytophthora* root rot of chickpea caused by *Ph. medicaginis* and found that both isolates promote vegetative growth of chick pea and successfully suppressed *Phytophthora* root rot when coinoculated with either *Mesorhizobium ciceri* WSM1666 or Kaiuroo 3 (Misk and Franco 2011).

7.3.7 Disease Suppression by Other BCAs

There are a few other bacterial genera which have been proved to be effective BCAs against peronosporomycete pathogens, but have been used in a limited study. Tu (1978) reported that *Phytophthora* root rot of soybean was lessened in the green house when rhizobia were applied to the potted soil immediately after planting. *Serratia plymuthica* HRO-C48 isolated from the rhizosphere of oilseed rape was effective in suppressing damping-off of cucumber caused by *Py. aphanidermatum* (Pang et al. 2009). *Stenotrophomonas maltophilia* W81, isolated from a sugar beet rhizosphere is capable of conferring protection against *Py. ultimum*-mediated damping-off (Dunne et al. 1997).

7.4 Mechanism of the Biological Control

Biological control of peronosporomycete phytopathogens is a multifaceted process in which several mechanisms are involved. This section reviews current knowledge on widely recognized mechanisms of disease suppression by the biocontrol bacteria.

7.4.1 Direct Antagonism or Antibiosis

A condition in which one or several metabolites that are excreted by an organism have a harmful effect on other organisms is known as antibiosis (Haas and Defago 2005). The microbial metabolites that can suppress growth and reproduction or kill other microorganisms at low concentration are known as antibiotics. A large number of structurally diverse chemical compounds (antibiotics) have been identified as principles of biocontrol of peronosporomycetal diseases by antagonistic bacteria

Table 7.2 Some antiperonosporomycetal compounds (antibiotics) produced by antagonistic bacteria

Antibiotic	Producing bacteria	Peronosporomycete	Bioactivity	References
2,4-diacetylphloroglucinol (DAPG)	*Ps. fluorescens* strain F113, M114, Pa 21, CHAO, and CHAO/pME3090	*Py. ultimum*	Mycelial growth inhibition	Shanahan et al. (1992), Fenton et al. (1992), Delany et al. (2001), Bainton et al. (2004), Maurhofer et al. (1995)
	Ps. fluorescens CHA0r	*Peronospora parasitica*	Induction of ISR in host plant	Iavicoli et al. (2003)
	Ps. fluorescens ECO-001	*Ap. cochlioides* AC-5	Growth inhibition, excessive branching, and disruption of F-actin in AC-5 hyphae	Islam and Fukushi (2010)
		Ap. cochlioides AC-5 and *Pl. vitivola*	Inhibition of zoosporogenesis and the motility of zoospores	Islam and von Tiedemann (2011)
Phenazine	*Ps. aeruginosa PNA1*	*Py. splendens*	Mycelial growth inhibition	Anjaiah et al. (1998)
		Py. splendens, *Py. myriotylum*	Mycelial growth inhibition. Induced aggregation of cell content and vacuolization within the hyphae	Perneel et al. (2008)
	Ps. fluorescens 2-79	*Py. aristosporum*	Mycelial growth inhibition	Gurusiddaiah et al. (1986)
	Ps. chlororaphis Tx-1 (*Ps. aureofaciens PA 147–2*)	*Ph. megasperma*	Mycelial growth inhibition	Carruthers et al. (1995)
Pyoluteorin (4,5-dichloro-1H-pyrrol-2yl-2.6-dihydroxyphenyl ketone)	*Pseudomonas fluorescens* Pf5 *Ps. fluorescens* CHA0 *Ps. fluorescens* strain CHAO and CHAO/pME3090	*Py. ultimum*	Mycelial growth inhibition	Howell and Stipanovic (1980), Kraus and Loper (1992), Maurhofer et al. (1994, 1995)
Oomycin A	*Ps. fluorescens* strain Hv37aR2	*Py. ultimum*	Decrease production of secondary inoculum	Howie and Suslow (1991)
Anthranilate	*Ps. aeruginosa* PNA1 and mutant FM13	*Py. splendens*	Mycelial growth inhibition.	Anjaiah et al. (1998)

HCN	*Pseudomonas* spp. strain group A1-A4 *Pseudomonas* sp. DSS73	*Py. ultimum*	Mycelial growth inhibition	Nielsen et al. (2002) Andersen et al. (2003)
	Ba. subtillis (R13, R33)	*Ph. capsici*	Mycelial growth inhibition	Lee et al. (2008)
	Streptomyces spp.	*Ph. medicaginis*	Mycelial growth inhibition	Misk and Franco (2011)
Massetolide A	*Ps. fluorescens* strain SS101	*Py. intermedium*	Had surface activity and caused lysis of zoospores	de Souza et al. (2003b)
		Ph. infestans	Lysis of zoospores and induce systemic resistance	Tran et al. (2007)
Viscosinamide	*Ps. fluorescens* DR54	*Py. ultimum*	Mycelial growth inhibition	Nielsen et al. (1998)
			Aiding in surface colonization of plant roots, surfaces of soil, and reduces growth and aerial mycelium development	Nielsen et al. (1999)
	Pseudomonas spp. strain group V1	*Py. ultimum*	Surface tension reduction and mycelial growth inhibition	Nielsen et al. (2002)
Amphisin	*Pseudomonas* spp. strain group A1 *Pseudomonas* sp. DSS73	*Py. ultimum*	Surface tension reduction and mycelial growth inhibition	Nielsen et al. (2002) Andersen et al. (2003)
Lokisin, hodersin, and tensin	*Pseudomonas* spp. strain group A2, A3, and A4, respectively	*Py. ultimum*	Surface tension reduction and mycelial growth inhibition	Nielsen et al. (2002)
Rhamnolipid A	*Ps. aeruginosa* PNA1 (B5)	*Ph. capsici*, *Py. splendens*, *Py. myriotylum*	Inhibition of hyphal growth and germination of zoospores and lysis of zoospores	Kim et al. (2000), Perneel et al. (2008)
Mycosubtilin	*Ba. subtillis* BBG100 (Transformant)	*Py. aphanidermatum*	Mycelial growth inhibition	Leclère et al. (2005)

(continued)

Table 7.2 (continued)

Antibiotic	Producing bacteria	Peronosporomycete	Bioactivity	References
Iturin A	*Ba. subtilis* KS1 *Acinetobacter* sp. LCH001 *Ba. subtilis* ME488	*Pl. viticola* *Ph. capsici* *Ph. capsici*	Mycelial growth inhibition	Furuya et al. (2011) Liu et al. (2007a) Chung et al. (2008)
Zwittermicin A	*Bacillus cereus* UW85	*Ph. megasperma* f. sp. *medicaginis*, *Ph. sojae*	Inhibition of germ tube length and mycelial growth	Handelsman et al. (1990), Silo-Suh et al. (1994), Osburn et al. (1995)
Antibiotic B	*Ba. cereus* UW85	*Ph. megasperma* f. sp. *medicaginis*	Inhibition of germ tube elongation and swollen and deformation of germ tube tips	Handelsman et al. (1990), Silo-Suh et al. (1994)
Bacilysin	*Ba. subtilis* ME488	*Ph. capsici*	Mycelial growth inhibition	Chung et al. (2008)
4-hydroxyphenylacetic acid	*L. antibioticus HS124* *Burkholderia* sp. MP-1	*Ph. capsici*	Deformation, lysis, and bending of hyphae, inhibition of mycelial growth	Ko et al. (2009) Mao et al. (2006)
Xanthobaccin A	*Lysobacter* (*Stenotrophomonas*) sp. strain SB-K88	*Pythium* spp., *Ap. cochlioides*	Inhibition of mycelial growth, motility of zoospores, and lysis of zoospores	Nakayama et al. (1999), Islam et al. (2005b), Islam (2008, 2010)
Pyrrolnitrin	*Serratia plymuthica* HRO-C48	*Py. apahnidermatum*	Mycelial growth inhibition	Pang et al. (2009)
Paromomycin	*Streptomyces* sp. AMG-P1	*Phytophthora* and *Pythium* spp.	Mycelial growth inhibition	Lee et al. (2005)
Khatmiamycin	*Streptomyces* sp. ANK313	*Pl. viticola*	Motility inhibition and lysis of zoospores	Abdalla et al. (2011)
Staurosporine	*Streptomyces* sp. strain B5136	*Pl. viticola*	Inhibition of motility of zoospores by inhibiting protein kinase C activity	Islam et al. (2011)
Isocoumarins	*Streptomyces* sp. ANK302	*Pl. viticola*	Motility inhibition and lysis of zoospores	Zinada et al. (2011)
(+)-4,5-Didehydroacaterin and 3[(1R)-hydroxyhexyl]-5-methylene-2(5H)-furanone	*Ps. jessenii* EC-S101	*Aphanomyces cochlioides*	Induction of hyperbranching and inhibition of hyphal growth	Deora et al. (2010), Hatano et al. (2007)

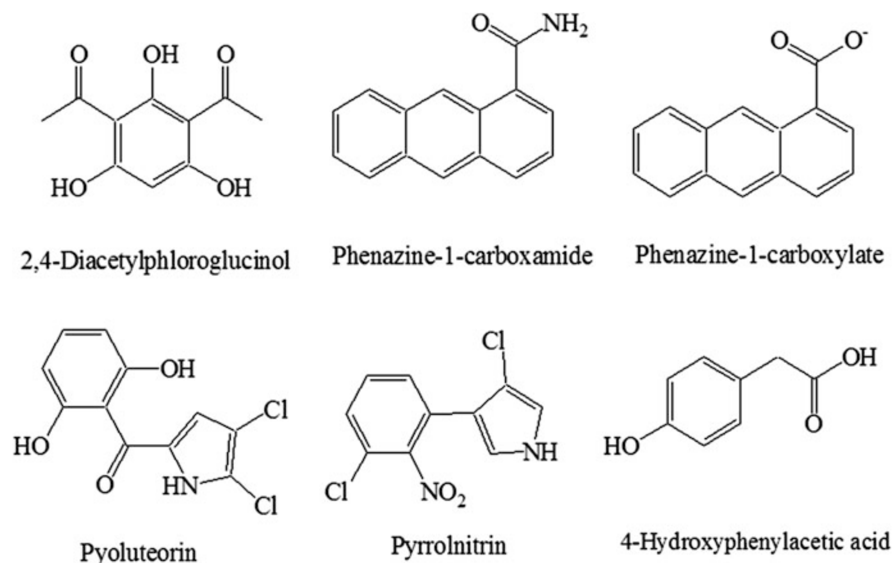

Fig. 7.3 Bioactive compounds from different biocontrol strains of *Pseudomonas* spp. against Peronosporomycete phytopathogens

(Figs. 7.3, 7.4, 7.5, and 7.6). The action mechanisms of antibiotics are very diverse and sometime very specific. Indeed, antibiosis is one of the most-studied mechanisms of biological control by bacterial antagonists. Antibiotics such as DAPG, phenazines, pyoluteorin, hydrogen cyanide, oomycin A, anthranilate, and cyclic lipopeptides have been reported to involve in suppression of peronosporomycete phytopathogens by *Pseudomonas* spp. (Table 7.2). The polyketide phenolic antibiotic, DAPG is produced by different strains of *Ps. fluorescens* to suppress the growth of peronosporomycetes (Fig. 7.3) (Shanahan et al. 1992; Fenton et al. 1992; Keel et al. 1992; Maurhofer et al. 1995; Delany et al. 2001; Iavicoli et al. 2003; Bainton et al. 2004; Islam and Fukushi 2010; Islam and von Tiedemann 2011).

DAPG has shown a wide range of inhibitory activities such as antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (Bainton et al. 2004). Production of DAPG is considered as one of the major determinants of disease suppression by *Ps. fluorescens*. It inhibits a diverse group of peronosporomycetes such as *Py. ultimum* (Fenton et al. 1992; Shanahan et al. 1992; Delany et al. 2001), *Ap. cochloides* (Islam and Fukushi 2010), *Peronospora parasitica* (Iavicoli et al. 2003), *Pl. viticola* (Islam and von Tiedemann 2011). DAPG-induced disease suppression is associated with alteration or disruption of a variety of cellular peronosporomycetes. For example, mycelial growth inhibition of *Py. ultimum* and *Ap. cochlioides* through excessive branching and curling (Shanahan et al. 1992; Islam and Fukushi 2010); disruption of the organization of cytoskeletal filamentous actin in *Ap. cochlioides* hyphae (Islam and Fukushi 2010); disorganization in hyphal tips of *Py. ultimum* var. *sporangiiferum*, including alterations (proliferation, retraction, and disruption) of the plasma membrane, vacuolization, and cell content disintegration

(de Souza et al. 2003a); inhibition of zoosporogenesis and the motility of zoospores of *Ap. cochlioides* and *Pl. vitivola* (Islam and von Tiedemann 2011); and induction of systemic resistance in the host plants (Iavicoli et al. 2003). DAPG has recently been reported to inhibit the mitochondrial function in yeast (Gleeson et al. 2010). As cleavage of nuclei and dramatic differentiation of sporangia during zoosporogenesis require supply of energy from the mitochondria, impairment of the mitochondrial function in the *Pl. viticola* sporangia and zoospores by DAPG might be associated with suppression of zoospore release and motility inhibition of zoospores, respectively (Islam and von Tiedemann 2011). To understand structure–activity relationships, Islam and von Tiedemann (2011) tested several phloroglucinol derivatives, namely, phloroglucinol (PG), monoacetylphloroglucinol (MAPG), 2,4,6-triacetylphloroglucinol (TAPG), and 2,4-dipropylphloroglucinol (DPPG) structurally related to the DAPG on zoosporogenesis and motility behavior of two peronosporomycetes, *Pl. viticola* and *Ap. cochlioides* (Fig. 7.3). According to their bioassay results, the activities of the tested compounds ranked DPPG > TAPG > DAPG > MAPG > PG for both zoosporogenesis and motility inhibition of the zoospores. It appeared that (1) the degree of substitution of hydrogen atoms in the benzene ring of phloroglucinol by acyl groups (acetyl or propyl) increased bioactivity; and (2) substitution with a larger aliphatic group (propyl) showed higher activity than the shorter aliphatic group (acetyl).

Phenazines are low molecular weight nitrogen-containing heterocyclic antimicrobial compound consisting of brightly colored pigment produced by the bacterial genera *Pseudomonas* (Gurusiddaiah et al. 1986; Carruthers et al. 1995; Anjaiah et al. 1998; Perneel et al. 2008). Phenazine compounds have antibiotic activity against a wide range of bacterial and fungal pathogens including several peronosporomycetes that cause important root diseases of plants (Carruthers et al. 1995; Anjaiah et al. 1998). Two phenazine antibiotics, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (oxychlororaphine), and an anthranilate produced by *P. aeruginosa* PNA1 showed a dominant role in suppressing *Pythium* damping-off diseases in chick pea, bean, and lettuce (Fig. 7.3) (Anjaiah et al. 1998; Perneel et al. 2008). PCA produced by *Ps. fluorescens* 2-79 (Gurusiddaiah et al. 1986) and *Ps. chlororaphis* Tx-1 (Carruthers et al. 1995) showed excellent activity against *Py. aristosporum* and *Ph. megasperma*, respectively. The mode of action of phenazines in antiperonosporomycete interactions includes mycelial growth inhibition of *Pythium* (Anjaiah et al. 1998; Perneel et al. 2008; Gurusiddaiah et al. 1986), and *Phytophthora* (Carruthers et al. 1995) and aggregation of cell content and vacuolization of *Pythium* hyphae (Perneel et al. 2008). Another antibiotic, pyoluteorin (4,5-dichloro-1H-pyrrol-2yl-2.6-dihydroxyphenyl ketone) is a chlorinated polyketide antibiotic secreted by the rhizosphere bacterium *Ps. fluorescens* Pf-5 (Howell and Stipanovic 1980; Kraus and Loper 1992) and *Ps. fluorescens* CHA0 (Maurhofer et al. 1994, 1995). This antibiotic inhibits growth of mycelia of a seed- and root-rotting peronosporomycete, *Py. ultimum* (Howell and Stipanovic 1980; Kraus and Loper 1992). *Ps. fluorescens* strain Hv37aR2 produces oomycin A, which is linked to in vivo biocontrol of *Py. ultimum* infection on cotton (Howie and Suslow 1991).

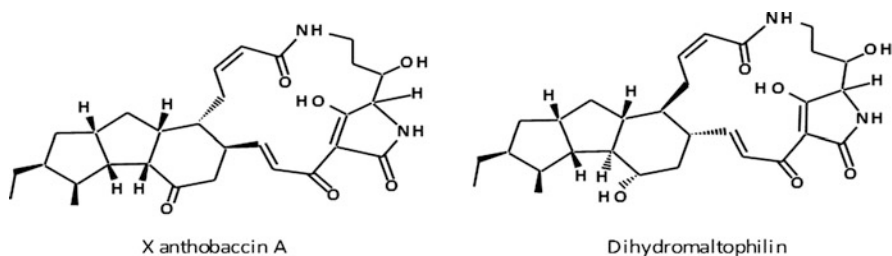

Fig. 7.4 Structures of two tetramic acid-containing macrocyclic lactam antibiotics, xanthobaccin A and dihydromaltophilin produced by *Lysobacter* sp. SB-K88 and *L. enzymogenes* C3, respectively

Zwittermicin A is a linear aminopolyol, which is involved in suppression of *Phytophthora* diseases on alfalfa and soybean by *Ba. cereus* UW85 (Handelsman et al. 1990; Silo-Suh et al. 1994; Osburn et al. 1995). It inhibits elongation of germ tube of zoospores and mycelial growth of *Phytophthora* spp. (Osburn et al. 1995). Four compounds, namely, phenylacetic acid (PA), hydrocinnamic acid (HCA), 4-hydroxyphenylacetic acid (HAA), and 4-hydroxyphenylacetate methyl ester (HPME) were isolated from the culture broth of *Burkholderia* sp. strains MP-1, PA, HCA, and HPME, which moderately inhibited *Ph. capsici*. However, HAA isolated from the culture supernatant of *Lysobacter antibioticus* HS124 induces abnormal hyphae of *Ph. capcisi* (Ko et al. 2009).

The biocontrol bacterium *Lysobacter* sp. SB-K88 suppresses damping-off disease in sugar beet and spinach caused by *Ap. cochlioides* and *Pythium* sp. through production of at least three lytic antibiotics, xanthobaccin A, B, and C (Nakayama et al. 1999; Islam et al. 2005b). Direct application of purified xanthobaccin A to seeds suppressed damping-off disease in sugar beet in soil naturally infested with *Pythium* spp. (Nakayama et al. 1999). The predominant antibiotic, xanthobaccin A produced by SB-K88 inhibits mycelial growth, impairs motility, and causes lysis of zoospores of *Aphanomyces cochlioides* (Nakayama et al. 1999; Islam 2008, 2010) (Figs. 7.4 and 7.5).

The mode of action of this macrocyclic lactam antibiotic includes disruption of ultrastructure and organization of filamentous actin in the cells of *Ap. cochlioides* (Islam 2008). The plane structure of xanthobaccin A is the same as that of a known antibiotic maltophilin, which was isolated from a rhizobacterium of rape *Stenotrophomonas maltophilia* R3089 (Jacobi et al. 1996). Both xanthobaccin A and maltophilin belong to a group of tetramic acid containing macrocyclic lactam antibiotics. An analogue of xanthobaccin A, dihydromaltophilin was identified as a heat stable and potent antiperonosporomycetal compound in the culture fluid of *L. enzymogenes* strain C3 (Yu et al. 2007). This compound exhibits a wide range of antimicrobial activities and shows a novel mode of action by disrupting the biosynthesis of a distinct group of sphingolipids (Giesler and Yuen 1998).

Suppression of *Py. aphanidermatum* damping-off in cucumber by *Serratia plymuthica* HRO-C48 is found to be responsible for its ability to produce antibiotic

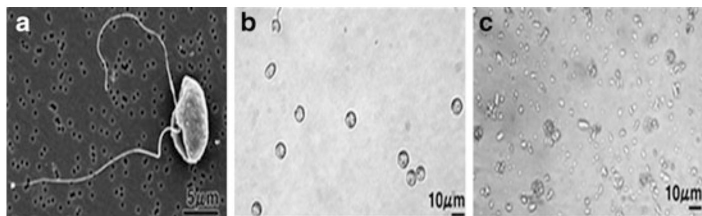

Fig. 7.5 Light and scanning electron micrographs showing *Aphanomyces cochlioides* zoospore-lytic activity of xanthobaccin A isolated from the biocontrol bacterium *Lysobacter* sp. SB-K88 (adapted from Islam et al. 2005b). (**a**) Micrograph of a biflagellate *A. cochlioides* zoospore (untreated control); (**b**) no lysis of zoospore in control. A small portion of (10–15 %) of motile zoospores in the control dish were stopped and changed into round cystospores and then settled to the bottom of the dish; (**c**) complete lysis of all halted zoospores by xanthobaccin A at 1.0 μg/ml

pyrrolnitrin (Pang et al. 2009). The pyrrolnitrin belongs to phenylpyrrole group and inhibits growth, synthesis of protein, RNA, DNA, and uptake of metabolites of pathogens (Pang et al. 2009).The aminoglycoside antibiotic, paromomycin is detected in *Streptomyces* sp. AMG-P1 as a candidate for the control of tomato and potato diseases caused by *Pythium* spp. and *Ph. infestans* (Lee et al. 2005). A new antiperonosporomycetal compound, khatmiamycin was recently isolated from the culture broth of a terrestrial *Streptomyces* sp. ANK313, which exhibits potent motility inhibitory (100 %) and lytic (83 ± 7 %) activities against zoospores of the grapevine downy mildew pathogen, *Pl. viticola* (Fig. 7.6) (Abdalla et al. 2011).

Similarly, four isocoumarins have been isolated from the terrestrial *Streptomyces* sp. ANK302, namely 6,8-dimethoxy-3-methylisocoumarin, 6,8-dihydroxy-3-methylisocoumarin, 6,8-dihydroxy-7-methoxy-3-methylisocoumarin, and 6,7,8-trimethoxy-3-methylisocoumarin displayed varying levels of motility inhibitory effects against *Pl. viticola* zoospores (Zinada et al. 2011). Although mechanism is not known, several other antibiotics such as nonactin, oligomycin F, and antimycin A isolated from marine *Streptomyces* spp. also displayed potent motility inhibitory and lytic activities against zoospores of the grapevine downy mildew pathogen, *Pl. viticola* (Fig. 7.7) (Islam et al. unpublished).

Recently, staurosporine was identified as the active principle in the ethyl acetate extracts of a marine *Streptomyces* sp. strain B5136 that rapidly impaired the motility of zoospores of the grapevine downy mildew pathogen *Pl. viticola* (Fig. 7.6) (Islam et al. 2011). The indolocarbazole antibiotic, staurosporine is a known broad-spectrum inhibitor of protein kinases, including protein kinase C (PKC). To understand the role of specific protein kinase in the maintenance of flagellar motility of zoospores, Islam et al. (2011) tested 22 known kinase inhibitors. Interestingly, the PKC inhibitor chelerythrine was the most potent to arrest the motility of zoospores at concentrations starting from 5 nM. Inhibitors that targeted kinase pathways other than PKC pathways did not practically show any activity in impairing zoospore motility. Both staurosporine and chelerythrine also inhibited the release of zoospores from the *Pl. viticola* sporangia in a dose-dependent manner. In addition, staurosporine completely suppressed downy mildew disease in grapevine leaves at

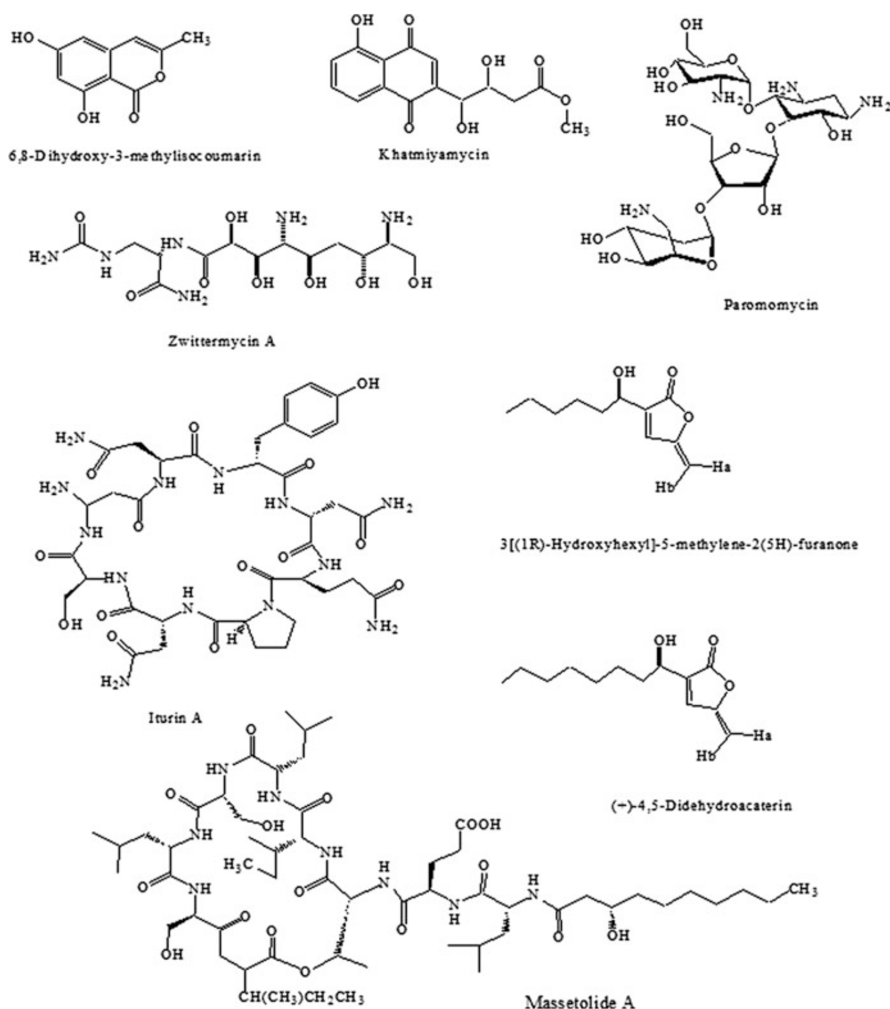

Fig. 7.6 Bioactive compounds from various biocontrol bacteria against peronosporomycete phytopathogens

2 μM, suggesting the potential of small-molecule PKC inhibitors for the control of peronosporomycete phytopathogens (Fig. 7.8). This study for the first time discovered that PKC is as a key signaling mediator associated with zoosporogenesis and the maintenance of flagellar motility in peronosporomycete zoospores (Islam et al. 2011). Interestingly, this finding parallels earlier work on the role of PKC in flagellar motility of mammalian sperm and spermatozoa of aquatic vertebrates (Rotem et al. 1990; White et al. 2007). Because motility is critical for the life cycles and pathogenicity of pathogens, elucidation of the details of signal transduction pathways might help us to design strategies for biorational management of the notorious peronosporomycete phytopathogens.

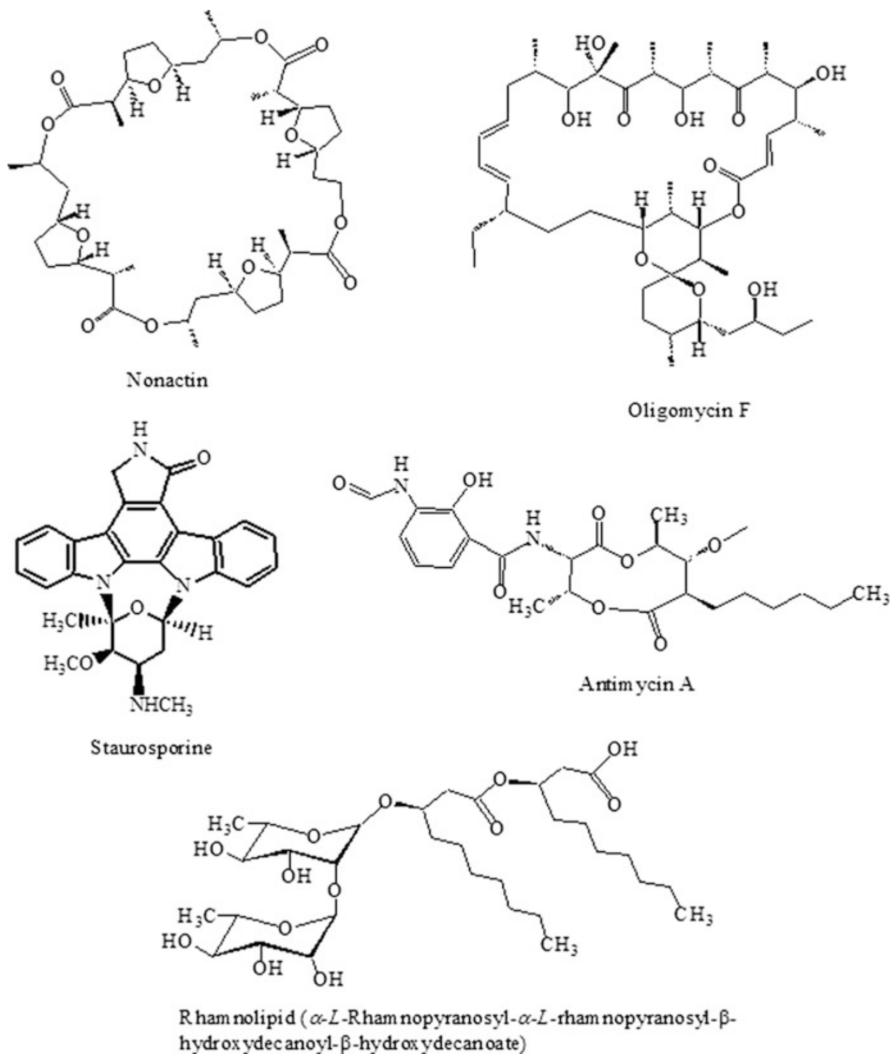

Fig. 7.7 Some antibiotics that affect motility, viability, and developmental transitions of zoospores of phytopathogenic peronosporomycetes

7.4.2 Biosurfactants as Antiperonosporomycetal Agent

Several strains of *Ps. fluorescens* were reported to produce antibiotics with surface-active properties. These antibiotics are designated as biosurfactants, which belong to a family of closely related cyclic lipopeptides (CLP). CLP has been shown destructive effects on zoospores of *Phytophthora* and *Pythium* spp. (Stanghellini and Miller 1997; Nielsen et al. 1999; de Souza et al. 2003b; de Bruijn et al. 2007;

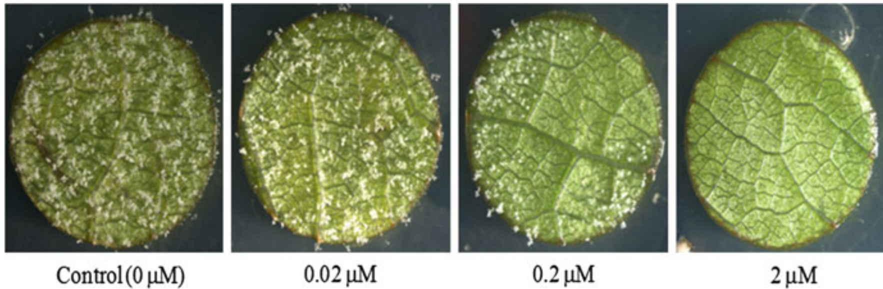

Fig. 7.8 Suppression of sporangial growth of a downy mildew pathogen, *Plasmopara viticola* on grapevine leaf disks by varying doses of staurosporine isolated from *Streptomyces* sp. B 5136 (adapted from Islam et al. 2011). Application of staurosporine at 2 mM concentration completely suppresses downy mildew disease on grapevine leaf. Staurosporine was suspended in aqueous DMSO (1 %) and an appropriate dose was sprayed on leaf disks placed on 1.5 % water agar 12 h before (pre-) or after (post) inoculation with *P. viticola* sporangia (5×10^3/ml). Inoculated leaf disks were incubated at 25 °C in 95 % relative humidity for 6 days

Perneel et al. 2008). Massetolide A, a cyclic lipopeptide with a nine-amino-acid peptide ring linked to 3-hydroxydecanoic acid was isolated from the culture broth of *Ps. fluorescens* strain SS101 (de Souza et al. 2003b). It is a metabolite with versatile functions causing lysis of zoospores, providing significant control of *Ph. infestans*, both locally and systemically via induced resistance and contribute to the colonization of tomato plants by *Ps. fluorescens* SS101 (Tran et al. 2007). Further study by van de Mortel et al. (2009) demonstrated that massetolide A induced the formation of transmembrane pores with an estimated size of between 1.2 and 1.8 nm. Zoospores were found to be most sensitive to massetolide A followed by mycelium and cysts. Massetolide A significantly reduced sporangium formation and caused increased branching and swelling of hyphae. Interestingly, a loss-of-function transformant of *Ph. infestans* lacking the G-protein subunit was more sensitive to massetolide A, whereas a gain-of-function transformant required a higher massetolide A concentration to interfere with zoospore aggregation which suggests that the cellular responses of *Ph. infestans* to this cyclic lipopeptide are, in part, dependent on G-protein signaling. Genome-wide expression profiling by microarray analysis may help to unravel the mode of action of massetolide on *Ph. infestans*.

Another metabolite, viscosinamide is an important component of activity of *Ps. fluorescens* DR54 against *Py. ultimum* (Nielsen et al. 1998, 1999). It aids in surface colonization of plant roots and soil surfaces and as antibiotic, reduces growth and aerial mycelium development of *Py. ultimum* and *Rhizoctonia solani* (Nielsen et al. 1999). Some strains of *Pseudomonas* sp. produce amphisin, lokisin, hodersin, and tensin (Nielsen et al. 2002). The ability of *Ps. fluorescens* DSS73 to efficiently control root-pathogenic *Py. ultimum* is shown to arise from secreting amphisin (Andersen et al. 2003). The biosurfactants, rhamnolipids produced by *Ps. aeruginosa* PNA1 suppressed plant-pathogenic peronosporomycetes through lysis of zoospore and inhibition of mycelia growth (Kim et al. 2000; Perneel et al. 2008). Although an increasing number of CLPs with surfactant properties have

been described in *Pseudomonas* spp., a few are also produced by *Bacillus* spp. *Ba. subtilis* ATCC 6633 produces surfactant mycosubtilin, a member of the iturin family (Leenders et al. 1999). Leclère et al. (2005) showed that a derivative of the *Ba. subtilis* strain BBG100 that overproduces mycosubtilin showed increased activity against *Pythium* on tomato seedlings. Iturin A, another antimicrobial lipopeptide structurally very similar to mycosubtilin has been reported to be produced by several *Bacillus* strains. This compound showed strong inhibitory activity against various peronosporomycetes (Chung et al. 2008; Furuya et al. 2011). Iturin A is also produced by a strain of *Acinetobacter* (Liu et al. 2007a). The cell free filtrate of *Acinetobacter* sp. LCH001 was strongly inhibitory against several phytopathogens including *Ph. capsici*, *F. graminearum*, and *R. solani*, and the bioactive compounds identified were as isomers of iturin A, namely, iturin A2, iturin A3, and iturin A6.

7.4.3 Lytic Enzyme as a Means of Biocontrol

Production of extracellular lytic enzymes has been implicated in plant protection by many biocontrol bacteria (Table 7.3). Exposure of phytopathogens to lytic enzymes can result in the degradation of the polymeric compounds such as chitin, proteins, cellulose, hemicellulose, glucans, and DNA of cell walls of the pathogen and use these as a carbon and energy source (Leah et al. 1991). Proteases, chitinases, glucanases, endopeptidase, lipases, lysoamidase, phospholipases, keratinases, lactamases, and phosphatases produced by bacterial antagonists can degrade structural matrix of cell wall of many phytopathogens including the peronosporomycetes (Lim et al. 1991; Fridlender et al. 1993; Valois et al. 1996; Dunne et al. 1997; Nielsen et al. 2002; Andersen et al. 2003; Hong and Meng 2003; Koch et al. 2002; Palumbo et al. 2005).

It has been reported that some fluorescent pseudomonad produced protease, siderophore, and HCN acted as antimicrobial agents against *Pythium* sp. and *Ph. nicotianae*. Suppression of late blight disease in pepper through secretion of lytic enzymes such as protease, chitinase, β-1,3-glucanase, and lipase in concert with the release of antibiotic compound 4-hydroxyphenylacetic acid by *L. antibioticus* strain HS124 has been demonstrated (Ko et al. 2009). However, chitinases are less essential in causing lysis of cell walls of the peronosporomycetes as they do not contain significant amount of chitin. For example, *Stenotrophomonas maltophilia* strain W81 produces extracellular enzymes chitinase and protease; however, commercially purified chitinase or cell-free supernatants from cultures of the protease-negative mutant W81M1 or the chitinase- and protease-negative mutant W81A1 had no effect on integrity of the essentially chitin-free *Pythium* mycelium and did not prevent subsequent growth of this peronosporomycete (Dunne et al. 1997). The proteolytic enzyme was identified as serine protease and

Table 7.3 Some important secreted lytic enzymes and antimicrobial protein produced by biocontrol bacteria against the peronosporomycete phytopathogens

Enzyme	Producing bacteria	Peronosporomycete	Mode of action	Reference
Protease	*Stenotrophomonas maltophilia* strain W81, *Pseudomonas* sp. DSS73	*Py. ultimum*	Irreversible loss of mycelial growth ability Degradation of proteinaceous cell-wall components and leakage of cell constituents	Dunne et al. (1997, 2000), Nielsen et al. (2002), Koch et al. (2002)
β-1,3-glucanases	*L. enzymogenes* strain C3, *Burkholderia* (*Pseudomonas*) *cepacia*	*Py. ultimum*	Lyse hyphae and/or inhibit hyphal growth	Palumbo et al. (2005) Fridlender et al. (1993)
	Paenibacillus sp., *St. sioyaensis*	*Py. aphanidermatum*	Inhibit mycelial growth and causes degradation or lysis of cell wall of hyphae	Hong and Meng (2003)
β-1,3-, β-1,4- and β-1, 6-glucanases	*Streptomyces* spp. (Strain EF-72, EF-22, EF-34, EF-14, EF-97, EF-76, EF-27, EF-43, DVD3, EF-25, and DVD4)	*Ph. fragariae* var. *rubi*	Hydrolyze glucans from *Phytophthora* cell walls and cause lysis of *Phytophthora* cells	Valois et al. (1996)
SAP (A novel antimicrobial protein)	*Streptomyces* sp. AP77	*Py. porphyrae*	Mycelial growth inhibition	Woo et al. (2002)

the bacterial mutants capable of over producing serine protease showed improved biocontrol activity against *Pythium* spp. on sugar beet (Dunne et al. 2000). Serine protease is antiperonosporomycetal against *Py. ultimum*, causing irreversible loss of mycelial growth ability, degradation of proteinaceous cell-wall components, and leakage of cell constituents (Dunne et al. 1997, 2000). Similarly, the ability of strain DSS73 to inhibit the growth of the root pathogenic peronosporomycete, *Py. ultimum* is believed to originate from the production and excretion of proteases and other biocontrol traits by the bacterium (Nielsen et al. 2002; Koch et al. 2002).

Bacterial enzymes with glucanolytic activity have a very significant role in the suppression of peronosporomycete diseases because β-glucans constitute 80–90 % of the wall dry weight of the peronosporomycete phytopathogens. *L. enzymogenes* strain C3 produces multiple extracellular β-1,3-glucanases encoded by the *gluA*, *gluB*, and *gluC* genes and are thought to contribute to the biological control activity against *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet (Palumbo et al. 2005). *Bu. cepacia* synthesizes β-1,3-glucanase that destroys the integrity of *R. solani*, *S. rolfsii*, and Py. *ultimum* cell walls (Fridlender et al. 1993). Valois et al. (1996) reported that the antagonistic actinomycetes that suppressed the mycelial growth of *Ph. fragariae* var. *rubi* were shown to produce glucanases cleaving β-1,3, β-1,4, and β-1,6. These enzymes could hydrolyze glucans from cell wall structure of growing mycelia of *Pythium* and *Phytophthora*, and cause lysis of hyphal cells (Valois et al. 1996; Hong and Meng 2003; Palumbo et al. 2005). *Streptomyces* sp. AP77 produces an extracellular protein to suppress *Pythium*. Surprisingly, this anti-*Pythium* protein, designated as SAP, had neither *Pythium* cell wall-degrading activity nor any other polysaccharolytic activity, implying that it has a unique inhibitory function different from those of the polysaccharolytic enzymes used in the higher terrestrial plants (Woo et al. 2002).

7.4.4 Competition Between Plant-Associated Bacteria and Peronosporomycete Phytopathogens

Rhizosphere is a battle field or playground for diverse microorganisms including plant pathogens and beneficial bacteria. Competition between plant-associated bacteria and phytopathogens has long been thought to be an important means of suppressing plant diseases. In rhizosphere and spermosphere habitats, common critical resources are shared by both the pathogen and introduced microbial biocontrol strains. They compete with each other for nutrient and/or space. Root and seed exudates are primary source of nutrient for the rhizosphere microorganisms. Rhizosphere competence implies that bacterial antagonists are well adapted to their utilization (Lugtenberg et al. 1999). Germination and hyphal growth of pathogen propagules are stimulated by root and seed exudates (Stanghellini and Burr 1973; Nelson 1990). However, degradation of exudate stimulants by introduced microbial strain limits germination of pathogen propagules and hyphal growth of the pathogen. As a result, chances of the disease development are reduced.

For example, biocontrol of *Py. ultimum* by pseudomonads could be mediated through competition for seed volatiles. Hyphal growth from soilborne sporangia of *Py. ultimum* is stimulated by volatile compounds such as ethanol and acetaldehyde from the germinating seeds of pea and soybean.

However, this stimulation is reduced when seeds are treated with *Ps. putida* NIR. NIR uses these volatiles as carbon source and reduce their concentration in the spermosphere through metabolism. Heungens and Parke (2000) reported that *B. cepacia* AMMDR1 controls *Py. aphanidermatum* largely through antibiosis, but competition for zoospore-attracting compounds can also contribute to the effect. The bacterium AMMDR1 metabolizes the zoospore attractant. Zoospores of *Pythium* spp. are also attracted to water soluble sugars, amino acids, and fatty acids exuded by seeds or seedling (van Dijk and Nelson 1998). van Dijk and Nelson (1998) have shown that *E. cloacae* strain EcCt-501 can utilize seed exudates from a number plant species as a sole carbon source and reduces stimulatory activity of exudate to *Py. ultimum* sporangia by metabolizing the active stimulatory molecules including linoleic acid present in the exudates. Therefore, this trait is important for biological control of *Pythium* seed rot by this bacterial antagonist.

Fatty acids from seeds and roots are required to elicit germination responses of *P. ultimum* (van Dijk and Nelson 2000). Two mutants of *E. cloacae* EcCT-501R3, Ec31 (*fadB*) and EcL1 (*fadL*), reduced in β-oxidation and fatty acid uptake, respectively, fail to metabolize linoleic acid, to inactivate the germination-stimulating activity of cotton seed exudate and linoleic acid, and to suppress *Pythium* seed rot (van Dijk and Nelson 2000). This suggests that *E. cloacae* prevents *Py. ultimum* seed infections by preventing the germination of *Py. ultimum* sporangia through efficient metabolism of fatty acid components of seed exudate. However, the success of *E. cloacae* as a biological control organism is directly related to its ability to rapidly interfere with the early responses of *Pythium* propagules to germinating seeds. *E. cloacae* fails to suppress *Pythium* damping-off, if bacterial cells are added after full sporangial activation, but suppresses *Py. ultimum* seed infections if sporangial activation and germination happens within the first 30–90 min after sowing (Windstam and Nelson 2008a). It has been reported that *E. cloacae* can reduce the stimulatory activity of cucumber but not corn seed exudates (Kageyama and Nelson 2003). This is due to exudate inactivation by *E. cloacae* occurs in the cucumber spermosphere but not in the corn spermosphere (Windstam and Nelson 2008a). Other components of the seed exudates such as elevated level of sugars in the corn spermophere prevent degradation of long-chain unsaturated fatty acids by *E. cloacae*, leading to its failure to suppress *Py. ultimum* sporangial activation, germination, and subsequent disease development (Windstam and Nelson 2008b).

7.4.5 Production of Siderophores by Biocontrol Bacteria

Siderophores are small, high-affinity iron chelating compounds secreted by grasses and microorganisms such as bacteria and fungi. Iron is an important mineral

element essential for growth of all living organisms including microorganisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces leads to a furious competition (Loper and Henkels 1997). Under iron-limiting conditions, some antagonistic bacteria produce low molecular weight compounds called siderophores to competitively acquire ferric ion (Whipps 2001). These metabolites chelate the ferric ion and serve as vehicles for the transport of Fe(III) into bacterial cells. There are various types of bacterial siderophores, which differ in their abilities to sequester iron. However, in general, they deprive pathogenic peronosporomycetes of this essential element since their siderophores have lower affinity (Loper and Henkels 1999; O'Sullivan and O'Gara 1992). Some plant growth promoting microorganisms increase plant growth by supplying the plant with sequestered iron where microbial siderophores are used by plants as a source of iron. In a number of cases, the growth promotion and biocontrol effect of rhizosphere-inhabiting bacteria has been attributed to siderophore-mediated iron acquisition (Kloepper et al. 1980a; Loper 1988). Siderophore production was detected in the isolates of *Ba. subtilis* that increase shoot and root length of red pepper plants and suppress *Phytophthora* blight of the plants (Lee et al. 2008).

Becker and Cook (1988) reported that the plant growth-promoting activity of some strains of fluorescent pseudomonads on wheat results from ability of the strains to suppress *Pythium* by production of siderophores. Siderophore, pyoverdin production has been proved to be important in the biological control of *Pythium*-induced damping-off of cotton by *Ps. fluorescens* 3551(Loper 1988) and in the inhibition of *Ph. parasitica* by *P. fluorescens* and *P. putida in vitro* (Yang et al. 1994). *Ps. aeruginosa* 7NSK2 improves the growth of several crops (Höfte et al. 1991) by producing three siderophores, salicylic acid (Buysens et al. 1996), pyochelin (Höfte et al. 1993), and the fluorescent pyoverdin (Höfte et al. 1993) under iron limiting condition. Production of either pyochelin or pyoverdin by 7NSK2 is necessary to achieve high levels of protection against *Py. splendens*-induced postemergence damping-off in tomato (Buysens et al. 1996). The action of pyoverdin and pyochelin seems to be interchangeable because a mutant producing only pyochelin or pyoverdin is equally antagonistic and with both mutants, wild-type levels of protection are obtained. Mycelial growth (Loper 1988) but not sporangial germination (Paulitz and Loper 1991) of *Pythium* spp. is inhibited by iron starvation. *Streptomyce*s species are known for the production of hydroxamate type siderophores, which inhibit phytopathogen growth by competing for iron in rhizosphere soils (Khamna et al. 2009). Suppression of *Ph. medicaginis* as well as enhancement of plant growth by actinobacterial strains is attributed not only to their antibiotic production, but also to the ability to produce siderophores (Misk and Franco 2011).

7.4.6 Induced Systemic Resistance

Some biocontrol bacteria elicit a phenomenon that is known as induced systemic resistance (ISR) in the host plant. ISR of plant against pathogen is a widespread phenomenon that has been intensively investigated with respect to the underlying

signaling pathways as well as to its potential use in plant protection (Heil and Bostock 2002). Elicited by a local infection or colonization of nonpathogenic bacteria, plants respond with a salicylic-dependent signaling cascade that leads to the systemic expression of a broad spectrum and long-lasting disease resistance that is efficient against peronosporomycetes, fungi, bacteria, and viruses. Changes in cell wall composition, de novo production of pathogenesis-related proteins such as chitinases and glucanases, and synthesis of phytoalexins are associated with resistance (Kloepper and Tuzun 1996; Van Loon et al. 1998).

Bacterial agents mediated ISR against peronosporomycete pathogens such as *Ph. infestans*, *Ph. capsici*, *Py. aphanidermatum*, *Pl. halstedii*, *Sclerospora graminicola*, *H. parasitica*, and *Peronospora tabacina* have been demonstrated in many plant species including tomato, sunflower, squash, chili, pearl millet, apple seedling, tobacco, and Arabidopsis (Umesha et al. 1998; Ongena et al. 1999, 2005; Yan et al. 2002; Zhang et al. 2001, 2010; Van der Ent et al. 2008; Mazzola et al. 2007; Muthukumar et al. 2011). Zhang et al. (2010) reported that some strains of *Bacillus* spp. are effective in inducing ISR against *Ph. capsici* on squash, and improved disease control can be achieved by multiplexing them.

Two strains of plant growth-promoting rhizobacteria, *Ba. pumilus* SE34 and *Ps. fluorescens* 89B61, elicited systemic protection against late blight on tomato (Yan et al. 2002). Induced resistance by SE34 and 89B61 is associated with reduction in disease severity and germination of sporangia and zoospore on the leaf surface. Although physical separation between tested bacteria and target pathogens are necessary for ISR to be elicited, strain SE34 is detected in the leaves, suggesting the involvement of additional mechanism beside ISR during SE34-mediated protection (Yan et al. 2002). Localized stimulation of one part of a plant can result in the systemic expression of resistance in other parts; therefore, it has been hypothesized that a signal is generated and mobilized from the initial infection site (Dean and Kuc 1986).

Salicylic acid (SA), jasmonic acid (JA), and ethylene apparently are involved in signaling pathways. Both *Ba. pumilus* SE34 and *Ps. fluorescens* 89B61 elicit ISR in a JA-dependent manner. From a molecular point of view, the onset of rhizobacteria-mediated ISR has not generally been associated with major changes in gene expression. The nonpathogenic rhizobacterial strain *Ps. fluorescens* WCS417r has been shown to trigger ISR against *H. parasitica* in Arabidopsis (Ton et al. 2002). However, root inoculation with *Ps. fluorescens* WCS417r does not lead to an accumulation in the roots or in the leaves of the SA-responsive genes *PR*-1, *PR*-2, and *PR*-5, of the ET-inducible gene *Hel*, of the ET- and JA- responsive genes *ChiB* and *Pdf1.2*, or of the JA-inducible genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*. A change could only be observed in the potentiation of the expression of JA-dependent *Atvsp* after pathogen challenge of ISR-expressing plants (van Wees et al. 1999). Higher level of antimicrobial phenolics were accumulated in cucumber plants treated with *Ps. fluorescens* BTP1 and M3 and challenged with *Py. aphanidermatum* (Ongena et al. 1999). Similarly, potentiated activities of phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), and phenolics were observed in *Ps. fluorescens* Pf1 pretreated tomato and hot pepper

plants challenged with *Py. aphanidermatum* (Ramamoorthy et al. 2002). Combined application of talc-based formulation of *Ps. fluorescens* EBL 20-PF and *Trichoderma viridae* and challenge inoculated with *Py. aphanidermatum* recorded maximum induction of PO, PPO, PAL, β-1,3-glucanase, and the accumulation of phenolics in chili plants (Muthukumar et al. 2011).

Further studies using *H. parasitica* have shown that WCS417r-mediated ISR is elicited through JA-, ET-, and *NPR1*-dependent defenses (Ton et al. 2002). T-DNA knockout mutants *myb72*-1 and *myb72*-2 are incapable of mounting ISR against the pathogen *H. parasitica*, indicating that *MYB72* is essential to establish ISR against this pathogen (van der Ent et al. 2008). *MYB72* is an ethylene-inducible R2R3-*MYB*-like transcription factor gene and is specifically activated in the roots upon colonization by WCS417r (Verhagen et al. 2004). Root inoculation of *Arabidopsis thaliana* ecotype Columbia with *Ps. fluorescens* CHA0r also partially protect leaves from the *H. parasitica* where production of DAPG is thought to be the determinant of *Ps. fluorescens* CHA0r for this ISR (Iavicoli et al. 2003). Although DAPG is known for its antibiotic property, in this experiment DAPG produced by bacteria leads to physiological changes that subsequently induce ISR. Another study on pea plants indicates that DAPG can act also as a plant hormone-like substance, inducing physiological and morphological changes that enhance nodulation by *Rhizobium*. ISR induced by CHA0r depends on *NPR1* and JA signaling pathways, indicating that ISR induced by strains WCS417r and CHA0r against *H. parasitica* differ with respect to the sensitivity to ET.

Bacteria-mediated ISR against peronosporomycete phytopathogen is also associated with changes in plant defense-related enzymes (Ongena et al. 2005; Muthukumar et al. 2011). Disease protection by *Ba. subtilis* M4 against *Py. aphanidermatum* in tomato is associated with significant changes in gene transcription in the host plant (Ongena et al. 2005). These enzymes are known to have antifungal properties and thought to play an important role in plant defense by restricting the growth and development of pathogens (Boller 1992). Although several hundred articles on ISR have been published, however, many questions are still unanswered and require further investigation. Strong efforts are required to identify the compounds causing resistance, and future studies should quantify these compounds in combination with the biologically detectable resistance to characterize the induced stage (Heil and Bostock 2002).

7.4.7 Hyperparasitism

Parasitism is a symbiosis in which two phylogenetically unrelated organisms coexist over a prolonged period of time. An avirulent pathogen could be hyperparasite on more virulent pathogens. The activities of various hyperparasites that parasitize virulent plant pathogens can lead to biocontrol. Several bacterial antagonists have been reported to be hyperparasites on several peronosporomycetes. *Actinoplanes* spp. are filamentous bacteria that produce minute sporangia, which when hydrated

release motile spores capable of parasitizing *Pythium* spp. or related microorganisms. Khan et al. (1993) reported that oospores of *Py. aphanidermatum*, *Py. arrhenomanes*, *Py. irregulare*, *Py. myriotylum*, and *Py. ultimum* were parasitized by *Ac. azureus*, *Ac. brasiliensis*, *Ac. caeruleus*, *Ac. ferrugineus*, *Ac. ianthinogenes*, *Ac. italicus*, *Ac. minutisporangius*, *Ac. rectilineatus*, *Ac. teichomyceticus*, *Ac. utahensis*, *Ac. violaceous*, *Ac. yunnahenis*, plus 15 strains of unspeciated *Actinoplanes*. Parasitized oospores had disorganized cytoplasms and hyphae of *Actinoplanes* sp. emerging from them. Filonow and Dole (1999) showed that strains of *Actinoplanes* spp. that are hyperparasites of oospores of *Pythium* spp. reduce root rot severity and increase plant stand of poinsettia and geranium.

Ac. missouriensis and *Pseudomonas* spp. infect oospores of *Ph. megasperma* var. *sojae*, *Ph. cactorum*, *Pythium* sp., and *Ap. euteiches* in natural soil, reducing populations of oospores in soil. Tu (1978) reported that *Rh. japonicum* reduced *Phytophthora* root rot of soybean by parasitizing hyphae of the peronosporomycete. The bacteria colonize growing hyphal tips and prevent contact between *Ph. megasperma* and host root tissue, and thereby reduce the chance of *Ph. megasperma* infection. *Rhizobium* reduces fungal sporulation and causes extensive surface and internal colonization of mycelia of *Ph.megasperma* and *Py. ultirnum* (Tu 1978).

7.4.8 *Plant and Hyphal Colonization*

Antagonistic bacteria are thought to protect against root pathogens more effectively if they have a strong ability to colonize the root system (Weller 1988). Islam et al. (2005a, b) investigated plant colonization behavior of a *Lysobacter* sp. SB-K88 by the aid of scanning electron microscopy (SEM). SB-K88 colonizes both the root and leaf surfaces in a characteristic perpendicular fashion. In colonized regions, a semitransparent film apparently enveloping the root and microcolonies was observed on the sugar beet root surface (Islam et al. 2005a). That *Lysobacter* strain also efficiently colonized the roots of several plants, including spinach, tomato, *A. thaliana*, and *Amaranthus gangeticus*. Interestingly, the SB-K88 also colonized *Ap. cochlioides* hyphal surface in the same perpendicular manner when grown together on liquid medium. Detailed transmission electron microscopic analysis revealed that the SB-K88 has long (~6 μm) brush-like, fragile fimbriae at one pole of the dividing bacterial cells. As fimbriae are known to function in bacteria to adhere to the substrates, Islam et al. assumed that brush-like fimbriae help SB-K88 to attach perpendicularly on plant and hyphal cell walls as well as for gliding motility. Presence of fimbriae appears to be characteristic structural features of bacteria having gliding motility (Spormann 1999).

In cotton, control of *Pythium* seed rot and preemergence damping-off by *E. cloacae* and *E. herbicola* strains were correlated with suppression of seed colonization by *Pythium* spp. (Nelson 1988). Seed pericarps are favorable ecological niches for species of *Pseudomonas*. *Ps. fluorescens-putida* ML5 and *Ps. putida* R20 readily

colonize pericarp of sugar beet seed. Occupation of this competitive site in spermosphere accounts for the effectiveness of these strains in reducing the incidence of seed rot and damping-off caused by *Py. ultimum* (Osburn et al. 1989). Fukui et al. (1994a, b) also demonstrated the importance of high initial pericarp colonization by *Ps. fluorescens* for antagonism against *Py. ultimum*. The dynamics of bacterial colonization of plant tissues vary among bacteria as influenced by various environmental factors (Loper et al. 1985). For *Ps. fluorescens* B5, the total population size per plant and downward colonization of the root (below 40 mm depth) increased significantly with increasing its inoculum density applied to the seeds, while for *Ps. corrugata* 2140, no significant influence of initial inoculum density on root colonization was observed (Schmidt et al. 2004a). Soil matrix potential and temperature had pronounced influence on seed or root colonization and biological control of *Pythium* spp. by pseudomonads (Mathre et al. 1994; Schmidt et al. 2004b). Population density of *Ps. fluorescens B5* per seedling as well as downward colonization and biocontrol performance by B5 were significantly reduced at high temperatures (25–35 °C), while *Ps. aureofaciens* AB254 (Mathre et al. 1994) showed good biological control against *Py. ultimum* at temperatures above 22 °C. This strain may therefore complement *Ps. fluorescens* B5 in a combined inoculum, although compatibility would have to be confirmed.

In addition to the colonization of plant roots, the colonization of hyphae could be an important mechanism for maintaining a close association between antagonistic bacteria and peronosporomycetal pathogens. Biological control of *Pythium* seed rot and preemergence damping-off of cotton by *E. cloacae* and *Erwinia herbicola* have been attributed to the ability of bacteria to attach to the hyphae and to inhibit the growth of *Py. ultimum* (Nelson 1988). *Ps. putida* 06909 grew extensively on hyphae of *Ph. parasitica* that cause citrus root rot and inhibited, but did not kill, the pathogen *in vitro* (Yang et al. 1994). Hyphal colonization-deficient *Tn5* mutants of *P. fluorescens* and *P. putida* were nonflagellated and were defective in colonization and inhibition of colonies of *Ph. parasitica in vitro*, confirming the linkage between the loss of flagella and the loss of the ability to colonize and inhibit peronosporomycetal mycelia. The loss of flagella in *Tn5* mutants of *P. fluorescens* has also been associated with an inability to colonize potato roots (de Weger et al. 1987). In another study, nonflagellated *Tn5* mutants of *P. fluorescens* were defective in adhesion to sand, suggesting that the flagella, or other outer membrane proteins associated with flagella, were involved in adhesion to surfaces (Deflaun et al. 1990). Islam et al. (2005b) demonstrated that a biocontrol strain *Lysobacter* sp. SB-K88 equally colonize both plant roots and hypahe of damping-off pathogen *Aphanomyces cochlioides* in perpendicular fashion. The SB-K88 does not show penetration of hyphal cells, however, disrupts ultrastructure and cytoskeleton of the *Ap. cochlioides* cells by secreting macrocyclic lactam antibiotics.

7.5 Conclusion and Perspectives

The peronosporomycetes have remarkable biological features including β-1,3-glucan polymers and cellulose made cell wall, energy storage carbohydrate mycolaminarin, diploidy at the vegetative stage, motile zoospores, and sexual cycle, which make them ubiquitous inhabitants in the diverse environment. Application of bacterial antagonists for the management of peronosporomycetal diseases of various plants is strongly connected to our understanding of bacterial diversity, host specificity, mechanism of action, active ingredient identification, formulation, and application. Information on potential biocontrol agents, their *in vitro* antagonistic activity, detection and isolation techniques of antipathogenic compounds, mode of action, in vivo disease suppressive activity, and practical formulation will facilitate to more efficient application methods of inoculant strains and strategy to reduce disease caused by peronosporomycete phytopathogens. A wide diversity of bacterial strains have been reported with antagonistic activity and some have shown high promise in vivo biocontrol effect on economically important peronosporomycete plant diseases through different mechanisms including antibiosis, high plant colonization, induced systematic resistance, and lytic enzyme production. This wealth of information has provided a firm foundation for broader incorporation of these bacterial antagonists into sustainable strategies for the management of peronosporomycete phytopathogens.

Although knowledge on ecological impact of biocontrol agents as alternative means of chemical-based plant disease management has been enhanced in the past decades, however, the greatest challenge facing BCAs is to release them for practical use. BCAs are needed to be transformed from niche market products into mainstream crop protection agents. More technical developments are desired to recognize the high-throughput production process of quality biocontrol products and to identify factors that affect efficacy and shelf life of these living biopesticides. Aside from the fundamental research for biocontrol agent identification, application, and efficacy improvement, continued effort should be taken to establish close linkage between researchers and entrepreneurs to facilitate technology promotion and acceptance by end users. Recent advances on whole genome sequences of several peronosporomycetes and biocontrol bacteria will provide the future basis for a comprehensive understanding of BCA–plant–pathogen interactions and development of improved strains with customized properties that will potentially function as effective biocontrol agents in low input sustainable agriculture.

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Chapter 8
Pseudomonas Inoculants as Agents for Plant Disease Management

Nobutaka Someya, Seishi Ikeda, and Kenichi Tsuchiya

8.1 Introduction

Pseudomonas is one of the most ubiquitous bacterial genus. It has been isolated from diverse environments, including soils, water, animals, plants, and clinical samples. A total of 128 species is officially recognized for this genus (Peix et al. 2009). Most species are saprophytic, but some are pathogens of both plants and animals. It is reported that 23 and 16 species are known to be causal agents for plant and animal (including humans) diseases, respectively (Höfte and Altier 2010).

However, some *Pseudomonas* species are agriculturally beneficial for controlling phytopathogens and enhancing plant growth. Although some of these species also are plant and animal pathogens, others are saprophytic when appropriate host plants are absent. Some isolates have been used in commercial products in the form of biofungicides and biofertilizers (Fravel 2005). In the following chapters, we summarize previous studies of plant-associated *Pseudomonas* spp. from an agricultural perspective, and then discuss the application of beneficial *Pseudomonas* spp. with an emphasis on the latest advancements in molecular microbial ecology.

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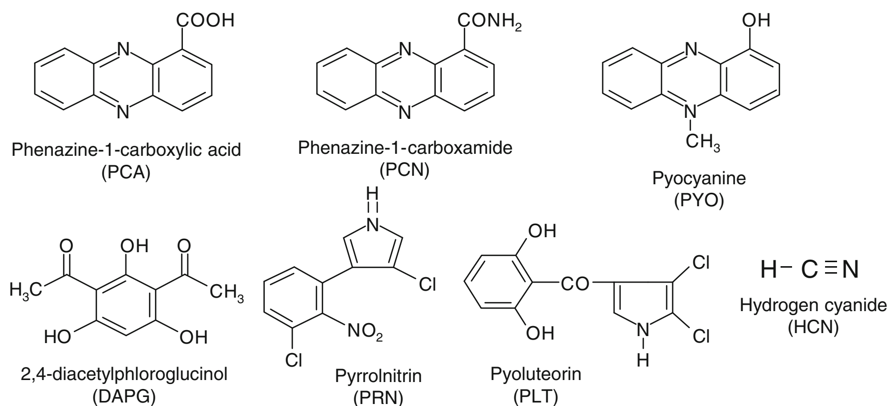


Fig. 8.1 Structures of major antibiotics produced by biocontrol *Pseudomonas* spp. that have activity against phytopathogens

8.2 *Pseudomonas* as a Biocontrol Agent

The best known application of *Pseudomonas* in agriculture is biocontrol of phytopathogen. *Pseudomonas* spp. are effective against a wide range of phytopathogens, and function by both direct and indirect mechanisms. Antibioses involving antibiotics and fungal cell wall degrading enzymes are direct mechanisms of biocontrol. On the other hand, competition for space and nutrient resources in the phytosphere and the induction of disease resistance in plants are indirect mechanisms of plant growth promotion.

Previous studies have empirically demonstrated that most biocontrol capabilities in this genus occur in fluorescent pseudomonads, such as *P. chlororaphis*, *P. fluorescens*, and *P. putida*. Extracellular diffusible pigments, such as pyoverdine siderophores, are responsible for the fluorescence of these organisms. Antibiotics are primary agents by which fluorescent pseudomonads control phytopathogens. Most fluorescent pseudomonads that have been isolated for biocontrol produce one or more antibiotics (Gross and Loper 2009; Haas and Keel 2003; Ligon et al. 2000; Raaijmakers et al. 2002, 2006). Among these bacteria, isolates that produce 2,4-diacetylphloroglucinol (DAPG), phenazines [i.e., phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCA), and pyocyanin (PYO)], pyoluteorin (PLT), pyrrolnitrin (PRN), and hydrogen cyanide (HCN) have biocontrol efficacy against a wide range of phytopathogens (Fig. 8.1). These and other antibiotics that are produced by biocontrol *Pseudomonas* spp. are listed in Table 8.1. In addition, the genes for several novel antibiotics have been identified in the genomes of biocontrol *Pseudomonas* (Paulsen et al. 2005).

The importance of antibiotic biocontrol by pseudomonads has been demonstrated using antibiotic-deficient mutants in biocontrol assays (Tran et al. 2007; Voisard et al. 1989). In some bacteria that are capable of producing multiple antibiotics, a series of mutants in the same isolate has demonstrated that the biocontrol activity of

Table 8.1 Antibiotics produced by biocontrol *Pseudomonas* spp. that have activity against phytopathogens

Compound[a]	Producer[b]	Target pathogens[c]	References
Aerugine	*P. fluorescens*	*Co*, *Pc*, *Pu*	Lee et al. (2003)
N-butylbenzenesulphonamide	*Pseudomonas* sp.	*Bc*, *Fo*, *Pc*, *Pu*, *Rs*	Raaijmakers et al. (2002)
Butyrolactones	*P. chlororaphis* subsp. *aureofaciens*	*Pcr*, *Pu*	Raaijmakers et al. (2002)
Cyclic lipopeptide (Viscosin group, Amphisin group, Tolaasin group, Syringomycin group)	*P. asplenii* *P. fluorescens* *P. fuscovagina* *P. koreensis* *P. syringae*	*Gc*, *Pd*, *Pi*, *Pim*, *Pu*, *Rp*, *Rs*	Hultberg et al. (2010), Raaijmakers et al. (2006)
2,3-Deepoxy-2,3-didehydroehizoxin	*Pseudomonas* sp.	*Fc*, *Mn*, *Pt*, *Tc*	Raaijmakers et al. (2002)
2,4-Diacetylphloroglucinol (DAPG)	*P. aeruginosa* *P. brassicacearum* *P. chlororaphis* *P. fluorescens* *Pseudomonas* sp.	*Ac*, *Cg*, *Fo*, *Ggt*, *Pc*, *Pu*, *Rs*, *St*, *Tb*	Chung et al. (2008), Haas and Keel (2003), Raaijmakers et al. (2002), Ryu et al. (2000)
Fluviol	*P. fluorescens*	*Ec*, *Hg*, *Ps*, *Vd*	Smirnov et al. (1997)
Gluconic acid	*Pseudomonas* sp.	*Ggt*	Kaur et al. (2006)
3-(1-Hexenyl)-5-methyl-2-(5H)furanone	*P. chlororaphis* subsp. *aureofaciens*	*Fo*, *Fs*, *Pu*, *Rs*, *Tb*	Paulitz et al. (2000)
2-Hexyl-5-propyl resorcinol	*P. fluorescens*	*Dn*, *Rs*, *Pu*	Ligon et al. (2000), Cazorla et al. (2006)
Hydrogen cyanide (HCN)	*P. corrugata* *P. fluorescens* *Pseudomonas* sp.	*Bc*, *Tb*	Gross and Loper (2009) Guo et al. (2007) Haas and Keel (2003)
N-mercapto-4-formylcarbostyril	*P. fluorescens*	*Cc*, *Cl*, *Fc*, *Fo*	Fakhouri et al. (2001)
Oomycin A	*P. fluorescens*	*Pu*, *Pythium* spp.	Ligon et al. (2000)
Organic volatile compounds (Undecene, Undecadiene, Benzyloxybenzonitrile)	*P. fluorescens* *P. trivialis*	*Rs*	Kai et al. (2007)
Phenazines (PCA, phenazine-1-carboxylic acid; PCN, phenazine-1-carboxamide; 2-OH-PCA; Pyocyanin)	*P. aeruginosa* *P. chlororaphis* *P. fluorescens* *P. oryzihabitans* *Pseudomonas* sp.	*Bs*, *Cgr*, *Co*, *Fo*, *Ggt*, *Lk*, *Mp*, *Psp*, *Pu*, *Pythium* spp., *Rs*, *Sh*, *Ti*	Haas and Keel (2003) Kapsalis et al. (2008) Ligon et al. (2000) Powell et al. (2000) Raaijmakers et al. (2002)
Phenylacetic acid	*Pseudomonas* sp.	*Bc*, *Fo*, *Pc*, *Pu*, *Rs*	Kang et al. (1999)
Pyrrolnitrin (PRN)	*P. fluorescens* *Pseudomonas* sp.	*Alternaria* spp., *Bc*, *Fsa*, *Pe*, *Ptr*, *Rs*, *Ss*, *Tb*, *Vd*	Haas and Keel (2003), Ligon et al. (2000), Raaijmakers et al. (2002)

(continued)

Table 8.1 (continued)

Compound[a]	Producer[b]	Target pathogens[c]	References
Pyoluteorin (PLT)	*P. fluorescens* *Pseudomonas* sp.	*Pu*, *Rs*, *Tb*	Haas and Keel (2003) Raaijmakers et al. (2002)
Rhamnolipids	*P. aeruginosa* *P. chlororaphis*	*Pythium* spp.	Ligon et al. (2000)
Trigliceropeptides	*P. chlororaphis* *P. putida*	*Aa, Fusarium* spp., *Hs*, *Pf*	Chetverikov and Loginov (2005)

[a]Structurally related compounds such as cyclic lipopeptide and phenazines are listed as single groups.
[b]Species that are responsible for the production of each antibiotic.
[c]Representative phytopathogens that are biocontrolled by antibiotics. *Abbreviations*: *Aa Alternaria alternata*, *Ac Aphanomyces cochlioides*, *Bs Bipolaris sorokiniana*, *Bc Botrytis cinerea*, *Cc Cladosporium cucumerinum*, *Cgr Colletotrichum graminicol*, *Cg Colletotrichum gloeosporioides*, *Cl Colletotrichum lagenarium*, *Co Colletotrichum orbiculae*, *Dn Dematophora necatrix*, *Ec Erwinia carotovora*, *Fc Fusarium culmorum*, *Fo Fusarium oxysporum*, *Fsa Fusarium sambucinum*, *Fs Fusarium solani*, *Ggt Gaeumannomyces graminis* var. *tritici*, *Gc Geotrichum citriaurantii*, *Hg Helminthosporium gramineum*, *Hs Helminthosporium sativum*, *Lk Leptosphaeria korrae*, *Mp Magnaporthe poae*, *Mn Microdochium nivale*, *Pd Penicillium digitatum*, *Pe Penicillium expansum*, *Pf Penicillium funiculosum*, *Pc Phytophthora capsici*, *Pcr Phytophthora cryptogea*, *Pi Phytophthora infestans*, *Ps Pseudomonas syringae*, *Pt Pyrenophora teres*, *Ptr Pyrenophora triticirepentis*, *Pim Pythium intermedium*, *Psp Pythium splendens*, *Pu Pythium ultimum*, *Rs Rhizoctonia solani*, *Rp Rhodotorula pilimanae*, *Sh Sclerotinia homoecarpa*, *Ss Sclerotinia sclerotiorum*, *St Septoria tritici*, *Tb Thielabiopsis basicola*, *Tc Tilletia caries*, *Ti Typhyla incarnata*, *Vd Verticillium dahliae*.

an isolate depends on the production of specific antibiotics. For example, *P. chlororaphis* PA23 produces the antibiotics PCA, 2-hydroxyphenazine, and PRN. However, only PRN was required for biocontrol activity against *Sclerotinia sclerotiorum* (Selin et al. 2010). In contrast, *P. fluorescens* 2-79 requires phenazine for biocontrol of take-all on wheat (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006). Moreover, *P. aeruginosa* PNA1 produces phenazines and rhamnolipids, which are biodetergents that act synergistically against *Pythium* spp. (Perneel et al. 2008). In these cases, the biochemical mechanisms of antibiotic effects on pathogens are similar to those of chemical pesticides. Recent reports have demonstrated that there is intra-species variability in phytopathogen sensitivity to specific antibiotics (Kwak et al. 2010; Mazzola et al. 1995; Schouten et al. 2004). This implies that, similar to chemical fungicides, the effects of antibiotic biocontrol agents on phytopathogens must be assessed on a case-by-case basis.

Although detailed mechanisms of action are not known for most antibiotics, the mechanisms of biocontrol activity have been demonstrated for some. One of the better known antibiotics produced by biocontrol *Pseudomonas* isolates, DAPG, causes growth inhibition and excessive branching of hyphae in fungi by disrupting the filamentous actin cytoskeleton (Islam and Fukushi 2010). Phenazines, such as PCA, PCN, and PYO, also are toxic to a wide range of organisms. Although their antifungal mechanisms are not well understood, they are known to act as reducing

agents on cell membranes (Chin-A-Woeng et al. 2003). Phenazines are structurally related compounds. However, PCA and PCN have distinct effects on *Fusarium oxysporum* f. sp. *radicis-lycopersici*, which illustrate the difficulty of structure-based prediction of antifungal activity (Chin-A-Woeng et al. 2001). HCN is a potent inhibitor of cytochrome c oxidase and other metalloenzymes (Blumer and Haas 2000). Cyclic lipopeptide (CLP) causes immobilization and subsequent cell lysis of the entire zoospore of *Phytophthora infestans* (de Bruijn et al. 2007). Redox-active phenazines that are produced by fluorescent pseudomonads contribute to natural soil suppression of Fusarium wilt disease and may act synergistically with resident non-pathogenic *F. oxysporum*, which competes for carbon with pathogenic *Fusarium* (Mazurier et al. 2009).

Methods for screening antibiotic-producing fluorescent pseudomonads have progressed over several decades, and many antibiotic biosynthetic loci have been identified. Conserved regions of these loci have been utilized as polymerase chain reaction (PCR) primers to detect antibiotic-producing isolates. Therefore, it is straightforward to detect isolates that contain biosynthetic genes of target antibiotics. Interestingly, there are differences among *Pseudomonas* isolates in the number of antibiotic biosynthetic loci, ranging from none to several. Depending on the sampling environment, 1–20 % of rhizospheric pseudomonads produce antibiotics. Antagonistic isolates of antibiotic-producing *Pseudomonas* spp. comprise about 1 % of culturable bacterial populations in both tomato and avocado rhizospheres (Cazorla et al. 2006). Some primers that are used to detect antibiotic biosynthetic genes in biocontrol *Pseudomonas* spp. are summarized in Table 8.2.

Isolates that possess antibiotic biosynthetic genes are not always capable of producing antibiotics under test conditions. Thus, their productivity largely depends on their environment. For example, the *phlD* and *prnD* biosynthetic genes for DAPG and PRN, respectively, can be routinely detected by PCR (Fig. 8.2a, b). However, the production of the corresponding antibiotics varies widely among isolates depending on environmental conditions, as shown by thin-layer chromatography (Fig. 8.2c, d), which suggests that environmental conditions are crucial for production of antibiotics. An alternative exploitation for the absence of antibiotic production is non-functionality of target genes or biosynthetic loci. As a result, antibiotic production should be examined in conditions in which biocontrol agents are expected to be functional.

Recent molecular ecological studies have revealed the presence of a dominant genotype for antibiotic-producing *Pseudomonas* spp. in disease-suppressive soils (Mazurier et al. 2009; de Souza et al. 2003). The detection of such genotypes in environments or their screening in isolate collections has become a routine technique. However, the establishment of efficient and stable disease control using these isolates under field conditions is still a challenging task. One of the main problems is the presence of a huge biodiversity in field environments. Elucidation of biological networks in field environments may contribute to the reliable and efficient use of biocontrol agents.

Antibiotic production by bacteria is primarily a mechanism of interference competition that allows species to oust others from their preferred environments. Hence, it

Table 8.2 Polymerase chain reaction primers used for detecting major antibiotic biosynthetic genes in *Pseudomonas*

Target antibiotic[a]	Primer	Sequence	Reference
DAPG	Phl2a Phl2b	GAGGACGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	Raaijmakers et al. (1997)
DAPG	B2BF BPR4	ACCCACCGCAGCATCGTTTATGAGC CCGCCGGTATGGAAGATGAAAAAGTC	McSpadden Gardener et al. (2001)
HCN	ACa ACb	ACTGCCAGGGGCGGATGTGC ACGATGTGCTCGGCGTAC	Ramette et al. (2003)
HCN	PM2 PM7-26R	TGCGGCATGGGCGTGTGCCATTGCTGCCTGG CCGCTCTTGATCTGCAATTGCAGGCC	Svercel et al. (2007)
PCA	PCA2a PCA3b	TTGCCAAGCCTCGCTCCAAC CCGCGTTGTTCCTCGTTCAT	Raaijmakers et al. (1997)
PCA	PHZ1 PHZ2	GGCGACATGGTCAACGG CGGCTGGCGGCGTATTC	Delaney et al. (2001)
PCN	PhzH-up PhzH-low	CGCACGGATCCTTTCAGAATGTTC GCCACGCCAAGCTTCACGCTCA	Mavrodi et al. (2001a)
PLT	plt1 plt2	ACTAAACACCCAGTCGAAGG AGGTAATCCATGCCCAGC	Mavrodi et al. (2001b)
PLT	PltBf PltBr	CGGAGCATGGACCCCCAGC GTGCCCGATATTGGTCTTGACCGAG	Mavrodi et al. (2001a, b)
PLT	PLTC1 PLTC2	AACAGATCGCCCCGGTACAGAACG AGGCCCGGACACTCAAGAAACTCG	de Souza and Raaijmakers (2003a)
PRN	PrnCf PrnCr	CCACAAGCCCGGCCAGGAGC GAGAAGAGCGGGTCGATGAAGCC	Mavrodi et al. (2001a, b)
PRN	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	de Souza and Raaijmakers (2003a)

[a]*DAPG* 2,4-diacetylphloroglucinol, *HCN* hydrogen cyanide, *PCA* phenazine-1-carboxylic acid, *PCN* phenazine-1-carboxamide, *PLT* pyoluteorin, *PRN* pyrrolnitrin

follows that biocontrol is the outcome of microbial competitive abilities. Arguments on the role of antibiotics in biocontrol pseudomonad of phytopathogens have been resolved by experiments that use antibiotic-deficient mutants of biocontrol *Pseudomonas* spp. (Keel et al. 1992; Kraus and Loper 1992; Voisard et al. 1989). Antibiotic production by soil bacteria is several orders of magnitude lower for phytopathogen eliminations; however, local antibiotic concentrations on the epidermis of host plants may be sufficient to prevent invasion by sensitive phytopathogens (Haas and Keel 2003).

Most *Pseudomonas* spp. do not have antimicrobial activity, possibly because the cost of resource consumption by antibiotic biosynthetic pathways exceeds the benefits gained in competition for resources. Currently, it is difficult to determine the ecological significance of antibiotic production by biocontrol *Pseudomonas* spp., especially

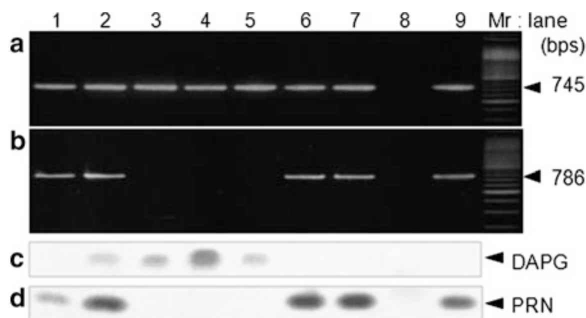


Fig. 8.2 Detection of antibiotic biosynthetic genes and products from different pseudomonad isolates with specific primer sets and thin layer chromatography. *Panel* **a**: detection of the *phlD* gene in 2,4-diacetylphloroglucinol (DAPG) biosynthesis; *Panel* **b**: Detection of *prnD* gene in pyrrolnitrin (PRN) biosynthesis. *Panel* **c**: Detection of DAPG by thin-layer chromatography (TLC); *Panel* **d**: Detection of PRN by TLC. Reproduced with permission from Someya and Tsuchiya (2009)

for biocontrol agents that have multiple biosynthetic loci. Of course, the presence of biosynthetic genes for multiple antibiotics would be favorable in the application of biocontrol bacteria to the treatment of diverse diseases. However, these isolates rarely produce multiple antibiotics at the same time; instead, activation of the biosynthesis for one antibiotic often suppresses the biosynthesis of others. Although one antibiotic may be effective against phytopathogens, synergistic antagonistic activity would be expected in a combined application of several antibiotics. Moreover, antibiotic production is not neutrally regulated in each bacterium. Some biocontrol *Pseudomonas* isolates, such as *P. fluorescens* CHA0 and *P. fluorescens* Pf-5, produce both DAPG and PLT. The biosyntheses of these two antibiotics are regulated by a positive feedback system (Brodhagen et al. 2004; Schnider-Keel et al. 2000). However, in other *Pseudomonas* strains, the biosynthesis of antibiotics may exclusively inhibit each other.

Antibiotic production by *Pseudomonas* is controlled by several global regulatory systems. For example, PLT production in *P. fluorescens* Pf-5 is controlled by *pltR*, a member of the LysR family of transcriptional activators (Whistler et al. 2000). The stationary-phase sigma factor δ^S, the GacS/GacA system, and the Lon protease also regulate antibiotic biosynthesis in pseudomonads (Whistler et al. 2000). GacA in *Pseudomonas* sp. M18 positively regulates PLT biosynthesis, but negatively regulates PCA biosynthesis (Ge et al. 2004). GacS also regulates the production of 2R, 3R-butanediol, which in *P. chlororaphis* is an elicitor of induced systemic resistance in host plants (Han et al. 2006). The global regulator RsmA in *Pseudomonas* sp. M18 controls PLT production negatively but not PCA production (Zhang et al. 2005). In *P. chlororaphis*, phenazine biosynthesis is regulated by the sigma factor RpoN or by the TetR/AcrR regulator Pip (phenazine inducing protein) (Girard et al. 2006; Liu et al. 2008).

N-Acylhomoserine lactone (AHL) is the best known autoinducer for quorum sensing systems in Gram-negative bacteria; it also regulates various antibiotic biosyntheses in *Pseudomonas* (Pierson et al. 1998; Someya et al. 2009; Venturi

2006). Biosyntheses of phenazines, PRN, HCN, and rhamnolipids in *Pseudomonas* are regulated by a quorum sensing system via AHLs (Pierson et al. 1998; Venturi 2006). Exogenous AHL increases the production of the antibiotics PLT and DAPG in *P. fluorescens* S272 (Nakata et al. 1999), suggesting that the potential for biosynthesis of DAPG is also under the control of quorum sensing via AHL.

Biosyntheses of antibiotics in *Pseudomonas* are influenced by both abiotic and biotic factors (Duffy and Défago 1999; Humair et al. 2009; van Rij et al. 2004, 2005). The expression levels of antibiotic biosynthesis genes vary significantly in the rhizospheres of diverse plant species. Physical stress and pathogen infection lower *phlA* expression (de Werra et al. 2008). Indigenous microorganisms, including phytopathogens, also have the potential to both positively and negatively affect antibiotic production by biocontrol *Pseudomonas* (Morello et al. 2004). Fusaric acid and phenylacetic acid, which are phyto- and mycotoxins that are produced by microorganisms including phytopathogens, block the biosynthesis of antibiotics, such as DAPG and PCA, in *Pseudomonas* (Duffy et al. 2003, 2004; Siddiqui and Shaukat 2005). The inhibition of DAPG biosynthesis by this mycotoxin can be released in the presence of zinc (Duffy et al. 2003). Furthermore, the sensitivity against the mycotoxin varies among different isolates of antibiotic-producing fluorescent pseudomonads (Notz et al. 2002). The mycotoxin produced by *Fusarium* inhibits phenazine biosynthesis by inhibiting the biosynthesis of the QS signal molecule AHL (van Rij et al. 2005). These examples clearly indicate that commercial application of biocontrol by antibiotic-producing *Pseudomonas* spp. should consider environmental conditions where biocontrol effects are expected. Among environmental conditions, natural biological interactions are of paramount importance.

Population densities of antibiotic-producing fluorescent pseudomonads play key roles in the suppression of some soil diseases (Raaijmakers et al. 1997). These phenomena are partly demonstrated by bacterial regulation of antibiotic production under the control of a quorum sensing system via autoinducers. Thus, when population density of antibiotic-producing *Pseudomonas* spp. reaches a threshold, antibiotics are produced by the activation of biosyntheses through the quorum sensing system, leading to successful disease control. Interestingly, enrichment of antibiotic-producing fluorescent pseudomonads in rhizospheres is influenced by genetic differences among the host plants at the species and cultivars levels (Landa et al. 2006; Latour et al. 1996; Mazzola et al. 2004; Okubara and Bonsall 2008). Selective pressure exerted by one or more plant species on certain genotypes of beneficial microbes may account for disease suppression of soils in which the plants are cultivated. Indeed, farming practices may alter the relative abundance and incidence of antibiotic-producing pseudomonads in the field (Rotenberg et al. 2007), which suggests that disease-suppressive soils may be produced by agricultural practices.

In addition to antibiotics, lytic enzymes also play important roles in the suppression of phytopathogens by pseudomonads. For example, chitinases are hydrolases that degrade chitin, a homopolymer of *N*-acetyl-D-glucosamine, the major structural component of the fungal cell wall. These enzymes are present in a wide range of organisms including bacteria. Although bacteria do not contain chitin, numerous

bacteria are able to hydrolyze it. Thus, chitinolytic bacteria may utilize chitin as a carbon source via the activation of chitinolytic enzymes. Most plant diseases are caused by phytopathogenic fungi. Similar to other filamentous fungi, phytopathogenic fungi contain chitin as the main component of their cell walls. Thus, chitinolytic bacteria have potential antagonistic activity against phytopathogenic fungi by degrading their cell walls. Some *Pseudomonas* isolates produce chitinase (Gupta et al. 2006; Nielsen and Sørensen 1999). In addition to chitinases, other cell wall degrading enzymes such as proteases have been identified as potential agents for biocontrol of fungal diseases (Jha et al. 2009; Naik and Sakthivel 2006). The presence of these cell wall degrading enzymes enhances antifungal activity of antibiotics used in the biocontrol of phytopathogens (Woo et al. 2002). The endophytic bacteria *P. aeruginosa* GSE 18 and GSE 19 control stem rot disease of groundnut (caused by *Sclerotium rolfsii*) by inhibiting the activities of the cell wall-degrading enzymes polygalacturonase and cellulase, which are produced by *S. rolfsii* (Kishore et al. 2005).

Some isolates of *Pseudomonas* control phytopathogens by competing for nutrients and other resources in phytospheres. Siderophores are metal-chelating metabolites (especially iron) that are produced by the genus *Pseudomonas*. Pyoverdine is the most common and extensively studied siderophore (Pandey et al. 2005; Visca et al. 2007). Some *Pseudomonas* isolates acquire iron from the environment via siderophores which causes iron deficiency in phytopathogens. Some pathogenicity-deficient mutants of phytopathogenic *Pseudomonas* have been utilized as biocontrol agents. In such cases, resource competition between pathogenic and mutant *Pseudomonas* isolates is likely to be a major factor in disease suppression. Hence, it is reasonable to expect disease suppression (by resource competition) by treatment with non-pathogenic *Pseudomonas* spp. that are phylogenetically related to pathogenic congeners.

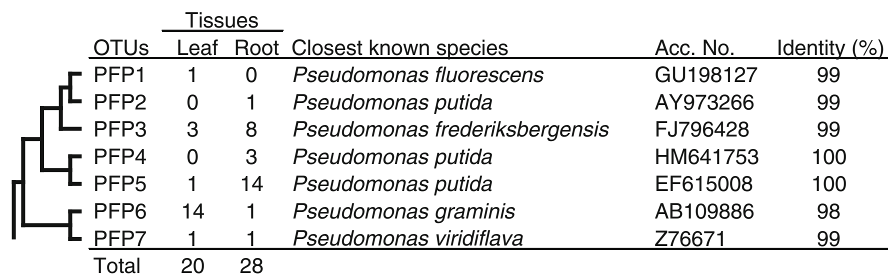
Some rhizobacteria are able to induce systemic resistance against various phytopathogens in multiple plant species. This type of induced resistance by rhizobacteria is distinct from systemic acquired resistance by phytopathogens and is called “induced systemic resistance” (ISR) (Van Loon and Bakker 2005). Numerous factors that induce ISR in host plants have been reported. For example, bacterial determinants include siderophores, salicylic acid, lipopolysaccharides, bacterial autoinducers [e.g., *N*-acylhomoserine lactone (AHL)], bacterial volatiles (e.g., *2R*, *3R*-butanediol and acetoin), and antibiotics (e.g., DAPG) (Schuhegger et al. 2006; Van Loon and Bakker 2005). Many isolates of *Pseudomonas* induce systemic resistance in various plants. Infection with diverse *Pseudomonas* genotypes may trigger ISR. Moreover, various factors for disease resistance are induced in plants by the several signaling pathways for each ISR (Van Loon and Bakker 2005). For example, ISR triggered by PGPR (plant growth-promoting rhizobacteria, including *Pseudomonas* spp.) was originally defined as a system that (1) comprises ethylene- and jasmonic acid-dependent signaling pathways and (2) is salicylic acid independent. ISR also was defined as a disease resistance system that does not accumulate PR proteins. However, recent studies indicate that these definitions are not applicable in all cases of ISR.

Pseudomonas isolates from most environments do not produce antibiotics or produce only a few antibiotics. There have been attempts to introduce genes encoding antibiotic biosyntheses into *Pseudomonas* isolates to improve biocontrol activity (Huang et al. 2004; Voisard et al. 1989). Transformed *Pseudomonas* cells have antifungal activity against phytopathogens and are able to control plant diseases. Hence, this bacterium has potential as a recipient of beneficial genes for biocontrol; it may also be possible to transfer genes among *Pseudomonas* spp. under natural conditions. Molecular evidence supporting the possibility of horizontal transfer of antibiotic biosynthesis genes has been obtained from a wide range of *Pseudomonas* spp. and from bacteria in other genera. Although antibiotic biosynthesis genes usually are located on bacterial genome DNA, recent studies have demonstrated that they also can occur on a plasmid DNA (Salman 2010). *Pseudomonas* spp. harbor plasmids containing various functional genes. Plasmids from fluorescent pseudmonads can be transferred into *Echerichia coli* cells, which have the potential to produce DAPG, PLT, and phenazines (Salman 2010). As described above, *Pseudomonas* spp. have developed diverse mechanisms for regulating antibiotic production. These regulatory mechanisms can be bioengineered to enhance the production of antibiotics in biocontrol agents (Ligon et al. 1999; Schnider et al. 1995). The antimicrobial spectrum of antibiotics produced by *Pseudomonas* varies among antibiotics. For example, PCA is one of the most effective antibiotics for controlling a wide range of phytopathogens, but it does not control tomato foot and root rot, which are caused by *F. oxysporum* f. sp. *radicis-lycopersici*. PCN, a derivative of PCA, is biosynthesized from PCA in *Pseudomonas* via the biosynthesis gene *phzH*. Chin-A-Woeng et al. (2001) introduced a *phzH* gene into PCA-producing isolates of *Pseudomonas*, which enabled PCA producers to produce PCN instead of PCA and suppress tomato foot and root rot.

The genome sequences of many bacteria have been either completed or are in progress. In addition, the complete genome sequence of *P. aeruginosa* PAO1 has been determined (Stover et al. 2000). Among *Pseudomonas* isolates that are beneficial to plants, the genome sequences of *P. fluorescens* Pf-5 and *P. fluorescens* SBW25 are known (Paulsen et al. 2005; Silby et al. 2009). *P. fluorescens* Pf-5 is a well-characterized biocontrol isolate that produces multiple antibiotics. Although its genome sequence shows that this bacterium has the potential to biosynthesize three novel secondary metabolites, further study is need to understand whether they have biocontrol properties.

8.3 Ecology of *Pseudomonas* in Plants

Although there have been many studies on the use of beneficial *Pseudomonas* spp. as biological agents in controlled environments, practical application to plant disease control has been rarely reported (Höfte and Altier 2010), most likely because beneficial effects are unstable under field conditions. In the field, biotic and abiotic environmental factors positively and negatively affect the diversity and



OTUs	Tissues: Leaf	Tissues: Root	Closest known species	Acc. No.	Identity (%)
PFP1	1	0	*Pseudomonas fluorescens*	GU198127	99
PFP2	0	1	*Pseudomonas putida*	AY973266	99
PFP3	3	8	*Pseudomonas frederiksbergensis*	FJ796428	99
PFP4	0	3	*Pseudomonas putida*	HM641753	100
PFP5	1	14	*Pseudomonas putida*	EF615008	100
PFP6	14	1	*Pseudomonas graminis*	AB109886	98
PFP7	1	1	*Pseudomonas viridiflava*	Z76671	99
Total	20	28			

Fig. 8.3 Phylogenetic tree of *Pseudomonas* isolates from potato leaves and roots. The dendrogram (*left*) indicates the phylogenetic relationships among seven representative sequences of operational taxonomic units (OTUs) that are defined by ≥99 % identity. The table (*right*) indicates the number of isolates in each OTU. Acc. No.: Genbank accession number

functionality of beneficial *Pseudomonas* spp. in plant disease control. Since there also are social concerns about the impacts of releasing genetically modified bacteria (GMB) into natural environments, environmental risk assessment should be a prerequisite for such releases into open fields. Ecological study is required to tackle these problems. Determination of the ecological traits of plant-associated microbes, including those of *Pseudomonas* spp., is essential for stable and effective use of biological control agents in practical agronomic circumstances. For example, there are distinct types of habitats among plant-associated *Pseudomonas* spp. in the potato phytosphere (Someya et al. 2009) (Fig. 8.3). The data clearly indicate that operational taxonomic units (OTUs) PFP5 and PFP6 (closely related to *P. putida* and *P. graminis*, respectively) strongly prefer inhabiting the roots and leaves, respectively, while OTU PFP3 (with high identity to *P. frederiksbergensis*) seems capable of inhabiting both the environments. Interestingly, the representative sequences of three OTUs (PFP2, PFP4, and PFP5; Fig. 8.3) are identical to *P. putida* isolates with different accession numbers, which suggests that there is genetic diversity in *P. putida* or that the OTUs distinctly different at the species level (Fig. 8.4). Identification of taxonomic units is now a powerful means for providing unambiguous ecological information that is highly objective. These ecological assessments provide very useful data for efficient screening of beneficial microbes and for reliable and stable application of microbiological agents under field conditions.

The dominant environmental factors that regulate the diversity and functionality of environmental microbes, including *Pseudomonas* spp., can be discerned using microbial community analysis. Conventional microbial community analysis has been mainly based on plate counts, using selective media for specific groups of microbes. These culture-dependent methods are limited by strong inherent biases that are due to medium selection and culture conditions. More importantly, most of the microbes in nature cannot be cultured (Amann et al. 1995). Culture-independent methods have been introduced to overcome these drawbacks to assess the structural and functional diversities of microbial communities. Most recent methods involve nucleic acid extraction from an environmental sample and analysis of genetic diversity in the microbial community with a series of molecular techniques (Ikeda et al. 2006).

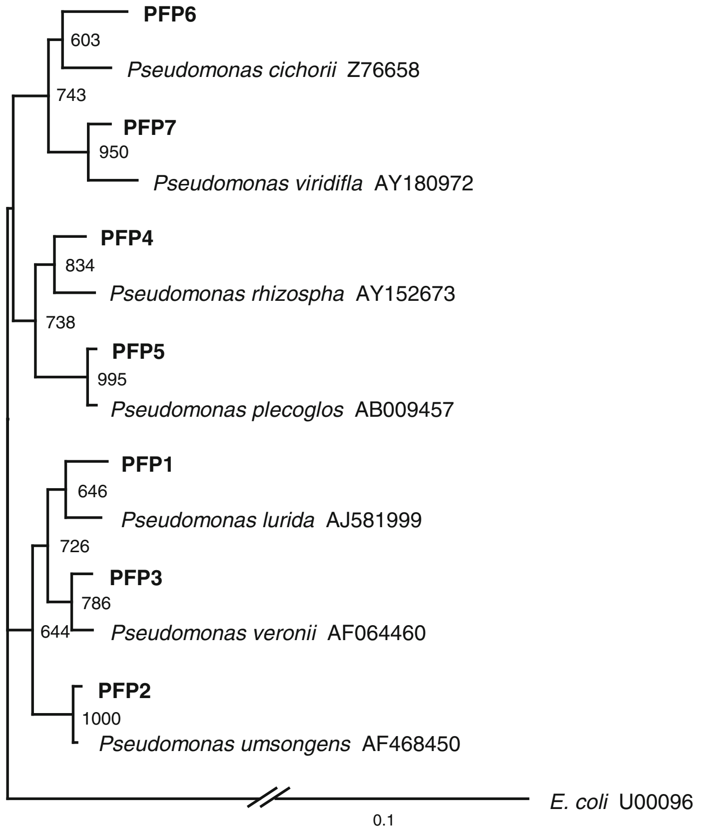


Fig. 8.4 Phylogenetic tree of the 16S rRNA gene sequences of the seven representative isolates shown in Fig. 8.3. The corresponding OTUs are boldfaced. *E. coli* K-12 (U00096) was used as an outgroup. The *scale bar* represents 0.1 substitutions per nucleotide position. Bootstrap values of >500 (from 1,000 replicates) are indicated at the nodes

Pseudomonas spp. are ubiquitous bacteria wherever there are plants. Using culture-independent methodologies, it has been shown that *Pseudomonas* spp. comprise <4 % of total microbes in soil whereas they comprise about 20 % of the total culturable bacteria in rhizospheres (Sørensen and Nybroe 2004). The phytosphere, particularly the rhizosphere component, is an attractive habitat for microorganisms because of the availability of many nutrients and the environmental stability provided by plants. Recent application of culture-independent methodologies has revealed that the diversity of plant-associated microbes is drastically different from those obtained by culture-dependent methods (Saito et al. 2007). Although the pseudomonads are generally considered easily culturable, the majority of a wild bacterial population may be viable but non-culturable (Troxler et al. 1997). Therefore, culture-independent analyses have become more popular and have improved the knowledge about the ecology of pseudomonads in agronomic environments. These new methods provide both qualitative and quantitative data for statistical and ecological analyses at a reasonable cost and within an acceptable time frame.

The abundance of *Pseudomonas* spp. is about 1.6 % of the total soil bacterial community, as shown by clone library analysis of the 16S rRNA gene (Janssen 2006). The population dynamics of *Pseudomonas* spp. in the rhizosphere also have been elucidated by whole community or *Pseudomonas*-specific community analysis using culture-independent methodologies. Kaiser et al. (2001) reported the absence of *Pseudomonas* spp. in a clone library constructed from a rhizoplane sample of oilseed rape, while 14.5 % of cultivated isolates from the rhizoplane belonged to *Pseudomonas* spp. Conversely, Reiter et al. (2003) reported that the majority of pseudomonads identified by a culture-independent procedure were not isolated in the rhizosphere of potato. Rhizospheres in some plants appear to selectively accumulate *Pseudomonas* spp., which account for up to 20 % of total clones in 16S rRNA gene libraries (Marilley and Aragno 1999). These studies also demonstrated unexpectedly large diversity in *Pseudomonas* spp., even at the species level.

Most community analyses of *Pseudomonas* spp. have been performed by 16S rRNA gene analysis. These species produce a variety of antibiotic substances. Several genes relating to antibiotic production in the PGPR of this bacterial group have been characterized. The *phlD* gene may be considered as a marker for evaluation of genetic diversity and population dynamics of DAPG producers; application of this marker procedure will promote efficient screening of isolates' evaluation for disease suppression in soils (De La Fuente et al. 2006; Mavrodi et al. 2007). Although the biocontrol characteristics in *Pseudomonas* spp. are not clearly reflected in phylogenetic relationships (Höfte and Altier 2010), detailed analyses of genetic diversity may help (1) explore compatible relationships between host plants (Mavrodi et al. 2007) and a particular beneficial *Pseudomonas* isolate and (2) identify the genetic diversity (at the subspecies levels) in terms of beneficial characteristics, such as phosphate solubilization (Browne et al. 2009).

An environmentally conservative procedure for biocontrol lies in the use of indigenous microbial populations in field soils. Some studies have hypothesized that some plant genotypes have the ability to stimulate beneficial microbes into suppressing pathogens in their phytospheres (Höfte and Altier 2010; Sessitsch et al. 2002). The idea is still not widely applicable to practical disease control. However, accumulating scientific evidence supports this hypothesis. Garbeva et al. (2004) have reported that agricultural regimes influence *Pseudomonas* population structure in soils, with specific antagonistic subpopulations being stimulated in grassland as opposed to arable land. Sanguin et al. (2008) proposed a possible use for microarray targeting of known beneficial *Pseudomonas* spp. as soil quality indicators in disease management. The concept might be developed for the identification of suppressive soils by monitoring populations of beneficial microbes.

Culture-independent community analyses may also allow extensive assessment of the impacts of introduced inoculants in agricultural environments (Saito et al. 2007). A DAPG-producing isolate induced a major shift in the composition of the resident culturable fluorescent *Pseudomonas* community (Moënne-Loccoz et al. 2001). This shift was spatially limited to the surface of the root. The compositional shift in the natural *Pseudomonas* community following introduction of a biocontrol inoculum resulted from niche overlap between natural residents and the introduced

isolates (Johansen et al. 2005). Götz et al. (2006) demonstrated the effects of a biocontrol *Pseudomonas* on non-target *Pseudomonas* spp. in the rhizosphere of tomato. The effects were apparent for more than 3 weeks after root inoculation. In contrast, a commercial product that contained *Pseudomonas* inoculants only had minor and transient effects (for 3 weeks postinoculation) on an indigenous rhizosphere bacterial community (Buddrus-Schiemann et al. 2010). These studies revealed the complexity of interactions between inoculants and biotic and abiotic environmental factors. This information will be crucial for the development of more efficient and reliable biocontrol systems. The data also will help assess the ecological impacts of introduced biocontrol agents. Due to the ecological importance of the *Pseudomonas* spp. assemblage, it is now used as an indicator group for environmental assessments (Gyamfi et al. 2002).

Culture-independent methodologies are indeed powerful tools for characterizing microbial communities; however, there are some problems and technical limitations. For example, fingerprinting-based methods, such as denaturing gradient gel electrophoresis (DGGE), have limited resolution across the wide spectrum of microbial diversity. Similarly, Costa et al. (2006) demonstrated the presence of intraspecies diversity in *P. putida* populations in a genomic diversity survey using BOX-PCR fingerprinting. This diversity was not detectable by either physiological characterization or DGGE. Sequencing-based methods have the advantage of providing maximum resolution of biodiversity analysis, but they are too expensive for ecological studies, which usually require large sample sizes for statistical accuracy. Furthermore, as pointed out by Garbeva et al. (2004), it is difficult to estimate the total number of pseudomonad types from DGGE profile patterns because of the limited resolution of gel electrophoresis and the lack of a clear match between sequence types and polymorphic DNA band positions. Moreover, excess amplification of plant organelle DNAs (chloroplast and mitochondria) in PCR analysis interferes with the community analyses of root-associated bacteria (i.e., rhizoplane and endorhizosphere) (Saito et al. 2007).

Pseudomonas spp. also occur in the endosphere (van den Broek et al. 2005) and phyllosphere (Hirano and Upper 2000). There are reports of culture-independent analyses using genus-specific primers for examining the *Pseudomonas* assemblage in the endosphere (Garbeva et al. 2001; Reiter et al. 2002; Sessitsch et al. 2002). Saito et al. (2007) employed nested PCR because of the low biomass of bacteria and the presence of large amounts of plant organelle DNAs (mitochondria and chloroplast DNAs) that interfere with PCR amplification of bacterial 16S rRNA genes in the phytosphere. The use of nested primers may skew the actual structures of bacterial communities because of biases caused by PCR amplifications and the selectivity of primers. It was previously impossible to evaluate whole bacterial community structures using universal primers. However, the development of a bacterial cell enrichment method for analyzing plant-associated bacteria has helped to solve this problem (Ikeda et al. 2009).

Culture-dependent community analyses have demonstrated that *Pseudomonas* spp. are dominant on soybean stems. Kuklinsky-Sobral et al. (2004) showed that proportions of *Pseudomonas* spp. among endophytes and epiphytes of stems were 36.4 % and

17.6 %, respectively. Similarly, Okubo et al. (2009) reported that ≥40 % of endophytes in soybean stems were identified as *Pseudomonas* spp. In contrast, culture-independent based community analysis recently demonstrated that only 0.7 % of total clones in a library constructed from soybean stems belonged to *Pseudomonas* spp. (Ikeda et al. 2010b). These remarkably different results between community analysis procedures raise questions about the significance of ecological roles that are attributed to *Pseudomonas* spp. in the phytosphere when only culture-based methods are used. The ecology of plant-associated pseudomonads clearly requires reassessment using a combination of culture-independent techniques and bacterial cell enrichment methodologies. A number of advanced molecular techniques now make it possible to determine the complex interactions between bacterial species and various environmental factors in phytospheres under field conditions (Ikeda et al. 2010a). Such challenging work will create new possibilities for efficiently screening beneficial microbes and developing stable and reliable applications of biocontrol agents in agricultural environments.

8.4 Risks of Using *Pseudomonas* in Agriculture

As mentioned earlier, some *Pseudomonas* species are pathogenic for both plants and animals. Thus, there is a potential risk of pathogenicity in the application of uncharacterized *Pseudomonas* spp. *P. aeruginosa* is a typical pathogenic species and its congeners include 8 and 11 pathogenic species of fish and humans, respectively (Peix et al. 2009).

There are 25 species of *Pseudomonas* that are plant pathogens. There are other deleterious congeners that have no clear pathogenicity; among these, there are many isolates of *Pseudomonas* spp. and *P. fluorescens* that are deleterious rhizosphere bacteria (Nehl et al. 1996). Their metabolites, especially the antibiotics HCN, DAPG, and 2,4,6-trihydroxyacetophenone inhibit the growth of plants (Chung et al. 2008). Studies by some researchers identified so-called "helper bacteria" for pathogens (Dulla et al. 2010; Fernando et al. 1994; Newton and Toth 1999). Although these bacteria are not pathogenic, they enhance the severity of pathogen's infections. Some *Pseudomonas* isolates have the potential to enhance diseases caused by phytopathogens. They act as deleterious bacteria that stimulate the action of mycoherbicides that contain phytopathogens (Fernando et al. 1994). The antibiotics produced by biocontrol *Pseudomonas* spp. also have phytotoxic effects. Hence, careful consideration is required before this bacterial genus is used for biocontrol.

Biocontrol without the risk of genetically acquired resistance in pathogens is a very attractive option. However, when biocontrol is antibiotics based, there is a potential risk of promoting acquired resistance in pathogens. Phytopathogen-acquired resistance against chemical fungicides in agriculture is a major concern. Biocontrol of plant diseases by antibiotic-producing *Pseudomonas* depends on the sensitivity of phytopathogens against antibiotics. Some species of phytopathogens have already acquired resistance against antibiotics produced by *Pseudomonas*

(Schoonbeek et al. 2002; Schouten et al. 2004). Recently, Ajouz et al. (2010) demonstrated how a fungal pathogen acquired resistance against PRN, one of the major antibiotics produced by biocontrol *Pseudomonas* spp. This is clearly of ecological significance, and ecological approaches will become more important in the development of practical biocontrol agent applications.

In the past, it was difficult to discriminate pathogenic from non-pathogenic species using conventional microbiological techniques, largely because of ambiguities in species discrimination within the *Pseudomonas* genus. However, current technical advances in molecular techniques permit precise genetic identification of plant and animal pathogens quickly and easily.

8.5 Concluding Remarks

Biocontrol of plant diseases is an attractive alternative to chemical control of phytopathogens. *Pseudomonas* and *Bacillus* are the best biocontrol bacteria and some isolates have been used as commercial products (Mark et al. 2006). Continued progress in understanding the mechanisms and application of these organisms to disease control will help select and produce better candidates for reliable commercial products. Indeed, there are already five registered *Pseudomonas* biocontrol agents in the United States (Höfte and Altier 2010). Nevertheless, the application of biopesticides in agriculture is still limited and has not yet provided a viable alternative to chemical pesticides.

An emerging literature shows that there is little doubt about the usefulness of beneficial *Pseudomonas* spp. as biocontrol agents. Further research using metagenomics and molecular microbial ecology should accelerate the identification of beneficial microbes and facilitate the practical application of biocontrol agents in future food production.

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Chapter 9
Use of *Bacillus*-Based Biocontrol Agents for Promoting Plant Growth and Health

Yu Zheng, Fang Chen, and Min Wang

9.1 Introduction

One of the major challenges for the twenty-first century will be an environmentally sound and sustainable crop production for the increasing human population (Berg 2009). Many species of pathogens, such as *Fusarium* spp. and *Pythium* spp., caused significant yield losses in horticulture and agriculture crops worldwide (Mao et al. 1997). Crop rotation and breeding for resistant plant varieties are insufficient to control diseases of important crop plants (Johri et al. 2003). Chemical fungicides have long been used as active agents in reducing the incidence of soil-borne plant diseases. However, some chemicals such as metham sodium and carbofuran used as soil fumigants lead to environmental pollution and toxic effects on human health and provide a possibility of building up resistance among pathogenic microorganisms (Baysal et al. 2008). Besides, the chemical treatments are only effective for a short time in the growing season. Alternatively, antimicrobial compounds produced by microorganisms proved to be quite effective in combating pest, pathogens, etc. and be effective as biocontrol agents. Therefore, the strategy of biological control has become an important approach to create a long-lasting effect to raise disease-free crops that ultimately help in facilitating sustainable crop production (Nagorska et al. 2007).

During the past decades, researches and applications of biocontrol agents for plant disease control have gained wide publicity and attention among farmers. A number of bacteria, fungi, and protozoa have been evolved to control disease and pests in below- and aboveground plant parts. Several kinds of biocontrol agents

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are now commercially available in the market in many countries. It has been clearly shown that *Bacillus* spp. are one of the most effective microbes in controlling various plant diseases and proved potentially useful as biocontrol agents (Nagorska et al. 2007). Plant growth and health supporting bacteria of the *Bacillus* group due to their ability to form heat- and desiccation-resistant spores have been suggested to provide a biological solution to the disease suppression of phytopathogenic fungi (Maheshwari 2011). Antagonistic *Bacillus* spp. not only produce various lytic enzymes (e.g., chitinase and β-1,3-glucanase) and antibiotics (Yu et al. 2002), but could also induce a systemic resistance of plant (Choudhary and Johri 2009; Kloepper et al. 2004), by increasing the activities of plant defense-related enzymes of peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) (Chen et al. 2010).

With the advances in molecular microbiological techniques, new insights into the underlying mechanisms by which introduced bacteria function have been developed. Many antibiotics produced by *Bacillus* have been identified, and many genetically modified strains with better biocontrol ability have been constructed. From the large amount of antibiotics produced, lipopeptides stand among the most representative. *Bacillus* lipopeptides are linear or cyclic in nature, divided into three families, iturin, fengycin, and surfactin. They frequently contain some amino acid residues, which are unique and not commonly found in proteins, with high stability to pH, heat, and protease (Kavitha et al. 2005). The specific hydrophobous section of the antifungal peptides could insert into the cell wall of hyphae and result in the leakage of cytoplasm. Antifungal peptide TasA produced by *B. subtilis* is a translocation-dependent antimicrobial spore component, which could inhibit the growth of certain bacteria (Stover and Driks 1999). These antifungal peptides produced by *Bacillus* have been proved safe to people due to no adverse effect on environment (Chen et al. 2008; Tsuge et al. 2005). Hence, it was proved highly effective as antisepsis agents in medicine and food besides their role in biological control. In this review, attention has been paid on *Bacillus* as biocontrol agents for promoting plant growth and health.

9.2 Diversity of *Bacillus* spp. in Agro-Ecosystem

Multiple *Bacillus* spp. can be readily cultured from all plant-associated microenvironments; especially from the rhizosphere, on solid medium cultivable counts of these bacteria generally range from log 3 to log 6 cells per gram fresh weight (Vargas-Ayala et al. 2000). Changes induced in the soil by the growing root provide additional niches for soil microbes. Rhizosphere conditions sustain communities which differ from those found in bulk soil. Hence, these microbial communities exhibit a "rhizosphere effect" (Curl and Truelove 1986; Lynch and Hobbie 1988). When testing the microbial isolates from these plant-associated habitats, 1–35 % showed antagonistic ability to inhibit the growth of pathogens in vitro (Berg et al. 2006).

The abiotic stress caused by nutrient limitation is a common phenomenon occurring in the rhizosphere and is responsible for the presence of a wide variety of physiologically different chemical metabolites. Application of the media low in nutrients thus provides the recovery of "novel" species, naturally residing in the rhizosphere. Novel cultivation-independent techniques are now rapidly changing this panel of rhizobacteria to include non-culturable microorganisms by providing a different representation of bacterial community composition, including the uncultured diversity in 16S rDNA lineages. Several reports have indicated that the sequences of dominant *Bacillus* species present in different soils are not the same as those present in easily cultured isolates. Interestingly, the substantial effort leading to the isolation of those previously uncultured lineage also led to the isolation of even more microdiversity (phylogenetically similar but physiologically dissimilar), not detected earlier as far as their genome sequences obtained from the same soil are concerned (Felske et al. 2003). The phylogenetic relation of three *Bacillus* species, *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains was initially established by both phenotypic and genotypic characterization (Priest 1993). More recently, the degree of homogeneity of this group has been further established by analyses of multiple gene loci and amplified fragment length polymorphism (AFLP) (Ticknor et al. 2001).

The biocontrol involving microbial agents or biochemicals to control plant pathogens is an eco-friendly and cost-effective component of an integrated disease management program. Initial researches on biocontrol agents were mainly focused on application of Gram-negative bacteria, such as *Pseudomonas*, *Agrobacterium*, *Erwinia*, etc., but now-a-days, more and more researches are aimed to investigate the *Bacillus* species with their characteristics of forming spores that allow them for easy cultivation and readily colonization in the soil or plants. Many studies have shown that *Bacillus* is one of the most effective genera among PGPR controlling various plant diseases, especially the seed- and soil-borne diseases, and acted as potentially useful as biocontrol agents (Nagorska et al. 2007).

9.3 Biocontrol *Bacillus* and Plant Health

A number of *Bacillus* strains express activities to suppress necrotizing pathogens/parasites or otherwise promote plant growth. The function of improvements in plant health and productivity due to biocontrol bacteria is mainly mediated by three different ecological mechanisms: (1) antagonism of pest and pathogens, (2) promotion of host nutrition and growth, and (3) stimulation of plant host defenses (induced systematic resistance, ISR) (Choudhary and Johri 2009). Based on their mode of action and effects, these biocontrol agents can be used as biofertilizers, plant strengtheners, phytostimulators, biopesticides, and sometimes in bioremediation. With a substantial increase in the popularity, there is a strong and growing market for microbial inoculants worldwide with an annual growth rate of approximately 10 %. The diverse range of *Bacillus*-based microbial inoculants is already available in the market (Thakore 2006; Berg 2009), as listed in Table 9.1.

Table 9.1 Some commercial products of *Bacillus* spp. for biological control in the USA

Product	Biocontrol agent	Target pathogen or disease	Crop
Green Releaf	*B. licheniformis* SB3086	Many fungal species especially those causing leafspot and blight diseases	Turf, lawns, golf courses, ornamental plants, conifers, and tree seedlings
EcoGuard	*B. licheniformis* SB3086	Dollar spot	Turf
GB34 Biological Fungicide	*B. pumilus* GB34	Fungal pests *Rhizoctonia* and *Fusarium*	Soybean seeds
Ballad	*B. pumilus* QST2808	Asian soybean rust	Soybeans
Companion	*B.subtilis*GB03, *B. subtilis*, *B. lichenformis*, *B. megaterium*	*Rhizoctonia*, *Pythium*, *Fusarium*, and *Phytophthora*	Greenhouse and nursery
HiStick N/T	*B. subtilis* MBI600	*Fusarium*, *Rhizoctonia*, *Aspergillus*	Soybean, alfalfa, dry/snap beans, and peanuts
Kodiak (several formulations)	*B. subtilis* GB03	*Rhizoctonia solani*, *Fusarium* spp., *Alternaria* spp., and *Aspergillus* spp. that attack roots	Cotton and legumes
Mepplus	*B. cereus* BP01	Uniform plant height, decrease in vegetative growth, larger cotton bolls	Cotton plants
Serenade/ Rhapsody/ Serenade Garden	*B. subtilis* QST713	Powdery mildew, downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight, and others	Cucurbits, grapes, hops, vegetables, peanuts, pome fruits, stone fruits, and others
Sonata	*B. pumilus*	Powdery mildew, downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight, and others	Cucurbits, grapes, hops, vegetables, peanuts, pome fruits, stone fruits, and others
Subtilex	*B. subtilis* MBI600	*Fusarium*, *Rhizoctonia*, and *Pythium* that cause seed and root rots	Field, ornamental, and vegetable crops
Taegro, Tae-Technical	*B. subtilis* var. *amyloliquefaciens* FZB24	*Rhizoctonia* and *Fusarium*	Only in greenhouses and other indoor sites on shade and forest tree seedlings, ornamentals, and shrubs. Not permitted for use on food crops
YiedShield	*B. pumillus* GB34	Soil-borne fungal pathogens	Soybean

Source: APS biological control committee. http://www.oardc.ohio-state.edu/apsbcc

Many studies have reported direct antagonism by some other species including *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *Paenibacillus polymyxa*, and *B. pumilus* together with isolates of unidentified species from the genus (Yu et al. 2002). In addition, several species of *Bacillus* are now known to produce toxins that could inhibit the growth and/or activities of fungal pathogens and plant parasitic nematodes. Among various *Bacillus* species, most thoroughly studied species includes *B. subtilis* (Pinchuk et al. 2002). General suppression effects frequently invoked by *Bacillus* can be demonstrated with the reduced incidence or severity of plant diseases (Mazzola 2004), due to antifungal agents produced by *B. subtilis*. These antifungal molecules are polypeptides, including surfactin, iturins A–E, bacillomycins D, F, and L (Baysal et al. 2008). In particular, *B. subtilis* is known to produce a number of antifungal compounds including alboleutin, bacitracin, botrycidin, clorotetain, fengycin, iturins, and rhizocticins (Zuber et al. 1993). In fact antifungal peptides could inhibit the growth of a large number of fungi, such as *Aspergillus*, *Penicillium*, and *Fusarium* species (Munimbazi and Bullerman 1998). Moreover, these compounds are heat stable, and are resistant to proteolytic degradation; however, their activity is reduced in the presence of cholesterol. Such characteristics indicate that those antifungal compounds commonly belong to the lipopeptide antibiotics, which are known to kill the fungi by interacting with sterols of the cytoplasmic membrane. Iturins are cyclic lipopeptides characterized by the presence of seven amino acids (Chitarra et al. 2003). Iturin A with members of bacillomycin D and mycosubtilin belongs to the iturin family of lipopeptide. It is a cyclic lipopeptide with an amino fatty acid moiety and is synthesized nonribosomally according to the multicarrier thiotemplate mechanism. Iturin A has been shown to increase the permeability of lipid membranes of fungal cells by pore formation, resulting in the loss of essential macromolecular compounds (Thimon et al. 1995). In addition to the peptide antibiotics, catabolic enzymes (e.g., proteases, chitinases, and glucanases) and a few small molecules can be secreted by various *Bacillus* species, which may contribute to the pathogen suppression.

In addition, antagonistic microorganisms also play an important role in competition for niche space and nutrients with other chemoheterotrophs in the rhizosphere wherein motile and chemotactic strain of *B. megaterium* shown to be better on colonizing roots and suppressing *Rhizoctonia solani* (Zheng and Sinclair 2000). Root colonization is an essential step for both soil-borne pathogenic and beneficial rhizobacteria. Colonization patterns have showed that rhizobacteria act as biocontrol agents or as plant growth-promoting bacteria (PGPB) forming the microcolony or biofilm at preferred sites of root. Soil–plant–microbe interactions beneath the ground are complex, since the interaction may be harmful, beneficial, or neutral to the plants. There are many ways in which the outcome can influence the plant health and productivity (Kennedy 1998). Beneficial plant–microbe interactions in the rhizosphere are the determinants of plant health and soil fertility (Jeffries et al. 2003). Thus, the beneficial interaction of plants and microbes should be extensively exploited.

The mechanisms of *Bacillus*-mediated enhancement of plant growth and yields of many crops are not yet fully understood (Dey et al. 2004). However, the possible explanations include as follows. (1) The ability to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase to reduce the level of ethylene in the roots of the developing plants, thereby increasing the root length and growth (Lee et al. 2008). (2) The ability to produce hormones like indole acetic acid (IAA), gibberellins, and cytokinins, which mediate the processes including plant cell enlargement, division, and enlargement in symbiotic and nonsymbiotic roots as well. Using a combined approach of chemical and genetic analyses, Kamilova et al. (2006) found that biosynthesis of IAA in the biocontrol bacterium *B. amyloliquefaciens* FZB42 affects its ability to promote plant growth. Moreover, the ability of IAA production is dependent on the presence of precursor tryptophan, which is one of the main compounds present in several plant exudates. (3) Antagonism against phytopathogenic microorganisms involved by producing siderophores, β-1,3-glucanase, chitinases, antibiotics, and cyanide (Lee et al. 2008; Singh et al. 2008; Leelasuphakul et al. 2006). (4) Due to solubilization of phosphates and mineralization of other nutrients. For example, phytases secreted by some *Bacillus* species, are enzymes that sequentially remove phosphate groups from myoinositol 1, 2, 3, 4, 5, 6-hexakisphosphate (phytate), the main storage form of phosphate in plants. Besides their ability to make phytate phosphorus available, elimination of chelate-forming phytate, which is known to bind nutritionally important minerals (Zn^{2+}, Fe^{2+} and Ca^{2+}), is another beneficial effect of extracellular phytase activities of *Bacillus* spp. (Kerovuo et al. 1998). Phytase activities of bacteria inhabiting the plant rhizosphere may contribute to their plant growth promoting effect (Idris et al. 2002). Researchers have shown that biofertilization exerted by extracellular bacterial phytase under conditions of phosphate limitation and in the presence of phytate can contribute to the PGP (plant growth promoting) activity of *B. amyloliquefaciens* FZB strains (Idris et al. 2002; Makarewicz et al. 2006).

Bacillus populations may also contribute to promote plant health by inducing host resistance pathways locally and systemically. Induced systematic resistance (ISR) is a physiological “state of enhanced defensive capacity” elicited by specific environmental stimulation, whereby the plant’s innate defenses against subsequent biotic challenges are potentiated. This enhanced state of resistance effectively inhibits a broad range of pathogens and parasites. Protection resulting from ISR has been reported against leaf-spot fungal and bacterial pathogens, systemic viruses, a crown-rot fungal pathogen, root-knot nematodes, and a stem-blight fungal pathogen as well as damping-off, blue mold, and late blight diseases (van Loon 2000). Some species, such as *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus*, have been reported to cause significant reductions in the disease incidence or severity of various diseases on a diversity of hosts. Elicitation of ISR by these strains has been demonstrated in greenhouse or field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, mustard, cucumber, loblolly pine, and two tropical crops (long cayenne pepper and green kuang futsoi). Several PGPRs, including *Pseudomonas*

putida, *B. pumilus*, and *Serratia marcescens*, which colonize root systems with seed applications could protect plant against foliar disease (Thomma et al. 2001). Previous studies showed that about 25 % of rhizobacteria isolated from composts could suppress *Pythium* spp., while less than 1 % of isolates suppressed bacterial leaf spot on radish. Thus, the suppression of *Pythium* sp. may be due to antibiosis, whereas the suppression of bacterial leaf spot is resulted from ISR (Kloepper et al. 2004). Elicitation of ISR by *Bacillus* strains may act with different mechanisms and signaling pathways. It has been suggested that mixtures of PGPR strains with different mechanisms of interactions may more reliably benefit plants than would individual PGPR strains (Raupach and Kloepper 1998). This suggestion is predicated upon finding out differences in host responses elicited by PGPR strains and subsequently demonstrating that mixtures of strains are compatible. Studies of *B. pumilus* T4 and SE34 on various mutant lines of *Arabidopsis* spp. revealed that in agreement with the results of signaling during ISR elicited by *Pseudomonas* spp., ISR elicited by strain SE34 was dependent on nonexpressor of pathogenesis-related genes 1 (NPR1), jasmonic acid (JA), and ethylene (ET), while ISR elicited by strain T4 was dependent on ethylene and independent on NPR1 and JA. Besides, when a salicylic acid (SA)-independent pathway was dominant in the tests, a salicylic acid-dependent pathway appeared to be activated during ISR elicited by strain SE34 against one pathovar (Ryu et al. 2003).

Besides ISR, there is another defined form of induced resistance, the so-called systemic acquired resistance (SAR), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved (Choudhary and Johri 2009). SAR can be triggered by exposing the plant to virulent, avirulent, or non-pathogenic microbes. Depending on the plant and elicitors, a set period of time is required for the establishment of SAR wherein the accumulation of pathogenesis-related proteins (chitinase and glucanase) and SA takes place. Unlike ISR, SAR does not rely on the pathways regulated by jasmonate and ethylene, but instead, involves the accumulation of pathogenesis-related proteins or SA (Yan et al. 2002). The specific signal transduction pathway that is promoted during ISR by *Bacillus* spp. depends on the strain, the host plant and at least in one case, on the pathogen used on a given host.

9.4 Biocontrol *Bacillus* and Agro-Ecology

Research on rhizosphere microbiology has a long history, but there are still many unresolved questions. One of these concerns the spatial and temporal distribution of microorganisms along the root and the mechanisms underlying this distribution. This field of research is important for various reasons, including a basic understanding of microbial dynamics as well as the potential application in sustainable agriculture. Although originating from plant-associated microenvironments, beneficial bacteria, if applied to plant roots in sufficient amounts, may perturb indigenous microbial populations and other associated important ecological functions (Bankhead et al.

2004; Winding et al. 2004). Studies on their non-target effects on soil microbial community structure are needed if the bacterial biocontrol agents were used in a large amount during field applications, even though some studies have found no significant alterations in soil microbial community structure followed by inoculation of bacterial antagonists (Bankhead et al. 2004).

Microorganisms present in the rhizosphere play an important role in the growth and in the ecological fitness of their host plants. Various microbial processes that are expected to occur in the rhizosphere include pathogenesis and its counterpart, plant protection, as well as the production of antibiotics, geochemical cycling of minerals, and root colonization (Kent and Triplett 2002). Plant roots exert strong effects on the rhizosphere through "rhizodeposition" (root exudation, production of mucilages, and release of shoughed-off root cells) and by providing suitable ecological niches for microbial growth (Bais et al. 2006). It has been proposed that bacterial populations residing in the rhizosphere oscillated along root axes in a wave-like fashion (Semenov et al. 1999). The bacterial community temporarily benefits from the nutrients released by younger roots, and the wave-like fluctuation in cell number was observed, which can be explained by death and lysis of bacteria upon starvation when nutrients become depleted, then followed by cell divisions in surviving and thus viable populations as promoted by the release of nutrients from dead and decaying cells (Semenov et al. 1999).

Although many *Bacillus* strains have been reported a decline in the population size of colonization, the rhizosphere could guarantee a high and stable survival of the bacterium for a long period, which will lead to a suppressive effect on plant diseases (Acea et al. 1988). Usually, biocontrol of *Bacillus* spp. exhibits an aggressive root colonization ability and imparts more resistance in soil than do some other species. The high level of stable colonization of *Bacillus* spp. is the result of spores formed during inoculation in the soil and the efficient germination of spores at the suitable niches in rhizosphere (Bankhead et al. 2004). Research on the survival of *B. subtilis* NB22 (Tokuda et al. 1995) showed that spores were tolerant not only against abiotic stresses but also against biotic stress such as predation, parasitism, or lytic enzyme reactions by the indigenous microbial community. Also, partial suppressive effect against plant pathogens in soil by the bacterium is thought to be triggered by the antifungal peptide, iturin, which is produced by the vegetative cells after spore germination (Tokuda et al. 1995).

However, the ecological significance of altering the structure of soil and rhizosphere microbial populations remains unclear. In fact, the changes that reduce the abundance and activities of soil pathogens are desirable, but changes that alter other mutualistic associations or biogeochemical cycling may be undesirable. Besides, the non-target effects of biocontrol bacteria are usually confined to one growth season, when the effects would likely be smaller than the effects caused by plant aging or agricultural practices. If non-target effects are not detectable in a short term, then long-term non-target effects clearly even less likely occur. From the point of environmental risks, large effects could be tolerable within the growth season, while only small non-target effects would be tolerable if the effect extended beyond a growth season (Winding et al. 2004). The minimal impact of the biocontrol bacteria on

rhizosphere bacterial populations suggests that the introductions of such strains into the environment may result in improved biological control because the beneficial effects of indigenous bacteria may not be compromised (Bankhead et al. 2004). Indeed, research has shown that a number of managed and unmanaged variables can have significant effects on microbial community structure and activities, including soil structure, fertilizer use, crop species, their genotypes, host development, and climate (Bankhead et al. 2004). However, so far the evidence for significant, reproducible, and lasting disturbances in rhizosphere microbial community structure is lacking, and that seems to indicate a tremendous resilience of the microbiological communities in agricultural systems to human disturbance (Lynch 2002).

9.5 Development of Engineered *Bacillus* spp. for Biocontrol

During the last decade, the availability of new genetic technologies has accelerated the development of biological control agents. Many efforts have been made to improve the biocontrol bacteria activity using genetic tools. The characteristics of recombinant microorganisms and their ability on the biocontrol have also been explored. The development of genomic technologies leads to the product with more predictable and consistent effects. There is some advancement in the development of engineering biocontrol bacteria based on the *B. subtilis* 168, since it is a clear background and is known as a safe and non-spreading strain (Kunst et al. 1997; Julkowska et al. 2004).

Different isolates of *B. subtilis* can be distinguished using a variety of genotypic and phenotypic tests, but biocontrol functions are not corresponded to above attributes. However, even within species, genetic diversity still exists. For example, among *B. subtilis* isolates, around 30 % of the predicted genes in the type strain 168, appear to be absent in genomes of other isolates (Marten et al. 2000; Stein et al. 2004; Gardener 2009). In this section, the research trend of genetic engineering of *Bacillus* genus in creating an effective combination of a new recombinant strain which displays a higher or wider inhibitory effect against pathogens or pests is summarized.

B. subtilis could produce nonribosomally synthesized lipopeptide antibiotics by large multienzymes nonribosomal peptide synthetases (NRPSs) (Finking and Marahiel 2004). However, *B. subtilis* 168 does not produce lipopeptide though the genes encoding synthetases for production of surfatin and fengycin, because of lacking Phosphopantetheinyl transferases (PPTases). By introducing a native *sfp* gene, encoding PPTases, into *B. subtilis* 168, the production of surfactin and fengycin was provoked (Tsuge et al. 1999). A genome segment, containing the complete iturin A operon, was transferred to the genome of *B. subtilis* 168, which did not produce iturin A originally, by means of double-crossover homologous recombination. The recombinant strain was then converted into an iturin A producer due to the introduction of an *sfp* gene (Tsuge et al. 2005). The productivity of iturin A was increased sevenfold and was restored to about half that of the donor strain by

inserting the gene of pleiotropic regulator *degQ* (Tsuge et al. 2005). The genetically engineered strain was constructed to produce lipopeptide by transferring the *ipaB3* gene into *B. subtilis* 168. The resulted strain produced only one lipopeptide, surfactin, but no fengycin, and showed significantly effective inhibition against fungi *Rhizoctonia solani* and *Pyricularia oryzae* (Gao et al. 2003).

Iturin A and its derivatives are lipopeptide antibiotics produced by *B. subtilis* and several closely related bacteria. Interestingly, three iturin group operons (i.e., iturin A, mycosubtilin and bacillomycin D) of those antibiotic-producing strains could horizontally transfer (Tsuge et al. 2005). The production of bacillomycin D of *B. subtilis* and *B. amyloliquefaciens* is regulated in multiple layers (Duitman et al. 2007; Koumoutsi et al. 2007; Chen et al. 2009). By strengthening the positive regulator or weakening some depressor, the productivity of some antibiotics could be substantially increased. For example, increased expression of the pleiotropic regulator DegQ in *B. subtilis* 168 enhances the antibiotic production (Tsuge et al. 1999). To improve the production of the antibiotic subtilin in *B. subtilis* ATCC 6633, additional copies of subtilin self-protection genes *spaIFEG* were integrated into the genome of the producer strain, showed in the 1.7-fold increase in subtilin yield. With the other strategy, genetic engineering of *B. subtilis* by deletion of the transition state regulator protein AbrB resulted in a very strong increase of subtilin production (Heinzmann et al. 2006).

The mutagenesis, strain breeding or over-expression of the genes associated with antibiotics formation, could enhance the antibiotics production. When the promoter of iturin A operon was replaced by the *repU* promoter originating from the replication gene of the *Staphylococcus aureus* plasmid pUB110, the production of iturin A increased threefold (Tsuge et al. 2001). Likewise, a mycosubtilin overproduction strain was achieved. The derivative has a 15-fold higher mycosubtilin production rate than the parental strain. And the engineered strain has significant antagonistic properties against phytopathogenic fungi, *Botrytis cinerea*, *Fusarium oxysporum*, and *Pythium aphanidermatum*, and yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae* (Leclere et al. 2005).

The gene coding for endo-β-1,3–1,4-glucanase from *B. circulans* was overexpressed in the *B. subtilis* and *B. megaterium*. The enzymes were 7 and 83 times more active in engineered *B. subtilis* and *B. megaterium*, respectively, than that of the gene donor strain (Kim 2003). The *chiA* gene, encoding chitinase (ChiA) from *B. circulans*, which was ligated into a shuttle vector, was transformed into *B. subtilis* F29-3, an antagonistic bacterium against a wide range of fungal species. The recombinant strain expressing *chiA* exhibited a greater inhibition of spore germination of *Botrytis elliptica* (Chen et al. 2004). Updated researches of development in genetic engineering of *Bacillus* spp. have involved rational design of peptide antibiotics. Symmank et al. (2002) reported the genetic engineering of the surfactin biosynthesis resulting in the production of a novel lipohexapeptide with altered antimicrobial activities. Genetic modifications of the surfactin biosynthesis machinery resulted in the production of a lipohexapeptide with reduced toxicity against erythrocytes and an increased inhibition of bacterial cells (Nagorska et al. 2007).

B. thuringiensis, which are generally regarded to be among the most important entomopathogens, has been used worldwide for more than 60 years to control agricultural and forestry pests, and it accounts for more than 90 % of all biopesticide use (Lambert and Peferoen 1992). Their insecticidal activities primarily result from the synergistic action of crystal protein toxins (Cry) and the cytolytic protein (Cyt1A), which are effective against lepidopterans, coleopterans, and nematocerous dipterans (Crickmore 2005). During the past two decades there has been considerable interest in improving the efficacy of *B. thuringiensis* products by engineering strains to synthesize novel combination of entomotoxins, and the advances in genetic engineering and molecular biology led to the cloning of a Cry gene for the construction of more efficient biocontrolling strains (Casique-Arroyo et al. 2007). For being effective against both the *Scarabaeidae* and *Chrysomelidae* pests, an engineered *B. thuringiensis* strain was constructed by introducing the gene *cry3Aa7* from *B. thuringiensis* 22, which could inhibit *Anomala corpulenta*, into the wild-type *B. thuringiensis* strain containing the gene *cry8Ca2*, which was effectively against *Leptinotarsa decemlineata* and *Colaphellus bowringi*. The recombinant strain co-expressing of Cry8Ca2 and Cry3Aa7 protein showed the insecticidal activities against all three pests (Yan et al. 2009).

B. thuringiensis could synthesize ChiA used for control of various phytopathogenic fungi (Regev et al. 1996). However, the expression of ChiA is rather low and needs induction by chitin, which limits its field application (Hu et al. 2009). To use chitinolytic enzymes in combination with entomopathogenic bacteria for an increased potency due to the synergistic interaction between them, the gene of ChiA was heterologously expressed in an anti-*Coleopteran B. thuringiensis* (Okay et al. 2008). Casique-Arroyo et al. (2007) transformed *B. thuringiensis* subsp. *kurstaki* HD-73 with the homologous endochitinase gene *chiA74* from *B. thuringiensis* subsp. *kenyae* LBIT-82 under the regulation of its original promoter. The recombinant strain showed an improvement in chitinolytic activity three times than that of the parent strain. However, the expression of ChiA74 resulted in the decrease of sporulation and Cry1Ac production in rich medium, and no significant increase in the toxicity of the recombinant bacterium toward *Plutella xylostella* was detected. When a signal peptide-encoding sequence-deleted *chiA* gene from *B. thuringiensis* strain 4.0718 was introduced into an acrystalliferous *B. thuringiensis* strain Cry-B, the ChiA was overexpressed within the sporangial mother cells in the form of spherical crystal like inclusion bodies. The insecticidal activity of Cry1Ac protoxin in combination with chitinase inclusion bodies was higher when compared with individual Cry1Ac protoxin (Hu et al. 2009).

9.6 Concluding Remarks and Future Directions

Chemical pesticides are harmful to the health of people, destroy microorganisms and friendly insects, and make the crop more susceptible to the attack of diseases, and they also cause soil, water, and air pollution (Babalola 2010). In the wake of an

increased attention on the environment-friendly biopesticides and the increasing market demand for organic produce, *Bacillus*-based biocontrol agents exhibited an excellent performance for promoting plant growth and health. The use and the exploitation of beneficial *Bacillus* biocontrol agents offer a promising and environmentally friendly strategy for conventional and organic agriculture worldwide. In the past years, many *Bacillus* species, including *B. subtilis*, *B. cereus*, *B. polymyxa*, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, and *B. thuringiensis*, have been studied under greenhouse or field conditions on several plant species. The future success of the biological control industry will benefit from interdisciplinary research, e.g., on mass production, formulation, interactions and signaling with the environment, as well as on innovative business management, product marketing, and education (Berg 2009).

The efficacy of biocontrol products largely depends on their stability and persistence in the field. However, some researchers have shown the unpredictable nature of biological colonization of biocontrol microorganism (Callaghan et al. 2001; Bloemberg 2007; Choudhary and Johri 2009). Thus, the characteristic of each biocontrol agent and their environments should be determined for the permanence and success of biological control. The availability of new analysis method, for example, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), has positioned to determine the nature of microbial metabolites as practical aspects (Ibekwe and Grieve 2004; Campbell et al. 2009). Another aspect that should be considered is the safety of the natural and the engineered biocontrol bacteria, particularly the release of genetically modified organisms to field. Furthermore, it will be interesting to discover the multifactorial mechanisms of *Bacillus*-based biocontrol agents for biological control and plant growth promotion (Jaronski 2010).

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Chapter 10
Use of PGPR for Controlling Soilborne Fungal Pathogens: Assessing the Factors Influencing Its Efficacy

Blanca B. Landa, Miguel Montes-Borrego, and Juan A. Navas-Cortés

10.1 Introduction

Crop losses may be caused by abiotic and biotic environmental factors, leading to the reduction of crop performance and resulting in a lower actual yield than the site-specific attainable yield/production of crops. Many of those biotic factors may be controlled by applying physical (cultivation, mechanical weeding, etc.), biological (cultivar choice, crop rotation, antagonists, predators, etc.), and chemical measures (pesticides) (Oerke 2006). However, currently the main aim of crop protection is to move towards Integrated Crop Management (ICM) which may be defined as the economical production of high quality crop, giving priority to ecological safe methods of crop cultivation, minimizing the undesirable side effects of use crop protection chemical products. Within those ecological safe or environmental friendly methods the use of biocontrol agents, including Plant Growth Promoting Rhizobacteria (PGPR), is currently increasing worldwide as an alternative for the control of plant pathogens, mainly in sustainable and organic agriculture systems.

Thus, the application of PGPR in plant cultivation is one of the most promising methods for increasing agricultural productivity through plant growth stimulation, biological control of plant pathogens, as well as for increasing efficiency of soil pollutant biodegradation (e.g., Cummings 2009; Haas and Défago 2005; Lugtenberg et al. 2002; Raaijmakers et al. 2009; van Elsas and Heijnen 1990; van Veen et al. 1997; Weller et al. 2007).

The history of PGPR application spans more than 100 years. For a long time, PGPR isolated from soil have been used for microbial inoculation of the rhizosphere in different regions including China, European countries, the former Soviet Union, and the United States, where some countries have initiated practical programs to

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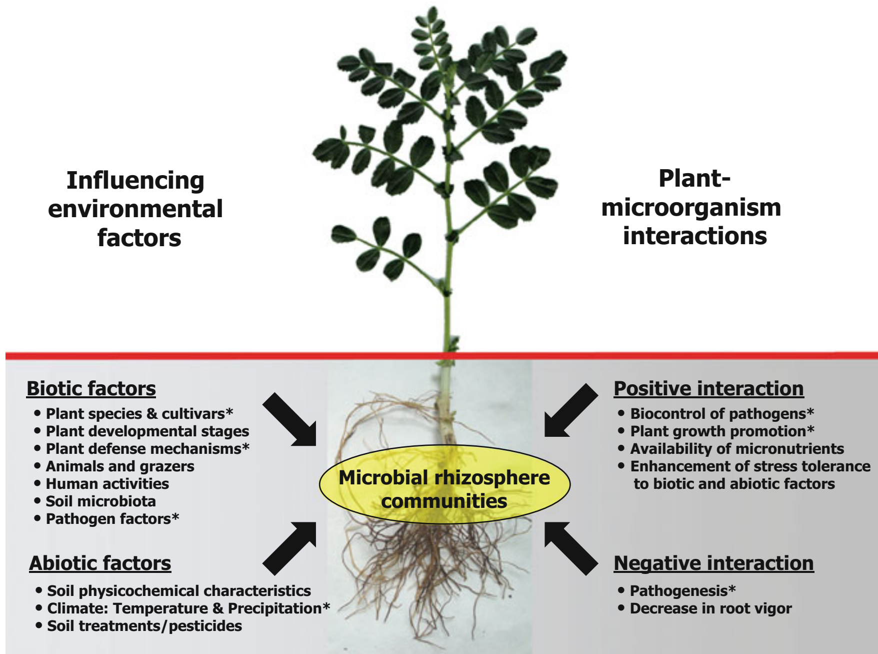


Fig. 10.1 Factors influencing rhizosphere microbial communities and positive and negative interactions resulting from them. *Asterisk*: Factors that have been specifically addressed in the Fusarium wilt–chickpea pathosystem by the authors (Modified from Berg and Smalla 2009)

develop PGPR applications at a large scale in agriculture (Cummings 2009; Jagnow et al. 1991; Zehnder et al. 2001). Currently, although most of the future research focuses on the development of genetic engineering approaches that may provide genetically modified (GM) PGPR strains with enhanced activity and additional biocontrol mechanisms (Blouin-Bankhead et al. 2004; Glick and Bashan 1997), an effort should be done to better understand the environmental factors that specifically govern and affect PGPR activity and biocontrol mechanisms of wild strains (Berg and Smalla 2009; Landa et al. 2004a, b; Picard and Bosco 2008) (Fig. 10.1). This would be especially important to hamper their inconsistent performance, specifically due to the fact that those environmental factors do not necessarily may affect GM- and wild-type PGPR strains in the same direction or with the same intensity.

10.2 Importance of PGPR for Biocontrol of Soilborne Fungal Pathogens

Productivity of crops grown for human consumption and animal feeding is at risk due to the incidence of abiotic stresses, pests, weeds, pathogens and animal pests, among others. Among crop losses due to pathogens, those caused by soilborne fungi

are considered one of the main limitations for crop productivity (Oerke 2006). Furthermore, whereas genes encoding resistance to foliar pathogens are abundant in many plant species, resistance to many of the most common and widely occurring soilborne plant pathogenic fungi (e.g., *Gaeumannomyces graminis, Pythium* spp., *Rhizoctonia* spp., and many *Fusarium* spp.) is lacking. Consequently, there is a need to look for alternative environmental friendly management strategies such as the application of PGPR as biocontrol agents (Weller et al. 2007).

The use of PGPR specifically as biocontrol agents of soilborne fungal plant pathogens as an alternative or complementary strategy to physical and chemical disease management have been investigated for over a century (Berg and Smalla 2009; Haas and Défago 2005; Weller 1988). Thus, PGPR have been shown to protect crops against a broad range of soilborne fungal pathogens. However, one of the main limitations for using PGPR extensively in agricultural production is that their biocontrol performance or beneficial effect under field conditions is often not consistent enough. Thus, in practice some of the main problems are that the applied PGPR strains sometimes do not survive in the place where they were applied, or do not execute as expected their specific biocontrol activity (Berg and Smalla 2009; Jagnow 1987; Jagnow et al. 1991; Landa et al. 2001, 2004a, b; van Elsas and Heijnen 1990; van Veen et al. 1997).

One of the main clues for that inconsistency is that successful introduction of a PGPR strain to one soil plant environment does not guarantee its survival in another; e.g., a different soil type or host plant genotype (Berg and Smalla 2009; Jagnow et al. 1991; Kravchenko et al. 1993; Landa et al. 2002b, c, 2004a, b, 2006a; Picard and Bosco 2008; Pillay and Nowak 1997; van Elsas and Heijnen 1990). Most of the biocontrol inconsistency has been attributed to variability in the physical and chemical properties of the natural niches where PGPR were applied, but to a lower extent to biotic factors such as host plant genotype or to agricultural practices (Landa et al. 2001; 2004a, b; Picard and Bosco 2008; Whipps 2001) (Fig. 10.1).

Biocontrol mediated by PGPR may involve one or more natural processes (e.g., antibiosis, parasitism, competition, etc.) that can be influenced by biotic and abiotic environmental factors (Haas and Défago 2005; Weller et al. 2002, 2007). Those factors often modify the plant–pathogen–antagonist interactions, thus reducing the efficiency of biocontrol agents on pathogen and/or disease suppression (Berg and Smalla 2009; Cook and Baker 1983; Landa et al. 2004a, b). Although many abiotic soil factors may influence the mechanisms of biocontrol (e.g., pH, temperature, moisture, texture, inorganic and organic constituents, etc.) (Fig. 10.1), there is little experimental evidence of how specific factors affect the interactions among soilborne plant pathogens and their antagonists (Berg and Smalla 2009; Burpee 1990; Duffy and Défago 1999; Landa et al. 2001, 2004a, b; Picard and Bosco 2008). Furthermore, factors that influence dynamics of PGPR populations may not necessarily affect the biocontrol mechanisms that govern the PGPR efficacy. In fact, the specific activities occurring during PGPR growth are probably more important in pathogen suppression than proliferation of the PGPR population itself (Landa et al. 2004a, b; Lewis and Papavizas 1985). In this chapter we will examine some of the main biotic and abiotic factors that may influence efficacy of PGPR for controlling

soilborne fungal pathogens using examples from research conducted in our research group over the last 15 years as well as selected studies conducted in other well-known laboratories focused in the use of PGPR for controlling soilborne fungal pathogens (Fig. 10.1). Furthermore, the significance that environmental factors associated to climate change, particularly temperature increase, may have on interactions among soilborne fungal pathogens and PGPR, as well as on biocontrol efficacy mediated by PGPR, will be addressed using a model PGPR biocontrol strains-fungal pathogen–host plant pathosystem.

10.3 Abiotic Factors Affecting PGPR Biocontrol Activity

There are various factors that can influence the biocontrol activity.

10.3.1 Soil Physical and Chemical Characteristics

Identification of soil factors that exert a major influence on PGPR establishment in the plant rhizosphere, PGPR activity or disease suppression is essential to improving biocontrol and extensive use of PGPR in agriculture. Although the effects of various minerals, growth factors, carbon and nitrogen source, and pH, on growth or production of antimicrobial metabolites by biocontrol strains of PGPR in defined liquid media have been examined (e.g., Duffy and Défago 1999; Landa et al. 2004a; Slininger and Jackson 1992; Slininger and Shea-Wilbur 1995), there is a lack of knowledge concerning analysis of the overall effect of soil physicochemical properties on PGPR biocontrol activity. Thus, some studies have reported that the survival, density, and structure of populations of both indigenous and introduced PGPR may vary in different soil types and with their crop history (Bashan et al. 1995; Cook and Papendick 1970; Landa et al. 2003, 2006a; Latour et al. 1996, 1999; Lemanceau et al. 1995); however the specific factors governing those effects were not fully identified.

The effects of single soil parameters such as soil texture and clay content on the survival and proliferation of soil and rhizosphere PGPR are known for some plant–PGPR systems (Foster 1988; van Elsas 1992; van Elsas and van Overbeek 1993). However, the relative effects of each of those physicochemical factors on the overall effect of PGPR performance are poorly known, nor is it known whether manipulation of some of those parameters can alter survival and activity of PGPR in soil and rhizosphere ecosystems. Bashan et al. (1995) evaluated the survival of the PGPR *Azospirillum brasiliense* in the rhizosphere of tomato and wheat plants in 23 soil types. In all soils, clay, nitrogen, and organic matter content were positively correlated with PGPR viability, but high $CaCO_3$ content and fine or rough sand had a highly negative effect on viability. On the other hand, the silt, pH, phosphorus or potassium content, the electrical conductivity, and the C/N ratio had no apparent

effect on bacterial viability in the soil. In a different study Bashan and Vazquez (2000) found that increased levels of $CaCO_3$, and fine and rough sand, had significant detrimental effects on the survival of five *Azospirillum* species, whereas increased organic matter content improved survival. In contrast, when the bacterial strains were incubated in the rhizosphere of tomato seedlings, all *Azospirillum* species survived well in the tomato rhizosphere under conditions that were previously shown detrimental. This study also indicates that although most cells of the five species of *Azospirillum* tested tend to die out over time in different soils, only the major soil components affected *Azospirillum* survival in soil, with small differences in mortality rates occurring among the species in a particular soil, and thus suggesting that survival of the PGPR strains is mostly related to the soil type and not to *Azospirillum* species.

Some studies have indicated that the physiological state of PGPR cells may affect their survival in the soil (Vandenhove et al. 1993), and how some PGPR can survive in the soil independently of the soil type (Allende-Molar et al. 2004; Jacoud et al. 1998; Raaijmakers and Weller 2001). However, in general, it is thought that a change in the physicochemical properties of the soil may have a much greater effect on bacterial survival in the soil than changes of the PGPR itself or the host plant cropped (Stotzky 1997). Some studies have provided evidence that soil type can be an important determinant in the composition of PGPR communities. For example, Latour et al. (1999) studied the effect of host plant type (flax and tomato) and soil type on the genetic diversity of the populations of cultivable fluorescent *Pseudomonas* spp. Both factors, soil type and host plant affected the diversity of fluorescent *Pseudomonas* species; however, soil type was clearly the main factor that accounted for the diversity found. One of the hypothesis raised to explain those results were attributed to differences in clay content in these soils.

The influence of clay content on the density and diversity of PGPR in soils had been reported in other studies (Heijnen et al. 1992, 1993; van Elsas et al. 1986). Some works have reported that soils rich in clay, naturally or after amendment, positively influence the survival and increase the density of populations of indigenous or introduced PGPR, mainly fluorescent pseudomonads (Heijnen et al. 1992, 1993; Hoper et al. 1995; van Elsas et al. 1986). In southern Spain were soils with a high clay content are common, such as cambisols and vertisols, population densities of indigenous fluorescent *Pseudomonas* spp. in olive rhizospheric soil are quite high (i.e., $>10^6$ cfu/g of soil). Furthermore, a significant and positive correlation between population densities of those bacteria and clay content in the soil has been recently established (Landa and Montes-Borrego unpublished results). However, the mechanisms responsible for this specific response of populations of fluorescent *Pseudomonas* to clay content in all the above-mentioned studies has to be determined yet.

Finally, it seems that some genotypic and phenotypic traits of specific strains of a PGPR sometimes are more important in their survival and rhizosphere competence than soil or host-associated factors. That is the case of aggressive root colonizer strains such as *Pseudomonas fluorescens* Q8r1-96, that is able to survive in dried bare soils for long periods of time and reach high population densities as soon as a

host crop is sown (Allende-Molar et al. 2004). Furthermore, strain Q8r1-96 and strain *P. fluorescens* F113, another aggressive colonizer are able to reach higher population densities ($>10^7$ cfu/g of root) than that reached by other closely related strains, in different natural and agricultural soils (Allende-Molar et al. 2004; Landa et al. 2002a, b, 2003, 2006a; Raaijmakers and Weller 2001; de Leij et al. 2002).

It is important to point out that few studies have been able to establish a significant correlation between the ability of a PGPR strain to suppress a soilborne fungal pathogen with specific soil factors. Ownley et al. (1992) were pioneers in establishing a direct relationship between soil pH and suppression of *Gaeumannomyces graminis* var. *tritici* by the PGPR bacterium strain *P. fluorescens* 2-79 both in vitro and in vivo. Their results showed that in vitro inhibition by this strain was greatest at pH 6.0–6.6, and lowest at pH 4.9–5.8; however, the bacterium was able to suppress disease development across the entire range of soil pH values tested (from 4.9 to 8.0). Interestingly, there was a trend to increase the amount of protection by the PGPR strain 2-79 as the soil pH increased (Ownley et al. 1992). This was correlated with the general ability of pseudomonads to grow well at neutral or slightly alkaline conditions (Stolp and Gadkari 1981) with activity being sharply curtailed at pH <5.5 (Brady 1984). This may be correlated to the fact that bacteria are thought to be more efficient competitors than fungi at higher soil pH ranges (Brady 1984). Similar to that findings, Howie (1985) found that soil pH within the range of 6.0–6.5 were optimum for both rhizosphere colonization and take all suppression by PGPR strain 2-79.

In a different study, an elegant work conducted by Ownley et al. (2003), the authors identified the relative importance of 28 soil properties that exerted the greatest influence on suppression of take-all, by a PGPR bacterium that produces the antibiotic phenazine-1-carboxylate (PCA). Wheat seeds bacterized with the PGPR were sown in ten different soils, and 16 soil properties were correlated with disease suppression. Biocontrol activity of PCA strains was positively correlated with ammonium-nitrogen, sand content, soil pH, sodium (extractable and soluble), sulfate-sulfur, and zinc. In contrast, biocontrol was negatively correlated with cation-exchange capacity (CEC), exchangeable acidity, iron, manganese, clay, organic matter (OM) and silt content, total carbon, and total nitrogen. Furthermore, a model was selected using a step-wise regression analysis that included six key soil properties: ammonium-nitrogen, CEC, iron, silt content, soil pH, and zinc. The regression model allowed predicting that biocontrol activity of the PGPR strain should be improved by amending a soil low in Zn with 50 μg of zinc-EDTA/g of soil. The authors also investigated the negative correlation of OM with disease suppression and found that addition of OM (as wheat straw) at rates typical of high-OM soils significantly reduced biocontrol activity of the PGPR biocontrol strain.

Additionally, to complicate more the scenario soil factors can affect the susceptibility of the host plant to the pathogen by altering the host plant nutrition status (Huber and Graham 1999) making the plant more able to support the biocontrol activity of PGPR. For instance, in wheat rhizosphere, PGPR biocontrol strains of *Bacillus subtilis* reduced Mn^{+4} to Mn^{+2}, making it more available to the wheat plant, inducing in parallel a decrease in take-all severity and an increase in yields (Huber and McCay-Buis 1993).

10.3.2 Temperature

Temperature increases metabolic processes and biomass of plants and microorganisms within their respective biological limits (Beauchamp et al. 1991), and consequently may modify interactions between them. Temperature is a key factor influencing composition and functioning of soil microbiota (Braker et al. 2010), and consequently may affect both plant–PGPR interactions as well as success of biocontrol (Beauchamp et al. 1991; Burpee 1990). Soil temperature has been reported to influence plant disease biocontrol by (1) affecting the natural disease suppressiveness of soils (Broadbent et al. 1971; Olsen and Baker 1968), (2) predisposing pathogens to microbial antagonism (Henis and Papavizas 1983; Munnecke et al. 1976), (3) regulating the growth or production of metabolites by specific antagonists (Adams and Ayers 1980; Landa et al. 2004a; Paulitz and Baker 1987; Raaijmakers et al. 2002), or (4) modulating disease development and consequently the level of disease suppression achieved (Landa et al. 2001, 2004b).

PGPR activities specifically involving a direct interaction with the plant may be greatly influenced by temperature. Thus, Landa et al. (2004a) demonstrated that inoculation of chickpea seeds or soil with four PGPR strains delayed chickpea emergence but increased chickpea growth; however, the extent of plant growth promotion decreased and emergence increased as temperature was raised from 20 to 30 °C. Furthermore, the four PGPR strains colonized the chickpea rhizosphere and grew as endophytes within the chickpea stem tissues at 20, 25 and 30 °C, however while the rhizospheric bacteria population increased with a significant linear trend as temperature increased from 20 to 30 °C, endophytic stem colonization by bacteria was highest at 25 °C.

Published results of temperature effect on plant growth promotion mediated by PGPR or on colonization by PGPR inoculants in some circumstances are sparse and sometimes conflicting. For example, Turner and Backman (1991) found that peanut seed treatments with *B. subtilis* increased seedling emergence only at low soil temperatures, but accelerated germination and increased root growth at 20 °C but not at 27 °C. In other studies the PGPR strains *Mycobacterium* sp. 44, *P. fluorescens,* and *Pantoea agglomerans* significantly increased the root and shoot growth of winter wheat at 16 °C compared with that at 26 °C in loamy sand soil; however, *Mycobacterium phlei* strain MbP18 as well as *Mycoplana bullata* MpB46 performed well at both soil temperatures, indicating genotype-specific preferences for certain environmental conditions (Egamberdiyeva and Höflich 2003). Other studies have indicated better colonization by introduced PGPR at high temperature (Davies and Withbread 1989; Egamberdiyeva and Höflich 2002), but the opposite effect occurred in most cases (Bowers and Parke 1993; Loper et al. 1985; Pillay and Nowak 1997; Schmidt et al. 2004; Weller 1988; Weller and Thomashow 1994) despite that optimum temperature for in vitro bacterial growth occurs at higher temperatures. A study with the endophyte *Burkholderia phytofirmans* strain PsJN demonstrated that a temperature increase from 10 to 30 °C reduced the colonization of this strain in the tomato rhizosphere, whereas endophytic abundance was not

affected (Pillay and Nowak 1997). Similarly, sugar beet root colonization by the biocontrol strain *P. fluorescens* B5 was reduced at high temperatures (25–35 °C) compared to that reached at lower temperatures (7–25 °C) (Schmidt et al. 2004). On the contrary, rhizosphere colonization and plant growth promotion of *Cellulomonas* sp. 21/2 and sp. 43 in association with winter wheat and pea were more efficient at 16 °C than at 26 °C (Egamberdiyeva and Höflich 2002). Higher rhizosphere colonization at low temperature probably might reflect that indigenous soil microbial activity competing with the introduced PGPR in the rhizosphere declines with temperature (Beauchamp et al. 1991; Weller and Thomashow 1994).

Biosynthesis of antifungal compounds is a primary mechanism of disease suppression by PGPR (Edwards et al. 1994; Haas and Défago 2005; Weller et al. 2002; Wulff et al. 2003). However, biosynthesis of those metabolites may be modulated by a number of factors including temperature. Temperature has been shown to have a direct effect on antagonism of PGPR on the target pathogen. In this context, Landa et al. (2004a) showed how temperature significantly influences the ability of different PGPR including *P. fluorescens*, *Bacillus megaterium,* and *Paenibacillus macerans* to produce inhibitory metabolites in culture media. Thus, they evaluated the effect of temperature (ranging from 12.5 to 35 °C) on inhibition of *Fusarium oxysporum* f. sp. *ciceris* mycelial growth and conidial germination by the PGPR and their culture filtrates. Results in their study indicated that the interacting microorganisms (PGPRs and the pathogen) differed in the optimum temperature for growth, and that the production of inhibitory metabolites in liquid cultures by the PGPRs was higher at the optimum temperature for growth. Pathogen colony radial growth was greatly reduced by the PGPRs at temperature ranging from 22.5 to 32.5 °C (Fig. 10.2). However, the optimum temperature enabling the highest pathogen inhibition differed among bacterial isolates, being higher for strains *B. megaterium* RGAF 51 and *Pa. macerans* RGAF 101 than for *P. fluorescens* RGAF 19 and RG 26 (Fig. 10.2). Similarly, inhibition of conidial germination and mycelial growth by the PGPR cell-free culture filtrates was influenced by the incubation temperature and showed the similar trend (Fig. 10.3). Interestingly, the *P. fluorescens* strains produced pyoverdine at all incubation temperatures tested except for 37.5 °C, with the amount of pyoverdine increasing with the incubation temperature as it approached the optimum for PGPR growth being coincident with maximum pathogen inhibition (Landa et al. 2004a).

Some in planta experiments conducted under growth chamber conditions have also demonstrated the effect of temperature on disease suppression by PGPR. Thus, incubation temperature strongly interacted in modulating the suppression of Fusarium wilt in chickpea (*Cicer arietinum*) by the four PGPR strains referred above (Landa et al. 2001). Interestingly, Fusarium wilt of chickpea was suppressed by these rhizobacteria only at 20 or 30 °C, but not at 25 °C, the temperature at which disease developed more severely (Landa et al. 2001) (Fig. 10.4). Disease suppression by the PGPR decreased as conditions became more favorable for disease development. Thus, at 25 °C the disease potential was too high to be countered by the biocontrol PGPR strains (Landa et al. 2001) (Fig. 10.4).

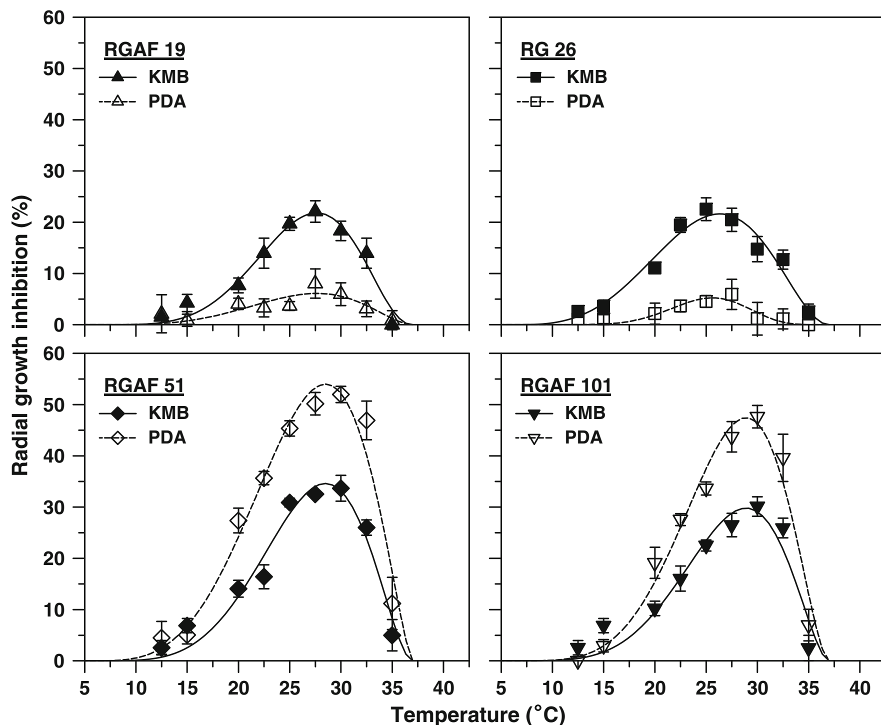


Fig. 10.2 Effect of incubation temperature on inhibition of *Fusarium oxysporum* f. sp. *ciceris* radial growth by *Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51, and *Paenibacillus macerans* RGAF 101 on King's B medium agar (KMB) and potato-dextrose agar (PDA) (Source: Modified from Landa et al. 2004a)

Those results of the effect of temperature on disease suppression by introduced PGPR bacteria were corroborated under field conditions (Landa et al. 2004b). Thus, they conducted a 3-year study in microplots infested with *F. oxysporum* f. sp. *ciceris* race 5 at southern Spain, aimed to assess the efficacy of an integrated management strategy for Fusarium wilt of chickpea that combined the choice of sowing date, use of partially resistant chickpea genotypes, and seed and soil treatments with biological control agents including the PGPR *B. megaterium* RGAF 51, *B. subtilis* GB03, and *P. fluorescens* RG 26 as well as a nonpathogenic *F. oxysporum* isolate Fo 90105 (Landa et al. 2004b). Advancing the sowing date from early spring to winter significantly delayed disease onset, reduced the final disease intensity and increased chickpea seed yield. A significant linear relationship was found between disease development over time and weather variables at the experimental site, with epidemics developing earlier and faster as mean temperature increased. The increase in chickpea seed yield was the most consistent effect of the biocontrol agents. However, that effect was primarily influenced by sowing date (i.e., temperature regimes) which also determined disease development. A small effect in disease suppression occurred in January sowings, when conditions were

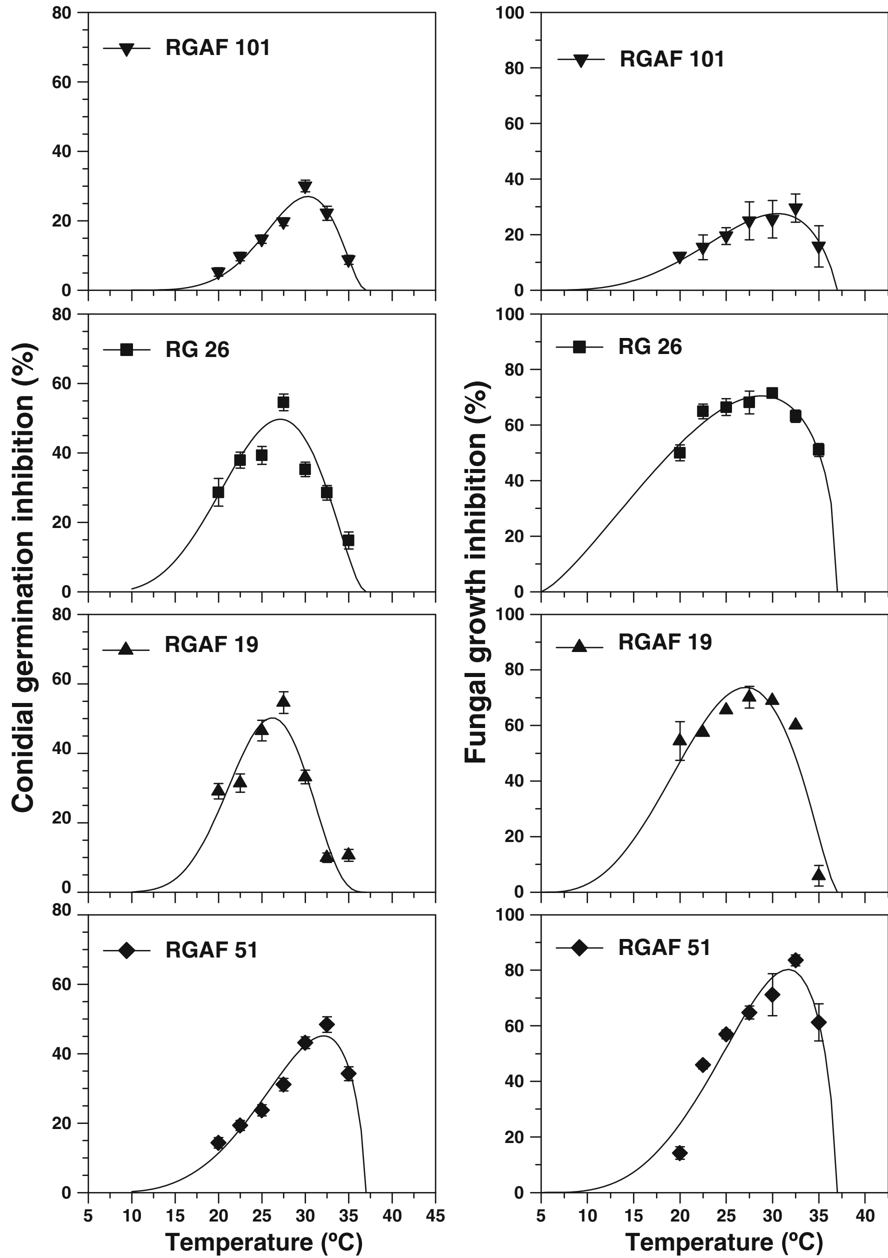


Fig. 10.3 Effect of cell-free crude culture filtrates of *Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51, and *Paenibacillus macerans* RGAF 101 at different temperatures on inhibition of conidia germination (*left panels*) or fungal growth (*right panels*) of *Fusarium oxysporum* f. sp. *ciceris* (Source: Modified from Landa et al. 2004a)

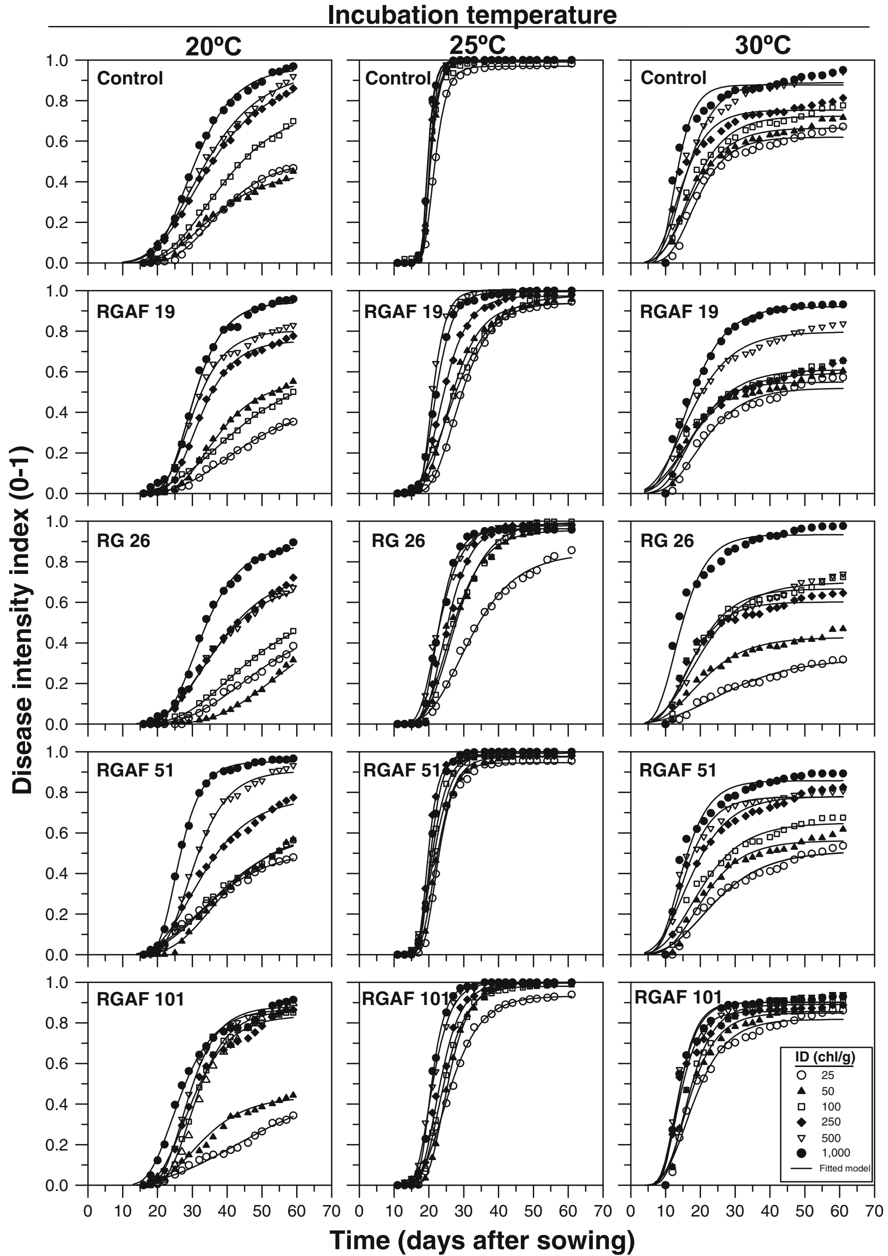


Fig. 10.4 Effect of treatments with four PGPR on development of Fusarium wilt in chickpea cv. PV 61 grown in soil infested with an inoculum density (ID) of 25, 50, 100, 250, 500, and 1,000 chlamydospores (chl)/g of soil of *Fusarium oxysporum* f. sp. *ciceris* and incubated at 20, 25, and 30 °C. PGPR tested were *Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51, and *Paenibacillus macerans* RGAF 101. Nontreated seeds sown in nontreated soil served as controls (Source: Landa et al. 2001)

less favorable for Fusarium wilt development; whereas a maximum increase in chickpea seed yield by biocontrol agents occurred in February sowings, even with a moderate reduction in disease intensity. For sowings in March, when environment was most conducive to disease, the biocontrol agents provided relatively low disease suppression, but delayed the onset of disease as well as increased seedling emergence. The authors postulated that environmental conditions (average temperatures) less suitable for Fusarium wilt also interfered with activities of the biocontrol agents related to biocontrol. This might explain why the lower level of disease occurring in the first year of the experiments, and particularly in January sowings, made difficult to detect a significant suppression of disease. Nevertheless, although treatments with the biocontrol agents provided a moderate level of Fusarium wilt suppression, a significant increase in chickpea seed yield was obtained only under environmental conditions moderately conducive for disease development, indicating a potential benefit of those PGPR strains as a component for integrated Fusarium wilt management.

10.3.3 Water Availability

There are many convincing evidences of the strong effect that water availability has on microbial survival in the soil and plant growth. However, there is a lack of knowledge concerning the specific either direct or indirect effects of water availability on efficacy of PGPR in suppressing plant pathogens or promoting plant growth.

Water availability, irrigation regimes, and drought stress may affect several soil properties that may affect directly or indirectly PGPR activities that may be of ecological significance including percolation, adsorption/desorption in soils, growth rate, sedimentation, chemotaxis, motility, and transport through different soil types of PGPR (e.g., Bowers and Parke 1993; Briettenbeck et al. 1988; Howie et al. 1987; Hyusman and Verstraete 1993; Toyota et al. 2000). Thus, it has been shown that the PGPR *Azospirillum* spp. is adsorbed firmly to soil particles, especially clays and organic matter in the topsoil, but barely washed downward (Bashan and Levanony 1988a, b). In temperate areas, in soils under conditions of water stress or old bacterial age, the bacterium took on a cyst-like form which is believed to be more resistant than the common vegetative cells and therefore may serve as a survival form during dry periods (Bashan et al. 1991a; Lamm and Neyra 1981). In sandy soils, the bacterium may produce fibrillar material which helps to immobilize it into the soil (Bashan et al. 1991b; Levanony and Bashan 1991). This particular feature of *Azospirillum brasilense* differentiates it from several other PGPR such as biocontrol pseudomonads, which can be washed down with percolating water (Liddell and Parke 1989).

In a complex soil environment, PGPR motility and chemotaxis seem to play an important role in movement towards the food resource, exudates from plant roots, eukaryotic organisms (target pathogens), or nutrient release from decaying/dead

plant parts (Bashan 1986; Arora and Gupta 1993; Scher et al. 1985; Singh and Arora 2001). Recently, Singh et al. (2010) demonstrated chemotaxis in *P. aeruginosa* PN1 towards the root exudates of chir–pine. Thus, it seems that bacterial motility and chemotaxis towards fungal propagules involves the most critical step in initiation of the fungal–bacterial interaction in antagonism or parasitism mediated biocontrol by PGPR (Hyakumachi and Arora 1998). One of such poorly understood traits in the mechanism is lack of sufficient knowledge about the motility behavior and chemotactic response of such biocontrol bacteria in the soil environment and their possible roles in plant–bacterial and fungal–bacterial interactions. Earlier and recent studies on fluorescent pseudomonads showed the important role of bacterial motility and water availability in plant–microbe and fungal–bacteria interactions, demonstrating that such bacteria can (1) be carried passively by water flowing through saturated soil; (2) move vertically through percolating water; or (3) be attracted chemotactically towards roots or fungal propagules (Arora and Gupta 1993; Bashan and Holugin 1994; Singh and Arora 2001; Singh et al. 2002). Singh et al. (2002) used two wild-type motile antagonistic *P. fluorescens* isolates and a non-motile Tn*5* mutant to evaluate the vertical migration through percolating water in three soil types (sandy, sandy loam, and clay loam). The authors showed that greater motility was observed in irrigated sandy soil, followed by sandy loam and clay loam. In general, filtration coefficient of *P. fluorescens* was higher in soils irrigated with 5 cm of water than that with 25 cm. Furthermore, the horizontal movement of *P. fluorescens* strains in sandy soil adjusted at different matric potentials showed a marked reduction with the decrease in matric potential (Singh et al. 2002).

10.4 Biotic Factors Affecting PGPR Biocontrol Activity

Various biotic factors can influence the efficacy and ability of a PGPR to control the deleterious pathogens.

10.4.1 Target Pathogen

The rhizosphere, rhizoplane, and pathogen infection court are the playgrounds and battlefields where fungal soilborne pathogens and PGPR interact and where as a result of those interactions the outcome of pathogen infection may take place (Raaijmakers et al. 2009). Most of the efforts and research on biology and ecology of interactions between PGPR and pathogens in soil and rhizosphere have focused on the mechanisms that govern PGPR activity and efficacy against the target pathogen. Thus, to date, most of those microbial interactions are viewed from the perspective of how PGPR inhibit the growth of a pathogen or suppress a disease on a certain host plant. In contrast, few studies have focused on the "pathogen perspective," i.e., responses of the pathogen to microbial antagonists (Duffy et al.

2003), although there is increasing evidence that pathogens populations are not static, but rather, dynamic entities, capable of rapidly adapting to and coping with adverse conditions generated or induced by a biocontrol agent. Furthermore, pathogens may also affect directly the PGPR activity and the outcome of biocontrol by the PGPR. Thus, disease suppression by PGPR have been shown to be dependent on (1) some mechanisms that the pathogen may use to counteract with the antagonism mediated by the PGPR; (2) the inoculum density of the pathogen, and (3) the race, strain, or isolate of the pathogen (e.g., Duffy and Défago 1997; Duffy et al. 2003; Hervás et al. 1997, 1998; Landa et al. 1997, 2001, 2002a; Mazzola et al. 1995; Schouten et al. 2004).

There are various mechanisms described in some plant–pathogen interactions that enable microorganisms to resist toxic compounds including enzymatic degradation or inactivation of antibiotic compounds, alteration of the target sites, and active efflux (Fleissner et al. 2002; Morrissey and Osbourn 1999; Schoonbeek et al. 2002; Schouten et al. 2004; VanEtten et al. 2001). However, relatively little is known about the role of these mechanisms in fungal defense against microbial antagonism, although some studies have shown that, within fungal populations, there is variation in sensitivity to antifungal metabolites produced by antagonistic PGPR (Duffy et al. 2003, 2004; Landa et al. 1997, 2002a; Mazzola et al. 1995).

For example, Landa et al. (1997) studied the antagonistic potential of four PGPR, three *Bacillus* spp. and *Pseudomonas chlororaphis* 30-84, against a collection of 18 isolates of races 0, 1, and 5 of *F. oxysporum* f. sp. *ciceris*, four isolates of races 0, 1, and 2 of *F. oxysporum* f. sp. *melonis*, three *F. oxysporum* f. sp. *phaseoli,* and four nonpathogenic *F. oxysporum*. Interestingly, *Bacillus* isolates obtained from chickpea rhizosphere differed in their antagonistic activity and inhibited mycelial growth of the chickpea fungal pathogen *F. oxysporum* f. sp. *ciceris* in a lower extension as compared to that of other *F. oxysporum* isolates from other hosts. Furthermore, the extent of growth inhibition of *F. oxysporum* f. sp. *ciceris* was influenced by race and geographical origin of the pathogen. Mazzola et al. (1995) studied a total of 66 individual isolates of the take-all fungus *G. graminis var. tritici,* obtained from a single wheat field, for their variation in sensitivity to the broad-spectrum antibiotics 2,4-diacetylphloroglucinol (DAPG) and phenazine-1-carboxylic acid (PCA), that are produced by multiple strains of antagonistic PGPR *Pseudomonas* spp. The authors found substantial variation in sensitivity to both antimicrobials and interestingly, in interactions with antagonistic *Pseudomonas* strains producing either DAPG or PCA, the antibiotic-insensitive pathogen isolates could not be controlled effectively in the rhizosphere of wheat plants by those PGPR. In a different study, Schouten et al. (2004) analyzed a collection of 76 plant-pathogenic and 41 saprophytic *F. oxysporum* strains for sensitivity to 2,4-DAPG. The authors found that approximately 17 % of the *F. oxysporum* strains were relatively tolerant to high 2,4-DAPG concentrations, but tolerance to 2,4-DAPG did not correlate with the geographic origin of the strains, formae speciales, genetic group, or fusaric acid production levels by the fungi. Biochemical analysis also showed that some tolerant *F. oxysporum* strains were capable of metabolizing 2,4-DAPG.

On the other hand, Duffy and Défago (1997) using liquid culture screening demonstrated that fusaric acid produced by the phytopathogenic fungus, *F. oxysporum* f. sp. *radicis-lycopersici* acts as a repressor of antibiotic production by the PGPR *P. fluorescens* CHA0, demonstrating that fusaric acid acts as a negative signal in the biocontrol of Fusarium crown and root rot of tomato inhibiting antibiotic production in situ and that fusaric acid-insensitive strains are more suitable for controlling this disease. Recent studies have further shown that certain *Pseudomonas* biocontrol strains are relatively insensitive to fusaric acid and that fusaric acid acts as a negative signal in 2,4-DAPG synthesis (Duffy et al. 2003, 2004). Thus, fusaric acid has been shown to act as a repressor of *phlA* expression both in vitro and in situ (Notz et al. 2001; Schnider-Keel et al. 2000). Blocking fusaric acid production by the pathogen via zinc amendment relieved *phl*A repression and improved the biocontrol activity of strain CHA0 (Duffy and Défago 1997). Other studies have shown a great diversity among strains of PGPR for their ability to grow in the presence of fusaric acid (Landa et al. 2002a). Thus, *Bacillus* spp. and *Pa. macerans* were completely inhibited at low concentrations (5 μg/ml). On the contrary, 29 strains of *Pseudomonas* spp. showed four patterns of tolerance to fusaric acid being all strains able to grow at a concentration of 5 μg/ml, and five of them tolerated very high concentrations of fusaric acid (up to 500 μg/ml) (Fig. 10.5). Besides bacterial growth, fusaric acid also affected the production of the siderophore pyoverdine by those *Pseudomonas* spp. isolates (Landa et al. 2002a). All those results indicate the importance of taking into consideration the ecology of interactions between PGPR and plant pathogens from the perspective of both partners, not forgetting that those interactions occurs specifically on the third partner of the disease triangle (the host plant) as it will be described in the next section.

Some studies have shown that the amount of disease suppression achieved by introduced biocontrol agents is influenced by the specific strain and the inoculum density of the pathogen. It has been shown that effective disease suppression by a biocontrol agent can be obtained only under low to moderate disease pressure or environmental conditions moderately favorable for disease development. For instance, *P. putida* WCS358 and *P. fluorescens* WCS374, and *B. subtilis* isolate GB03 and *B. megaterium* RGAF 51 reduced Fusarium wilt of radish and chickpea, respectively, only if disease incidence or pathogen inoculum density were low (Fuchs et al. 1999; Hervás et al. 1997, 1998; Raaijmakers et al. 1995). In a different study, Landa et al. (2001) showed that efficacy of PGPR in suppression of soilborne fungal pathogens decreased as conditions became more favorable for disease development (i.e., optimal temperature for disease development and high inoculum density of the pathogen). Thus, Fusarium wilt development in chickpea was greater at 250–1,000 chlamydospores/g of soil compared to that reached at 25–100 chlamydospores/g of soil. In parallel, the four PGPR tested only suppressed disease when inoculum density of the pathogen was below 250 chlamydospores/g of soil, and when temperature was less favorable for disease development (Fig. 10.4). There are several possibilities that can account for the differential effect of pathogen inoculum density on suppression of Fusarium wilt by those PGPR. One is that the pathogen inoculum potential at optimum temperature and high inoculum

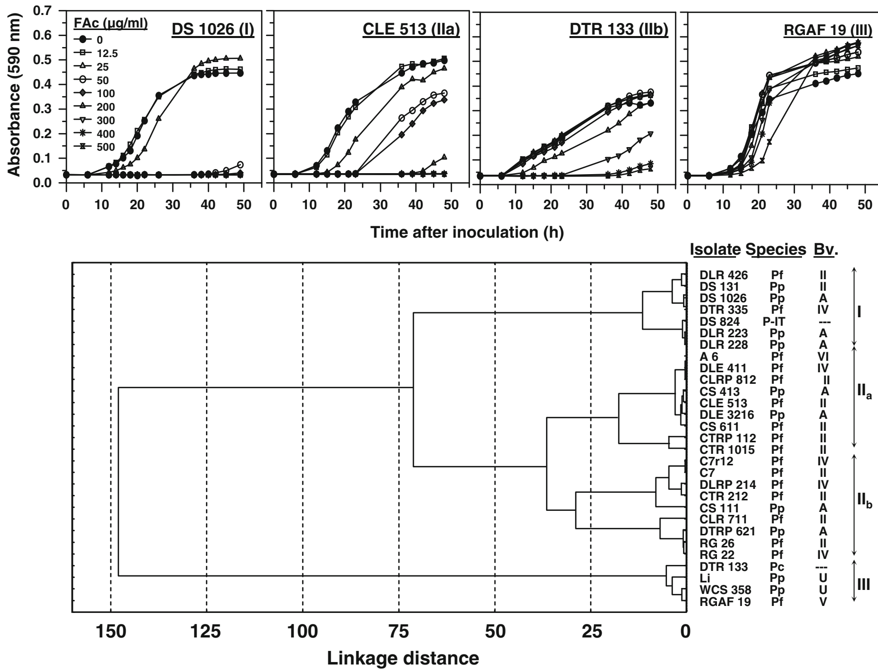


Fig. 10.5 Growth curves of four pseudomonad strains (*Pseudomonas putida* DS 1026, *P. fluorescens* CLE 513, *P. putida* DTRP 621, and *P. fluorescens* RGAF19) in response to Fusaric acid (FAc) concentrations (*upper panels*) representative of the four groups (I, IIa, IIb, and III) derived from cluster analysis of 29 isolates of fluorescent pseudomonads in response to FAc tolerance. Species (Pc = *Pseudomonas chlororaphis;* Pf = *P. fluorescens;* Pp = *P. putida;* P-IT = Intermediate type *P. fluorescens–P. putida*). *Bv*. Biovar. Tolerance to FAc: Strains were inhibited at FAc concentrations <100 µg/mL (I), between 100 and 400 µg/mL (II_a and II_b) and still growing at 500 µg/mL (III) (Source: Modified from Landa et al. 2002a)

density is just too high to be counteracted by the biocontrol agents. At 25 °C (the optimum temperature for Fusarium wilt development; Navas-Cortés et al. 2000, 2007), the disease potential seemed saturated with the lowest of the inoculum densities of *F. oxysporum* f. sp. *ciceris* in the study, since increasing inoculum density did not increase disease. When PGPR were introduced with seed and soil, the pathogen inoculum potential must have been reduced since the disease onset and rate of increase were delayed and slowed down, respectively, compared with the control. However, those effects by the introduced PGPR occurred only at low inoculum densities of the pathogen, which suggests a threshold in the extent of reduction of inoculum potential by the PGPR (Landa et al. 2001).

Several authors have reported the utility of dose–response models in driving biocontrol research approaches and interpretations (Johnson 1994; Larkin and Fravel 1999; Montesinos and Bonaterra 1996; Paulitz 2000; Schisler et al. 1997). However, pathogen dose and environmental factors may influence the level of pathogen inactivation occurring per unit of biocontrol agent, consequently the

resulting dose–response curves are predicted to radically change with those factors (Johnson 1994; Montesinos and Bonaterra 1996; Smith et al. 1997). Application of epidemiological and mathematical models to data of biocontrol efficacy of PGPR and its dependency on pathogen inoculum density should help to clarify the complexities of these interactions and reveal fundamental ecological and biological principles of the microbial interactions taking place between pathogen and PGPR (Paulitz 2000). Montesinos and Bonaterra (1996) pointed out that there is a need for objective means to fit data on infectivity titration of pathogens and biocontrol agents to suitable models that may provide dose–response surfaces and parameters describing the virulence of the pathogen and the efficiency of the biocontrol agent.

In a study conducted by Landa et al. (2001), use of response surface analysis and epidemiological dose–response models were useful for describing, clarifying, and characterizing differences in the efficacy of PGPR on the suppression of Fusarium wilt in chickpea as influenced by inoculum density of the pathogen and temperature (Fig. 10.6). PGPR treatments increased the disease onset, reduced the Gompertz relative rate of disease increase, and reduced the overall amount of disease developed. Those models would help to identify conditions for which treatments with PGPR isolates would potentially improve efficacy in suppressing Fusarium wilt in chickpea. Recognition of conditions under which PGPR biocontrol agents fail in disease control is the first step towards designing approaches to combat the problem of inconsistency and improve efficacy.

10.4.2 Host Plant

Plant species, as well as the soil type as we saw in previous sections, have a substantial influence on the structure and function of root associated microbial populations, including PGPR. However, there is no general decision about the key player, as indicated for different studies. Thus, both factors can dominate depending on biotic and abiotic conditions, and the main effect of each of them can change with time. The rhizosphere represents a highly dynamic and complex interface for chemical, physical, and biological interactions. In the same way that many phytopathogenic organisms, bacteria as well as fungi, have coevolved with plants and show a high degree of host specificity (Raaijmakers et al. 2009), some PGPR have evolved the same strategy. The most well-known example is rhizobia–legume interactions, which are highly specific (Long 2001). However, up till now, there was no clear evidence that this host specificity might be occurring for root associated PGPR (Landa et al. 2003, 2006a; Weller et al. 2007). However, the molecular basis of this host specificity is not well understood.

Studies on the basis of cultivation-dependent and independent methods have provided some hints concerning the composition and genetic diversity of total bacterial populations as well as specific groups of PGPR on the rhizosphere of plants (e.g., Berg and Smalla 2009; Berg et al. 2002, 2005a, b, 2006; Germida et al. 1998; Latour et al. 1996, 1999; Landa et al. 2003, 2006a). Those results have shown

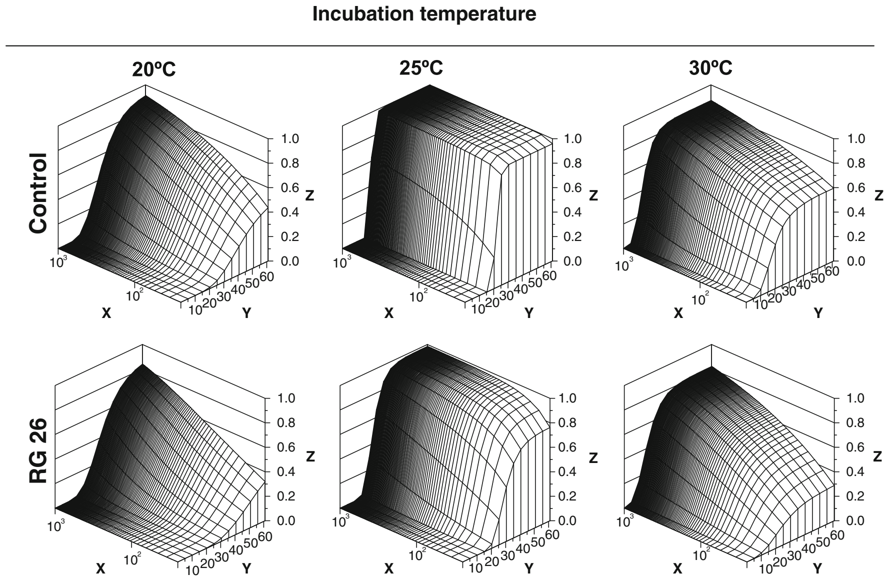


Fig. 10.6 Surface response for Fusarium wilt disease intensity (**Z**) in chickpea cv. PV 61 incubated at 20 °C, 25 °C, and 30 °C after treatment with the PGPR strain *Pseudomonas fluorescens* RG 26, as a function of both inoculum density (25, 50, 100, 250, 500, and 1,000 chlamydospores/g of soil) of *Fusarium oxysporum* f. sp. *ciceris* (**X**) and time (days) from sowing (**Y**). Nontreated seeds sown in nontreated soil served as controls (Source: Landa et al. 2001)

that the host plant is one, if not, the major factor in driving the PGPR community composition as compared to the soil type (e.g., Appuhn and Joergensen 2006; Grayston et al. 1998; Lemanceau et al. 1995; Merbach et al. 1999; Miller et al. 1989; Wieland et al. 2001). Thus, it is considered that the activity of plant roots through root deposits and exudates form a unique micro-environment that have an impact on the physicochemical conditions of soil as well as on the biological activity and composition of PGPR communities in the surrounding rhizosphere and endosphere, due to the important source of substrates that root exudates represent to support rhizosphere microorganisms (Berg and Smalla 2009; Raaijmakers et al. 2009; Rovira 2005; Ryan et al. 2009). Furthermore, other compounds that the plants produce on roots as defense mechanism against pathogens and may be excreted to the rhizosphere, for example phytoanticipins and phytoalexins, may interact with PGPR. Those compounds can reach high concentrations in root cells and soil (Cachinero et al. 2002; Landa et al. 2002a; Dakora and Phillips 1996). Consequently, PGPR used for disease suppression should also be tolerant to toxic compounds produced by the host plant.

Landa et al. (2002a) showed that phytoanticipins differ in their effects on specific PGPR strains and how the bacterial sensitivity to a given phytoanticipin varies from one PGPR strain to the other. Thus, whereas tomatine did not affect growth of bacteria, the presence of biochanin A appeared to enhance growth of four

Pseudomonas spp. strains and delayed growth of five isolates of *B. megaterium, Pa. macerans,* and *Bacillus circulans*, but coumarin inhibited growth of *Pseudomonas* spp. strains and had no effect on *B. circulans* and *Pa. macerans*. Those results agreed with those of Wyman and VanEtten (1978), who showed that sensitivity of bacterial isolates to six selected isoflavonoid phytoalexins varied widely, with pseudomonads being more tolerant to phytoalexins than xanthomonads or *Achromobacter* isolates.

It is well known that plant species play a fundamental role in the dynamic and structural composition of bacterial communities (Ahn et al. 2007; Aranda et al. 2011; Berg et al. 2005a, b; Germida et al. 1998; Mendes et al. 2007). This effect has been attributed to the fact that patterns, amount, and quality of root exudates are genetically regulated in plants; consequently it can be highly specific for a given plant species or even a particular genotype or cultivar. Some studies have suggested that these differences in exudates explain the specific influence of the plant genotype on rhizosphere and endosphere PGPR communities (Adams and Kloepper 2002; Appuhn and Joergensen 2006; Kowalchuk et al. 2002; Marschner et al. 2001; Meredith and Bais 2009; Rovira 2005; Smalla et al. 2001). Thus, in the recent years, a number of studies have obtained experimental evidence about the selective influence of specific plant genotypes, cultivars, and even ecotypes on the composition of bacterial communities in the rhizosphere and endosphere (de Long et al. 2002; Marschner et al. 2001; Mazzola et al. 2004; Micallef et al. 2009; Rumberger et al. 2004, 2007). Moreover, recent studies targeting specific microbial components of the rhizosphere such as strains of fluorescent *Pseudomonas* sp. producing the antibiotic 2,4-DAPG have demonstrated that successive cycles of crop monoculture of different plant species (Berg et al. 2002; Bergsma-Vlami et al. 2005; de la Fuente et al. 2006a, b; Landa et al. 2003, 2006a; Mazzola et al. 2004; Okubara et al. 2004) or of different genotypes (cultivars) of a given plant species (de la Fuente et al. 2006a, b; Landa et al. 2002b, c, 2006a; Mazzola and Gu 2002; Notz et al. 2001; Okubara and Bonsall 2008; Picard et al. 2004) differentially enrich and support specific populations of those PGPR bacteria. This effect of the plant cultivar on PGPR populations has been found also in other studies. For example, interestingly wild rice species and old rice varieties were preferred over modern rice cultivars by *Azoarcus* spp. endophytes (Engelhard et al. 2000), and rhizospheric *Pseudomonas* were found to be more abundant in older wheat cultivars, whereas the roots of newer wheat cultivars were colonized in higher extension by endophytic *Pseudomonas* (Germida and Siciliano 2001). In a very elegant set of studies Picard et al. (2004) and Picard and Bosco (2005, 2006) showed that maize heterozygosis stimulates the colonization of roots by abundant populations of 2,4-DAPG-producing *Pseudomonas* with a high level of genetic diversity as compared to the respective maize parental lines.

Plant species and cultivars may also have an effect on supporting biocontrol efficacy of PGPR. Van Peer et al. (1991) showed that *Pseudomonas* sp. strain WCS417 suppressed Fusarium wilt development in a moderately resistant carnation cultivar as compared to the disease suppression obtained on the susceptible cultivar, but Leeman et al. (1995) showed that *P. fluorescens* WCS374 protected each of six

radish cultivars differing in susceptibility to *F. oxysporum* f. sp. *raphani*. Smith et al. (1997) demonstrated that tomato lines differing in resistance to *Pythium torulosum* also differ in supporting the biocontrol activity by *Bacillus cereus* UW85. However, biocontrol was not supported in lines with low and high levels of resistance to the pathogen, suggesting that the two traits are independent. Hervás et al. (1997, 1998) found that two chickpea cultivars susceptible to the highly virulent race 5 of *F. oxysporum* f. sp. *ciceris*, with different genetic pool varied in the level of wilt suppression achieved when their roots were colonized by different PGPR antagonists. In these later studies, the extent of disease protection was always higher and more consistent in chickpea cv. PV 61 than in cv. ICCV 4 even though roots of both cultivars were colonized by the PGPR strains in the same extension. All those results indicate the need to understand the specific effect and influence that the host plant genotype have in supporting populations of PGPR in their rhizosphere as well as their activity against the specific target pathogen, in order to maximize the chance of success of biocontrol.

10.5 Effect of Climate Change on Biocontrol Mediated by PGPR

Climate change also affects the efficacy of a biocontrol agent in various ways.

10.5.1 Climate Change and Plant Disease Management

Climate change is expected to affect agriculture very diversely in different parts of the world (Parry et al. 1999). The resulting effects will depend greatly on current climatic and soil conditions, the direction of change and the availability of resources and infrastructure to cope with change (Olesen and Bindi 2002). The increase in mean temperatures, change in precipitation regimes, and a continuous increase in CO_2 concentration are likely the main scientific evidence of climate change in recent decades (IPCC 2007).

Plant disease epidemics result from interactions of a susceptible host plant, a prevalent and virulent pathogen, and a conducive environment. Their interactions can be conceptualized by the disease triangle model to assess how shifts in any one of these components can change disease expression in a given pathosystem (Fig. 10.7) (Scholthof 2007). Due to the integral role of environmental conditions in disease expression, outcomes of climate change in plant pathosystems include (1) modifications in host resistance, (2) altered stages and rates of pathogen development, and (3) changes in the physiology of host–pathogen interactions (Fig. 10.7). These effects have been predicted to result in shifts in (1) the geographical distribution of hosts and their pathogens, (2) altered crop losses due to disease,

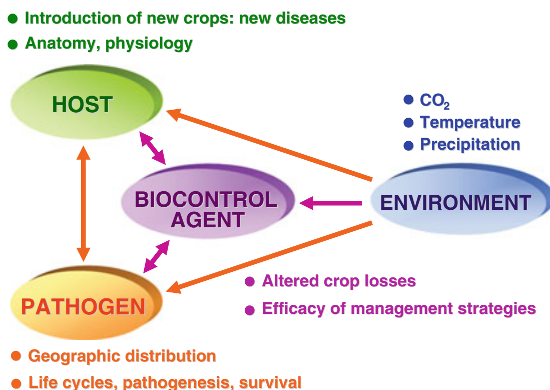


Fig. 10.7 Disease triangle for host plant–pathogen – environment–biocontrol agent interactions, and potential shifts in each component as a result of environmental changes associated to climate change (Source: Chakraborty 2005; Coakley et al. 1999; Juroszek and von Tiedemann 2011)

and (3) a change in the efficacy of management strategies with regard to timing, preference, and efficacy of chemical, physical, and biological control measures and their utilization within integrated pest management (IPM) strategies (Chakraborty 2005; Coakley et al. 1999; Juroszek and von Tiedemann 2011). As a consequence, current disease management strategies may require adjustment under the different scenarios of climate change (Garrett et al. 2006).

Research on the effects of climate change on biocontrol is limited and in most cases is focused on its impacts on the composition and dynamics of the microbial community of the rhizosphere and soil (Ghini et al. 2008). One of the major uncertainties of climate change on biological control would be the direct effect that might have on consistency of PGPR due to the vulnerability of their population levels to environmental variation and to the environmental extremes that would be associated to climate change (Garrett et al. 2006; Wong et al. 2002). If appropriate environmental conditions (i.e., temperature and moisture) are not consistently available, PGPR populations may reach densities that are too small to have important biocontrol effects, and may not recover as rapidly as pathogen populations when conducive conditions recur (Garrett et al. 2006; Gibson et al. 1999; Hannusch and Boland 1996). Therefore, a better understanding of effect of climatic change on PGPR–plant–pathogen interaction is consequently needed to select appropriate PGPR strains that may perform well under potential environmental altered conditions (Compant et al. 2010).

10.5.2 Effect of Climatic Change on Pathogen and PGPR Geographical Distribution: Biocontrol of Fusarium Wilt of Chickpea, A Case Study

Climatic mapping predicts the potential distribution of organisms based on their responses to climate in their home range (Baker et al. 2000). Different climate-based risk mapping systems have been used for pest risk analysis, e.g., BIOCLIM,

CLIMEX, DOMAIN, GARP, HABITAT (Kriticos and Randall 2001; Magarey et al. 2007). These tools may facilitate the geographic assessment of potential pathogen and PGPR distribution and how those factors may interact under the different scenarios of climatic change. The CLIMEX™ model (Hearne Scientific, Melbourne, Vic., Australia; Sutherst et al. 2007) is a widely used method to estimate the potential geographic distribution and risk assessments of arthropod pests, weeds, and diseases under current climatic conditions (e.g., Brasier et al. 1996; Brasier and Scott 1994; Matsuki et al. 2001) as well as under future climatic change scenarios (e.g., Kriticos et al. 2003; Potter et al. 2009; Watt et al. 2009). In contrast with the usual, statistically based, pattern-matching of meteorological data, CLIMEX takes also into account the possible mechanisms that limit the pathogen geographical distribution (Kriticos and Randall 2001). CLIMEX can model those geographic areas that are climatically suitable for the species (host plant, pathogens and PGPR), rather than habitat per se (Sutherst et al. 2007). Also, as CLIMEX includes a global meteorological database and process-based algorithms, it can project species potential distributions into novel or future climates with more confidence than regression-based models (Kriticos and Randall 2001).

We have developed risk models for the Mediterranean region in current and future climate change scenarios for the pathosystem chickpea × *F. oxysporum* f. sp. *ciceris*-race 5 (the most virulent race described) (Landa et al. 2001, 2004b; Navas-Cortés et al. 2000, 2007), as well as for two PGPR bacteria (*P. fluorescens* RGAF19 and RG26) that have been shown effective as biocontrol agents of Fusarium wilt of chickpea under both controlled (Landa et al. 2001) and field conditions (Landa et al. 2004b).

The CLIMEX™ model was run using a long term average meteorological data from a spatially interpolated (10″ degree grid) data set (CRU, University of East Anglia, Norwich, UK, Mitchel et al. 2002) adapted for its use in CLIMEX. Model parameters were fitted to distribution data for the pathogen and environmental suitability for the rhizobacteria under current and future climate change scenarios using an iterative adjustment and comparison method based on experimental data from controlled condition experiments in our previous studies (Landa et al. 2001, 2004b, 2006b; Navas-Cortés et al. 2000, 2007). The growth and stress indices are calculated weekly and then combined into an overall annual index of climatic suitability, the Ecoclimatic Index (EI), which gives an overall measure of the potential of a given location to support a permanent population of the species in the study (Sutherst et al. 2007). The model was estimated for current climatic conditions and forced by an effective greenhouse effect corresponding to the Intergovernmental Panel on Climate Change (IPCC) A2 scenario for the period 2011–2040 (SRES A2-2020) and 2070–2100 (SRES A2-2080) as well as for the B2 scenario for the period 2070–2100 (SRES B2-2080) (IPCC 2007) regionalized for Europe (Iglesias et al. 2007).

The model estimated that all the geographic areas where *F. oxysporum* f. sp. *ciceris* has already been described as climatically suitable for the occurrence of the Fusarium wilt. Thus, in current weather condition areas of Mediterranean climate (Southern Europe, and North and South of Australia and America), Middle East, the

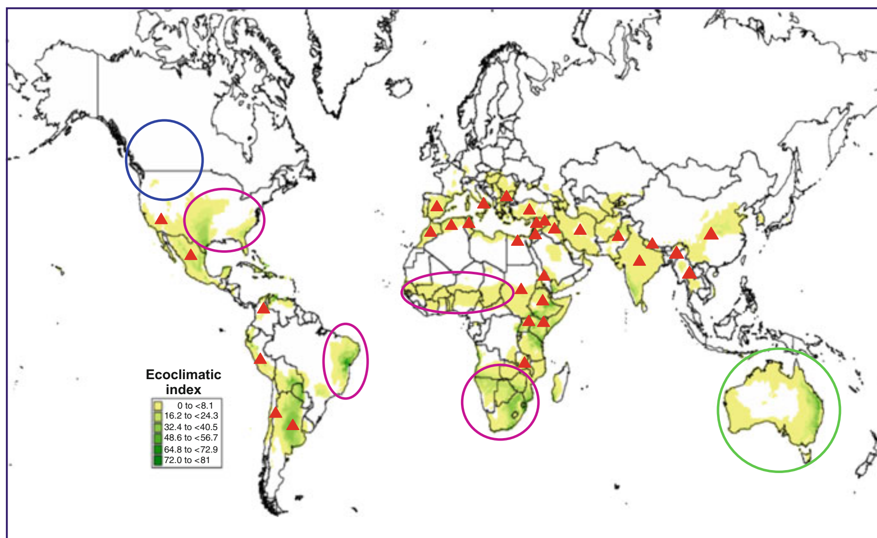
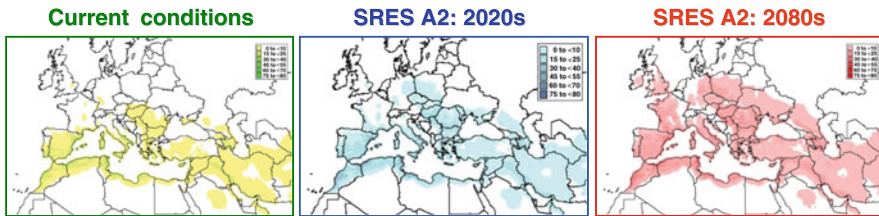


Fig. 10.8 World map of reported distribution of *Fusarium oxysporum* f. sp. *ciceris* and its potential distribution estimated by the CLIMEX™ model for current climatic conditions. *Circles* indicate areas where climatic conditions are suitable for the development of *F. oxysporum* f. sp. *ciceris* although it has not been described (*green*) or chickpea is not grown (*purple*); *blue circles* indicate areas where chickpea crops exit but climatic conditions are not favorable for *F. oxysporum* f. sp. *ciceris* development. *Red triangles* indicate countries where *F. oxysporum* f. sp. *ciceris* has been reported in the scientific literature

Indian subcontinent and Central and Southern Africa, have weather conditions suitable for the survival of *F. oxysporum* f. sp. *ciceris* and development of Fusarium wilt (Fig. 10.8). In the Mediterranean region, under current weather conditions, the geographic distribution *F. oxysporum* f. sp. *ciceris*-race 5 and the potential biocontrol of *P. fluorescens* RGAF19 and RG26 is mainly limited by the detrimental effect (stress) on their survival and development of low temperatures prevalent in central and northern Europe. On the contrary, drought conditions and high temperature limits their development in North African countries. As a result of Climate change, the model project an extension in the potentially favorable areas for the development of *F. oxysporum* f. sp. *ciceris*-race 5 as well as for the potential biocontrol of *P. fluorescens* RGAF19 and RG26 towards Central Europe, including south of France, central Italy, Czech Republic, Slovakia, central-east Germany, and southwest Poland for the SRES A2-2020 scenario. Similarly, for this same scenario in the last third of the twenty-first century, i.e., SRES A2-2080, this area would extend to most of France, north of Italy, south of United Kingdom, Ireland, and Ukraine (Fig. 10.9, upper panels). In summary, both the Fusarium wilt PGPR–biocontrol agents and the pathogen shared a similar geographical distribution; however, IE values are smaller for the PGPRs than that estimated for the pathogen, indicating a decrease in the biocontrol activity of the PGPRs as latitude increase (Fig. 10.9, lower panels). For the scenario SRES B2-2080, projections show intermediate

Fusarium oxysporum f.sp. *ciceris*

Current conditions SRES A2: 2020s SRES A2: 2080s



BCAs: *Pseudomonas fluorescens* RGAF19 & RG26

Current conditions SRES A2: 2020s SRES A2: 2080s

Fig. 10.9 Ecoclimatic Index estimated by CLIMEX™ for *Fusarium oxysporum* f. sp. *ciceris*-race 5 (*upper panel*) and biological control agents *Pseudomonas fluorescens* RGAF19 and RG26 (*lower panel*) under current climate and future climate change scenarios

values in both geographic range and IE than those specified for the other two scenarios. These results demonstrate that climate risk mapping tools may help in selection or designing of new biocontrol strategies for the efficient use and improved performance of well-performing PGPR as effective biocontrol agents of soilborne fungal plant pathogens under current or future climate scenarios.

10.6 Concluding Remarks and Future Perspectives

Genes encoding resistance to the most common and widely occurring soilborne fungal pathogens are normally scarce or lacking in many cultivated plants of economic importance. As an alternative, plants have evolved a strategy of stimulating and supporting specific groups of antagonistic microorganisms from the thousands of beneficial, neutral, and deleterious species in the rhizosphere environment. Thus, specific antagonistic microorganisms among which PGPR are the most important are selected and enriched by nutrients released from plant roots, providing the first line of defense against many soilborne pathogens causing root rots, crown rots, and wilts (Cook et al. 1995; Weller et al. 2007). Some of the results addressed in this review emphasize the need to obtain better insight into all of the biotic and abiotic factors involved in the success of biocontrol of soilborne fungi mediated by PGPR, including the biocontrol mechanisms utilized and genes governing the microorganism–plant interactions of significant importance for

disease suppression. Identifying the environmental factors that influence in the effectiveness of these PGPR in terms of disease control would provide a basis for improved integration of biocontrol treatments with other environmental friendly control practices, both under current or future climate scenarios, helping farmers to better manage diseases caused by soilborne pathogens and reduce inputs of chemical pesticides often applied to control them.

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Chapter 11
Plant–PGPR Interactions for Pest and Disease Resistance in Sustainable Agriculture

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11.1 Introduction

Plant growth in agricultural soils is influenced by many abiotic and biotic factors. There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere. The rhizosphere concept was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots where microbe populations are stimulated by root activities. A large number of microorganisms such as bacteria, fungi, protozoa, and other groups of microorganisms coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low (Garcia et al. 2001). Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plant's physiology to a greater extent, especially considering their competitiveness in root colonization (Barriuso et al. 2008).

Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect. In the last few years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Various species of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus,* and *Serratia* have been reported to enhance the plant growth (Joseph et al. 2007). There are several PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism: suppression of plant disease (termed bioprotectants), improved nutrient acquisition (biofertilizers), or phytohormone

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production (biostimulants). Inoculant development has been most successful to deliver biological control agents of plant disease, i.e., organisms capable of killing other organisms pathogenic or disease causing to crops (Saharan and Nehra 2011).

Microbial inoculants suppress plant disease through at least one mechanism: induction of systemic resistance and production of siderophores or antibiotics. Exposure to the PGPR triggers a defense response by the crop as if attacked by pathogenic organisms. Bioprotectants are currently being studied by the laboratories of Fernando and Daayf in the Department of Plant Science, University of Manitoba. Biofertilizer nitrogen-fixing bacteria are also available for increasing crop nutrient uptake of nitrogen from nitrogen-fixing bacteria associated with roots (*Azospirillum*). Phosphorus-oxidizing bacteria help in making the phosphorus available to the plants. The phytohormones they produce include indole-acetic acid, cytokinins, gibberellins, and inhibitors of ethylene production. Rhizoremediers PGPR also help in degrading organic pollutants (Nihorimbere et al. 2010). PGPR is a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Such an integrated system could be used for transplanted vegetables to produce more vigorous transplants that would be tolerant to nematodes and other diseases for at least a few weeks after transplanting to the field (Kloepper et al. 2004). Selected strains of beneficial PGPR trigger a plant-mediated-induced systemic resistance (ISR) response that is effective against a broad spectrum of plant pathogens. ISR is a plant-mediated mechanism; it resembles classic pathogen-induced resistance, in which noninfected parts of previously pathogen-infected plants become more resistant to further infection (Umashankari and Sekar 2011).

11.2 Plant-Growth-Promoting Rhizobacteria

About 2–5 % of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microflora, exert a beneficial effect on plant growth and are termed Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1978). PGPR are free-living bacteria (Kloepper et al. 1989), and some of them invade the tissues of living plants and cause unapparent and asymptomatic infections (Sturz and Nowak 2000). The recognition of plant-growth-promoting rhizobacteria (PGPR), a group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years to where today researchers are able to repeatedly use them successfully in field experiments. Commercial applications of PGPR are being tested and are frequently successful; however, a better understanding of the microbial interactions that result in plant-growth enhancement will greatly increase the success rate of field applications (Burr et al. 1984). PGPR, root-colonizing bacteria, are known to influence plant growth by various direct or indirect mechanisms. Several chemical changes in soil are associated with PGPR and are reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. Some

bacterial strains directly regulate cell physiology by mimicking synthesis of hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth.

Among the various fungal and bacterial biocontrol agents, PGPR play a significant role in the management of plant pests and diseases. PGPR are a group of free-living saprophytic bacterial microorganisms that live in the plant rhizosphere and aggressively colonize the root system and have been studied as plant-growth promoters for increasing agricultural production and as biocontrol agents against plant diseases. They survive in seed or soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids (Kloepper et al. 1992), attach to root surface (Suslow 1980), and become endophytic by colonizing in root cortex region. Rhizobacteria are distributed in the rhizosphere in a lognormal pattern and are sporadically dispersed along root segments. PGPR favorably affect plant growth, and yield of commercially important crops includes the bacteria belonging to the genera *Azotobacter*, *Azospirillum*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia,* and *Xanthomonas* (Vessey 2003). However, most of the reported PGPR strains are from *Pseudomonas* and *Bacillus* only (Ramamoorthy et al. 2001). More specifically, the soilborne fluorescent pseudomonads have received particular attention throughout the global science because of their catabolic versatility, excellent root-colonizing abilities, and their capacity to produce a wide range of antifungal metabolites.

11.2.1 PGPR as Biocontrol Agent and Biofertilizer

PGPR are indigenous to soil and the plant rhizosphere and play a major role in the biocontrol of plant pathogens. They can suppress a broad spectrum of bacterial, fungal, and nematode diseases. PGPR can also provide protection against viral diseases. The use of PGPR has become a common practice in many regions of the world. Although significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies, results in the field have been inconsistent. Recent progress in our understanding of their diversity, colonizing ability, and mechanism of action, formulation, and application should facilitate their development as reliable biocontrol agents against plant pathogens. Some of these rhizobacteria may also be used in integrated pest management programs. Greater application of PGPR is possible in agriculture (Maheshwari 2011) for biocontrol of plant pathogens and biofertilization (Siddiqui 2006). PGPR may induce plant-growth promotion by direct or indirect modes of action (Kloepper 1993; Lazarovits and Nowak, 1997; Kumar et al. 2011) (Table 11.1; Fig. 11.1).

Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non symbiotic nitrogen fixation), and stimulation of disease-resistance mechanisms (induced systemic resistance) (Umashankari and Sekar 2011). Indirect effects

Table 11.1 Terms adopted for classified mechanisms by which plant-growth-promoting bacteria stimulate plant growth

Term	Definition	Mechanisms
Biofertilizer	A substance which contains live microorganisms which, when applied on the seed, plant surface, or the soil, colonizes the rhizosphere or the interior of the plant and promotes growth through increased supply or availability of primary nutrients for the host plant	Biological nitrogen fixation—utilization of insoluble forms of phosphorus
Phytostimulator	Microorganism with the ability to produce or change the concentration of growth regulators such as indole-acetic acid, gibberellic acid, cytokinins, and ethylene	– Production of phytohormones (auxins, cytokinins, and gibberellins) – Decreased ethylene concentration (in the interior of the plant)
Biopesticide or biocontrol agent	Microorganisms that promote plant growth through the control of phytopathogenic agents, mainly for the production of antibiotics and antifungal metabolites	– Production of antibiotics (siderophores, HCN, antifungal metabolites) – Production of enzymes that degrade the cellular wall of the fungi – Competitive exclusion – Acquired and induced systemic resistance

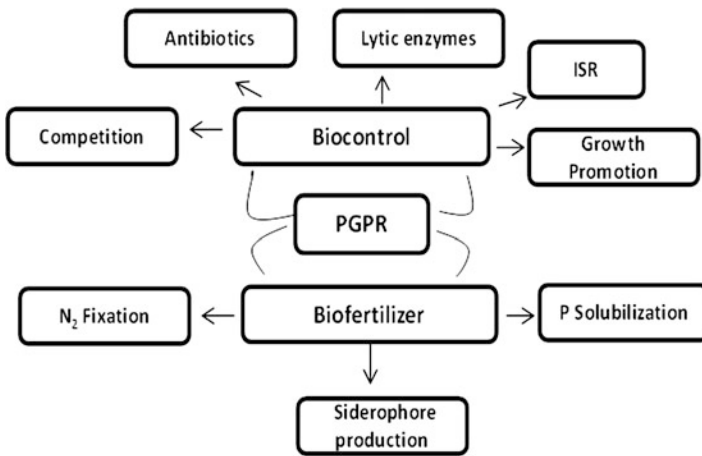

Fig. 11.1 Schematic illustration of important mechanisms known for plant-growth promotion by PGPR

originate, for example, when PGPR act like biocontrol agent-reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory-contaminated soils (Jacobsen 1997). Based on their

activities, Somers et al. (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants), and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites). 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. When studying beneficial rhizobacteria, the original definition of PGPR is generally used: it refers to the subset of soil and rhizosphere bacteria colonizing roots in a competitive environment, e.g., in non-pasteurized or non-autoclaved field soils (Kloepper 2003a, b; Reddy et al. (2003)). Furthermore, in most studied cases, a single PGPR will often reveal multiple modes of action including biological control (Kloepper 2003a).

11.2.2 Rhizobacteria in the Pest Management

Most of the researches carried out so far have been dealt with the utilization of PGPR in the management of plant pathogens (Ramamoorthy et al. 2001). Very few reports have been dealt with the management of insect pests by PGPR. However, induction of systemic resistance by PGPR against various pests was considered as the most desirable approach in crop protection. Generally, fluorescent pseudomonads influence the growth and development of insects at all stages including egg, larva, pupa, and adult. *Pseudomonas maltophilia* affects the growth of *Helicoverpa zeae*, the corn earworm, leading to 60 % reduction in adult emergence. Pupae and adults emerging from the bacteria-infected larvae remained smaller (Bong and Sikorowski 1991). Qingwen et al. (1998) reported that the relative growth rate, consumption rate, and digestibility of feed by larvae of *H. armigera* have been affected when they were allowed to feed on cotton plants treated with *Pseudomonas gladioli* due to increased polyphenol and terpenoid content in treated plants. As fluorescent pseudomonads are effective rhizosphere colonizers and are endophytic in nature in the plant system, attempts have been made to transfer the insecticidal crystal protein from *B. thuringiensis* to *P. fluorescens*. Thus, genetically engineered *P. fluorescens* has been found to be effective against lepidopteran insect pest. Transgenic *P. cepacia* strain 526 with the crystal protein gene has consistently shown insecticidal activity against tobacco hornworm (Stock et al. 1990).

When plants are attacked by insects and pathogens, volatile chemical signals are released. These signals have direct and indirect effect over insects, viz., affect the suitability of food or invite the parasites of the targeted insect. Volatiles molecules are released not only from the damaged parts but also systemically from other parts of the plant, and this continues after cessation of feeding by the insect. These signals are perceived by olfactory sensory mechanisms in both the herbivorous insects and their parasites and predators. Characterization of molecular structures involved in predator inviter showed the evidence that such signals also affect the neighboring

intact plants and initiate the defense by induction of further-signaling systems such as to increase the predator population. These discoveries provide the basis for new crop protection strategies that are either delivered by genetic modification of plants or by applying PGPR strains or synthetic chemicals, viz., 2,6-dichloroisonicotic acid to conventionally produced plants (Pickett et al. 2003). This phenomenon has been demonstrated by a number of laboratories, including those studying plant systems involving Lepidopterous caterpillars, broconid wasp parasitoids and herbivorous or predatory mites (Dicke and Bruin 2001). Similarly, Du and coworkers have reported the induction of volatile compound, viz., 6-methyl-5-hepten-2-one, conferring specificity to the parasitoid showing an innate response during aphid infestation (Du et al. 1998). Also Pickett et al. (2003) reported the induction of 5-methylfurfural, chrysanthenone, α-pinene, *cis*-jasmone, methyl salicylate, 1-octen-3-ol, and α-caryophyllene during pest attack in the forage crops. Interestingly, *cis*-jasmone was repellent to the aphid, *Phorodon humuli,* and it established the insect populations that are antagonistic to aphids including ladybirds and the parasitic wasp *Aphidius ervi* (Birkett et al. 2000). Furthermore, with lepidopterous larvae-attacking plants, there are signals specific to the herbivorous insect that are passed to the plant and in turn induce specific volatile production that is exploited by specialist parasitoids (De Moraes et al. 1998). Recently, it was established that plant volatiles released upon insect attack repel females and deter oviposition (De Moraes et al. 2001). Volatile signals generated by certain nonpathogenic bacteria have also been shown to trigger defense responses in *Arabidopsis* (Ryu et al. 2004).

In an another study, *Pseudomonas*-treated rice leaves altered the feeding behavior of leaf folder, reduced larval and pupal weight, increased the larval mortality and incidence of malformed adults under *in vitro* condition (Radjacommare et al. 2002). Similarly, application of *P. fluorescens* strain Pf1 consistently reduced the aphid and bollworm incidence within 5 days of spraying in cotton. PGPR treated leaves, altered feeding behavior of boll worm larvae, reduced the larval as well as pupal weight, and increased the larval mortality under *in vitro* conditions (Bhuvaneswari 2005).

11.2.3 Rhizobacteria in the Management of Diseases

Biological control by antagonistic organisms has been studied extensively, and rhizobacterial strains are emerged as potential biocontrol agents for the control of root and foliar diseases (Ramamoorthy et al. 2002). PGPR are having the ability to protect aboveground plant parts against viral, fungal, and bacterial diseases by induced systemic resistance (ISR) (Kloepper et al. 1992). Among the PGPR, fluorescent pseudomonads are the most exploited bacteria for biological control of soilborne and foliar plant pathogens. In the past three decades numerous strains of fluorescent pseudomonads have been isolated from the soil and plant roots by several workers and their biocontrol activity against soilborne and foliar pathogens was reported (Ramamoorthy et al. 2002).

PGPR are reported to survive in both rhizosphere and phyllosphere region (Kloepper et al. 1992). In groundnut, seed treatment and foliar spray with *P. fluorescens* Pf1 induced systemic resistance against late leaf spot by inducing various defense mechanisms in the host plant (Meena 2000). Seed treatment with *Bacillus pumilus* (SE 34) and *Serratia marcescens* strain 90-166 induced systemic resistance against fusiform rust incited by *Cronartium quercuum* f. sp. *fusiforme* in loblolly pine. In cucumber, PGPR strains with or without application of methyl bromide showed a higher level of induced resistance against foliar diseases, viz., angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* and anthracnose caused by *Colletotrichum orbiculare* (Zehnder et al. 2001). Viswanathan and Samiyappan (1999) reported PGPR-mediated ISR against red rot disease in sugarcane. Application of PGPR strains effectively reduced the infection of fruit rot and dieback of chilli incited by *Colletotrichum capsici* (Bharathi et al. 2004).

The phyllosphere microbial community is an open system; hence, biocontrol specifically in the phyllosphere has been extensively reviewed since 1980 (Andrews 1990). The reduction of leaf spot disease caused by *Cercospora moricola* in mulberry is through the application of *Pseudomonas maltophilia*. Interestingly, soilborne bacteria colonized pear flowers and gave good control of fire blight similar to that of commercial bactericides. Similarly, foliar application of PGPR strains against the bacterial spot (*Xanthomonas axonopodis* pv. *vesicatoria*) in tomato, angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*) in cucumber, and blue mold (*Peronospora tabacina*) and wildfire (*P. syringae* pv. *tabaci*) in tobacco was found to be effective in controlling the disease incidence (Reddy et al. 1999). Recently, Vivekananthan et al. (2004) reported the foliar application of fluorescent pseudomonad strain FP7 strongly reduced the incidence of anthracnose disease in mango.

Demeyer et al. (1999) reported that *Pseudomonas aeruginosa* 7NSK2 strain induced systemic resistance against *Tobacco mosaic virus*. ISR has been reported as one of the mechanisms by which PGPR reduce the plant disease, functioning through manipulation of the physical and biochemical properties of host plants (Ramos Solano et al. 2008). PGPR strains belonging to the genera *Pseudomonas*, *Bacillus,* and *Azospirillum* have been reported to elicit growth promotion and ISR. Secondary metabolites produced by PGPR strains induced systemic resistance (Gumede 2008) in many crops. Treatment with *P. fluorescens* enhanced the induction of chitinase and β-1,3 glucanase associated with defense mechanism. However, the fungicide metalaxyl did not show such induction against *Peronosclerospora sorghi* (Kamalakannan and Shanmugam 2009). Murphy et al. (2003) reported that combination of PGPR strains, viz., GBO3 (*Bacillus subtilis*) and IN937a (*B. amyloliquefaciens*), with the carrier chitosan to the tomato leads to protection against CMV in tomato. However, in certain cases, mixture of different strains has no synergistic effect. *Trichoderma virens* GL 21 applied as a granular formulation, in combination with *Burkholderia cepacia* BC-1 or *B. ambifaria* BC-F applied as a seed treatment, significantly improved suppression of cucumber damping-off caused by *Rhizoctonia solani* over individual applications of these microbes (Roberts et al. 2005).

Induction of systemic resistance by PGPR against viral diseases has been reported in cucumber and tobacco plants. Seed treatment with *P. fluorescens* strain 89B-27 and *S. marcescens* strain 90-166 has consistently reduced the number of Cucumber mosaic virus-infected plants (CMV) and delayed the development of symptoms in cucumber and tomato. Soil application of *P. fluorescens* strain CHAO has induced systemic protection against inoculation with Tobacco necrosis virus (TNV) in tobacco (Maurhofer et al. 1998). These experiments show that PGPR strains initiate ISR against a wide array of plant pathogens causing fungal, bacterial, and viral diseases.

Increased phenolic activity in the PGPR-treated plants against *Bitter gourd yellow mosaic virus* (Rajinimala et al. 2003), *Tomato-spotted wilt virus* (Kandan et al. 2002), and *Banana bunchy top virus* (Harish et al. 2009b) was reported. The PGPR is a group of rhizosphere-colonizing bacteria, which produce substances that increase the growth of plants and/or protect them against pathogens (Harish et al. 2009a). Karthiba et al. (2010) reported that combination of *Pseudomonas* strains and *Beauveria* isolate showed their ability to promote plant growth and their effectiveness against leaf-folder and sheath-blight disease on rice under glass house and field conditions. *P. fluorescens* strain, CHA0, in combination with chitin has the potential to increase growth, leaf nutrient contents, and yield of banana plants under perennial cropping systems (Kavino et al. 2010). The combination of PGPR strains with entomopathogenic fungi stimulates the plant growth by releasing of phytohormones and suppressing the pathogen (Senthilraja et al. 2010).

11.2.4 Rhizobacteria in the Management of Nematodes

PGPR also induce systemic resistance against nematode pests (Sikora 1992). *P. fluorescens* induced systemic resistance and inhibited early root penetration of *Heterodera schachtii*, the cyst nematode in sugar beet (Oostendorp and Sikora 1990). Similarly, *B. subtilis* has induced protection against *Meloidogyne incognita* and *M. arenaria* in cotton (Sikora 1988). Though attempts to use PGPR for nematode control are limited, the use of PGPR as biological control agents of plant-parasitic nematodes especially for sugar beet and potato cyst nematode has been reported as a successful strategy in management of these nematodes (Sikora 1992). Treatment of rice seed with PGPR alone or in combination with chitin and neem cake has reduced the root and soil population of the rice-root nematode, *Hirschmanniella oryzae* (Swarnakumari et al. 1999). The level of infestation of root-knot nematode *M. incognita* on tomato was reduced with fewer galls and egg masses in the soil, following root dipping with *P. fluorescens* strain Pf1 (Santhi and Sivakumar 1995). Similarly, application of the bacterium, *P. chitinolytica,* reduced the root-knot nematode infection in tomato crop (Spiegel et al. 1991). Recently, Kumar et al. (2009) discovered rhizosphere competent *Pseudomonas aeruginosa* in the management of *Heterodera cajani* on sesame.

Nematodes influence the colonization of roots by pathogenic and beneficial organisms, but little is known on the interactions with their natural antagonists in the rhizosphere (Kerry 2000). Based on phylogenetic studies, it was proposed that the origin of parasitism in the root-knot nematode *Meloidogyne* spp. may have been facilitated through horizontal gene transfer from soil bacteria. Root-knot nematodes and rhizobacteria occupy similar niches in the soil and roots, suggesting the possibility for genetic exchange (Bird et al. 2003).

Nonparasitic nematodes can also play an important role in the colonization of the rhizosphere by PGPR in the absence of percolating water. Three species of nematodes (*Caenorhabditis elegans*, *Acrobeloides thornei,* and *Cruznema* sp.) promote rhizosphere colonization of four strains of beneficial bacteria in sand-based microcosm system. Nematodes considered as important vectors for bacterial rhizosphere colonization (Knox et al. 2003). Soil application of *P. fluorescens* in tomato reduces the incidence of wilt disease (Sankari Meena et al. 2011; Kavitha et al. 2011).

11.2.5 Interaction Between Plants and Rhizobacteria

Plant roots offer a niche for the proliferation of soil bacteria that thrive on root exudates and cell lysates. Population densities of bacteria in the rhizosphere may be up to 100-fold higher than the bulk soil, and up to 15 % of the root surface may be covered by microcolonies of a variety of bacterial strains. While these bacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can act as signaling compounds that are perceived by neighboring cells within the same microcolony by cells of other bacteria that are present in the rhizosphere or by root cells of the host plant (Gray and Smith 2005). Plant species determined the population of rhizobacterial communities (Marschner et al. 2004) by the differences in the composition of root exudates. Root exudates offer carbon-rich nutrients to the rhizosphere microorganisms, viz., organic acids such as citrate, malate, succinate, pyruvate, fumarate, oxalate, and acetate and sugars such as glucose, xylose, fructose, maltose, sucrose, galactose, and ribose, which constitute the main source, whereas variable amounts of amino acids, nucleobases, and vitamins such as thiamine and biotin provide for the entry or dessert (Lugtenberg and Bloemberg 2004).

A root glycoprotein complex known as agglutinin involved in the short-term adherence of pseudomonads (Glandorf et al. 1994). After the movement and attachment of the biocontrol pseudomonads to the root zone, they will form microcolonies in the grooves between epidermal cells within few days, and other bacteria can reach the same site and intermingle with already existing microcolonies (Haas and Defago 2005). It is proposed that a root glycoprotein complex known as agglutinin involved in the short-term adherence of pseudomonads (Glandorf et al. 1994). So far, no genetic trait has been identified in biocontrol pseudomonads that would point to a mechanism allowing the bacteria to recognize specific plant surface receptors or to

interact with specific plant signals (Bais et al. 2004). Once biocontrol pseudomonads have moved and attached to a root zone, microcolonies form in a few days. Some biocontrol pseudomonads penetrate into intercellular spaces in the epidermis and cortex through damaged root cells (Troxler et al. 1997).

Strains of *B. subtilis* are beneficial rhizobacteria, feeding on plant exudates at the root surface and producing the lipopeptides as secondary metabolites detrimental to the plant pathogens. Lipopeptides surfactin and iturin A, two of the antibiotics produced by *B. subtilis,* enable the bacterium to minimize competition by other microbes for their food source and form stable, filmlike colonies on the root surface (Bais et al. 2004). Bais et al. (2004) found that lipopeptide production and biocontrol activity are directly related to the ability of *B. subtilis* to form stable biofilms on plant roots. Murali Gopal et al. (2005) observed presence of more number of silicate-solubilizing bacteria in the rhizosphere of field-tolerant coconut palms which could promote more silica uptake, thus enhancing tensile strength to the leaves to resist vector feeding.

11.2.5.1 Proteomics of Plant–PGPR Interaction for Pest Resistance

The development of biocontrol strategies involving a mixture of microbials is an emerging area in crop protection to reduce the damage caused by plant pests and pathogens in economically important crops. Reports are available on the induction of systemic resistance against insect pests by selected strains of PGPR in a number of crop plants (van Peer et al. 1991). Our earlier studies demonstrated that application of PGPR affected the preference of leaf-folder larva on rice leaves. In addition, application of PGPR had significant effect on growth and development of leaf-folder larva, and application of PGPR led to malformation of larva, pupae, and adults of the insects which delayed the population buildup (Saravanakumar et al. 2007b). Duffey and Stout (1996) showed that the application of PGPR had antibiotic effects on the larva which resulted in reduced larval weight and increased mortality. Evidences are also there for the induced antixenotic effects of PGPR which resulted in reduced feeding by certain insects on induced plants (Cooper and Goggin 2005). In a study using *Pseudomonas maltophilia*, it was reported that this rhizobacteria was found to affect the growth of *Helicoverpa zea* (Boddie), the corn earworm, to the extent of 60 %. Enhancement of predators and parasitoids population against insect pests using PGPR strains has attained significance in biological control of plant pests (Monnerat et al. 2007). Recently, Baskaran et al. (2004) reported the potential ability of spiders, *A. pulchella* and *Oxyopes* sp., against insect pests. From the above findings, it is assumed in the present study that greater occurrence of natural enemy population could be involved in the reduction of leaf-folder damage in rice plants treated with combination of bioagents.

In addition to this, the most important enzyme that involved in the synthesis of volatile compounds against insect pests is LOX (Vieira et al. 2001). The dioxygenation of polyunsaturated fatty acid by LOX in response to insects leads

to the formation of highly reactive LOX products (HPODE: hydroperoxy octadecatrienoic acid, HPOTE: hydroperoxy octadecadienoic acid). These are subsequently transformed into jasmonates that are involved in signaling events and regulate plant defense gene expression and synthesize hydroperoxide lyase products which behave as volatile phytoalexins (Thaler et al. 2001). The induction of jasmonic acid (JA) enhanced the predation rates by triggering the release of airborne volatiles that attract the natural enemies of insect herbivores. The tomato plants treated with JA have been shown to reduce the incidence of naturally occurring aphid infestations (Thaler 1999). Volatiles also have an indirect role in defense by attracting parasitoids of the foreign pest or repelling females and thus reducing oviposition. In addition to plant volatiles released during the day that serve as a chemical beacon for host location by parasitoids, a second blend of nocturnal volatiles repels females and deters oviposition (Kessler and Baldwin 2001).

The plant transcripts are modulated based on the application of biocontrol bacteria or the challenge inoculation of leaf-folder insect, which may serve as basis to elucidate the interaction between plant–microbe and herbivore. The transcript-profiling approach through DD-RT PCR revealed the molecular responses of the rice plant to PGPR and leaf folder. Upon treatment with biocontrol bacteria and inoculation with leaf-folder insect, a total of 165 transcripts were found to be differentially expressed in leaf sheaths of rice. Beneficial bacterial application shared more number of differential expressions compared to treatment with insect herbivore alone. Further sequence analysis of these differentially expressed cDNAs revealed that more than 90 % of the ESTs were predicted to have known functions. They belong to various functional categories, viz., protein metabolism, DNA/RNA metabolism, secondary metabolism, response to stress, defense response, resistance, etc. It was evident from this study that application of biocontrol bacteria is highly influencing the transcriptional reprogramming when the plant is facing the challenge from the plant–pathogen (Sarosh et al. 2009) and plant–pest (Park et al. 2007) interactions.

Damage caused by leaf folder is characterized by the presence of a large number of folds in the leaves. The larvae prior to feeding fold the leaves longitudinally and feed by scraping the green mesophyll tissue inside the fold. This leads to reduction in the vigor and photosynthetic ability of infested rice plant which ultimately results in yield loss. It has been observed that a 10 % increase in damage to the leaves reduces the yield by 0.15 g per tiller (Fraenkel et al. 1981). These adverse effects may be due to the effect of leaf folder on the expression of important genes involved in growth/metabolism of rice plant, viz., inositol-1-monophosphatase, phosphoenolpyruvate carboxylase 1, aspartic proteinase, protein translation factor SUI1, autophagy-related protein, zinc finger protein, and ankyrin-like protein, as revealed from this study. Upon biocontrol bacteria treatment, rice plants tried to defend themselves by overexpressing some defense-related genes and genes related to metabolism and signal transduction.

Signal transduction is an important molecular response which plays a major role in protecting the plants by modulating the stress-responsive gene expression. These signals may be transmitted to neighboring cells/plants by emitting volatile organic

compounds (Bruinsma et al. 2008). Emission of organic compounds from the PGPR applied plants selectively attract carnivorous insects (natural enemies) which help in reducing the pest incidence in the field conditions (Saravanakumar et al. 2008). PGPR treatment resulted in the overexpression of signaling-related transcripts like jasmonate *O*-methyltransferase, zinc finger protein CONSTANS-LIKE 2, 1-aminocyclopropane-1-carboxylate oxidase 1, and ubiquitin-protein ligase.

PGPR-mediated overexpression of zinc finger protein, protease 4, ubiquitin-protein ligase, and protein kinase is in line with earlier report on resistant barley–stem rust interactions (Zhang 2007). Moran and Thompson (2001) studied the molecular mechanisms underlying host plant resistance against herbivores mainly by examining interaction between the hosts and chewing insects. Extent of tissue damage caused by the chewing insects activated the jasmonic acid (JA)-mediated wound-signaling pathway. Aspartic proteinase has been shown to be involved in various functions like DNA binding, cellular process, hydrolase activity, and some metabolic processes (Athauda et al. 2004). Thiol protease SEN102 precursor is supposed to be involved in putative response to stress; ubiquitin-protein ligase is reported to be involved in intracellular, nucleus, signal transduction, cellular process, and in protein metabolic process. Peroxidases catalyze oxidation–reduction reactions.

Octadecanoid pathway plays major role in plants to defend against insect pests. Comparative Arabidopsis-herbivory transcriptome meta-analysis revealed the expression of most of the functional genes in the octadecanoid pathway. The role of octadecanoids in mediating herbivore-induced responses is well established, and it has been estimated that up to 80 % of all herbivore-induced Arabidopsis genes are octadecanoid regulated (Reymond et al. 2004). Most of our pest response genes, like aspartic proteinase, protein kinase, TPR domain-containing protein, glucan endo-1,3-beta-glucosidase 3, etc., were reported to be present in the octadecanoid pathway.

PGPR priming was found to induce the transcripts like jasmonate *O*-methyl transferase, ubiquitin-protein ligase (Stone et al. 2005), protease, peroxidase 66 precursor, farnesyl pyrophosphate synthetase, inositol-1-monophosphatase, and thiol protease which were involved in the signal transduction and ISR activities and other energy metabolism-related activities. Beyond this the PGPR priming overexpressed the transcript 1-aminocyclopropane-1-carboxylate oxidase 1 (Mirica and Klinman 2008) which is involved in growth regulation, metabolism, signal transduction, and modulating ethylene biosynthetic pathways.

Application of growth-promoting biocontrol bacteria is not involved in the disruption of normal defensive capacity of plant system. The overexpression of some of the defensive genes, viz., beta-glucosidase homolog precursor, kinesin motor domain-containing protein, 3-*N*-debenzoyl-2-deoxytaxol *N*-benzoyl-transferase, pathogenesis-related protein-PRB1-3 precursor, 2Fe-2S ferredoxin, glucan endo-1,3-beta-glucosidase 3 precursor, oxidoreductase, FAD binding, CIPK-like protein 1, TPR domain-containing protein, NB-ARC domain-containing protein, and 4Fe–4S ferredoxin, was noticed in all the treatments including the treatment that received the pest challenge alone. Likewise the transcript

downregulation (zinc finger A20 and AN1domain-containing protein, golgi transport 1 protein B, phosphatidylinositol-4-phosphate 5-kinase, family protein, phosphoenolpyruvate carboxylase1, sialin, conserved hypothetical protein, farnesyl pyrophosphate synthetase, and phosphatidylinositol-4-phosphate 5-kinase family protein) was also noticed in all the above-said treatment conditions (Saveetha 2009).

11.2.5.2 Proteomics of Plant–Rhizobacterial Interaction for Plant Diseases

In the context of plant pathology, DD-RT-PCR was first used to isolate both plant and pathogen genes specifically expressed in the interaction between tomato and the fungal pathogen *Botrytis cinerea*. More recently, it has allowed the isolation of genes upregulated during the pathogenic interactions between plant and viruses, plant and bacterium, and plant and nematode and during the symbiotic associations between plant arbuscular mycorrhiza and plant-growth-promoting rhizobacteria (PGPR) (Timmusk et al. 1999).

Proteomics usually pertains to three broad categories: identification and quantification of all the proteins, study of protein–protein interactions that affect the various complex pathways and networks, and structural characterization. Identifying the proteins helps in getting a complete picture of the proteome under study. Protein interaction study helps define the function of a specific protein in relation to the other proteins that together form the pieces in the network of pathways that control cellular processes. Finally, structural characterization gives a clue to the active sites in the protein, thus elucidating its functional attributes, in addition to providing valuable information. Each of these endeavors serves different purposes, though ultimately when the different units are pieced together, it serves the common goal of a better understanding of cellular processes.

Model systems have been employed to unravel mechanisms of plant-inducible biotic and abiotic stress tolerance and resistance activated by PGPR. Proteomics has been widely used in the field of understanding stress responses as well as in understanding constitutive differences between developmental stages or genotypes. First it provides the broad overview of proteins produced by both the partners. Second it allows the detection of signal transduction pathways after phosphorylation of protein, which decides protein function. The identified proteins are separated by their isoelectric point (pI) in the first dimension of electrophoresis and then separated by their molecular weight in the second dimension using SDS-PAGE. Using two-dimensional electrophoresis (2-DE), approximately 2,000 proteins can theoretically be separated and displayed on a single gel (O'farrell 1975). Recently, Shoresh and Harman (2008) characterized *Trichoderma harzianum* and maize interactive proteins and reported the positive changes in maize metabolic pathways induced by *T. harizianum*. Further characterizations of plant–microorganism interactions might use parallel analyses of interaction proteome.

Many rhizobacteria have been reported to stimulate plant growth under different conditions. Seed treatment with fluorescent pseudomonads increased plant-growth

promotion in tomato and hot pepper (Saravanakumar et al. 2007a, 2009). Overexpression of RuBisCO was observed in *P. fluorescens* KH-1-treated rice samples. It was reported that RuBisCO plays a significant role in photosynthesis of pathogen-infected plant cells (Agrios 2005). Similarly, during rice-sheath blight–pathogen interaction, the upregulated twenty-five percent of the differential proteins were reported as RuBisCO (Lee 2005). Thus, it is assumed that overexpression of RuBisCO strongly increase the photosynthetic activity in order to attain greater growth and possible link with plant defense. The another interactive protein chaperones are known as stress-related proteins that bind particularly to denatured proteins to prevent degradation and to assist in protein refolding of ATP (Rochester et al. 1986). In eubacteria and eukaryotic organelles, chaperonin 60 is presumably involved in numerous enzyme-folding functions (Lorimer 1996). In plant chloroplasts, the level of chaperonin 60, being involved in assembly of RuBisCO holoenzyme, is normally coordinate with RuBisCO (Avni et al. 1989). However, Holland et al. (1998) reported that the accumulation of chaperonin 60 in *N. tabacum* seedlings against salt, cold, and prolonged darkness while the RuBisCO large subunit was decreased. This negative correlation suggests the possible role of chaperonin 60 in stress responses. This protein binds hsp90 and participates in the folding of a number of cell regulatory proteins. The amino-terminal domain (N domain) of Hsp90 represents the ATP binding site and is important for interaction with its cochaperone, p23 (Zhu and Tytgat 2004). The differential expression in our study indicates the involvement of co-chaperones in the assembly of RuBisCO which is an important enzyme in chloroplast metabolism and photosynthesis.

The priming of rice with *P. fluorescens* KH-1 exhibited the overexpression of nucleoside diphosphate kinases (NDKs) which catalyze the exchange of phosphate groups between different nucleoside diphosphates (Cho et al. 2004; Saveetha et al. 2009). NDK activities maintain equilibrium between the concentrations of different nucleoside triphosphates. The expression of plant NDPKs in response to wounding (Harris et al. 1994), heat shock (Moisyadi et al. 1994), phytochrome B (Choi et al. 1999), UV-B light (Zimmermann et al. 1999), oxidative stress (Moon et al. 2003), and hormones (Novikova et al. 1999) has been reported by several research groups. These studies suggest that NDKs overexpression in the current study might play regulatory roles in addition to their primary metabolic function.

Moreover, the rice plants treated with strain KH-1 differentially expressed proteasome subunit alpha type-4-2 protein. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. These are known to be involved in the degradation of proteins modified by oxidation (Grune et al. 1995). In mammalian cells, the proteasome subunit proteins have been shown to recognize and selectively degrade oxidatively damaged proteins, such as hydrogen peroxide-modified hemoglobin (Giulivi et al. 1994). Amino acids of proteins can be modified by oxygen radicals or other activated oxygen that are produced as by-products of cellular metabolism or against abiotic and biotic stresses. Subsequently, oxidatively modified proteins can undergo

chemical fragmentation or form aggregates due to covalent cross-linking reactions and increased surface hydrophobicity (Dean et al. 1997). Thus, the expression of proteasome subunit proteins in the current study might involve in the cell cycle, the regulation of gene expression, and responses to oxidative stress as previously reported by Peters et al. (1994).

Similarly, the expression of GST is known to be involved in tagging toxic endogenous substrates with GSH conjugation to transport toxic substrates into the vacuole through a glutathione pump (Peters et al. 1994). GST has numerous roles in cellular processes with a common function, namely, the recognition and transport of a broad spectrum of reactive electrophilic compounds from both exogenous and endogenous origins (Ishikawa 1992). Many plant GST genes were reported to be auxin inducible where GTS binds auxin at the noncatalytic site or catalytic site, depending on different auxins, suggesting that GTS plays different roles in auxin function. GST has an important role in plant defense from oxidative damages caused by various biotic or abiotic stresses such as heavy metal, wounding, ethylene, ozone, and pathogen attack (Marrs 1996). From the known roles of GST, it is postulated that overexpression of GSTs in this study might have an essential role in the ISR by priming rice plants and protecting cells from oxidative damage.

In addition, the priming of rice plants with *P. fluorescens* KH-1 differentially expressed the thioredoxin proteins. The expression of *Arabidopsis* thioredoxin AtTRX3 in the *Saccharomyces* strain EMY63 has the ability to grow on methionine sulfoxide and H_2O_2 tolerance. The presence of this gene has been reported in most of the eukaryotic organisms that contain chlorophyll. This in turn implies the role of this enzyme in photosynthesis (Verdoucq et al. 1999). According to the literatures, the presumed functions of the identified proteins are related to antifungal activity, energy metabolism, photosynthesis, protein degradation, and antioxidation. This strongly implies the role of *P. fluorescens* KH-1 in various pathways including energy metabolism and plant defense. In addition to the current efforts, further studies using transcriptomics and proteomics on rice–*Pseudomonas* interactions will allow to manipulate the PGPR-based crop health and yield response in rice through genetic engineering.

In the case of *P. fluorescens* root colonization in *Arabidopsis*, disease suppression against *P. syringae* DC3000 is observed with up-(95) and down-(105) regulation for transcripts encoding metabolism, signal transduction, and stress response proproteins (Wang et al. 2005). With PGPR-induced salt tolerance, the sodium uptake transporter HKT1 has been shown to be organ specifically regulated in *Arabidopsis* by *B. subtilis* resulting in reduced endogenous sodium when plants are grown with elevated salt (Zhang et al. 2008). For growth promotion, PGPR have been linked with auxin redistribution from leaves to roots that in turn is associated with foliar cell expansion and lateral root proliferation in *Arabidopsis* (Xie et al. 2009).

PGPR was effective when plants were inoculated 9 days after planting that is also the incubation time necessary for chemical elicitation with benzothiadiazole, an activator of SAR (Jabaji-Hare and Neate 2005). While UFLA285 does produce

metabolites with antibiotic activity against AG4 (data not shown) and the time necessary for the onset of disease control is sufficient for UFLA285 to cause direct disease suppression, the upregulation of jasmonate and ethylene-related transcripts suggests that UFLA285 may also participate in induced systemic resistance (ISR).

UFLA285-induced transcripts encoding phenylpropanoid metabolism included phenylalanine ammonia lyase, 2-hydroxyisoflavone reductase, dihydroflavonol-4-reductase, caffeic acid O-methyltransferase, and cinnamoyl CoA reductase, which catalyze the production of undifferentiated phenylpropanoids, phytoalexins, catechins/anthocyanins, and phenolics/lignin, respectively (Zabala et al. 2006). The oxidation products of catechins and tannins are the main polyphenols in cotton and are produced in high amounts in response to *R. solani* infection. Since tannins can inhibit fungal polygalacturonases, responsible for the tissue maceration (Kirkpatrick and Rothrock 2001), overexpression of tannin synthesis may well serve in plant defense. Lignin, also a product of the phenylpropanoid pathway, can provide a physical barrier against additional microbial colonization (Vorwerk et al. 2004). Moreover other cell wall reinforcement strategies including downregulation of transcripts that participate in cell wall loosening (Zhang et al. 2007) and upregulation of callose synthase genes coding for callose deposition have been associated with the thwarting further fungal colonization. A downregulation of xyloglucan endoglycosyl transferase aids in reducing natural openings for fungal invasion (Zhang et al. 2007). Cotton defense responses induced with a combination of UFLA285 cottonseed treatment and AG4 infection including pathogenesis-related genes and genes encoding lignin biosynthesis have been previously observed when cotton hypocotyls were infected with *Fusarium oxysporum* f. sp. *vasinfectum*. The repression of drought-responsive proteins such as aquaporins has also been observed previously (Dowd et al. 2004) which may be specific to vascular wilt diseases.

Necrotrophic infections such as produced by *R. solani* as well as drought can generate reactive oxygen species (ROS) that are in turn scavenged by plant peroxidases and glutathione S-transferases. Other detoxifying strategies may also be operative in plant–pathogen interactions. To either eliminate the toxin from inside the cell or degrade such metabolites to nontoxic components, multidrug and toxin extrusion (MATE) transport proteins and cytochrome P450/endo-beta-*N*-acetylglucosaminidase are induced in host plant tissue (Ralston et al. 2001), as observed with UFLA285 treatment (Flavio et al. 2011).

Although *R. solani* has not been reported as being a xylem colonizer (Kirkpatrick and Rothrock 2001) from the initial infection, cushions form on the stem epidermis, the mycelium reach and damage the tracheary elements reducing the water conductivity (data not presented), and thus, with normal water transpiration, a negative water balance occurs leading irreversible plant wilting. On treated plants, in spite of the brownish necrotic lesions with no wilting, symptoms were observed possibly due to a lower extent of pathogen internal tissue colonization due to many possible factors including cell wall reinforcement (callose and lignin deposition), as shown for the binucleate *Rhizoctonia*-mediated biological control of damping-off.

11.2.6 Interaction of Defense Pathways in Response to Pathogen and Pest Attack

The number of insect species is estimated to be in the order of six million, 50 % of which are herbivorous (Schoonhoven et al. 1998). The biodiversity of pathogenic microorganisms is less well characterized, but generally, plant pathogens are a common threat to plants. To effectively combat invasion by microbial pathogens and herbivorous insects, plants have evolved sophisticated defensive strategies to perceive attack by pathogens and insects and to translate this perception into an appropriate defensive response (Pieterse and van Loon 2004). These induced defense responses are regulated by a network of interconnecting signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles (Dicke and Van Poecke 2002). SA, JA, and ET accumulate in response to pathogen infection or damage caused by insect feeding, resulting in the activation of distinct sets of defense-related genes (Reymond et al. 2004). Evidence for the significance of SA, JA, and ET in plant defense came from studies using mutant and transgenic plants affected in either SA, JA, or ET signaling (Pozo et al. 2005). For instance, SA-defective-signaling mutants and transgenics are often more susceptible to pathogen infection than wild-type plants (Sekar and Kandavel 2010). Blocking the response to JA generally renders plants more susceptible to herbivorous insects (Kessler et al. 2004). This also enhanced susceptibility of plants towards necrotrophic pathogen infection (Thomma et al. 1998). Furthermore, analysis of mutants affected in ET signaling demonstrated that ET plays a modulating role in many plant defense responses (Hoffman et al. 1999).

Although the importance of SA, JA, and ET in induced plant defense is clear, evidence is accumulating that their signaling pathways cross-communicate (Kunkel and Brooks 2002). The interaction among SA, JA, and ET defense-signaling pathways can be antagonistic, cooperative, or synergistic, depending on the plant species, the combination of organisms attacking the plant and the developmental, and physiological state of the plant (Rojo et al. 2003). For instance, activation of SA-dependent systemic acquired resistance (SAR) has been shown to suppress JA signaling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defense which is more effective against insect herbivory (Thaler et al. 2002). Pharmacological and genetic experiments have indicated that SA-mediated suppression of JA-inducible gene expression plays an important role in this process and sometimes can work in both directions (Glazebrook et al. 2003). The antagonistic effect of SA on JA signaling recently was shown to be controlled by a novel function of the defense regulatory protein NPR1 in the cytosol (Pieterse and van Loon 2004). Cross talk between defense-signaling pathways is thought to provide the plant with a powerful regulatory potential, which helps the plant to decide which defensive strategy to follow, depending on the type of attacker it is encountering. De Vos et al. (2005) demonstrated the dynamics of SA, JA, and ET signaling in a single plant species of *A. thaliana* in response to attack by a range of microbial pathogens and

herbivorous insects with very different modes of action. Surprisingly complex set of transcriptional alterations are induced in all cases showing stress-related genes, and they are overlapped in response to different modes of attack by pathogens and insects.

Plant defense system involves major two regulatory pathways: (1) SAR which is SA dependant can be activated through pathogen infestation and involves the novel function of NPR-1 gene present in the cytosol. (2) ISR which is JA/ET dependant can be activated through insect herbivory and thus involves the function of ubiquitin-ligase complex. Transcription family factor WRKY was shown to act as both an activator of SA responsive genes and a repressor of JA-inducible gene, thereby possible integration of signals from these antagonistic pathways. In addition, the transcription factors ERF1 and MYC2 were found to integrate signals from JA and ET in activating defense-related genes which are response to both the pathways. Cross talk between defense-signaling pathways is thought to provide the plant with a powerful regulatory potential, which helps the plants to decide on the most appropriate defensive strategy, depending on the type of attacker it is encountering. In fact, it was found that PGPR-mediated simultaneous expression of both ubiquitin-ligase complex protein and WRKY transcription factor family protein, implying the positive cross talk between all the three regulatory pathways, thus help plants to choose most appropriate defensive strategy.

11.2.7 PGPR in Sustainable Agriculture

Sustainable agriculture needs IPM, and part of the IPM approaches is the use of nonchemical control methods. The nonchemical methods include use of biocontrol microbes, predators and parasites, and plant products especially neem-derived products. Dissemination and adoption of eco-friendly approaches of pest management reached only a smaller section of farmers, and hence, future extension efforts should be concentrated to reach larger section of farmers.

Plant pest and disease management includes use of beneficial microorganisms for the effective and sustained production of agricultural and horticultural crops. Numerous species of soil bacteria and fungi which flourish in the rhizosphere of plants stimulate plant growth both directly and indirectly. Plant-growth-promoting rhizobacteria (PGPR), viz., *Pseudomonas*, *Bacillus*, and fungal antagonist *Trichoderma*, have been well exploited for the management of plant diseases in economically important agricultural and horticultural crops. Environmental factors, soil type, and amendment of organic and inorganic substances influence the survival and potentiality of the microbial agents used for the management of plant diseases. Hence, the identification, selection, and conservation of biocontrol agents itself fulfills the strategy for the enhanced management of plant diseases besides producing quality products. The mode of action includes increasing the availability of nutrients in the rhizosphere, positively influencing plant growth and inhibiting the soilborne and foliar pathogens. Thus, selection of effective biocontrol agents with

desired traits of (1) enhancing plant-growth promotion, (2) production of antibiotics and lytic enzymes, (3) rhizosphere colonization and endophytic nature, and (4) induction of systemic resistance in crop plants by activating defense enzymes, viz., chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase, and phenolics, will provide support for sustained crop production for a long term. In addition, commercial production of bioagents with the suitable carrier material either in single or combination, with or without chitin amendment and method of application, plays a major role in managing plant diseases. The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biopesticides.

Further research has to be oriented towards the identification of good and cheap substrate/carrier materials for mass production of biocontrol bacteria, viz., *Bacillus thuringiensis*, fluorescent pseudomonads, and *B. subtilis*, and biocontrol fungi, viz., *Trichoderma* spp. *Metarhizium* and *Beauveria* spp. Also research has to be focused on delivery system and identification of efficient biocontrol strains.

Molecular analyses helped to identify some of the important traits of biocontrol agents associated with suppression of important pests and diseases in crops. Certainly such types of molecular studies in future would help to develop superior biopesticide formulation which is going to become one of the major components in IPM system.

In the development of genetically modified microbial biocontrol agents, the main focus is the creation of new organisms that possess a functional advantage over unmodified ones. This implies that genetically modified microbes should have a fitness advantage within the target environment into which they are released. The experience gathered to date indicates that adverse nontarget effects, if any, are likely to be short-term or transitory events that can, if hazardous, be eliminated by suspending the use of genetically modified organisms. Several genes which are responsible for biocontrol efficacy in biocontrol bacteria or biocontrol fungi have been cloned and characterized. Few genes, viz., *Bt* from *B. thuringiensis* and endochitinase from *Trichoderma* spp., have been introduced into plant genome and transgenic crops have been developed.

11.3 Concluding Remarks and Future Trends

The use of PGPR inoculants to improve agricultural production has been demonstrated in numerous studies, and the basic mechanisms are now well understood. PGPR, in accordance with their mode of action, can be classified as biofertilizers, phytostimulators, and biopesticides, with certain bacteria having overlapping applications. It is becoming increasingly apparent that most PGPR can promote plant growth by several mechanisms, but most studies currently focus on individual mechanisms and have not been able yet to sort out the relative contributions of different processes that are responsible for plant-growth promotion. Screening strategies for selecting the best strains will require more

comprehensive knowledge of the traits required for rhizosphere competence and studies on the ecology of introduced PGPR with the resident PGPR and other microbial species in the plant rhizosphere. While inoculation is now viewed as a means to enhance plant growth, the effects of various management practices or soil amendments on PGPR activity of indigenous bacteria remain unknown (Martínez-Viveros et al. 2010).

Research into the mechanisms of plant-growth promotion by PGPB has provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents. Still, most of the focus has been on free-living rhizobacterial strains, especially to *Pseudomonas* and *Bacillus*. Much remains to be learned from nonsymbiotic endophytic bacteria that have unique associations and apparently a more pronounced growth-enhancing effect on host plants (Ping and Boland 2004). There is overwhelming evidence in the literature indicating that PGPR can be a true success story in sustainable agriculture. In fact, through their numerous direct or indirect mechanisms of action, PGPR can allow significant reduction in the use of pesticides and chemical fertilizers. These beneficial events producing biological control of diseases and pests, plant-growth promotion, and increases in crops yield and quality improvement can take place simultaneously or sequentially. Plant age and the soil chemical, physical, and biological properties will greatly influence the outcome of PGPR inoculation. Presently, the absence of a universal magic PGPR bioinoculant formulation for each important field crop simply reflects the complexity of the interactions and of the molecular signal exchanges taking place in the soil–plant–organisms ecosystems.

The use of PGPR inoculants in agriculture is already proceeding and offers many opportunities to improve plant nutrition, crop yields, and disease management while improving sustainability by reducing the need for chemical inputs. Nevertheless, as our understanding of the ecology of these bacteria improves, it should be possible to obtain a more informed explanation of the mechanisms that are involved in plant-growth promotion and identify situations in which bioaugmentation with soil inoculants may be useful for increasing crop yields.

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Chapter 12
Bio (Bacterial) Control of Pre- and Postharvest Diseases of Root and Tuber Crops

Ramesh C. Ray and Manas R. Swain

12.1 Introduction

There are approximately 400 vegetable crops (including root and tuber crops) that are commercially cultivated worldwide (Kays and Silva Dias 1996). The term "root and tuber crops" is a very general "catch-all" for a wide cross section of subterranean storage organs of which there are approximately 38 root, 23 tuber, 14 rhizome, 11 corm, and 10 bulb crops. Crops with an enlarged pseudostem or stem (e.g., leek, kohlrabi), even when subterranean, are generally not considered within the root and tuber crop category. Likewise, each of the crops included are commercially cultivated and marketed, though in some instances the volume is not great; species that are gathered from the wild are not included. All of the crops are utilized as food though in diverse ways: e.g., as staples, vegetables, sources of industrial products and condiments. Root and tuber crops are divided, for convenience, into temperate (i.e., potato (*Solanum tuberosum* L.), sugar beet (*Beta vulgaris* L.), onion (*Allium cepa* L.) and garlic (*Allium sativum* L.), etc.) and tropical (i.e., cassava (*Manihot esculenta* Crantz), sweet potato (*Ipomoea batatas* L.), yams (*Dioscorea* spp.), and edible aroids (*Colocasia esculenta* L.) Schott. and *Xanthosoma* spp.), based on the climate in which they are cultivated. Global productions of some of the important root and tuber crops are given in Table 12.1 (FAO 2003).

Pre- and postharvest losses of these crops are very high and, depending on the species cultivated and the storage environment, may be of the order of 30–60% during the course of 3–6 months (Proctor et al. 1981). The principle causes of loss include (1) weight loss due to desiccation, (2) loss of carbohydrate and water due to

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Table 12.1 Production statistics for selected root and tuber crops in 2002

Crop	Production (Metric tones/year)
Beet (sugar)	246,475,609
Carrot	21,020,436
Cassava	184,852,540
Chicory roots	960,700
Garlic	12,107,007
Ginger	988,182
Onion (dry)	51,914,247
Potato	307,440,446
Sweet potato	136,130,396
Taro	9,220,522
Yam	39,643,170

Source: FAO (2003)

respiration, (3) sprouting on breakage of dormancy, (4) losses due to rodents and insects, and (5) fungal, bacterial, and viral diseases. Biological control involving microorganisms is currently used to prevent various diseases, particularly fungal and to some extent bacterial diseases on seed potatoes, sugar beet, yams, and aroids (Lebot 2008).

This chapter focuses on the progress made in the recent years on the spectrum of bacteria used as antagonists for control of pre- and postharvest diseases of these crops, their modes of action, and methods to enhance biocontrol efficacy of the antagonists.

12.2 Microbial Control Strategies

There are two basic approaches for using microbial antagonists for controlling plant diseases:

1. Manipulation of epiphytic and endophytic microflora
2. Those that can be artificially introduced against pathogens.

12.2.1 Manipulation of Epiphytic and Endophytic Microflora

Identification and selection of effective antagonistic bacteria is the first and foremost step in biological control. Antagonistic bacteria are mostly searched among endophytic and epiphytic microflora of cultivated plants. However, the implication of endophytic bacteria in biological control has received less attention as compared to epiphyte bacteria (Helbig 2006). Few representative examples are cited below to reinforce this view.

Rhizoctonia solani causes stem, stolon canker and black scurf on potato tubers, reducing plant health, yield quality and quantity (Rauf et al. 2007). Lahlali et al. (2007) reported that endophyte and non-endophyte bacterial isolates from healthy potato plants and rhizosphere for their antagonistic activity against *R. solani*. Among a total of 220 bacterial isolates, only 25 showed a highly significant inhibition rate against *R. solani* in in vitro dual culture assays.

Pseudomonas putida, originally isolated from the tuber surface (geocaulosphere) of potato showed in vitro antibacterial activity to the bacterial pathogen, *Erwinia carotovora*. Similarly, the T4 lysozyme sensitive *Serratia grimesii* isolated from the rhizosphere of parental potatoes showed in vitro antagonism toward *Verticillium dahliae*. Both introduced isolates were able to colonize the rhizo- and geocaulosphere of transgenic plants and non-transgenic parental plants and established in the rhizosphere at 10^5 colony forming units/g fresh weight of root (Lottmann et al. 2000). These strains also significantly decreased Fusarium dry rot of potato at cell concentrations of 10^6 cells/ml (Lottmann et al. 1999). Various antagonistic bacteria were isolated from the phylloplane of onion crops. Among epiphytic microorganisms, *Bacillus amyloliquefaciens* BL-3, *Paenibacillus polymyxa* BL-4, and *Pseudomonas putida* Cha 94 were highly inhibitory to conidial germination of fungi such as *Fusarium oxysporum*, *Aspergillus* sp., and *Botrytis allii*, which were the basal and neck rot causing pathogens for onion during storage (Lee et al. 2001). Likewise, application of *Bacillus subtilis*, isolated from the epiphytic microflora of yam tuber, showed a drastic reduction in the number of spoilage fungi of yams during 5-month storage period. The surface fungi like *Botryodiplodia theobromae*, *Fusarium moniliforme*, and *Penicillium sclerotigenum* were displaced completely on the treated tubers (Okigbo 2002, 2005).

12.2.2 Introduction of Microbial Antagonists

Earliest efforts to control plant diseases involved the introduction of antagonistic bacteria, *Bacillus subtilis* and *Pseudomonas cepacia* and fungi, *Trichoderma* and *Rhodotorula* (Sadfi et al. 2001, 2002; Sharma et al. 2009) in plant–pathogen interactive environment. Since then, several other antagonists (including yeasts) have been identified and used for controlling various plant diseases. Table 12.2 shows exclusively the bacterial control of pre- and postharvest diseases of root and tuber crops.

12.2.2.1 Fungi

Fungi are also used as antagonist in disease control of roots and tubers. Studies showed that *Trichoderma reesei* and *T. viride* significantly reduced the incidence of *Rhizoctonia* symptoms on potato sprouts. *Gliocladium* (*Trichoderma*) *virens*,

Table 12.2 Microbial antagonists used for control of diseases of roots and tuber crops

Antagonists	Disease and pathogen	Crop(s)	Reference(s)
Bacillus licheniformis	Botrytis rot (*Botrytis allii*)	Onion	Lee et al. (2001)
Bacillus amyloliquefaciens	Fusarium rot (*Fusarium oxysporum*)		
Bacillus spp.	Fusarium rot (*Fusarium roseum* var. *sambucinum*)	Potato	Sadfi et al. (2002)
Bacillus subtilis	Sugar beet Cercospora leaf spot (*Cercospora beticola*)	Sugar beet	Collins and Jacobsen (2003)
Bacillus subtilis	Botryodiplodia rot (*Botryodiplodia theobromae*)	Yams	Okigbo (2002), Swain et al. (2008)
Bacillus subtilis	Fusarium rot (*Fusarium moniliforme*)	Yams	Okigbo (2002)
Pantoea agglomerans	Dry rot (*Gibberella pulicans*)	Potato	Schisler et al. (2000)
Pseudomonas fluorescens	Dry rot (*Gibberella pulicans*)	Potato	Schisler et al. (2000)
Pseudomonas sp.	Blue and Green rot (*Penicillium sclerotigenum*)	Yams	Okigbo (2002)
Pseudomonas fluorescens	Soft rot (*Erwinia carotovora* subsp. *atroseptica*)	Potato	Cronin et al. (1997)
Bacillus cereus, *Cellulomonas fimi*, *Kocuria varians*, *Pseudomonas putida*, *Rhodococcus erythropolis*, *Rhodococcus globerulus*	Silver scurf (*Helminthosporium solani*)	Potato	Martinez et al. (2002)
Burkholderia cepacia	Dry rot (*Fusarium sambucinum*, *Fusarium oxysporum* and *Fusarium culmorum*)	Potato	Al-Mughrabi (2010)
Pseudomonas putida	*Erwinia carotovora* ssp. *atroseptica*	Potato	Lottmann et al. (2000)
Pseudomonas fluorescens	*Pythium ultimum*	Sugar beet	Thrane et al. (2000)

Fusarium oxysporum, *F. lateritium*, *Penicillium tritinum*, and *Taralomyces* spp. have good potential for potato cyst nematode biocontrol (Nagtzaam and Bollen 1997; Sharma et al. 2009; Indarti et al. 2010).

From in vitro and in vivo screening tests for antagonism by isolates of *Trichoderma* against postharvest pathogens of yams (*Dioscorea* spp.), an isolate of *Trichoderma viride* was selected as the most promising candidate. Inoculation of

white yam (*Dioscorea rotundata* Poir.) with conidia of *T. viride* and subsequent storage of the tubers under the ambient environmental conditions of a traditional yam barn resulted in drastic reduction in the frequency of occurrence of the normal tuber surface mycoflora (*Aspergillus niger*, *Botryodiplodia theobromae* and *Penicillium oxalicum*) over a 4-month storage period (Okigbo and Ikediugwu 2008).

12.2.2.2 Yeast

Yeasts are mostly used for control of postharvest diseases of fruits, vegetables, roots, and tubers. Tian et al. (2005) have made several positive points in recommending yeasts as potential microbial agents for controlling the postharvest diseases including (a) they can colonize the wound surface for long period even under dry conditions; (b) they produce extracellular polysaccharides, which enhance their survivability and restrict the growth of pathogen propagules; (c) they can use nutrients rapidly and proliferate at a faster rate; and (d) they are the least affected by the pesticides. Of the various yeasts, *Candida sake*, *Candida albidus*, *Candida oleophila*, *Debaryomyces hansenii*, *Pichia anomala*, *Pichia guilliermondii*, *Issatchenkia orientalis*, *Metschnikowia pulcherrima*, *Cryptococcus laurentii*, etc., have exhibited a wide spectrum of biological activity against many postharvest plant pathogens (Sharma et al. 2009).

12.2.2.3 Bacteria

Bacterial flora have attracted enormous attention as agents for biocontrol plant diseases, particularly since they are easy to handle, generally stable, resistant, and ability to survive desiccation and inherently possess a quick generation time (Sharma et al. 2009). They are also known to affect life cycles of different plant pathogens or pests by diverse mechanisms including the production of extracellular metabolites and intracellular proteinaceous toxins. In general, spore-forming bacteria (e.g., *Bacillus* spp.) survive to a greater extent even in harsh environments, compared to the non-spore-forming bacteria. Among the *Bacillus* spp., the ones that have attracted the most attention are *Bacillus thuringiensis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* (Swain et al. 2008; Al-Mughrabi 2010). Other bacterial species of interest are *Pseudomonas fluorescens* (Thrane et al. 2000), *Pseudomonas putida* (Sharma et al. 2009), *Talaromyces flavus* (Nagtzaam and Bollen 1997), *Pantoea agglomerans* (Kim et al. 2006), etc. Bacteria are easily mass produced using a liquid fermentation process, although in some cases they may be more amenable to semi-solid or solid-state fermentation (Swain and Ray 2008).

12.3 The Mode of Action of Bacterial Agents

Biological control by bacteria uses naturally occurring mechanism to suppress plant pathogens. The modes of action are antibiosis, competition for space, nutrients, siderophore-mediated suppression, parasitism, cell-wall lytic enzymes, and induced systemic resistance (Sharma et al. 2009). In general, more than one mechanism is implicated, but in no case was a single mechanism found to be responsible/suitable for biological control.

12.3.1 Antibiosis

Production of antibiotics is the most important mechanism by which the bacterial antagonists suppress the pathogens (Fig. 12.1). Examples of antibiotics are iturins (a powerful antifungal peptide) produced by *Bacillus subtilis*, pyrrolnitril produced by *Pseudomonas cepacia*, trichothecenes produced by *Myrothecium roridium*, etc., (Bull et al. 1998).

Pseudomonas fluorescens DR54, isolated from sugar beet rhizosphere, had shown biocontrol of *Pythium* in planta (Nielsen et al. 1998). This bacterial isolate produced the antifungal cell-associated lipopolypeptide, viscosinamide (Nielsen et al. 1999), which induced physiological changes in *Pythium ultimum* and *Rhizoctonia solani* in vitro and in soil as studied by fluorescent microscopy (Hansen et al. 2000). Viscosinamide was detected in increasing amounts on both seed coats and in rhizosphere soil surrounding the sugar beet roots during 7 days of incubation (Thrane et al. 2000). In another study, *Pseudomonas aeruginosa* PNA1 strongly reduced root rot disease tissue culture derived cocoyam plantlets. Soil experiments involving the strain PNA1 in comparison to phenazine-deficient mutants suggested that the biocontrol activity of PNA1 against *Pythium myriotylum* might involve phenazines. Phenazine involvement was further strengthened by the fact that FM13 fed with exogenous tryptophan (so that phenazine production was restored) reduced disease severity on cocoyam. The efficiency of PNA1 to control *P. myriotylum* on cocoyam was improved when the strain and the pathogen were allowed to interact for 24 h prior to transplanting cocoyam plantlets, while doubling the inoculum density of the pathogen negatively affected its efficiency (Tambong and Hofte 2001).

Recep et al. (2009) showed that *Burkholderia cepacia* OSU-7 has great potential to be used as biocontrol agents for management of *Fusarium* species causing dry rot on potato. *Burkholderia cepacia*, formerly known as *Pseudomonas cepacia* (Yabuuchi et al. 1992), produces one or more antibiotics that are active against a broad range of plant pathogenic fungi (Rosales et al. 1995). Organisms of the *B. cepacia* complex produce inhibitory metabolites such as pyrrolnitrin (Hwang et al. 2002), siderophores (Stephan et al. 1993), cepaciamide A(B), cepacidine A(B), cepacin A(B), and lipopeptides (Kang et al. 1998). These antibiotics appear,

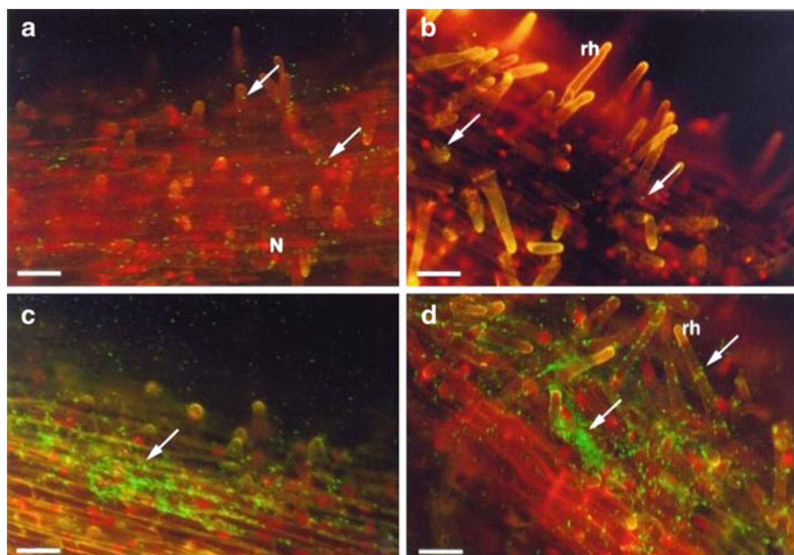

Fig. 12.1 Confocal microscopy image of carrot roots in the region of (**a**) and (**c**), emerging and, (**b**) and (**d**), fully developed root hairs. *Pseudomonas fluorescens* are visible as *small green spots* on the root surface. Plant nuclei are stained in *red*. Differences in the abundance of bacterial cells were observed when the wild-type CHA0 (**a** and **b**) or the mucoid mutant CHA211 (**c** and **d**) were used in the assay. *N* nucleus, *rh* root hair. *Bars* = 30 μm (Hansen et al. 2000)

in many cases, to be important for disease suppression. Compounds such as cepacin A and cepacin B exhibit only antibacterial activity, whereas pyrrolnitrin is effective against fungi, yeasts, and Gram-positive bacteria (Quan et al. 2006).

12.3.2 Siderophore Production

Effect of root-colonizing *Pseudomonas* to enhance crop yields is partly due to siderophore produced by them that make iron in the rhizosphere less available to deleterious fungi and rhizobacteria (Nautiyal et al. 2006; Pandey et al. 2005). Siderophores are low-molecular weight compounds synthesized under iron-deficient conditions by many microorganisms. They chelate Fe^{3+} and the resulting iron complex is transported into the cell through receptor mediation. The siderophores of fluorescent pseudomonads are commonly referred to as pseudobactins or pyroveridines.

12.3.3 Competition for Nutrients

Competition for nutrients is the most promising mode of action for several bacterial agents. Attachment by bacterial antagonist to the pathogen hyphae appears to be an important factor necessary for competition for nutrients as shown by the interactions

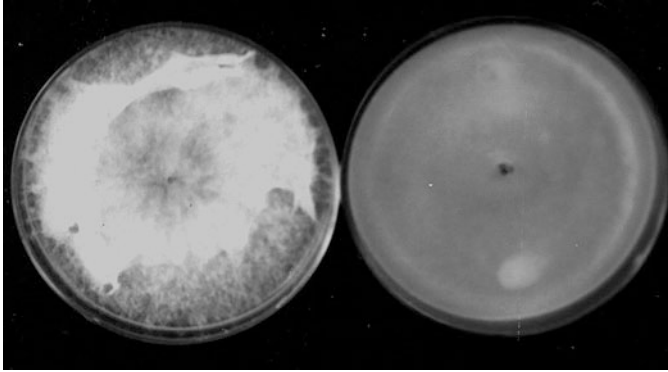

Fig. 12.2 Competition for Space between yam postharvest pathogen *Botryodiplodia theobromae* and biocontrol agent *Bacillus subtilis*. (**a**) control (*B. theobromae*), (**b**) treatment (*B. theobromae* + *B. subtilis*) (Swain and Ray 2009a)

of *Pseudomonas fluorescens* and *Pythium utimum* in sugar beet (Thrane et al. 2000). In vitro studies conducted on such interactions revealed that due to direct attachment, antagonistic bacteria take nutrients more rapidly than target pathogens and thereby prevent spore germination and growth of the pathogens. Nonpathogenic species of *Erwinia*, such as, *E. cypripedii*, showed antagonistic activity against various isolates of *Erwinia caratovora* sub sp. *caratovora*, the causal agent of soft rot of carrot, by competing for nutrients (Sharma et al. 2009).

12.3.4 Competition for Space

Competition for space is the competition for infection sites, which may occur if antagonists are able to occupy the specific places where recognition mechanisms between host and pathogen take place (Fig. 12.2). If these places are no more available for pathogens, the necessary procedure of recognition cannot take place and infection does not occur. Thus, microbial antagonists should have the ability to grow more rapidly than the pathogen (Lübeck et al. 2000).

Lübeck et al. (2000) studied *P. fluorescens* DR54 colonization of the sugar beet rhizosphere by confocal laser scanning microscopy and found that *P. fluorescens* DR54 was the dominating organism a few days after the inoculation. During their 20 days study, active micro-colonies of *P. fluorescens* DR54 could be detected on all parts of the roots. Lottmann et al. (2000) demonstrated that isolates of *P. putida* QC14-3-8 and L16-3-3 were able to colonize potato tuber surface of transgenic as well as that of non-transgenic potatoes, but the culturable population of both inoculants was about one exponential unit lower in the geocaulosphere than in the rhizosphere. This was expected, because the rhizosphere represents a more attractive habitat compared to the tuber surface due to the exudation of nutrients (Lottmann et al. 1999)

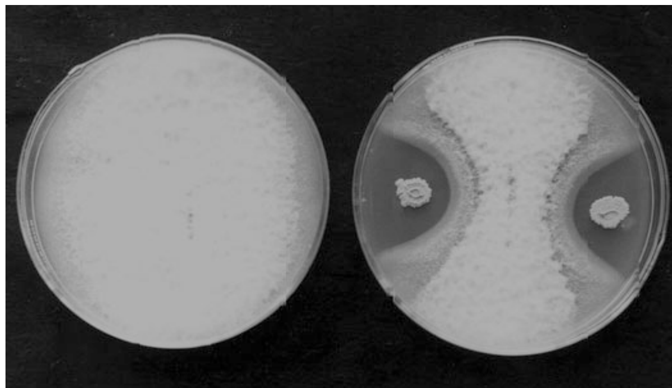

Fig. 12.3 Antibiotic activity between yam postharvest pathogen *Fusarium oxysporum* and bio-control agent *Bacillus subtilis*. (**a**) control (*F. oxysporum*), (**b**) treatment (*B. theobromae* + *B. subtilis*) (Swain and Ray 2009a)

12.3.5 Production of Cell-wall Lytic Enzymes

Microbial antagonists also produce lytic enzymes such as gluconase, chitinase, and proteinases that help in the cell wall degradation of the pathogenic fungi (Chernin and Chet 2002). The interaction between *Bacillus subtilis* and *Fusarium oxysporum*, the postharvest pathogens of yam (*Dioscorea* spp.) tubers, was studied by scanning electron microscopy (Swain et al. 2008). Lysis of fungus cell wall by *B. subtilis* was observed owing to the production of extracellular chitinase (Fig. 12.3).

12.3.6 Direct Parasitism

Antagonist and pathogen can interact also through a direct parasitism. Bianciotto et al. (2001) tested the biofilm forming ability of two mutant strains with increased production of acidic extracellular polysaccharides compared with the wild-type biocontrol strain *Pseudomonas fluorescens* CHA0. The anchoring of bacteria to axenic non-mycorrhizal and mycorrhizal roots of carrot as well as on extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* was investigated. The non-mucoid wild-type strain *P. fluorescens* CHA0 adhered very little on all surfaces, whereas both mucoid strains formed a dense and patchy bacterial layer on the carrot roots and fungal structures. Increased adhesive properties of plant-growth-promoting bacteria may lead to more stable interactions in mixed inocula and the rhizosphere.

12.3.7 Induce Resistance

Interactions between plants and pathogens can lead either to a successful infection (compatible response) or resistance (incompatible response). In incompatible interactions, infection by viruses, bacteria, or fungi will elicit a set of localized responses in and around the infected host cells. These responses include an oxidative burst (Lamb and Dixon 1997), which can lead to cell death (Kombrink and Schmelzer 2001). Thus, the pathogen may be "trapped" in dead cells and appears to be prevented from spreading from the site of initial infection. Further local responses in the surrounding cells include changes in cell wall composition that can inhibit penetration by the pathogen and de novo synthesis of antimicrobial compounds such as phytoalexins and pathogenesis related (PR) proteins (Hammerschmidt 1999).

Bacillus mycoides isolate Bac J (BmJ) was capable of inducing systemic acquired resistance (SAR) in sugar beet (Bargabus et al. 2002). Two molecular markers associated with pathogen-induced SAR, β-1,3-glucanase, and chitinase were found to be induced during BmJ–plant interactions. These host responses could, therefore, be used as a predictor of systemic resistance induction capability, provided the assay results are both accurate and precise (Bargabus et al. 2004).

12.4 Fields of Use for Bacterial Antagonists

Bacteria are generally more predominant microflora in nature, compared to yeasts and filamentous fungi. A large body of information is available concerning antagonism between bacteria and pathogenic fungi.

12.4.1 Seed Treatment

When seeds are treated with antagonists, the antagonist is present from the beginning of plant growth. Due to early presence antagonists are able to colonize the seed and the young roots successfully. Competition by other inhabitants of the rhizosphere is significantly reduced and antagonists are able to establish a high population density. *Bacillus subtilis* and *Pseudomonas* spp. are the antagonists mainly investigated for this purpose (Schmiedeknecht et al. 1998). For example, seed treatment of potatoes with *B. subtilis* successfully reduced incidence of *Rhizoctonia solani* and *Streptomyces scabies* in greenhouse and field trials. Reduction of disease incidence in field trials varied between different experimental years but reached a level of up to 50% for *R. solani* and up to 67% for *S. scabies* (Schmiedeknecht et al. 1998). Similarly, *Pseudomonas fluorescens–putida* and strain R20 of *P. putida*, when inoculated onto sugar beet seed, resulted in a markedly lower incidence of colonization by *Pythium ultimum*. The incidence of fungal colonization of beet seeds treated with *P. fluorescens* or *P. putida* was 6.7 and 35.7%, respectively, compared with 90% of untreated seeds after planting (Osburn et al. 1982).

12.4.2 Soil Application

Application of antagonists to soil has many advantages. The most important one might be the relatively low variation that occurs in soil environmental conditions. Extreme events such as heavy rainfall and dryness due to several hours of sunshine are buffered by soil. Therefore, antagonists have less stress and higher possibility of survival after application (Helbig 2006).

Extracellular polysaccharides play an important role in the formation of bacterial biofilms. Bianciotto et al. (2001) tested the biofilm forming ability of two mutant strains with increased production of acidic extracellular polysaccharides compared with the wild-type biocontrol strain of *P. fluorescens*. The anchoring of bacteria to axenic nonmycorrhizal and mycorrhizal carrot roots as well as on extra radical mycelium of the arbuscular mycorrhizal fungus, *Glomus intraradices*, was investigated. The non-mucoid wild-type strain *P. fluorescens* adhered very little on all surfaces, whereas both mucoid strains formed a dense and patchy bacterial layer on the carrot roots and fungal structures (Fig. 12.4).

12.4.3 Treatment of Aerial Plant Parts

Biological control of plant pathogens on aerial plant parts obviously is the most challenging task for microbial antagonists. Regarding crops in the field, aerial plant parts are exposed to environmental conditions such as heavy rainfalls, dryness, UV-radiation, wind, and other conditions. These unfavorable conditions hinder survival of large antagonist populations for longer periods. Foliar application of *Psudomonas fluoresens* and *P. putida* strain significantly reduced the number of angular leaf spots per leaf on susceptible clones in cassava (Hernandez et al. 1985).

12.5 Measures to Improve Performance of Antagonists

There are several approaches to improve the performance of antagonists; some representative examples are described below.

12.5.1 Addition of Nutrients

The addition of nutrients is aimed at increasing the performance of antagonists by providing nutrients. Entry et al. (2000) hypothesized that wood chip-polyacrylamide cores surrounding host plant roots could alter the soil environment to favor growth of introduced biocontrol microorganisms (*Streptomyces lydicus* or *Pseudomonas corrugata*), thereby reducing *Verticillium dahliae* infection of potato in greenhouse condition.

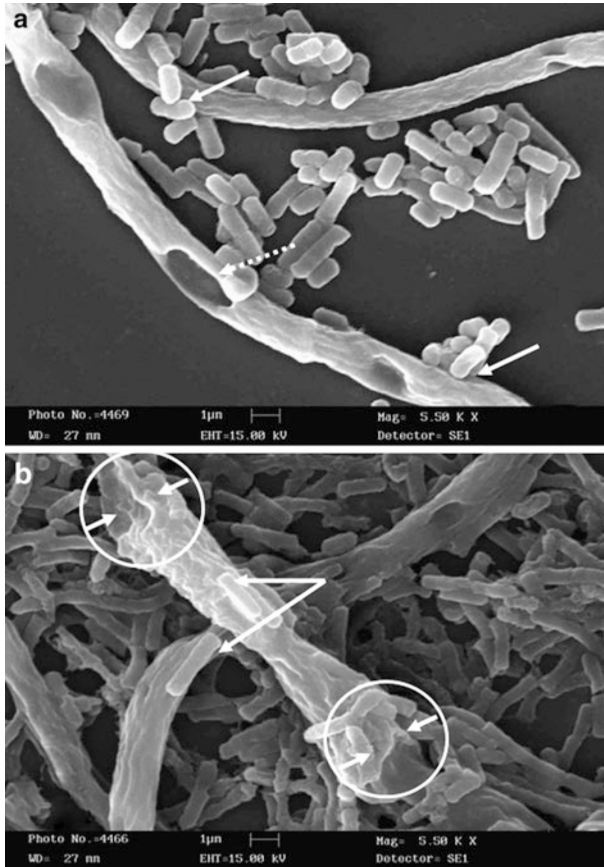

Fig. 12.4 Scanning electron micrograph of *Fusarium oxysporum* sample collected at 12 h (**a**) and 36 h (**b**) after interaction with *Bacillus subtilis* CM1. The *solid* and *dotted arrow* shows the bacterial attachment with fungal hyphae and lytic mark hyphae. *Circles* indicate the complete lysis of fungal mycelium after 36 h of interaction (Swain et al. 2008)

12.5.2 Use of Antagonist Mixtures

Because of the different sensitivities of fungi, yeasts and bacteria vis-à-vis environmental conditions, especially on aerial plant parts, the use of antagonist mixtures could contribute to consistency of performance of antagonist preparations. If one organism fails due to unfavorable conditions, the others should be effective under these conditions. Moreover, the combination might have a synergistic effect.

Pseudomonas fluorescens F113 and *Stenotrophomonas maltophilia* W81 when combined in a consortium improved the level of protection in comparison when used singly to protect sugar beet from *Pythium*-mediated damping-off (Dunnea et al. 1998). In another study, commercial biocontrol agents, microbial inoculants,

mycorrhizae, and an aerobic compost tea (ACT), were used in three different 2-year crop rotations (barley/ryegrass, barley/clover, and potato, all followed by potato). Biological amendments reduced soil-borne disease and improved yield in some rotations, but not others. Soil-applied ACT and the combination of ACT with a mixture of beneficial microorganisms (Mix) reduced stem canker, black scurf, and common scab on potato tubers by 18–33% and increased yield by 20–23% in the barley/ryegrass rotation, but not in the other rotations. Mix also reduced disease (20–32%) in the barley/clover rotation only (Larkin 2008).

12.5.3 Formulation of Antagonists

Formulation of antagonists should fulfill a variety of functions with the overarching goal to support viability and by this stabilize and increase performance of antagonists.

Seed treatment with non-sterilized powdered straws from 39 crops was tested for the control of *Pythium* damping-off of sugar beet. Four straws, including flax, coriander, pea, and lentil were effective in controlling the disease in soil artificially infested with *Pythium* sp. Sterilizing flax and pea straws eliminated the efficacy of these straws. Wheat straw powder coated on sugar beet seeds increased the incidence of *Pythium* damping-off, but this effect was reversed by the co-inoculation of wheat straws with the biocontrol agent *Pseudomonas fluorescens* 708. Coating sugar beet seeds with *P. fluorescens* 708 and flax or pea straws also increased the efficiency of the bacterial strain for the control of *Pythium* damping-off. Pea straws and to a lesser extent lentil straws produced volatile substances that affected mycelial growth of *Pythium* sp. (Bardin et al. 2004).

12.6 Bacterial Biocontrol for Root and Tuber Crops

Bacteria are the most promising biocontrol agents as compared to the fungi and yeast. Most of the fungal infections in root and tuber crops are controlled by bacteria-based biocontrol agents.

12.6.1 Temperate Root Crops

Plants cultivated for its swollen edible roots (which may or may not be a tuber roots) are called root and tuber crops. Crops that are mostly cultivated in temperate climate such as carrot, potato, beet root, etc., are called temperate tubers crops.

12.6.1.1 Potato

Potato tubers endophytic bacteria were examined from the tuber peel (periderm plus top 3 mm of tissue) of four cultivars (Russet Burbank, Kennebec, Butte, and Shepody). Endophytic bacteria from several layers of peel were challenged in in vitro bioassays to the soil-borne plant pathogens, *Fusarium sambucinum*, *Fusarium avenaceum*, *Fusarium oxysporum*, and *Phytophthora infestans* (mating types A1 and A2). In general, antibiosis of bacterial endophytes against these pathogens was significantly higher in isolates recovered from the outermost layer of tuber peel and decreased progressively toward the center of the tuber. Antibiosis against *P. infestans* was variable, with a progressive decrease in antibiotic activity from outer to inner layers of peel occurring in cultivars, "Russet Burbank" and "Kennebec," only. In all cases the inhibitory activity of endophytic bacteria was significantly greater against the A1 than the A2 mating type of *P. infestans*. In four of seven cases, where the same species of bacteria were recovered from all three peel layers, antibiosis to pathogens decreased significantly with depth of recovery (from the periderm to inside the tuber), indicating that in certain communities of endophytic bacteria, defense against pathogens may be related to bacterial adaptation to location within a host and may be tissue-type and tissue-site specific (Sturz et al. 1999).

Eight plant growth promoting rhizobacteria (PGPR) of different species (*Bacillus subtilis*, *Bacillus pumilus*, *Burkholderia cepacia*, *Pseudomonas putida*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus macerans*, and *Flavobacter balastinium*) were tested for antifungal activity in in vitro (on Petri plate) and in vivo (on potato tuber) conditions against *Fusarium sambucinum*, *F. oxysporum*, and *F. culmorum* that cause dry rot of potato. All PGPR strains were also tested on tubers of two potato cultivars "Agria" and "Granola" under storage conditions. Only, *B. cepacia* strain OSU-7 had significant effects on controlling potato dry rot caused by three different fungi (Al-Mughrabi 2010).

Bacillus thuringiensis, previously selected for their efficiency against insects, were tested in vitro and in vivo against *Fusarium roseum* var. *sambucinum*, the causal agent of dry rot of potato tubers. Results of the in vitro dual culture screening revealed that more than 50% of *Bacillus* spp. isolated from salty soils inhibited the growth of the pathogen in vitro. By contrast, other *B. thuringiensis* strains failed to inhibit the growth of the pathogen in vitro. On wounded potato tubers, the most effective isolates obtained from salty soils were X7, X9, X16, I32, and G7 Bacillus strains, with a percentage of dry rot reduction ranging from 66 to 89%. These effective *Bacillus* isolates were identified as belonging to one of the species *Bacillus cereus* (X9, X16 and G7), *B. lentimorbus* (X7), or *B. licheniformis* (I32). Although ineffective in vitro, *B. thuringiensis* strains inhibited dry rot development in vivo, with percentage inhibition scores ranging from 41 to 52%. While *Bacillus* isolates selected from salty soils best inhibited dry rot development when applied as young cultures (24 h), *B. thuringiensis* strains generally performed better as older cultures (48–72 h). The cell filtrates of *Bacillus* spp. were unable to inhibit the growth of *Fusarium*. By contrast, volatiles liberated by the antagonists seem to contribute to the inhibition of the pathogen (Sadfi et al. 2001).

The antagonistic potential of potato- (endophytic and ectophytic) associated, a total of 2,648 bacteria were screened by dual testing of antagonism to the soil-borne pathogens, *Verticillium dahliae* and *Rhizoctonia solani*. The rhizosphere and endorhiza were the main reservoirs for antagonistic bacteria and showed the highest similarity in their colonization by antagonists. The most prominent species of all microenvironments was *Pseudomonas putida*, and rep-PCR with BOX primers showed that these isolates showed microenvironment-specific DNA fingerprints. *P. putida* isolates from the rhizosphere and endorhiza gave nearly identical fingerprints confirming the high similarity of bacterial populations. The *phlD* gene, involved in the production of the antibiotic 2,4-diacetyl-phloroglucinol, was found only among *Pseudomonas* isolates from the rhizosphere and endorhiza. Evaluation of the bacterial isolates for biocontrol potential based on fungal antagonism and physiological characteristics resulted in the selection of five promising isolates from each microenvironment. The most effective isolate was *Serratia plymuthica* 3Re4-18 isolated from the endorhiza (Berg et al. 2005).

Twenty-eight potential biocontrol organisms were tested for efficacy against *Rhizoctonia solani* on potato in a series of greenhouse trials. Organisms tested consisted of field isolates of *Paenibacillus polymyxa*, *Pseudomonas fluorescens*, *Penicillium* sp., *Trichoderma* sp., and *Rhizoctonia zeae*; known biocontrol isolates including *Laetisaria arvalis*, *Verticillium biguttatum*, *Cladorrhinum foecundissimum*, and *Stilbella aciculosa*; and commercial products of *Bacillus subtilis* (Kodiak), *Trichoderma virens* (SoilGard), and *T. harzianum* (RootShield). Different formulations and rates of several fungal isolates and the efficacy of combinations of effective antagonists were also investigated. None of the treatments, including a chemical control (azoxystrobin), effectively controlled stem canker and black scurf in all trials. However, *B. subtilis* GB03, *R. zeae* LRNE17E, *S. aciculosa* 112-B, and the chemical control were most effective in reducing stem canker severity (40–49% reduction) relative to the infested controls over all trials. *L. arvalis* ZH-1, *R. zeae* LRNE17E, and the chemical control reduced black scurf severity 54–60% relative to the infested control. Other treatments also significantly reduced stem canker and black scurf; however, they were slightly less effective. Biocontrol amendment rate was not correlated with disease control, although the higher rates usually provided the best control. One combination of biocontrol organisms, *B. subtilis* and *T. virens*, demonstrated somewhat better control of stem canker than each organism alone, suggesting that this approach may provide improved biocontrol efficacy (Brewer and Larkin 2005).

12.6.1.2 Sugar Beet

Bacillus subtilis isolate, BacB, is extensively studied for the control of sugar beet Cercospora leaf spot, caused by *Cercospora beticola* Sacc. by examining application timing, biocontrol agent (BCA) concentration, use of the selective nutrient substrate β-glucan, and the form of the BCA at the time of application. Examining the effects of varying β-glucan concentrations and levels of BacB at application

demonstrated a complex interaction between β-glucan, BCA population, and disease control. Growth chamber experiments demonstrated that applying the bacteria as vegetative cells instead of spores or applying the BCA 1–5 days before infection could significantly increase disease control. Laboratory experiments demonstrated the ability to induce germination and vegetative growth of BacB from a spore formulation, without shaking or fermentation equipment. This shows promise for optimizing *Bacillus* sp. for biological control. In field trials the vegetative cells did not perform better than the spore application, though the potential for β-glucan to increase disease was demonstrated (Collins and Jacobsen 2003).

Pseudomonas fluorescens F113 and *Stenotrophomonas maltophilia* W81 protect sugar beet from *Pythium*-mediated damping-off through production of the antifungal secondary metabolite 2,4-diacetylphloroglucinol and extracellular proteolytic activity, respectively. Growth and in vitro production of 2,4-diacetylphloroglucinol by F113 and extracellular lytic enzymes by W81 were not affected when inoculated in combination. The abilities of W81 and F113 to colonize the rhizosphere of sugar beet were essentially similar when the two strains were applied singly or co-inoculated onto seeds in a 1:1 ratio, both in natural soil microcosms and under field conditions. Concomitantly, single inoculation with W81 or F113 effectively prevented colonization of sugar beet seeds by *Pythium* spp. in soil microcosms, without the necessity for combining both strains. However, this parity was not reflected in seed emergence where the combination of W81 and F113 significantly enhanced final sugar beet stands (to the level achieved with chemical pesticides) under microcosm conditions at 28 days after sowing. In a field experiment, the only inoculation treatment capable of conferring effective protection of sugar beet was that in which W81 and F113 were co-inoculated, and this treatment proved equivalent to the use of chemical fungicides. In conclusion, when compared with single inoculations of either biocontrol strain, the combined use of a phloroglucinol-producing *P. fluorescens* and a proteolytic *S. maltophilia* improved protection of sugar beet against *Pythium*-mediated damping-off (Dunnea et al. 1998).

In another study, dual compatibility of antagonists (*Pseudomonas* and *Bacillus* strains) were also evaluated against *Pythium* spp. Antagonist combinations did not show any superior biocontrol ability to individual antagonists and compatibility of bacteria in vitro did not correlate with compatibility in vivo (Georgakopoulos et al. 2002).

Bacillus mycoides isolate Bac J., a nonpathogenic, phyllosphere-inhabiting bacterium, reduces Cercospora leaf spot (*Cercospora beticola* Sacc.) of sugar beet by 38–91% in both glasshouse and field experiments. Disease control was attributed to the bacterium's ability to induce systemic resistance, which was demonstrated through classical induced resistance challenge experiments, western analysis, and enzyme activity assays. Enzyme assays following *B. mycoides* and acibenzolar-*S*-methyl treatment demonstrated increased activity of chitinase, β-1,3-glucanase, and peroxidase, all pathogenesis-related proteins and accepted indicators of systemic resistance. Western analysis detected numerous chitinase isoforms in *B. mycoides* and acibenzolar-*S*-methyl-treated plants that were not detected in the water controls. The active chitinase isoforms were identified using in-gel activity assays.

β-1,3-glucanase activity assays following native polyacrylamide gel electrophoresis revealed two unique isoforms produced following *B. mycoides* treatment, one of which was also found with acibenzolar-*S*-methyl treatment. The increase in peroxidase-specific activity following acibenzolar-*S*-methyl and *B. mycoides* treatment was due to production of two unique isoforms not found in the water-treated plants as shown by activity assays following native polyacrylamide gel electrophoresis (Bargabus et al. 2002).

Pseudomonas fluorescens–putida and strain R20 of *P. putida*, when inoculated onto seed, resulted in a markedly lower incidence of colonization by *P. ultimum*. The incidence of fungal colonization of beet seeds treated *P. fluorescens–putida* or *P. putida* was 6.7 and 36.7%, respectively, compared with 90% of untreated seeds 24 h after planting. *P. fluorescens* inhibited both mycelial growth and sporangial germination, whereas *P. putida* inhibited only mycelial growth (Osburn et al. 1982).

Pseudomonas fluorescens (biovars I to VI) were collected from the rhizosphere of field-grown sugar beet plants to select candidate strains for biological control of preemergence damping-off disease. The isolates were tested for in vitro antagonism toward the plant-pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani* in three different plate test media. Mechanisms of fungal inhibition were elucidated by tracing secondary-metabolite production and cell wall-degrading enzyme activity in the same media. Most biovars expressed a specific mechanism of antagonism, as represented by a unique antibiotic or enzyme production in the media. A lipopeptide antibiotic, viscosinamide, was produced independently of medium composition by *P. fluorescens* bv. I, whereas the antibiotic 2,4-diacetylphloroglucinol was observed only in glucose-rich medium and only in *P. fluorescens* bv. II/IV. Both pathogens were inhibited by the two antibiotics. Finally, in low-glucose medium, a cell wall-degrading endochitinase activity in *P. fluorescens* bv. I, III, and VI was the apparent mechanism of antagonism toward *R. solani*. The viscosinamide-producing DR54 isolate (bv. I) was shown to be an effective candidate for biological control, as tested in a pot experiment with sugar beet seedlings infested with *Pythium ultimum*. The assignment of different patterns of fungal antagonism to the biovars of *P. fluorescens* was discussed in relation to an improved selection protocol for candidate strains to be used in biological control (Nielsen et al. 1998).

The effectiveness of *Bacillus subtilis* isolate, in the field and growth chamber in the presence of the fungus, *Cercospora beticola*, was studied. The use of the selective biocontrol agent support substrate β-glucan, applied at 0, 0.5, and 1.0% of the spray solution, did not influence differences in total population numbers (spores + vegetative cells) of a spontaneous rifampicin resistant isolate of BacB (Rif+) over a 14-day spray period. BacB Rif+, applied as a spore formulation, declined from 10,000 CFU (Colony Forming Units)/cm^2 on day 0.5–100 CFU/cm^2 on day 14 at the three levels of β-glucan tested. Distribution of BacB Rif + populations was modeled on a leaf scale, with and without β-glucan. Higher populations of vegetative cells were more likely at 14 days with 1% β-glucan than with 0% β-glucan. BacB populations were more aggregated without β-glucan than with the nutrient substrate. There was no correlation between BacB density and Cercospora leaf spot disease severity, indicating that neither antibiosis nor parasitism is likely an important mechanism of disease control (Collins and Jacobsen 2003).

Nontarget effects of a bacterial (*Pseudomonas fluorescens* DR54) and a fungal (*Clonostachys rosea* IK726) microbial control agent (MCA), on the indigenous microbiota in bulk soil and rhizosphere of barley, and subsequently a sugar beet crop, were studied in a greenhouse experiment. MCAs were introduced by seed and soil inoculation. Bulk and rhizosphere soils were sampled regularly during the growth of barley and sugar beet. The soils were assayed for the fate of MCAs and various features of the indigenous soil microbiota. At the end of the experiment (193 day), DR54 and IK726 had declined by a factor of 106 and 20, respectively, and DR54 showed a short-lasting growth increase in the sugar beet rhizosphere. In general, the nontarget effects were small and transient. IK726 seemed to have general stimulating effects on soil enzyme activity and the soil microbiota, and resulted in a significant increase in plant dry weight. The plant growth promoting effect of DR54 was less pronounced and the DR54 displaced indigenous pseudomonads. DR54 stimulated growth of protozoans with a tolerance for the antifungal compound viscosinamide produced by DR54. Treatment with the fungicide Fungazil had no effects on plant growth or soil microorganisms. Phospholipid fatty acid (PLFA) analysis detected the perturbations of the soil microbial community structure in the MCA treatments as well as the return to non-perturbed conditions reflecting the decline of inoculant populations. The PLFA technique appeared to be suitable for in situ monitoring of MCA nontarget effects on the soil microbiota, but should be combined with assays for MCA survival and soil enzyme activity (Johansen et al. 2005).

Growth inhibition of the root pathogen *Pythium ultimum* by the biocontrol agent *Pseudomonas fluorescens* DR54 inoculated on sugar beet seeds was studied in a soil microcosm. Plant emergence was followed, together with bacterial rhizosphere colonization, antibiotic production, and effects on fungal growth. *P. fluorescens* DR54 inoculation of the *P. ultimum*-challenged seeds improved plant emergence after 7 days compared to a control without the biocontrol strain. At this time, *P. fluorescens* DR54 was the dominating colony-forming pseudomonad in rhizosphere soil samples from inoculated seedlings as shown by immuno-staining with a strain-specific antibody. Viscosinamide, which has previously been identified as a major antagonistic determinant produced by *P. fluorescens* DR54 and shown to induce physiological changes in *P. ultimum* in vitro, could be detected in the rhizosphere samples. The impact of *P. fluorescens* DR54 on the growth and activity of *P. ultimum* was studied by direct microscopy after staining with the vital fluorescent dyes Calcofluor white and fluorescein diacetate. *P. fluorescens* DR54 caused reduction in *P. ultimum* mycelial density, oospore formation, and intracellular activity. Further, *Pythium* oospore formation was absent in the presence of *P. fluorescens* DR54. A striking effect on zoospore-forming indigenous fungi was also observed in microcosms with *P. fluorescens* DR54 and, thus, where viscosinamide could be detected; a large number of encysted zoospores were seen in such microcosms both with and without *P. ultimum* infections. In vitro studies confirmed that purified viscosinamide-induced encystment of *Pythium* zoospores (Thrane et al. 2000).

12.6.1.3 Carrot

Diseased carrot seeds were treated with selected microorganisms isolated from soils, carrot seeds, and tap roots. The effects of those antagonists on the control of *Alternaria radicina* were evaluated by growing-on tests on water agar, filter paper, vermiculite, and in a potting medium. The germination percentage, emergence percentage, and the disease severity of those carrot seeds treated with *Burkholderia* (*Pseudomonas*) *cepacia* no.229 were significantly ($P = 0.05$) differed from the nontreated seeds and the seed treated with other antagonists. The effects of *B. cepacia* no.229 in promoting seed emergence and controlling disease were as good as those seeds treated with iprodione (100 ppm). Black rot lesions on carrot tap roots were significantly reduced ($P = 0.05$) in size when roots were treated with *B. cepacia* no 229 or *Bacillus amyloliquefaciens* no. 224 compared to the nontreated roots. Also, *B. cepacia* no. 229 significantly ($P = 0.05$) reduced black rot on the foliage of carrot compared to check (Chen and Wu 1999).

12.6.1.4 Onion

Bacillus subtilis (B-2) in the rhizospheres of onion seedlings grown from bacterized seeds in muck soil at various pH, moisture, and temperature regimes were monitored for 14 weeks. Seed bacterization significantly increased shoot dry weight (12–94%), root dry weight (13–100%), and shoot height (12–40%) of onion seedlings over controls. Increases in shoot height and shoot weight were greatest at low temperature and high moisture, under all pH regimes. Root weight was similarly affected by temperature and moisture, but was significantly increased at pH 6.5 compared to 5.5 and 4.5. Though *B. subtilis* B-2 failed to maintain high populations in the onion rhizosphere, it nevertheless caused significant growth effects on bacterized onion seedlings. The observed growth effects were not proportional to rhizosphere populations of B-2 (Reddy and Rahe 1989).

Bacillus amyloliquefaciens BL-3 and *Paenibacillus polymyxa* BL-4 were applied in the rhizoplane of onion at transplanting. BL-3 completely suppressed the neck rot of onion (Lee et al. 2001). Further, strain BL-3 produced a heat-stable antifungal protein (Bae 1999) that reduced decay regardless of the inoculum level or the isolate of pathogenic fungi tested and was effective at temperatures of 10–30 °C (Lee et al. 2001).

12.6.1.5 Ginger

Kahili ginger (*Hedychium gardnerianum*) is an invasive weed in tropical forests in Hawaii and elsewhere. Bacterial wilt caused by the ginger strain of *Ralstonia* (=*Pseudomonas*) *solanacearum* systemically infects edible ginger (*Zingiber officinale*) and ornamental gingers (*Hedychium* spp.), causing wilt in infected plants.

The suitability of *R. solanacearum* as a biological control agent for kahili ginger was investigated by inoculating seedlings and rooted cuttings of native forest plants, ornamental ginger, and solanaceous species to confirm host specificity. Inoculation via stem injection or root wounding with a bacterial–water suspension was followed by observation for 8 weeks. Inoculations on *Hedychium gardnerianum* were then carried out in ohia-lehua (*Metrosideros polymorpha*) wet forests of Hawaii Volcanoes National Park to determine the bacterium's efficacy in the field. No native forest or solanaceous species developed wilt or other symptoms during the study. The bacterium caused limited infection near the inoculation site on *H. coronarium*, *Z. zerumbet*, *Heliconia latispatha*, and *Musa sapientum*. However, infection did not become systemic in any of these species, and normal growth resumed following appearance of initial symptoms. All inoculated *H. gardnerianum* plants developed irreversible chlorosis and severe wilting 3–4 weeks following inoculation. Systemic infection also caused death and decay of rhizomes. Most plants were completely dead 16–20 weeks following inoculation. The destructiveness of the ginger strain of *R. solanacearum* to edible ginger has raised questions regarding its use for biological control. However, because locations of kahili ginger infestations were often remote, the risk of contaminating edible ginger plantings was unlikely. The ability of this bacterium to cause severe disease in *H. gardnerianum* in the field, together with its lack of virulence in other ginger species, contributed to its potential as a biological control agent (Anderson and Gardner 1999).

12.6.1.6 Garlic

Treatment of garlic cloves with tebuconazole achieved a significant reduction in the rate of disease progress and the final incidence of plant death by *Sclerotium cepivorum*; garlic yields were improved. In contrast, lower levels of disease control were obtained when selected isolates of *Trichoderma harzianum* and *Bacillus subtilis* were applied to the soil and cloves, respectively (Melero-Vara et al. 2000).

Pantoea agglomerans S59-4 isolate from rhizosphere or rhizoplane of *Allium* species was selected as a potential biocontrol agent against *Penicillium hirsutum*, when using an in vivo wounded garlic bulb assay. When the spore suspension (10^5 spores/ml) of *P. hirsutum* was co-inoculated with a cell suspension of *P. agglomerans* S59-4 (10^8 CFU/ml) isolate on wounded garlic, the isolate showed a highly suppressive effect on disease development. Bacterial density of *P. agglomerans* on wounded garlic cloves increased continuously both under room temperature and low temperature conditions until 30 days after application. In addition, *P. agglomerans* showed in vitro inhibitory effects against various postharvest diseases of citrus fruits, apples, onions, lettuces, and carrots. In particular Pa59-4 showed strong inhibitory effects against *Penicillium digitatum*, *Aspergillus niger*, *Sclerotinia sclerotiorum*, and *Geotrichum candidum* (Kim et al. 2006).

12.6.2 Tropical Tuber Crops

Tropical roots and tuber crops are important staples for food security for about a fifth of the world crop production. The most important tropical root and tuber crops include cassava, yam, cocoyam, sweet potato, colocasia (taro), and amorphophallus.

12.6.2.1 Cassava

Forty isolates of fluorescent *Pseudomonas* were isolated from the plants growing in five different ecosystems in Nigeria. Thirty-four of these isolates inhibited *Erpinia aarotovoroa* pv. *aarootovoroa*, in vitro, the causal agent of cassava stem rot. One-month old plantlets, produced by rooting the shoots of a cultivar in distilled water, were inoculated with suspensions (1×10^9 cells/ml) of each *Pseudomonas* isolate. Inoculated plants were free from symptoms of root pathogens and roots swelled earlier than controls. Microbial deterioration of bulked swollen roots was also reduced up to 60% when roots were dip treated in a bacterial suspension (1×10^9 cells/ml) of the above isolates and stored for 15 days in polyethylene bags. Taxonomic studies showed that these bacterial isolates were either *Pseudomonas putida* (90%) or *P. fluorescens* (10%) (Hernandez et al. 1985).

Cassava bacterial blight caused by *Xanthomonas campestris* pv *manihot's* is a serious problem in the cassava growing region of Nigeria. Several bacterial anatagonists such as *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas* spp., and some fungi such as *Trichoderma* spp. and *Gliocadium* spp. were applied successfully to control the blight (Amusa and Odunbaku 2007).

Sixty-seven endophytic bacteria isolated from cassava cultivated by Brazilian Amazon Indian tribes were subjected to 16S rRNA sequencing and FAME (fatty acid methyl ester) analysis. The bacterial profile revealed that 25% of all endophytic isolates belonged to the genus *Bacillus*. The isolate *B. pumilus* MAIIIM4a showed a strong inhibitory activity against the fungi *Rhizoctonia solani*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* causing cassava stem rot. Secondary metabolites of this strain were extracted using hexane, dichloromethane, and ethyl acetate (Pereira de Melo et al. 2009).

12.6.2.2 Yam

The leaf spot of yam (*Dioscorea cayennensis* Lam.), caused by *Curvularia eragrostidis* (Henn.) Meyer, is one of the most frequent and severe diseases in all yam growing areas of northeast Brazil. The disease causes a reduction of about 35–40% of the weight of the commercial tuber (Michereff et al. 1994). From a total of 162 bacterial isolates, 39 showed antagonism to the pathogen. The bacteria produced extracellular, nonvolatile, and diffusible metabolites in the membrane cellophane test. Seventeen isolates resulted in more than 75% inhibition of

C. eragrostidis mycelial growth. Among them, IF-26 showed the greatest antagonism. The isolates IF-82, IF-88, and IF-109 inhibited pathogen conidial germination, with average inhibition levels of 99.2, 98.2, and 96.2%, respectively. Under greenhouse conditions the antagonists were applied at three different time intervals relative to *C. eragrostidis* inoculation: 3 days before, at the same time, and 3 days after. IF-82 and IF-88 applied at the same time as pathogen inoculation both reduced disease severity to the extent of 75%. IF-82 showed the best persistence of antagonistic action, with an average of 96.3%. IF-82, identified as *Bacillus subtilis*, was the best biocontrol agent for the yam leaf spot disease in this study (Michereff et al. 1994).

Rot of yam tubers and setts may be caused by a wide variety of fungi including *Aspergillus niger*, *Botryodiplodia theobromae*, *Fusarium solani*, *Penicillium* spp. *Rhizopus stolonifer*, and *Mucor* spp. Twenty-four yam rhizobacteria were screened on potato dextrose agar–nutrient agar (PDA–NA) plates for antifungal activity toward the above rot fungi using the zone of inhibition test. The most promising bacterial antagonist was tested further against 22 fungi from different phyla. Nine rhizobacterial isolates, representing 38% of all bacterial isolates initially tested, exhibited antifungal activity. They were all Gram-negative rods, catalase positive, aerobic, endospore-forming rods, and tentatively identified as *Bacillus* spp. (Awuah and Akrasi 2007).

Bacillus subtilis (Enrenberg) Cohn was investigated for its antagonistic properties against surface mycoflora of yam (*Dioscorea rotundata* Poir) tubers in storage. Yam tubers inoculated with a spore suspension of *B. subtilis* in potato dextrose broth using a knapsack sprayer showed a drastic reduction in the range and number of mycoflora, including pathogens of the tuber surface in contrast to the control tubers, during the 5-month storage period in a traditional yam barn. However, *B. subtilis* maintained a high frequency of occurrence during the same period. *Botryodiploidia theobromae* Pat, *Fusarium moniliforme* Wollen and Reink., *Penicillium sclerotigenum* Yamamoto, and *Rhizoctonia* sp. were displaced completely on the treated tubers. The antagonism of *B. subtilis* was so effective that the normal tuber surface mycoflora was greatly reduced throughout the storage period of 5 months by a simple initial application of the antagonist (Okigbo 2002).

The biocontrol potential of *Bacillus subtilis* isolated from cow dung microflora was investigated in vitro and in vivo against two postharvest yam pathogenic fungi, *Fusarium oxysporum* and *Botryodiplodia theobromae*. *B. subtilis* strains inhibited the growth of *F. oxysporum* and *B. theobromae* in vitro in liquid medium in the range of 49.3–56.6% and in solid medium in the range of 31.0–36.0%, in comparison to the corresponding growth of fungi without bacterial inoculation. The interaction between *B. subtilis* CM1 and *F. oxysporum* was also studied by scanning electron microscopy (Fig. 12.3). In vivo study showed that *B. subtilis* strains inhibited the growth of fungi (*F. oxysporum* and *B. thobromae*) up to 83% in wound cavities of yam tubers (Swain et al. 2008). Likewise, these strains blocked the production of oxalic acid produced by these pathogenic fungi in yam tuber as well as in culture medium (Swain and Ray 2009b).

12.6.2.3 Cocoyams

Root rot of cocoyam (*Xanthosoma sagittifolium*) caused by *Pythium myriotylum* is the most devastating disease of this important tropical tuber crop with yield reductions of up to 90%. *Pseudomonas aeruginosa* PNA1 (wild type) produced phenazine-1-carboxylic acid and phenazine-1-carboxamide (oxychlororaphin), while its tryptophan auxotrophic mutant FM13 was phenazine negative and secreted anthranilate in vitro (Tambong and Hofte 2001). PNA1 and FM13 significantly inhibited growth of *P. myriotylum* in dual cultures, while their supernatants highly reduced mycelial dry weight in potato dextrose broth. However, in the presence of tissue culture derived cocoyam plantlets, only strain PNA1 strongly reduced root rot disease severity. Soil experiments involving strain *P. aeruginosa* PNA1 in comparison to phenazine-deficient mutants suggested that the biocontrol activity of PNA1 against *P. myriotylum* might involve phenazines. Phenazine involvement was further strengthened by the fact that FM13 fed with exogenous tryptophan (so that phenazine production was restored) significantly reduced disease severity on cocoyam. The efficiency of PNA1 to control *P. myriotylum* on cocoyam was significantly improved when the strain and the pathogen were allowed to interact for 24 h prior to transplanting cocoyam plantlets, while doubling the inoculum density of the pathogen negatively affected its efficiency (Tambong 2000). *Pseudomonas* CMR5c and CMR12a were identified as novel and promising biocontrol agents of *P. myriotylum* on cocoyam, producing an arsenal of antagonistic metabolites (Perneel et al. 2007). *Pseudomonas aeruginosa* PNA1 was considered as a promising biological control agent to solve the increasing problem of cocoyam root rot in Cameroon (Tambong and Hofte 2001).

12.6.2.4 Sweet Potato

Endophytic bacteria associated with sweet potato plants (*Ipomoea batatas* L. Lam.) were isolated, identified, and tested for their ability to fix nitrogen, produce indole acetic acid (IAA), and exhibit stress tolerance. Eleven such strains belonging to the genera, *Enterobacter*, *Rahnella*, *Rhodanobacter*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, and *Phyllobacterium*, were identified (Khan and Doty 2009). Other bacterial species have been reported from sweet potato endophytic included *Acetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, and *Pseudomonas* (Lodewyckz et al. 2002). These bacteria were associated with plant growth promoting activity and biological control of pathogens in sweet potato (Katarina et al. 2005).

12.7 Conclusion and Future Perspectives

Despite our understanding of the mechanisms by which bacterial antagonists offer disease resistance to root and tuber crops, the ecological significance of their presence as endophytes or epiphytes and in rhizosphere and phyllosphere is less understood. These factors need to be addressed sufficiently in order to develop biocontrol products of commercial interest. Further, most of the studies on biocontrol of diseases are concentrated on potato and sugar beet, and very fewer attentions have been given to other tuber crops particularly on tropical root crops like cassava and sweet potato. When considering possible improvements in biological plant protection, formulation of antagonists, use of such formulations in seed coating and aerial spray, and use of antagonists mixture and in combination with known and established biocontrol enhancing additives such as $CaCl_2$, NaCl are to be studied.

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Chapter 13
Bacteria in the Management of Plant-Parasitic Nematodes

Pravin C. Trivedi and Abhiniti Malhotra

13.1 Introduction

Nematodes are one of the most important constraints to crop productivity and cause 12 % annual loss in the yield of important food and fiber crops on a worldwide basis (Sasser and Freckman 1987; Barker et al. 1994). The control of nematode is far more difficult than any other kind of pest because they inhabit soil and usually attack the underground parts of plants. On account of eco-friendly plant protection drive, great emphasis has been given to the exploitation of potential bioagents for controlling nematodes. Soils being a complex environment, housing various flora and fauna, nematodes are generally exposed to many enemies. The most widely found enemies are fungi and bacteria, of which bacteria as a bioagent have several advantages, followed by fungi. Not only is bacterium eco-friendly, but it also takes a long time to develop resistance. Besides, biotechnological interventions for evolving efficient strains are possible in the organism as they possess a simple genome.

Nematodes in soil are subject to infections by bacteria and fungi. This creates the possibility of using soil microorganisms to control plant-parasitic nematodes (Mankau 1980; Jatala 1986). Bacteria are numerically the most abundant organisms in soil, and some of them, for example, members of the genera *Pasteuria*, *Pseudomonas*, and *Bacillus* (Emmert and Handelsman 1999; Siddiqui and Mahmood 1999; Meyer 2003), have shown great potential for the biological control of nematodes. Extensive investigations have been conducted over the last few years to assess their potential to control plant-parasitic nematodes.

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13.2 Bacteria in Nematode Control

Bacterial antagonists of plant-parasitic nematodes are grouped under the following categories: obligate parasites, antagonistic bacteria, and other soil bacteria.

13.2.1 Obligate Parasites

An obligate parasite is a parasitic organism that cannot live independently of its host. Members of the genus *Pasteuria* are obligate parasites of plant-parasitic nematodes. *Pasteuria penetrans* is a mycelial, endospore-forming, bacterial parasite that has shown great potential as a biological control agent of root-knot nematodes. Considerable progress has been made during the last 10 years in understanding its biology and importance as an agent capable of effectively suppressing root-knot nematodes in field soil. The biological control potential of *Pasteuria* spp. has been demonstrated on 20 crops; host nematodes include *Belonolaimus longicaudatus*, *Heterodera* spp., *Meloidogyne* spp., and *Xiphinema diversicaudatum*. The potential of predacious and nematoxic fungi and bacteria for the biological control of nematode parasites may offer a cheaper and more sustainable approach to reducing the damage caused by phytonematodes.

13.2.1.1 *Pasteuria penetrans*

Members of the genus *Pasteuria* are obligate, mycelial, endospore-forming bacterial parasites of plant-parasitic nematodes and water fleas (Sayre and Starr 1985; Bekal et al. 2001). A number of bacterial species in this genus have shown great potential as biocontrol agents against plant-parasitic nematodes. They occur worldwide and have been reported from at least 51 countries (Siddiqui and Mahmood 1999).

Taxonomy and Host Range of *Pasteuria penetrans*

Members of the genus have been reported to infect 323 nematode species belonging to 116 genera, including both plant-parasitic nematodes and free-living nematodes (Chen and Dickson 1998). The majority of economically important plant-parasitic nematodes have been observed to be parasitized (Bird et al. 2003). *Pasteuria* was first described as a protozoan and later classified into the bacterial genus *Bacillus* and then into *Pasteuria* (Sayre and Starr 1985). At present, the taxonomy within the genus *Pasteuria* is based mainly on morphological and pathological characteristics, including the size and shape of sporangia and endospores, and ultrastructures, life cycles, and host ranges (Atibalentja et al. 2000). Over the last few years, a number of molecular biological analyses have been used in the identification and classification of this genus. Recent analysis of a portion of the 16S rRNA gene showed that the genus

Pasteuria is a deeply rooted member of the *Clostridium–Bacillus–Streptococcus* branch of the Gram-positive Eubacteria (Anderson et al. 1999). Charles et al. sequenced the genome of *Pas. penetrans*, performed amino-acid-level analysis using concatenation of 40 housekeeping genes, and identified *Pas. penetrans* as ancestral to *Bacillus* spp. The results suggested that *Pas. penetrans* might have evolved from an ancient symbiotic bacteria associate of nematodes, possibly when the root-knot nematode evolved to a highly specialized parasite of plants (Charles 2005; Charles et al. 2005). So far, four nominal *Pasteuria* species have been reported. Among them, *Pasteuria ramosa* has been described from water fleas (Ebert et al. 1996). The other three nematode-infecting species are *Pas. penetrans*, which primarily parasitizes root-knot nematodes such as *Meloidogyne* spp.; *Pas. thornei*, which parasitizes root-lesion nematodes such as *Pratylenchus spp*.; and *Pas. nishizawae*, which occurs on cyst nematodes of the genera *Heterodera* and *Globodera* (Atibalentja et al. 2000). Recently, based on morphological characteristics, host specificity, and the analysis of 16S rRNA gene Giblin-Davis et al. (2001, 2003) proposed that strain S-1, which parasitizes the sting nematode *Belonolaimus longicaudatus*, represents a novel *Pasteuria* species, *Candidatus Pasteuria* usgae.

Mechanisms of Infection

The life cycle of *Pas. penetrans* is completed in four stages, viz., spore germination, vegetative growth, fragmentation, and sporogenesis. *Pasteuria penetrans* infects the root-knot nematode *Meloidogyne* spp. Spores of *Pasteuria* can attach to the cuticles of the second-stage juveniles and germinate about 8 days after the juvenile has entered roots and begun feeding (Sayre and Wergin 1977). The germ tubes can penetrate the cuticle, and vegetative microcolonies then form and proliferate through the body of the developing female. Finally, the reproductive system of the female nematode degenerates, and mature endospores are released into the soil (Mankau et al. 1976; Sayre and Wergin 1977). Attachment of the spores to the nematode cuticle is the first step in the infection process (Davies et al. 2001). However, spores of individual *Pasteuria* populations do not adhere to or recognize all species of nematode. The spores of each *Pasteuria* species usually have a narrow host range. For example, *Pas. penetrans* infects *Meloidogyne spp*., *Pas. thornei* infects *Pratylenchus spp*., and *Pas. nishizawae* infects the genera *Heterodera* and *Globodera* (Gives et al. 1999; Atibalentja et al. 2000). The specificity of spore attachment to the nematode cuticle has been intensively studied using biochemical and immunological methods. Monoclonal antibody studies have revealed a high degree of heterogeneity both within and among different populations of *Pas. penetrans* (Davies and Redden 1997).

The distribution on the spore of any particular epitopes that are thought to be involved in adhesion may differ among populations and species (Davies and Redden 1997; Davies et al. 2001). The distribution of an adhesin-associated epitope on polypeptides from different *Pasteuria* isolates provides an immunochemical approach to differentiating species and biotypes with specific host preferences

(Preston et al. 2003). The processes associated with the initial binding of the endospores of *Pasteuria* spp. to their respective hosts have been explored by several laboratories (Stirling et al. 1986; Persidis et al. 1991; Davies and Danks 1993; Charnecki 1997). These studies have led to a model in which a carbohydrate ligand on the surface of the endospore binds to a lectin-like receptor on the cuticle of the nematode host (Persidis et al. 1991). The fibers surrounding the *Pasteuria* spore core are thought to be responsible for the adhesion of the spore to the host cuticle (Sayre and Wergin 1977; Stirling et al. 1986; Persidis et al. 1991). Sonication can increase spore attachment by removing the sporangial wall and exposing the parasporal fibers (Stirling et al. 1986).

13.2.1.2 Opportunistic Parasitic Bacteria

In 1946, Dollfus investigated and documented bacteria within the body cavity, gut, and gonads of nematodes (Jatala 1986). Other reports have since suggested the association of some bacteria with the nematode cuticle. However, these studies were unable to specify whether these bacteria were parasites or saprophytes (Jatala 1986). In fact, most nematophagous bacteria, except for obligate parasitic bacteria, usually live a saprophytic life, targeting nematodes as one possible nutrient resource. They are, however, also able to penetrate the cuticle barrier to infect and kill a nematode host in some conditions.

They are described as an opportunistic parasitic bacteria here, represented by *Brevibacillus laterosporus* strain G4 and *Bacillus* sp. B16. As a pathogen, *Br. laterosporus* has been demonstrated to have a very wide spectrum of biological activities. So far, it has been reported that four nematode species (three parasitic nematodes, namely, *Heterodera glycines*, *Trichostrongylus colubriformis*, and *Bursaphelenchus xylophilus*, and the saprophytic nematode *Panagrellus redivivus*) could be killed by various *Br. laterosporus* isolates (Oliveira et al. 2004; Huang et al. 2005). Among these isolates, *Br. laterosporus* strain G4, which was isolated from soil samples in Yunnan province in China and parasitizes the nematodes *Panagrellus redivivus* and *Bursaphelenchus xylophilus*, has been extensively studied (Huang et al. 2005). After attaching to the epidermis of the host body, *Br. laterosporus* can propagate rapidly and form a single clone in the epidermis of the nematode cuticle.

The growth of a clone can result in a circular hole shaped by the continuous degradation and digestion of host cuticle and tissue. Finally, bacteria enter the body of the host and digest all the host tissue as nutrients for pathogenic growth (Huang et al. 2005). During bacterial infection, the degradation of all the nematode cuticle components around the holes suggests the involvement of hydrolytic enzymes (Cox et al. 1981; Decraemer et al. 2003; Huang et al. 2005). At present, the majority of research efforts on opportunistic nematode-parasitic bacteria have concentrated on understanding pathogenesis using free-living nematodes as targets. Such studies should allow us to identify new pathogenic factors and to learn more about infectious processes in nematodes. It is important to understand the mechanism that controls the switch from saprotrophy to parasitism in order to formulate effective commercial nematode control agents.

13.2.2 Antagonistic Soil Bacteria

Many species of soil bacteria are capable of decomposing plant and animal residues. A succession of these bacteria facilitates stepwise degradation of soil organic matter. The products released by the metabolic activity of the bacteria vary from complex to the simplest molecules. Some of these products accumulate in the soil and may be toxic, antibiotic, or inhibitory to plant-parasitic nematodes. During natural decomposition of plant residues, ammonifying bacteria apparently produce enough ammonia to influence nematodes.

Other compounds like hydrogen sulfate and ammonia produced by bacteria have also been found to have deleterious effects on *Hirschmanniella oryzae* in rice fields and root-knot nematodes (Jacq and Fortuner 1979; Zavalata 1985). Soil bacteria like *Bacillus thuringiensis* var. *thuringiensis* (Prasad and Tilak 1972) producing butyric acid, hydrogen sulfide, cyanide, and exotoxins, have been demonstrated to be antagonistic to nematodes. Ammonia produced by ammonifying bacteria during decomposition of nitrogenous organic materials can result in reduced nematode populations in soil (Rodriguez-Kabana 1986).

13.2.2.1 Cry-Protein-Forming Bacteria

Bacillus thuringiensis, a spore-forming aerobic, Gram-positive bacterium belonging to the genus *Bacillus*, is considered a potential biocontrol agent. More than 200 isolates of *B. thuringiensis* have been grouped into more than 12 stereotypes. The classification was done by combination of *Heterodera antigens* (stereotypes) and biotypes, particularly the esterase types (Norris 1964). *B. thuringiensis* occurs in the dead matter of insects, litter of sericulture form, and soils. Chahal and Chahal (1991) perhaps for the first time investigated *B. thuringiensis* toxic to eggs and larvae of *Meloidogyne* sp. Chahal and Chahal (1999) examined the effect of different strains of *B. thuringiensis* on wheat galls and on egg masses of *Meloidogyne incognita*. The result showed a drastic inhibition of egg masses and death of all J_2S of *M. incognita*. The gelatinous matrix of egg mass was disintegrated due to bacterial action which might be due to the ability of bacteria to produce enzyme chitinase (Chigaleichik 1976), an enzyme which hydrolyze chitin present in the egg shell and gelatinous matrix of egg masses (Spiegel and Cohn 1985), thereby affecting the permeability.

Bird and McClure (1976) and Ignoffo and Dropkin (1977) reported that a thermostable toxic of *B. thuringiensis* was found to be toxic to population *Meloidogyne*, *Panagrellus*, and *Aphelenchus* and prevented *M. incognita* juveniles from forming galls on tomato roots. *B. thuringiensis* (Bt) produces one or more parasporal crystal inclusions (Cry or d-endotoxins), which are known to be toxic to a wide range of insect species in the orders *Lepidoptera* (butterflies and moths), *Diptera* (flies and mosquitoes), *Coleoptera* (beetles and weevils), and *Hymenoptera* (wasps and bees) (Schenpe et al. 1998; Maagd et al. 2001). Some Cry proteins are also toxic to other invertebrates such as nematodes, mites, and protozoans

(Feitelson et al. 1992). To date, there are six Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21) known to be toxic to larvae of a number of free-living or parasitic nematodes (Alejandra et al. 1998; Crickmore et al. 1998; Marroquin et al. 2000; Wei et al. 2003; Kotze et al. 2005).

On the basis of amino acid sequence homology, these nematode-affecting Cry proteins (except for Cry6A) were assigned to a single cluster in the main Cry lineage, parallel to other main groups (Bravo 1997; Marroquin et al. 2000). Separate phylogenetic analysis of the three domains of Cry protein also generated a consensus tree result. The domain I and domain II trees showed that nematode-specific toxins (Cry5, Cry12, Cry13, Cry14, and Cry21) were arranged together in a single branch (Bravo 1997). Domain III from all the nematode-specific toxin trees are also clustered together (Bravo 1997). Nematicidal and insecticidal toxins of Bt are believed to share similar modes of action. Cry toxicity is directed against the intestinal epithelial cells of the midgut and leads to vacuole and pore formation, pitting, and eventual degradation of the intestine (Marroquin et al. 2000). The binding of pore-forming toxin to a receptor in the epithelial cell is a major event. In order to determine host receptors, a mutagenesis screen was performed with the genetically well-characterized nematode *Caenorhabditis elegans*. A detailed understanding of how the Bt toxins interact with nematodes should facilitate the production of more effective Bt biocontrol agents.

Other than Cry toxin, previous studies using *B. thuringiensis israelensis*, *B. thuringiensis kurstaki*, and another parasporal-crystal-forming bacterium, *B. sphaericus*, showed that some strains had significant activity on the eggs and larvae of the parasitic nematode *Trichostrongylus colubriformis* (Bottjer et al. 1986; Bowen et al. 1986a, b; Bowen and Tinelli 1987; Meadows et al. 1989). The toxicities of these strains were inhibited by antibiotics and neither correspond to the sporulation phase of the bacteria nor to their resistance to alkaline pH and heat, demonstrating that the pathogenic factors were not the parasporal crystal (Bottjer et al. 1986; Bowen et al. 1986a, b; Bowen and Tinelli 1987; Meadows et al. 1989). Subsequently, an unknown Bt isolate was also reported to have toxicity to root-lesion nematodes (Bradfish et al. 1991). However, the pathogenic factors of this strain have not been discovered.

13.2.2.2 Rhizobacteria

Rhizospheric bacteria mainly fluorescent *Pseudomonas* (Oostendrop and Sikora 1989; Spiegel et al. 1991) and certain others like *B. subtilis* and *B. cereus* (Oka et al. 1993), *B. sphaericus* (Racke and Sikora 1992), *Anthrobacter* (Kloepper et al. 1988), *Scroratia* (Kloepper et al. 1988), and *Agrobacterium* (Racke and Sikora 1992) play an important role in biocontrol of plant-parasitic nematodes. The rhizobacteria usually comprise a complex assemblage of species with many different modes of action in the soil (Siddiqui and Mahmood 1999). Rhizobacteria reduce nematode populations mainly by regulating nematode behavior (Sikora and Hoffmann-Hergarten 1993), interfering with plant–nematode recognition (Oostendorp and

Sikora 1990), competing for essential nutrients (Oostendorp and Sikora 1990), promoting plant growth (El-Nagdi and Youssef 2004), inducing systemic resistance (Hasky-Gunther et al. 1998), or directly antagonizing by means of the production of toxins, enzymes, and other metabolic products (Siddiqui and Mahmood 1999). Most rhizobacteria act against plant-parasitic nematodes by means of metabolic by-products, enzymes, and toxins. The effects of these toxins include the suppression of nematode reproduction, egg hatching, and juvenile survival, as well as direct killing of nematodes (Zuckerman and Jasson 1984; Siddiqui and Mahmood 1999). There are two commercial bionematicidal agents based on *Bacillus* species. Through a PGPR research program of the ARS (Agricultural Research Service, USA), a commercial transplant mix (Bio Yield TM, Gustafson LLC) containing *Paenibacillus macerans* and *Bacillus amyloliquefaciens* has been developed to control plant-parasitic nematodes on tomato, bell pepper, and strawberry (Meyer 2003). Another product, used in Israel, is BioNem, which contains 3 % lyophilized *Bacillus firmus* spores and 97 % nontoxic additives (plant and animal extracts) to control root-knot nematodes as well as other nematodes (Giannakou and Prophetou-Athanasiadou 2004). In extensive testing on vegetable crops (tomato, cucumber, pepper, garlic, and herbs), BioNem preplant applications significantly reduced nematode populations and root infestation (galling index), resulting in an overall increase in yield (Giannakou and Prophetou-Athanasiadou 2004). BioNem showed a higher effectiveness against root-knot nematodes in the field than did *Pas. penetrans*.

However, the excellent biocontrol effects of BioNem can be partially attributed to the stimulating effect that the animal and plant additives contained in the bionematicide formulation have on the microbial community of the rhizosphere. Previous studies have shown that the addition of manure or other organic amendments stimulates the activity of the indigenous soil microbial community (Giannakou and Prophetou-Athanasiadou 2004).

13.2.2.3 *Pseudomonas fluorescens*

In many crop–pathogen systems, the primary mechanism of biocontrol by fluorescent pseudomonads is production of HCN and antibiotics such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin, pyrrolnitrin, and phenazines, playing an important role in biocontrol of pathogens (Défago et al. 1990). It is not clear exactly how the plant-growth-promoting properties of *P. fluorescens* are achieved; theories include that the bacteria might induce systemic resistance in the host plant, so it can better resist attack by a true pathogen; the bacteria might outcompete other (pathogenic) soil microbes, e.g., by siderophores giving a competitive advantage at scavenging for iron; and the bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanide.

P. fluorescens produces some siderophores (iron-chelating substances) which act as growth factors and disease-suppressive siderophores like pseudoactin which can

presumably deliver iron to plants they benefit; otherwise, these plants would develop iron chlorosis and become susceptible to pathogens (Leong 1986). Rhizosphere *Pseudomonas* strains also exhibit diverse pathogenic mechanisms upon interaction with nematodes (Spiegel et al. 1991; Kloepper et al. 1992; Kluepfel et al. 1993; Westcott and Kluepfel 1993; Cronin et al. 1997a; Jayakumar et al. 2002; Siddiqui and Shaukat 2002, 2003a, b; Andreogloua et al. 2003; Siddiqui and Singh 2005). The mechanisms employed by some *Pseudomonas* strains to reduce the plant-parasitic nematode population have been studied. These mechanisms include the production of antibiotics and the induction of systemic resistance (Spiegel et al. 1991; Cronin et al. 1997a; Siddiqui and Shaukat 2002, 2003a, b).

P. fluorescens controlled cyst nematode juveniles by producing several secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG) which reduces juvenile mobility (Cronin et al. 1997a; Siddiqui and Shaukat 2003a, b). Additionally, mortality of root-knot and cyst nematode juveniles in culture filtrates of *P. fluorescens* has also been observed (Gokta and Swarup 1988). Mena and Pimentel (2002) reported that *Corynebacterium paurometabolum* inhibited nematode egg hatching by producing hydrogen sulfide and chitinase. Some other rhizobacteria reduce deleterious organisms and create an environment more favorable for plant growth by producing compounds such as antibiotics or hydrogen cyanide (Zuckerman and Jasson 1984). Recently, rhizobacteria-mediated induced systemic resistance (ISR) in plants has been shown to be active against nematode pests (Van Loon et al. 1998; Ramamoorthy et al. 2001). Plant-growth-promoting rhizobacteria (PGPR) can bring about ISR by fortifying the physical and mechanical strength of the cell wall by means of cell wall thickening, deposition of newly formed callose, and accumulation of phenolic compounds. They also change the physiological and biochemical ability of the host to promote the synthesis of defense chemicals against the challenge pathogen (e.g., by the accumulation of pathogenesis-related proteins, increased chitinase and peroxidase activity, and synthesis of phytoalexin and other secondary metabolites) (Van Loon et al. 1998; Siddiqui and Mahmood 1999; Ramamoorthy et al. 2001). Bacterial determinants of ISR include lipopolysaccharides (LPSs), siderophores, and salicylic acid (SA) (Van Loon et al. 1998; Ramamoorthy et al. 2001).

The mechanism involved in resistance development seems to be directly related to nematode recognition and penetration of the root (Reitz et al. 2001; Mahdy et al. 2001). However, Siddiqui and Shaukat (2004) found that SA-negative or SA-overproducing mutants induced systemic resistance to an extent similar to that caused by the wild-type bacteria in tomato plants. They concluded that fluorescent pseudomonads induced systemic resistance against nematodes by means of a signal transduction pathway, which is independent of SA accumulation in roots. Except for the nematophagous fungi and actinomycetes, rhizobacteria are the only group of microorganisms in which biological nematicides have been reported. Ganeshan and Kumar (2005) used *Pseudomonas fluorescens* as a potential biopesticide for augmentative biological control of many diseases of agricultural and horticultural importance. Biological control by plant-growth-promoting fluorescent pseudomonad*s* protects the plant from pathogens by activating defense

genes encoding chitinase, 1,3 glucanase, and peroxidase (Chen et al. 2000). *P. fluorescens* strain PF-1 was toxic to *R. reniformis*, with all tested concentration exhibiting toxic effects (Jayakumar et al. 2002).

Plant growth promotion by rhizobacteria can effect directly (Glick 1995; Presello-Cartieaux 2003) by fixation of nitrogen, solubilization of minerals, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (auxin, cytokinin, gibberellins, ethylene, or abscisic acid) that enhance plant growth at various stages of development, whereas indirect growth promotions occur when PGPR promotes plant growth by improving growth restricting conditions (Glick et al. 1995). Shanti et al. (1998) reported suppression in nematode multiplication (root-knot) in grapevine root even after 8 months (second-generation crop) with application of *P. fluorescens*. Fluorescent pseudomonad*s* have received much attention as biocontrol agents because they generally act through direct antagonism to pathogens, through antibiotic production, through competition with pathogen, or more directly through plant growth promotion (Gamlial and Katan 1993).

13.2.2.4 *Pseudomonas aeruginosa*

Siddiqui and Shaukat (2003a, b) reported on biocontrol agents *Pseudomonas aeruginosa* IE-6 and IE-6S$^+$ (previously shown to suppress several soil-borne plant pathogens) on soil microfungi and plant-parasitic nematodes as well as on the root-knot development and growth of tomato (*Lycopersicon esculentum*). The biocontrol agents significantly reduced root-knot development and enhanced shoot growth of tomato over the controls.

Gulnaz et al. (2008) used *P. aeruginosa* and *B. japonicum* alone or with mineral fertilizers significantly reduced infection of tomato roots by the root-rotting fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium solani*. Use of *P. aeruginosa* or *B. japonicum* alone or with mineral fertilizers suppressed the root-knot nematode *M. javanica* by reducing numbers of galls on roots, nematode establishment in roots, and nematode populations in soil. The tallest plants and maximum shoot fresh weight occurred due to treatment with *P. aeruginosa*. Siddiqui and Akhtar (2007) found that *P. aeruginosa* reduced galling and nematode multiplication the most followed by *A. awamori and G. intraradices*. Combined inoculation of these microorganisms caused the greatest increase in plant growth and reduced the root-rot index more than individual inoculations. Pathogens adversely effected root colonization by *G. intraradices*. However, root colonization and root nodulation were increased when co-inoculated with *P. aeruginosa* and *A. awamori* whether in the presence or absence of pathogens.

13.2.2.5 *Bacillus subtilis*

Numerous *Bacillus* strains can suppress pests and pathogens of plants and promote plant growth. Some species are pathogens of nematodes (Gokta and Swarup 1988;

Li et al. 2005). The most thoroughly studied is probably *B. subtilis* (Krebs et al. 1998; Siddiqui and Mahmood 1999; Siddiqui and Shaukat 2002). In addition, a number of studies have reported direct antagonism by other *Bacillus* spp. towards plant-parasitic nematode species belonging to the genera *Meloidogyne*, *Heterodera*, and *Rotylenchulus* (Gokta and Swarup 1988; Kloepper et al. 1992; Madamba et al. 1999; Siddiqui and Mahmood 1999; Insunza et al. 2002; Kokalis-Burelle et al. 2002; Meyer 2003; Giannakou and Prophetou-Athanasiadou 2004; Li et al. 2005).

B. subtilis improved plant growth by inhibiting nonparasitizing root pathogens, producing biologically active substances or by transforming unavailable minerals and organic compounds into forms available to plants (Broadbent et al. 1997). El-Hassan and Gowen (2006) found that the formulation of *B. subtilis* decreased the severity by reducing colonization of plants by pathogen, promoting their growth, and increasing the dry weight of lentil pea. *B. subtilis* is not a nematode parasite, but it has a high degree of larvicidal property (Siddiqui and Mahmood 1995a), and it produces many biological active substances. Gokta and Swarup (1988) also reported that isolates of *B. subtilis* and *B. pumilus* were found most effective against *M. incognita*, *H. cajani*, *H. zeae*, and *H. avenae*.

Other rhizobacteria reported to show antagonistic effects against nematodes include members of the genera *Actinomycetes*, *Agrobacterium*, *Arthrobacter*, *Alcaligenes*, *Aureobacterium*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Chromobacterium*, *Clavibacter*, *Clostridium*, *Comamonas*, *Corynebacterium*, *Curtobacterium*, *Desulforibtio*, *Enterobacter*, *Flavobacterium*, *Gluconobacter*, *Hydrogenophaga*, *Klebsiella*, *Methylobacterium*, *Phyllobacterium*, *Phingobacterium*, *Rhizobium*, *Serratia*, *Stenotrotrophomonas*, and *Variovorax* (Tables 13.1 and 13.2; Fig. 13.1) (Jacq and Fortuner 1979; Kloepper et al. 1992; Racke and Sikora 1992; Guo et al. 1996; Cronin et al. 1997b; Duponnois et al. 1999; Neipp and Becker 1999; Siddiqui and Mahmood 1999, 2001; Meyer et al. 2001; Mahdy et al. 2001; Hallmann et al. 2001; Insunza et al. 2002; Khan et al. 2002; Mena and Pimentel 2002; Meyer 2003).

13.2.2.6 *Azotobacter*

Another bacterium, *Azotobacter*, is an aerobic, nonsymbiotic Gram-negative nitrogen-fixing bacteria, which occurs in most of the cultivated soil, is gaining importance in controlling phytoparasitic nematodes. Verma and Bansal (1996) showed the inhibitory effect of *A. chroococcum* on hatching of *M. javanica*. Racke and Sikora (1992) found that out of 179 bacterial isolates isolated from roots and cysts, only six caused a significant reduction (>25 %) in *Globodera pallida* penetration of potato roots. Six of these isolates caused significant reductions in repeated greenhouse tests. The antagonistic activity was shown to be directly correlated with the number of colony-forming units (cfu) present on the tuber. The isolates *Agrobacterium radiobacter* and *Bacillus sphaericus* at densities of 9.7×10^8 and 3.16×10^9 cfu ml^{-1}, respectively, caused significant reductions in root infection of 24–41 % in repeated experiments.

Table 13.1 Antagonistic rhizosphere for the control of phytonematodes

Biotic agent	Nematode sp.	Crop	Reference
Bacillus licheniformis	*M. incognita*	–	Siddiqui and Hussain (1991)
Pseudomonas mendocina	*M. incognita*	–	Siddiqui and Hussain (1991)
Bacillus subtilis	*M. incognita*	–	Gokta and Swarup (1988)
B. pumilus, *B. cereus Pseudomonas* sp.	*H. cajani*, *H. zeae*, *H. avenae*		
P. fluorescens	*H. avenae*	Wheat	Kamra and Dhawan (1997)
P. fluorescens	*M. incognita*	Tomato	Verma et al. (1999)
P. stutzeri	*M. incognita*	Tomato	Khan and Tarannum (1999)
B. subtilis, *P. fluorescens*	*M. incognita*	Tomato	Santhi and Sivakumar (1995)
P. fluorescens	*M. incognita*	Black pepper	Eapen et al. (1997)
P. fluorescens	*H. cajani*	Pigeon pea	Siddiqui and Mahmood (1995a, b)
B. subtilis, *Bradyrhizobium japonicum*	*H. cajani*	Pigeon pea	Siddiqui and Mahmood (1995a)
P. fluorescens	*Hirschmanniella gracilis*	Paddy	Ramakrishnan and Sivakumar (1999)
B. subtilis	*M. incognita*	Chickpea	Siddiqui and Mahmood (1993)
P. fluorescens	*H. cajani*	Black gram	Latha and Shivakumar (1998)
P. fluorescens	*Tylenchulus semipenetrans*	Sweet orange and lime	Santhi et al. (1999)
P. fluorescens	*Globodera* sp.	Potato	Mani et al. (1998)

Ali (1996) found that the population density of nematode species was reduced by application of five bacterial isolates (*Arthrobacterium* spp., *Bacillus* spp., *Corynebacterium* spp., *Serratia* spp., and *Streptomyces* spp.). Reductions of nematode populations were ranged between 46 % and 100 %. Youssef et al. (1998) studied the potential of *A. chroococcum*, *Bacillus megaterium*, and *Rhizobium lupine* for the control of *M. incognita* infecting cowpea and tomato plants. They noticed a number of both root galls and egg masses of *M. incognita* were decreased in soil treated with *B. megatherium* and *A. chroococcum* except *R. lupine*-treated soil. El-Sherif et al. (1995) studied the effect of culture filtrates of 5 isolates for their nematotoxic effect against plant-parasitic nematode (*Bacillus* spp., *Corynebacterium* spp., *Serratia* spp., *Arthrobacterium* spp., and *Streptomyces* spp.). The authors determined the culture filtrate concentration as 0.1 % to inhibit the hatching of the eggs and 0.6 % to be highly toxic to the juveniles. The toxic effect of the filtrate varied with the different nematode species.

Siddiqui and Futai (2009) studied the effects of antagonistic fungi (*Aspergillus niger* v. Teigh, *Paecilomyces lilacinus* (Thom) Samson, and *Penicillium chrysogenum*

Table 13.2 Mode of action of various bacterial groups

Nematophagous bacterial group	Genus and species	Target nematodes	Pathogenic effects on nematodes	Action mode
Parasitic bacteria	Four species: *Pasteuria penetrans*, *P. thornei*, *P. nishizawae*, *Candidatus Pasteuria* usgae	323 Nematode species of 116 genera	Major economic important plant-parasitic nematodes have been observed to be parasitized by *Pasteuria*	Parasitism
Opportunistic parasitic bacteria	*Bacillus nematocida* (Bacillus sp. B16), *Brevibacillus laterosporus*, *Bacillus* sp. RH219, etc.	*Panagrellus redivivus* and *Bursaphelenchus xylophilus*	*Br. laterosporus* strain G4 could penetrate the nematode cuticles and eventually digest the target organism in the laboratory	Parasitism, production of enzymes and toxin
Rhizobacteria	Distribution in more than 29 genera, *Bacillus* (more than 15 species) and *Pseudomonas* (more than 11 species) are two of the most dominant populations	Reduce nematode populations in soil	Different rhizobacteria showed different degrees of suppression on nematodes in various conditions Three commercial bionematicides from bacteria all belong to this group	Interfering with recognition, production of toxin, nutrient competition, plant-growth promotion; induction of systemic resistance
Parasporal-crystal-forming bacteria	*Bacillus thuringiensis* (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21)	*Trichostrongylus colubriformis*, *Caenorhabditis elegans*, and *Nippostrongylus brasiliensis*	These Cry proteins showed toxicity to larval stages of free-living and parasitic nematodes	Cry proteins caused damage to the intestines of nematodes
Endophytic bacteria	The majority of *rhizobacteria* can also be identified as endophytic bacteria	Root-knot nematode and root-lesion nematode, etc.	Suppress root-knot nematodes and root-lesion nematode, etc.	Rhizobacteria and endophytic bacteria use some of the same mechanisms
Symbiotic bacteria of entomopathogenic nematodes	Two genera: *Xenorhabdus* and *Photorhabdus*	*Bursaphelenchus xylophilus*, *M. incognita*, and their eggs	Toxic to juveniles of root-knot and pinewood nematodes and inhibit egg hatch	Toxin production (ammonia, indole, and stilbene derivatives)

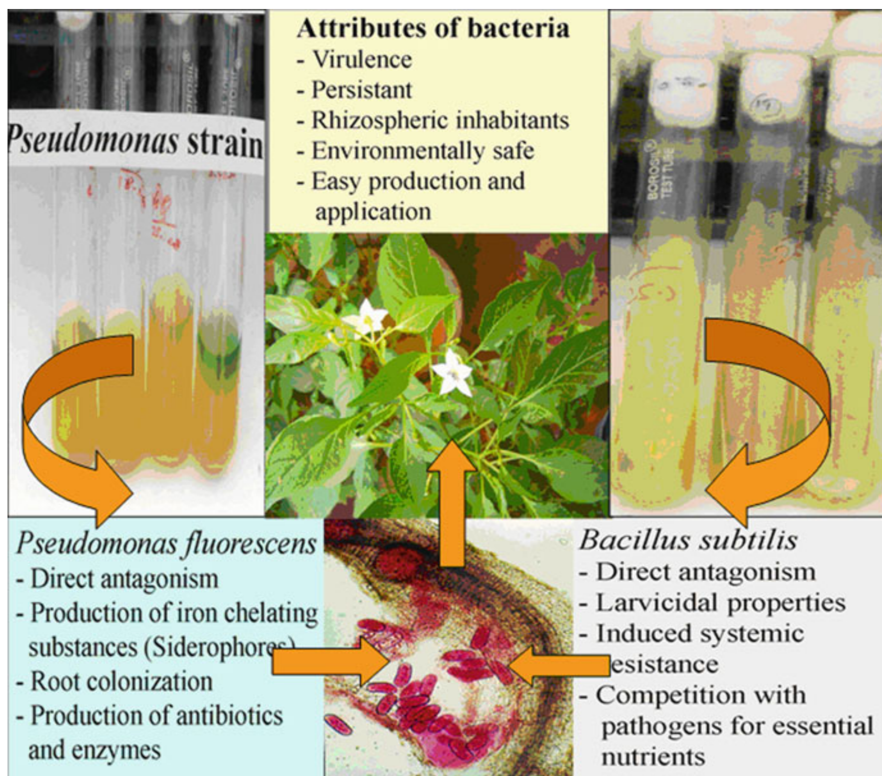

Fig. 13.1 Mechanism of biocontrol by *Pseudomonas fluorescens* and *Bacillus subtilis*

Thom), and plant-growth-promoting rhizobacteria (PGPR) (*A. chroococcum* Beijer, *B. subtilis* (Ehrenberg) Cohn, and *Pseudomonas putida* (Trev.) Mig.) were assessed with cattle manure on the growth of tomato and on the reproduction of *M. incognita* (Kof. and White) Chitwood. Application of antagonistic fungi and PGPR alone and in combination with cattle manure resulted in a significant increase in the growth of nematode-inoculated plants. Siddiqui (2004) conducted glasshouse experiments to assess the influence of *P. fluorescens*, *A. chroococcum*, and *Azospirillum brasilense* and composted organic fertilizers (cow dung, horse dung, goat dung, and poultry manure) alone and in combination on the multiplication of *M. incognita* and growth of tomato.

13.2.3 Other Soil Bacteria: Rhizobia

Nodulation is a complex symbiotic process between host plant and *Rhizobia*. For successful nodulation, the *Rhizobia* must multiply to a sufficient population level

and colonize the rhizosphere before making contact with the legume roots. Subsequently, the bacteria attach themselves to roots and penetrate root hairs and stimulate formation of nodules. This process can be disrupted by biotic stresses on either host plant or *Rhizobia*. Survival and colonization of *Rhizobia* in the rhizosphere are greatly influenced by root exudation of host plants (Bhagwat and Thomos 1982). Carbohydrates, amino acids, and a variety of nutrients by soybean roots as root exudates. Among amino acids, a variety of nutrients are released by soybean roots as root exudates. Among amino acids, tryptophan is easily converted by *Rhizobium* to indoleacetic acid that stimulates the formation and elongation of root hairs. This facilitates the bacteria to enter soybean roots via epidermal cells of root hairs and initiate the bacterial nodulation (Barker and Hussey 1976). However, plant-parasitic nematodes have been shown to alter quality and quantity of root exudates of infected plants (Wang and Bergeson 1974). These changes have an impact on the efficacy of tryptophan in formation and elongation of root hairs.

Plant lectins are the specific carbohydrate-binding proteins, constituting approximately 10 % of the extractable nitrogen in the seeds of leguminous plants and have been extensively used in the study of cell surface architecture. Earlier work on lectin distribution in plant tissues as well as lectin-mediated cell–cell interactions provides strong evidence for their involvement in the defense of plants against infection and also in *Rhizobium*–legume symbiosis. During the symbiotic biological nitrogen fixation, the bacteria of the genus *Rhizobium* living in the rhizospheric region of the leguminous plants adhere to the legume roots and are subsequently internalized to form nitrogen-fixing nodules. The *Rhizobium*–legume interactions are specific, and the specificity is achieved through the action of plant lectins.

It has been demonstrated that the lectin in beans extract could help bind the specific bacteria to the roots of *Phaseolus vulgaris*. Systematic studies in this direction were subsequently made in soybean–*Rhizobium japonicum* and clover–*Rhizobium trifolii* systems (Musarrat and Akhtar 2000). Plant lectins extruded by soybean roots are proteins capable of binding sugar or sugar containing proteins. Several studies suggest that *Rhizobia* bind to soybean roots via soybean lectins on the root surface (Bohlool and Schimt 1974). Soybean cyst nematode *Heterodera glycines* may affect the bacterial binding sites on the root to limit bacterial establishment for nodulation. An interaction between root surface lectins and surface carbohydrates of the nematode may be prerequisite for the nematode penetration (Zuckerman and Jasson 1984). *Rhizobium japonicum* cells also bind with soybean lectins (Balasubramanium 1971). Hence, there is a competition between *Meloidogyne* spp. and *R. japonicum* for binding to soybean root surface lectin. It also causes reduction of bacterial nodulation. Few studies have assessed the effect of *Meloidogyne* infection on bacterial nodulation of legume crops, and these studies have shown that *Meloidogyne* spp. have retarded the development of root system and the bacterial nodulation of legume crops (Balasubramanium 1971; Huang et al. 1984).

The presence of sugars such as *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, and mannose and/or glucose on the cuticle surface of plant-parasitic nematodes may play an important role in the interaction between

nematodes and their hosts. It has been demonstrated that the binding of *Rhizobia* to nematode-free roots was inhibited only after pretreatment with certain sugars. Studies on the interference of nematodes with soybean lectin metabolism showed the reduced binding of *Rhizobia* to *H. glycines*-infected soybean roots, suppressing the nodule formation. Furthermore, the root-knot nematodes *M. incognita* infecting mungbean, chickpea, cowpea, wandopea, and green gram; *M. hapla* infecting white clover; and *Meloidogyne* spp. infecting horsebean, lupin, clover, and pea have been reported to inhibit nodulation. Interrelationship between *M. incognita*, *Heterodera cajani*, and *Rhizobium* sp. on cowpea (*Vigna sinensis*) has been investigated. Hussaini and Seshadri (1975) reported that *M. incognita* and *H. cajani*, singly or in concomitant inoculum, significantly reduce the growth of cowpea; *M. incognita* reduced N-content to a greater extent than *H. cajani*. Similarly, Hussaini and Seshadri (1975) reported that *M. incognita* inoculated before and after or simultaneously with *Rhizobium* caused significant decrease in plant height, fresh and dry weight of shoot and root, number of nodules on root, and nitrogen content of root when compared to nematode-free plants. Presumably, the common sugars on the cuticle surface of nematodes compete for the plant lectins, resulting in reduced rhizobial binding sites (Musarrat and Akhtar 2000).

The association of rhizobia with legume hosts has a beneficial effect on plant nutrition and growth. In contrast, the plant–nematode relationship has adverse effects on plant growth. The role of plant-parasitic nematodes on rhizobial nodulation and nitrogen fixation of host plants has been reviewed by a number of workers (Huang 1987; Khan 1993). As a result of nematode infection, the nodulation and nitrogen fixation has been reported to be suppressed (Hussaini and Seshadri 1975), or stimulated (Hussey and Barker 1976), or remain unaffected (Taha and Raski 1969). The role of rhizobia in the control of plant diseases of various leguminous crops has already been discussed (Sawada 1982), and biological control of plant diseases is now increasingly capturing the imagination of plant pathologists (Papavizas and Lumsden 1980). Some of the possible reasons for the reduced nematode reproduction caused by root-nodule bacteria are physiological and biochemical changes, change in host nutrition, and histopathological numbers.

13.2.3.1 Physiological and Biochemical Changes

The root-nodule bacteria which fix atmospheric nitrogen are reported to produce toxic metabolites inhibitory to many plant pathogens (Haque and Gaffar 1993). *Rhizobium japonicum* secretes rhizobitoxine, which is inhibitory to charcoal root fungus *Macrophomina phaseolina* (Chakraborty and Purkayastha 1984). Chakraborty and Chakraborty (1989) reported an increased level of phytoalexin (4-hydroxy-2,3,9-trimethoxypterocarpan) when pea seeds were bacterized with *R. leguminosarum* prior to inoculation with *Fusarium solani* f. sp. *pisi*. This phytoalexin may have an important role in cross-protection against many pathogens. Siddiqui and Mahmood (1994) observed higher activity of peroxidase, nitrate reductase, and catalase in pigeon pea plants inoculated with *Bradyrhizobium*

japonicum than in plants without *B. japonicum*. An increase in peroxidase activity due to *B. japonicum* inoculation indicates increasing resistance of the plant because it catalyzes the polymerization of phenolic compounds and forms cross-links between extensin, lignin, and feruloylated polysaccharides (Siddiqui and Mahmood 1994). An increase in nitrate reductase and catalase may be correlated with the rate of protein synthesis and resistance of the plant to pathogens, respectively (Siddiqui and Mahmood 1994). Roslycky (1967) reported production of an antibiotic bacteriocin by rhizobia bacteria. Some properties of antibiotics of rhizobia bacteria have also been reported by others (Drapeau et al. 1973; Schwinghamer and Belkengren 1968; Tu 1978, 1988). Antibiotics and phytoalexin produced by rhizobia bacteria probably reduce damage from nematodes and other pathogens.

13.2.3.2 Change in Host Nutrition

Damage to plant growth by nematodes can be lessened by the application of nitrogen fertilizer (NH_4, NO_3) (Ross 1969), indicating that combined nitrogen can improve growth of diseased plants. Combined nitrogen, such as nitrate, at a high level is a powerful inhibitor of nodulation (Dart 1977) and also has an adverse effect on the development of nematodes (Barker et al. 1972). Barker and Huisingh (1970) observed necrosis in nodular tissues following invasion by nematodes; this may in part account for reduced nematode development. All this suggests that application of rhizobia bacteria which increase nitrogen content and plant growth can also reduce nematode populations.

13.2.3.3 Histopathological Changes

Endo (1964) indicated that nematodes, especially males, often caused plant necrosis and degeneration of syncytia as the nematodes matured. Endo (1965) found that nematodes induced much necrosis in resistant plants. Some reactions that he observed were very similar to those of nodular tissues where the surrounding tissues, as well as the nematode, died. This type of reaction may partially explain the reduced number of nematodes obtained when nematodes and *Rhizobium* were added simultaneously to soybean (Barker et al. 1971). Sharma and Sethi (1976) reported that both the nematodes, namely, *M. incognita* and *H. cajani*, either singly or in combination, significantly reduced the growth of cowpea and addition of *Rhizobia* tended to reduce this damage to some extent.

Mishra et al. (1994) reported improved plant growth in *R. leguminosarum*-inoculated *Phaseolus aureus* L. plant as compared to reniform-nematode-infected plant. Datal and Bhatti (2002) studied the interaction between *H. cajani* and *Rhizobium* in different combinations and revealed that alone or prior addition of *Rhizobium* enhanced nodulation but reduced multiplication on mungbean and

cluster bean. Sharma and Sethi (1976) and Khan and Hussain (1990) reported that the addition of rhizobia tends to reduce the damage caused to the host plant in combined inoculation of phtyoparasitic nematodes.

Siddiqui and Singh (2005) conducted glasshouse experiments to assess the ash amendments (0, 20, and 40 % with soil), a phosphate-solubilizing microorganism *Pseudomonas striata* and a root-nodule bacterium *Rhizobium* species on the reproduction of root-knot nematode *M. incognita* along with the growth and transpiration of pea. Amendments of fly ash with soil had no effect on transpiration. However, *M. incognita* reduced the rate of transpiration from first week onward after inoculation, while inoculation of *Rhizobium* sp and *P. striata* increased transpiration from first week onward after their inoculation both in nematode-inoculated and nematode uninoculated plants. *Rhizobium* sp. had greater adverse effect on galling and nematode multiplication than *P. striata*. Use of both organisms together had greater adverse effect on galling and nematode multiplication than caused by either of them alone. Highest reduction in galling and nematode multiplication was observed when both organisms were used in 40 % fly-ash-amended soil.

13.2.4 Other Nematophagous Bacterial Groups: Endophytic Bacteria

Endophytic bacteria have been found internally in root tissue, where they persist in most plant species. They have been found in fruits and vegetables, and are present in both stems and roots, but do no harm to the plant (McInory and Kloepper 1995; Hallmann et al. 1997, 1999; Azevedo et al. 2000; Hallmann 2001; Surette et al. 2003). They have been shown to promote plant growth and to inhibit disease development and nematode pests (Sturz and Matheson 1996; Hallmann et al. 1999; Azevedo et al. 2000; Munif et al. 2000; Shaukat et al. 2002; Sturz and Kimpinski 2004). For example, Munif et al. (2000) screened endophytic bacteria isolated from tomato roots under greenhouse conditions. They found antagonistic properties towards *M. incognita* in 21 out of 181 endophytic bacteria. Several bacterial species have also been found to possess activity against root-lesion nematode (*Pratylenchus penetrans*) in soil around the root zone of potatoes. Among them, *Microbacterium esteraomaticum* and *Kocuria varians* have been shown to play a role in root-lesion nematode suppression through the attenuation of host proliferation, without incurring any yield reduction (Munif et al. 2000). Despite their different ecological niches, rhizobacteria and endophytic bacteria display some of the same mechanisms for promoting plant growth and controlling phytopathogens, such as competition for an ecological niche or a substrate, production of inhibitory chemicals, and induction of systemic resistance (ISR) in host plants (Hallmann 2001; Compant et al. 2005).

Symbionts of entomopathogenic nematodes *Xenorhabdus* spp. and *Photorhabdus* spp. are bacterial symbionts of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabdus* spp., respectively (Paul et al. 1981). They have been thought to contribute to the symbiotic association by killing the insect and providing a suitable nutrient environment for nematode reproduction (Boenare et al. 1997). In recent years, a potentially antagonistic effect of the symbiotic complex on plant-parasitic nematodes has been reported (Bird and Bird 1986; Grewal et al. 1997, 1999; Perry et al. 1998; Lewis et al. 2001). Further investigation demonstrated that the symbiotic bacteria seemed to be responsible for the plant-parasitic nematode suppression via the production of defensive compounds (Samaliev et al. 2000). To date, three types of secondary metabolites from symbiotic bacteria have been identified as nematicidal agent: ammonia, indole, and stilbene derivatives (Hu et al. 1995, 1996, 1997, 1999). They were toxic to second-stage juveniles of root-knot nematode (*M. incognita*) and to fourth-stage juveniles and adults of pinewood (Bursaphelenchus xylophilus) and inhibited egg hatching of *M. incognita* (Hu et al. 1999).

13.3 Some Important Molecular Genetic Techniques Used in Studying Bacterial Pathogenesis in Nematodes

A number of bacteria have been shown to exhibit a variety of effects on nematodes in natural environments and laboratory conditions. However, studies on the mechanisms of bacterial pathogenicity have lagged behind those assessing their roles in biological control and resource potential. Over the past few years, a number of molecular genetic methods in bacterial pathogenicity have been developed, and it is now possible to introduce these successful techniques to the study of bacterial pathogenesis in plant-parasitic nematodes (Hensel and Holden 1996; Aballav and Ausube 2002; Tan 2002; Barker 2003). Although some technologies have been reported not to be successful in studying plant-parasitic nematodes, knowledge from studying bacterial pathogens of *C. elegans* and other animal pathogens may enhance knowledge of bacterial pathogenesis in plant-parasitic nematodes and provide a basic methodology for studies on plant-parasitic nematodes.

Reverse genetics is a common approach in identifying and determining functions of virulence determinants. This method involves the isolation of virulence proteins involved in pathogenicity and cloning of the corresponding genes. Mutational analysis, this tool can be divided into directed and random mutagenesis. In directed mutagenesis, a putative virulence determinant encoding a gene postulated to be responsible for a certain pathogenic trait is disrupted or replaced to construct a mutant strain. Comparative genomics, this technique can identify pathogenic genes by comparing genomic sequences of pathogenic and nonpathogenic strains or other sequences from strains of interest of the same genus.

13.4 Conclusion and Future Perspectives

Over the past 20 years, a large number of studies have been undertaken to investigate the use of microorganisms as biocontrol agents against nematode pests. All these groups of bacteria have undoubtedly generated a lot of interest in acting as natural enemies and for their role in biological control of phytoparasitic nematodes. However, the major constraints in the development of effective biocontrol agents have been the mass production, storage and distribution of fresh materials, and effect of abiotic factors like pH, moisture, and soil types which influence the activities of these microbial biopesticides, host range, and virulence of the inoculum. For instance, the major attributes which favor *Pas. penetrans* as a successful biocontrol agent are long viability of spores, resistance to heat and desiccation, persistence in soil, compatibility with chemical nematicides, nontoxicity to plants and other soil biota, and easy storage, but major hurdles are the following: lack of a technique enabling culture of the bacterium in vitro on any of standard biological media; neither vegetative cells nor spores of the organism can be harvested in sufficient numbers to test extensively in laboratory conditions or to infest soil in large-scale field tests to determine its influence; and with the currently available methods of mass multiplication, its commercial use may be limited to glasshouse crops or horticultural crops only, and more research is required to be conducted in order to exploit important aspects of bacterium–nematode interaction with particular emphasis on the mechanism of action for the control of plant pathogens and nematodes.

Only a few commercial biocontrol products from the bacteria with nematicidal potentials have been developed and used in the agriculture system (Whipps and Davies 2000; Gardener 2004; Schisler et al. 2004). The development of biocontrol agents is often unpredictable and too variable for large-scale implementation (Meyer 2003). No matter how well suited a commercial nematode antagonist is to a target host in a laboratory test, in order to realize ideal biocontrol effects in practice, an intensive exploration of the mechanisms of the antagonist against nematode populations and a thorough understanding of the interactions among biocontrol strains, nematode target, soil microbial community, plant, and environment must be developed.

An increased understanding of the molecular basis of the various bacterial pathogenic mechanisms on nematodes not only will lead to a rational nematode management decision but also could potentially lead to the development of new biological control strategies for plant-parasitic nematodes. For example, it has been recognized that the attraction between bacteria and their hosts is governed by chemotactic factors emanating from the hosts or pathogens (Zuckerman and Jasson 1984). Knowledge of these mechanisms could be used to attract or target nematodes intentionally by modified nematicidal bacteria or to regulate nematode populations by the chemotactic factors produced by these nematophagous bacteria.

Advances in molecular biology have allowed us to obtain important information concerning molecular mechanisms of action, such as the production of nematotoxins, the signaling pathways that induce the host-plant defense mechanism, and the

infection process. Such information should provide novel approaches to improve the efficacy of nematophagous bacteria for biological control applications, to increasing the expression of toxins or enzymes from the microorganisms, and for formulation of commercial nematicidal agents. For example, the developing genomic–bioinformatic approach may help to solve the difficulty of culturing the nematode parasite *Pasteuria* in vitro. This may allow mass production of spores for commercial use.

Microorganisms as biocontrol agents have a relatively narrow spectrum of activity compared with synthetic pesticides (Barker 1991; Janisiewicz 1996) and often exhibit inconsistent performance in practical agriculture. Application of a mixture of inoculated biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity. A good colonization capacity and compatibility of inoculated microorganisms constitutes an important prerequisite for successful development of biocontrol (Barker 1990). Phosphate-solubilizing microorganisms improved the growth of plants possibly through an inhibitory effect on nematode development as reported by Becker et al. (1988), Kloepper et al. (1992) and Hasseb et al. (2005). Pseudomonads may improve plant growth by suppressing parasitic and nonparasitic root pathogens (Oostendorp and Sikora 1990) by the production of biologically active substances (Gamlial and Katan 1993) or by converting unavailable minerals and organic compounds into forms available to plants (Broadbent et al. 1997; Siddiqui and Mahmood 1999). *Bacillus* and *Pseudomonas* are known to suppress diseases by inhibition of pathogens by competition of Fe (III), inhibition of pathogen by diffusible or volatile products, induction of resistance in plants, and aggressive root colonization and stimulation of plant growth (Kloepper et al. 1988; Weller 1988; Siddiqui and Mahmood 1999). Similarly, the presence of rhizobia in the rhizosphere presumably protects the host roots from pathogens, besides fixing atmospheric nitrogen. The use of these symbionts will reduce the damage without use of chemical pesticides, which are costly and have health hazards. Therefore, using a consortium of rhizobia and other phosphate-solubilizing microorganisms such as fluorescent *Pseudomonas* and *Bacillus* species could provide a better solution against phytonematodes.

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Chapter 14
Rhizobacteria for Management of Nematode Disease in Plants

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14.1 Introduction

Plant-parasitic nematodes form an important niche in agro-ecosystems. They feed on plant organ as ectoparasites, semiparasites, and endoparasites and cause reduction in plant productivity. About 2,500 species are known to parasitize the lower as well as higher plants at global level. In India, so far, about 600 nematode species belonging to about 85 genera have either been described or reported to be associated with more than 700 plant species growing in diverse agro-climatic

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zones. Many of these have been proven to be highly pathogenic causing considerable economic yield losses in cereals, pulses, vegetables, fruits, ornamentals, and forest crops. Almost every crop is being parasitized by one or more plant pathogenic nematode species.

Nematodes live in soil and interact with various other plant pathogens, many of which cause plant diseases (*Ditylenchus*, *Tylenchorhynchus*, *Rotylenchulus*, *Heterodera*, *Meloidogyne*, *Paratylenchus*, *Tylenchulus*, *and Trichodorus*).The high cost of conventional nematicides; withdrawal of nematicides from the market due to detrimental side effects such as residual effect, contamination of ground water, and their harmful effects on nontarget species, including humans; and the development of nematode-resistant host cultivars further aggrieved the situation of nematode control (Jatala 1986; Kerry 1995) and led to an increase in interest in biological control of plant-parasitic nematodes. Interest in biological control arose in 1920s and 1930s, when some plant pathogens were suppressed by introducing some antibiotic-producing microbes to the natural habitats. DeBach (1964) had defined biological control as the "action of parasites, predators and other pathogens in maintaining other organism's population density at a lower average than would occur in their absence." The definition of biological control was further elaborated by Garrett (1965), "any condition under which, or practice where by survival or activity of a pathogen is reduced" through the agency of any other living organisms, results in the reduction of disease incidence. On the other hand, Beirner (1967) proposed, "biological control is the induced or natural, direct or indirect limitation of harmful organism or its effect by another organism or group of organisms." But the most comprehensive definition of biological control is "the reduction of inoculum density or disease producing activities of pathogen or parasite in its active or dormant state, by one or more organisms, host or antagonists or by mass interaction of one or more antagonists" (Baker and Cook 1974).

14.2 Nematode Diseases in Plants

Nematodes feed on all parts of the plant, including roots, stems, leaves, flowers, and seeds. Nematodes feed from plants in a variety of ways, but all use a specialized spear called a stylet. Note the differences in stylet length and shape (Fig. 14.1). The size and shape of the stylet is used to classify nematodes and also can be used to infer their mode of feeding. All three nematodes in Fig. 14.1 are ectoparasites, but *Belonolaimus* and *Longidorus* feed deep within the roots using their long stylets, while *Helicotylenchus* feeds on the exterior of the root or partially burrows into the root to feed using its short stout stylet. Some nematodes do not kill the plant cells they feed upon but "trick" the plant cells to enlarge and grow, thus producing one or more nutrient-rich feeding cells for the nematode. These feeding cells enable long-term feeding associations and form by repeated nuclear division in the absence of cell division (giant cells) or by the incorporation of adjacent cells into a syncytium formed by the breakdown of neighboring cell walls. Collectively, nematodes can feed on

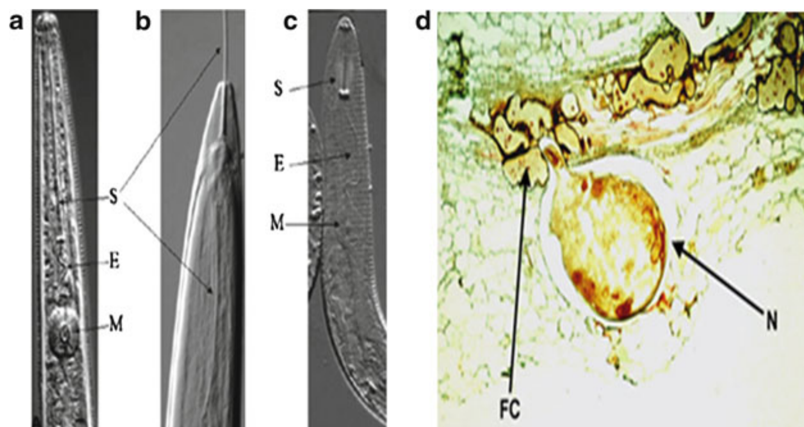

Fig. 14.1 Plant-parasitic nematode, (**a**), (**b**), and (**c**) shows ectoparasitic nematodes that feed deep within the roots using their long stylets, while (**d**) shows photomicrograph of the feeding cells of *Meloidogyne* (root-knot nematode)

almost any plant cell type and form a variety of feeding cell types. The number of feeding cells can vary from one to a half dozen depending on the nematode species. Many plant-parasitic nematodes feed on the roots of plants. The feeding process damages the plant's root system and reduces the plant's ability to absorb water and nutrients. Typical nematode damage symptoms are a reduction of root mass, a distortion of root structure, and/or enlargement of the roots. Nematode damage of the plant's root system also provides an opportunity for other plant pathogens to invade the root and thus further weakens the plant. Direct damage to plant tissues by shoot-feeding nematodes includes reduced vigor, distortion of plant parts, and death of infected tissues depending upon the nematode species.

Root-knot nematodes are plant-parasitic nematodes from the genus *Meloidogyne*. *Meloidogyne incognita* is widely distributed in India (Khan 1997). In addition to direct effect, it helps several fungi and bacteria by predisposing host plants for greater pathogenic damage (Webster 1985). According to Webster (1985), in a disease complex, nematode acts as a primary pathogen and modifies the host in a way that it becomes more vulnerable for the invasion by secondary pathogen. Association of *M. incognita* not only aggravates the wilt severity but also breaks the resistance of cultivars against *F. oxysporum* such as seen with the disease complex of pigeon pea involving *M. incognita* and *F. oxysporum*.

It has long been apparent that different populations of the root-knot nematode varied in their pathogenicity to different species of crop plant, but it was not until 1949 that B.G. Chitwood described five separate species of the root-knot nematode, at the same time separating them from the genus *Heterodera* and placing them in the genus *Meloidogyne* which now comprises of more than a dozen species (Roberts 1995). At present, it contains species affecting all the major vegetable crops by forming a gall- or tumor-like structure in the root system. This is why it is called as root-knot nematode.

Trivedi and Mathur (1985) studied the life cycle of *M. incognita* on chilies and observed that second stage juveniles penetrated the roots within 24 h after inoculation through the terminal end apart 1 cm of root. The juveniles after penetration migrated intercellularly through root cortex. They observed that penetrated juveniles became sedentary 3 days after penetration. Second molt of L_2S occurred 7 days after inoculation forming third stage juveniles whereas fourth occurred in 21 days produced young female. The ovipositing females were observed in 27 days and males in 24 days, and emergence of second generation juveniles took place in 42 days. Root penetration by L_2S of *M. incognita* and *M. javanica* in resistant germplasm was significantly less than the susceptible cultivars of the vegetables. The second stage juveniles of *Meloidogyne* spp. penetrate the plant root apex without inducing any necrosis of host cells (Nemec 1910). However, the other regions of root are not immune to attack (Christie 1936). Castillo et al. (1973) noted superimposed second, third, and fourth molting of root-knot nematode in peanut after 13 days of inoculation. On the other hand, Davies and Fisher (1976) reported on the factors influencing penetration, behavior, low inoculum density, exposure duration, temperature, distance of juvenile from root tips, and number of available root tips which were considered important determinants and observed positive correlation between number of juveniles penetrating and increase in inoculum density, exposure time, and number of root tips available.

The presence of galls on the root system is the primary symptom associated with *Meloidogyne* infection. In galls formed by one female, a swelling of the central cylinder, highly deformed fascular elements, and the spherical part of the female surrounded the parenchyma can be easily observed in stained roots. The size and form depends on the species involved, number of nematodes in the tissue, host, and plant. When plants are severely infected by *Meloidogyne*, the normal root system is reduced to limited number of severely galled roots with a completely disorganized vascular system. Rootlets are almost completely absent. The roots are seriously hampered in their functions of uptake and transport of water and nutrients. Plants wilt rapidly under growing conditions and are often stunted. Although roots are the main plant attacked by these nematodes, it has been shown by many workers that leaves and stems are also attacked by root-knot nematodes (Wong and Willetts 1969).

Meloidogyne spp. has been reported to parasitize and cause knots or galls on the root of almost all pulse crops grown in India. Their pathogenicity has been proved in chickpea, cowpea, mung bean, black gram, pea, lentil, soybean, and other pulse by several workers (Mani and Sethi 1984; Reddy et al. 1979; Nath et al. 1979; Alam et al. 1985; Gupta et al. 1986; Bhagwati and Phukan 1991). Hussaini and Seshadri (1975) showed that the infection of *M. incognita* caused significant decrease in heights of plants, fresh and dry weight of shoot and root, and number of nodules in primary and secondary root systems of *Phaseolus aureus* and in nitrogen content of the shoot and root. Sen and Dasgupta (1977) published an additional list of hosts of root-knot nematodes *M. incognita* causing root-knot on *Curcuma amada*, *Acroclinium roseum*, *Phlox drummondii*, *Linum grandifolium*, *Paper somniferum*, *Veridium decurrens*, and *Chrysanthemum coronarium*.

Various workers observed that penetration occurred within 24 h in chili, fenugreek, cumin, soybean, and coriander, respectively (Trivedi and Mathur 1985; Sharma 1989 and Midha 1990). Earlier, Krusberg and Nelsen (1958) observed that in roots of sweet potato, the larvae penetrated at the apex as well as away from the apex. Nematode entrance through hair cells has also been observed (Wyss et al. 1992). Khan and Khan (1991) studied penetration and post penetration development of *M. incognita* and *M. javanica* in susceptible and resistant vegetable crops such as cabbage, cauliflower, cucumber, pepper, and tomato. Significant differences in rate of juvenile penetration and development of juveniles into mature females were found between susceptible and resistant germplasm.

Pandey (1992) found numerous genera of plant-parasitic nematodes, viz., *Meloidogyne* (94.23%), *Tylenchorhynchus* (71.15%), *Helicotylenchus* (53.84%), *Rotylenchulus* (59.61%), and *Heterodera* (48.07%) associated with *Capsicum annum*. Growth reduction in plants due to *M. incognita* was also reported by several workers (Kalita and Phukan 1993; Fazal et al. 1996 and 3–4 more upto 2007). Significant decrease in early vegetative and late reproductive plant parameters were observed at the inoculum level of 1,000 J_2/kg soil (damaging threshold level) supported by Mahapatra et al. (1999). On the other hand, Anver and Alam (1994) screened 25 accessions of *Cicer arietinum* for their reaction to *M. incognita*. Significant loss in plant weight in most of the test accessions of chickpea was noticed due to the root-knot nematode. Mahapatra et al. (1999) found that the J_2 of *M. incognita* started penetrating roots of blackgram as early as 12 h after inoculation and continued up to 6 days. Sharma et al. (2000) conducted an experiment to find out the number of days required by *M. incognita* to complete its life cycle on *Arachis hypogea*. Under the experimental conditions, the root-knot nematode completed its life cycle from J_2 to the formation of adult female with egg mass in 29 days. Further, Mohanty et al. (1997) described that *M. incognita* besides being pathogenic to green gram was found to interfere with the functioning of nodules and normal root biochemistry. The total chlorophyll contents, leghemoglobin, and nitrogenase activities were also lowered in nematode-infected plants.

Goyal and Trivedi (1999) used freshly hatched J_2 in different lots of 10, 100, 1,000, and 10,000 per lot and recorded root, shoot length, and their fresh and dry weights to find out economic threshold level. They inferred that number of galls increased corresponding that of increasing inoculum level. Significant reduction in the number of nodules was also observed at the inoculum density of 1,000 (J_2)/kg second stage juveniles. Such adverse effects on nodulation in black gram were observed by Verdejo et al. (1988). Singh and Goswami (2000) applied 10, 100, 1,000, and 10,000 nematodes (J_2 stage) per 500 g soil. Significant plant growth reduction was observed with an initial population of 1,000 nematode per 500 g soil which was established as potential pathogenic level of *M. incognita*. Williams-Woodward et al. (2000) evaluated resistance to peanut root-knot nematode infestation on seven dwarf holly cultivars and one Japanese boxwood (*Buxus microphylla* var. *japonica*) cultivar in two separate field tests. One week after planting, the soil surrounding each plant was inoculated with 8,000 *M. incognita* or *M. arenaria* eggs per plant in 8 ml of water. The highest gall was indexed rating for *M. arenaria* infestation followed by *M. incognita*. A nonpolar, heat-stable product from root

exudates of legumes (Factor N) is known to trigger a transient, reversible state of quiescence in certain plant-parasitic and free-living nematodes. Effect of root exudates on the mobility and infectivity of *M. incognita*, alfalfa, cucumber, and other plants produced root tip exudates that induced quiescence in *M. incognita*, *Ditylenchus dipsaci*, *Caenorhabditis elegans*, and *S. glaseri*.

Singh and Trivedi (2007) noted wide distribution of nematodes *M. incognita*, *Hoplolaimus* sp., *Tylenchorhynchus* sp., and *H. cajani* in the surveyed areas and their association not only with *Vigna mungo* L. but also with other crops which were under mix cropping with *Vigna mungo* L. Patel and Dave (2000) revealed that *M. incognita* and *F. oxysporum* either individually or in combination reduced chickpea length and fresh root and shoot weights significantly, but the reduction was more due to *M. incognita* as compared to *F. oxysporum*. Simultaneous inoculation of both the pathogens had maximum suppressive effect on growth of chickpea plants as compared to preceding or succeeding inoculation of fungus and nematodes. Root galling and nematode multiplications on chickpea were maximum when nematodes were inoculated alone, but it was reduced in the presence of fungus. The incubation period for fungal disease development was reduced, and severity of disease increased when root-knot nematode was present with fungus. Among various pests and diseases, nematode–fungus disease complex particularly of *M. incognita* and *F. oxysporum* posed great problem to the cultivation of pulse crops (Perveen et al. 1999; Mahapatra and Swain 2001). In India, Shukla and Haseeb (2002) conducted survey of farmer's field, growing pigeon pea at different localities of Uttar Pradesh (India). Establishment of wilt disease complex of *Fusarium udum*, with *Heterodera/Meloidogyne* spp., was observed as the most severe problem in this survey.

Sharma (2004) assessed the occurrence and severity of *Meloidogyne–Fusarium* complex and found 4% to 52% damage to cumin crop. Desaeger et al. (2005) evaluated the effects of inoculation of *M. javanica* and rhizobial strains on nematode infections, nodulation, and growth of *Sesbania sesban* (L.) Merr. Heavy nematode infection was noted and plant growth was affected negatively at high nematode inoculum level. Few big and irregular galls were also recorded on infected roots, and root-knot and cyst infestation had a pronounced effect on rhizobial nodule formation. The nematode larvae interfered in formation of nodules by bacteria, and reduction of nodule size due to interaction between rhizobial and nematode population was observed by number of workers (Singh and Goswami 2000; Deka et al. 2003). To conclude this section, the nematode disease in plants and associated disease complex is a severe problem affecting many crops and attributes to yield losses as observed by reviewing the literature available depicting works by several researchers in this field.

14.3 Use of Chemicals in Control of Nematode Diseases

Nematicides used in control of root-knot nematodes are either fumigants or non-fumigants. In most cases, the fumigants are broad spectrum contact nematicides effective against juveniles and eggs as well as fungal pathogens and weeds.

Non-fumigant nematicides have either contact or systemic activity. For instance, carbofuran belonging to the carbamates group is a non-fumigant and had been reported to cure root-knot nematodes on various crops (Butool et al. 1998). It mainly affects nematode neuromuscular activity by inhibiting the enzyme cholinesterase resulting in reduced movement and ability of invasion and multiplication (Wright 1981).

Nematicides can be applied effectively by surface and drip irrigation (Johnson 1985). The fumigant metham-sodium was effective in controlling root-knot nematodes through drip irrigation (Roberts 1988). Jones and Overman (1976) found that carbofuran consistently controlled sting, stubby, and root-knot nematodes. Johnson (1978) revealed that when phenamiphos and carbofuran were applied through water in a sprinkler irrigation system, yields of marketable tomato transplants were increased by 50% and 35%, respectively, as compared with yields from non-treated plots. While Ethoprop was applied in similar manner, severe stunting in tomato transplants occurred, and it was found that yields of marketable transplants were reduced by 67% below the yields from non-treated plots. All nematicides significantly reduced the number of root-knot nematodes. Khan and Alam (1985) revealed that aldicarb or carbofuran at 100 and 1,000 ppm were highly toxic to *M. incognita*, and it inhibited larval hatching when applied to tomato seedlings. Whitehead (1986) briefly discussed the various methods of applying the fumigant and non-fumigant nematicides through seed, seed furrow, crop row, seed bed, planting hole, localized, top soil, and top soil treatment.

Maqbool et al. (1987) tested the influence of different concentrations of $NaCl_2 \cdot 2H_2O$ on juvenile hatching and found that juvenile hatching was inversely proportional to the salt concentration. Rahman et al. (1988) reported that pesticides, viz., Dimecron and Metasystox-R showed highly significant inhibitory effect on juvenile hatching of the root-knot nematode, *M. incognita*. The chemicals also brought about high enhancement in mortality of the juveniles; however, interestingly, metasystox-R was more effective than Dimecron. Use of nematicides has been found as an effective means to control nematodes all over the world. A few nematicides like aldicarb, Furadan, and Tenekil have been used for control of nematodes, but sometimes, contradictory results have been found (Hussain et al. 1993; Maqbool et al. 1987; Qamar et al. 1985). Khan et al. (1991) found that Tenekil was less effective as compared with Furadan and more effective than aldicarb in reducing nematode populations. Qamar et al. (1985) observed that Tenekil was more effective than aldicarb and Furadan, whereas maximum reduction in gall formation and greater shoot weight was observed with Furadan than aldicarb, Tenikil, or phenomiphos as also observed by Hussain et al. (1993). Cucumber rootstocks which have shown resistance to soil-borne diseases were tested to reveal any resistance/tolerance to root-knot nematodes, and integration of these rootstocks with nematicides was investigated. Metham-sodium and 1,3-dichloropropene (1,3-D) provided significantly good control of nematode populations when their application was followed by non-fumigant nematicide such as cadusafos or oxamyl (Ioannis and Karpouzas 2001).

Winter and Macpherson (2002) stated that low doses of the acetylcholinesterase-inhibiting carbamate nematicides disrupt chemoreception in plant-parasitic nematodes. Fluorescein isothiocyanate (FITC)/dextran conjugates up to 12 kDa are taken up from the external medium by certain chemosensory neurons in *Caenorhabditis elegans*. Similar chemoreceptive neurons of the non-feeding infective stage of *Heterodera glycines* (soybean cyst nematode) fill with FITC, and the nuclei of their cell bodies selectively stain with bisbenzimide. The widely used nematicide aldicarb disrupts the chemoreceptive response of *H. glycines* with 50% inhibition at very low concentrations (ca. 1 pM), some 10.6-fold lower than required to affect locomotion. Nematode control in the large commercial banana plantations is currently based on the application of two to four nematicide treatments per year for the best control.

Nematicides have some limitations as most of the nematicides have been found to be volatile and toxic having poor target specificity leading to ground water contamination or atmospheric zone depletion. In modern era, continuing problems by the use of nematicides have introduced a sense of urgency into research for alternative methods of nematodes management (Thomason 1987). Moreover, use of methyl bromide, a soil fumigant, is being restricted because of this chemical's deleterious effects on stratospheric ozone concentrations. Several products, some of which are currently used as soil fumigants, are being considered as possible replacements for methyl bromide, alone and in various combinations. Among these, 1,3-dichloropropene, methyl isothiocyanate generators such as metham-sodium and chloropicrin are currently registered, while others such as methyl iodide and sodium azide are at different stages of the registration process (Luis 2005). To conclude this section, it is imperative that chemical control of nematodes be supplemented or completely replaced by organic or biological means to overcome the damage caused by synthetic agents.

14.4 Organic Material in Control of Nematode Disease

Considerable progress has been made in the utilization of organic materials as soil amendments for the control of plant-parasitic nematodes (Singh and Sitaramaiah 1966; Muller and Gooch 1982; Akhtar and Alam 1993; Akhtar and Mahmood 1993; Akhtar 1999). Various kinds of organic and inorganic wastes have been shown to reduce nematode populations (Walia and Gupta 1995; Addabbo and Sassanelli 1998; Jonathan et al. 2000; Khan et al. 2001). To maintain a low inoculums load by continuous application of systemic nematicide alone is not practical for the control of disease. To cope with this, neem (*A. indica*) seed powder and other bio-agents may be applied in suitable formulation. The seed powder of neem was found to be effective against the plant-parasitic nematodes and their disease complex involving pathogenic fungi. This may be due to the presence of an array of complex compounds, triterpenes, or more specifically limonoids (Singh and Sitaramaiah 1970).

The incorporation of organic material into the soil reduces nematode disease (Muller and Gooch 1982). A wide range of oil cakes, sawdust, urea, and bagasse have been used with some success (Singh and Sitaramaiah 1966; Sikora et al. 1973). Chitin in combination with waste products from the paper industry has been used to reduce nematode disease (Culbreath et al. 1985). Pillai and Desai (1976) stated after his observation that undecomposed cake of *Callophyllum inophyllam* gave best control of *M. javanica* on tobacco, but cake allowed to be decomposed for 15 days before application gave better plant growth. Nematode control was directly proportional to the quantity of cake added. Haseeb et al. (1977) observed highest reduction in nematode population when soil was amended with chopped leaves of *Calotropis procera* while *Iserin herbstii* was highly effective in promoting the growth of egg plant. Muller and Gooch (1982) reduced nematode population and increased yield by applying organic amendments such as sawdust, compost, green manure, and chicken manure. Akhtar and Alam (1984) showed that the soil amendments (chopped flowers of different plants) brought about reduction in population of all genera of plant-parasitic nematodes. Maqbool et al. (1987) found that the incorporation of chopped shoots of latex-bearing plants significantly suppressed the population buildup of *Rotylenchulus reniformis*, *Tylenchorhynchus brassicae*, and root-knot nematode.

Akhtar and Alam (1990) showed that agro-wastes of some harvested crop, viz., marigold, mustard, and sunflower showed highly significant inhibitory effect on development of root-knot nematode and population of other plant-parasitic nematodes. Marigold plant residues were more effective followed by mustard and sunflower. Moreover, the increasing doses showed better results, and the growth of one of the cultivated crops of potato plants was improved with treated soil. In addition to their suppressive effects on root-disease complex, organic amendments improve soil structure and water holding capacity, but a large quantity is generally needed, which is the major hindrance in its popularization with farmers (McSolrey and Ghallar 1995).

14.5 Rhizobacteria in Management of Nematode Disease

Rhizobacteria are a subset of total rhizosphere bacteria which have the capacity, upon reintroduction to seed or vegetative plant parts (such as potato seed pieces), to colonize the developing root system in the presence of competing soil microflora (Kloepper et al. 1999). The premier example of rhizobacterial biocontrol agents occurs in many genera including *Actinoplanes*, *Agrobacterium*, *Alcaligenes*, *Amorphosporangium*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Cellulomonas*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Micromonospora*, *Pseudomonas*, *Rhizobia*, *Serratia*, *Streptomyces*, *Xanthomonas*, etc. as stated by large number of microbiologists (Kloepper et al. 1989; Tang 1994; Weller and Thomashow 1994; Glick et al. 1995; Lucy et al. 2004). The rhizobacteria are also inhibiting phytoparasitic nematodes that occur in many genera including *Pseudomonas*

(Oostendorp and Sikora 1989; Spiegel et al. 1991a, b), *Bacillus subtilis*, *B. cereus* (Oka et al. 1993), *B. sphaericus* (Racke and Sikora 1992), *Arthrobacter* (Kloepper et al. 1988), *Serratia* (Kloepper et al. 1988), and *Agrobacterium* (Racke and Sikora 1992).

Specific rhizobacteria have the ability to improve plant growth and/or root health (Kloepper et al. 1980; Suslow and Schroth 1982; Schippers et al. 1987; Sikora 1988; Weller 1988; Schroth and Hancock 1992). A key factor of all PGPR is that they all colonize seed and root or behave as endophytes. Such traits are desirable for considering them suitable for biocontrol activity (Lugtenberg and Bloemberg 2004; Compant et al. 2005). The significance of PGPR bacteria has been reported by several workers, viz., Deshwal et al. (2003), Compant et al. (2005), Ester et al. (2004), Kumar et al. (2005a, b), Gupta (2006), Kumar (2006) and Kokalis-Burelle et al. (2006).

Soil bacteria like *Clostridium butyricum* (Hollis and Rodriguez-Kabana 1966, 1967), *Disulfovibrio desulficans* (Rodriguez-Kabana et al. 1965), *Chromobacterium* sp. (Wilt and Smith 1970), and *Bacillus thuringiensis* var. thuringiensis (Prasad and Tilak 1972) producing butyric acid, hydrogen sulfide, cyanide, and exotoxins, respectively, have been demonstrated to be antagonistic to nematodes. *Bacillus thuringiensis* suppressed root-knot nematodes in soil under natural conditions (Prasad and Tilak 1972). In vitro studies established that the exotoxin produced by the bacterium during growth and sporulation had high nematicidal activity against juveniles and eggs of *Meloidogyne* spp. (Prasad and Tilak 1972). Among the rhizobacteria, *Pseudomonas* spp. have been investigated for antagonism to nematodes more extensively than others and found effective against *H. cajani*, *H. avenae* and *H. zeae* (Gokte and Swarup 1988; Kumar et al. 2005b), *H. schachtii* (Oostendorp and Sikora 1989), and *Meloidogyne* spp. (Gokte and Swarup 1988; Kumar et al. 2005a).

Fluorescent *Pseudomonas spp*. are among the most effective rhizosphere bacteria in reducing soil-borne diseases in disease-suppressive soils (Weller 1988), where disease incidence is low, despite the presence of pathogens and environmental conditions conductive to disease prevalence. *Pseudomonas* isolates caused greater inhibitory effect on hatching and penetration of *M. incognita* than caused by isolates of *Bacillus* (Siddiqui et al. 2009). It was reported by Siddiqui et al. (2005) that *P. fluorescens* CHAO mutant resulted in reduced biocontrol activity against the root-knot nematode *M. incognita* during tomato and soybean infection. Exposure of root-knot nematode to culture filtrates of *P. fluorescens* under in vitro conditions significantly reduced egg hatch and caused substantial mortality of *M. javanica* juveniles (Siddiqui and Shaukat 2003).

Ali (1996) found that the population density of nematode species was reduced by application of five bacterial isolates (*Arthrobacterium* sp., *Bacillus* sp., *Corynebacterium* sp., *Serratia* sp., and *Streptomyces* sp.). Reductions of nematode populations were ranged between 46% and 100%. Nematode multiplication reduced in the presence of *Rhizobium* in some legume plants (Siddiqui and Mahmood 1995b). *Rhizobium etli* G12 has been repeatedly demonstrated to be capable of suppressing

Table 14.1 Effect of rhizobacteria in combination with other biocontrol agent on nematode reproduction and plant growth

Management combination	Nematodes	Effect of nematodes and plant growth	References
Bacillus subtilis + *Bradyrhizobium japonicum* + *Glomus fasciculatum*	*Heterodera cajani*	Application of all the three management agents resulted in greater plant growth and greater reduction in nematode multiplication	Siddiqui and Mahmood (1995c)
Pseudomonas fluorescens + *Glomus mosseae* in different soil types	*M. javanica*	Reduced nematode multiplication and morphometrics of root-knot females in different soil types	Siddiqui and Mahmood (1998)
Bacillus subtilis + *P. lilacinus*	*M. incognita* race 3	Combined application improved plant growth and reduced nematode multiplication more than individual inoculation on chickpea	Siddiqui and Mahmood (1993)
Bacillus subtilis + *P. lilacinus* + *Eicchornia crassipes*	*M. incognita*	Application of all the three together resulted in greater reduction in nematode multiplication	Gautam et al. (1995)
Bacillus subtilis + culture filtrate of fungi	*M. incognita* race 3	Increase in plant growth and reduction in nematode multiplication was greater when *B. subtilis* was used with *A. niger* or these two combined with other filtrates	Siddiqui and Mahmood (1995a)
Bacillus licheniformis + *Pseudomonas mindocina* + *Acrophialophora fusispora* + *Aspergillus flavus*	*M. incognita*	Combination of all four biocontrol agents was best for reducing root-knot index. *P. mindocina* had adverse effect on plant growth while others increased plant growth	Siddiqui and Hussain (1991)

early infection by the potato cyst nematode *Globodera pallida* and the root-knot nematode *M. incognita* (Hallmann et al. 2001) (Table 14.1).

Corynebcterium flaccumfaciens (Schuster 1959) and *Pseudomonas solanacearum* (Reddy et al. 1979) were observed to control *M. incognita*. Earlier, *Bacillus thuringensis* prevented *M. incognita* from forming galls on tomato (Ignoffo and Dropkin 1977). Brown et al. (1985) described *Bacillus penetrans* to be pathogenic organism against *M. incognita* under field conditions in which tobacco and soybean were planted. Both tobacco and soybean showed increasing yields with decreasing

pathogenicity of *M. incognita*. It was interesting to note that *B. penetrans* reduced yield loss caused by *M. incognita* by 23% for tobacco and 35–55% for soybean. However, Jansson and Nordbring-Hertz (1988) examined role of nematophagous fungi in trapping nematodes. Both rhizobacteria and endophytic bacteria showed their potential to reduce plant damage due to ecto- and endoparasitic nematodes such as *Criconemella xenoplex* (Kluepfel et al. 1993), *M. incognita* (Becker et al. 1988), *Heterodera schachtii* (Oostendorp and Sikora 1989), *H. glycines* (Kloepper et al. 1992), *Globodera pallida* (Racke and Sikora 1985), and *Pratylenchus penetrans* (Hackenberg et al. 1997). Gokte and Swarup (1988) showed that *Bacillus subtilis* and *B. pumilus* were most effective against *M. incognita*. Their noncellular extract exhibited a high degree of larvicidal properties.

Different methods have been used to control nematodes, the most used cultural practices being solarization, chemical, and biological control. Biological control of nematodes has been developed successfully during the last few years. PGPR belonging to *Pseudomonas* and *Bacillus* spp. has also been used in bioantagonism (Aksoy and Mennan 2004). There has been large body of literature describing potential uses of PGPR against control of plant-parasitic nematodes of different crops (Zavaleta-Mejia and Van Gundy 1982; Becker et al. 1988; Oostendorp and Sikora 1989; Kloepper et al. 1992; Insunza et al. 1999) (Tables 14.2 and 14.3).

For many pseudomonads, production of metabolites such as antibiotics, siderophores, and hydrogen cyanide (HCN) is the primary mechanism of biocontrol (Weller and Thomashow 1994). *Pseudomonas* produce a polar substance heat labile, sensitive to extreme pH values causing in vitro juvenile mortality of *M. javanica* (Ali et al. 2002). Moreover, many strains of pseudomonads can indirectly protect the plants by inducing systemic resistance against various and disease (Mazzola et al. 1992). Fluorescent pseudomonads play a critical role in naturally occurring soil that is suppressive to plant disease (Siddiqui and Shaukat 2004).

Crude suspensions of twenty-two *Bacillus* spp. were evaluated for their potentiality in the control of *M. incognita* and *Tylenchulus semipenetran* (Ismail and Fadel 1997). These treatments exhibited higher mortality of both the nematode juveniles than water. In an experiment when different laboratory formulations of *B. thruingiensis* were tested against *T. semipenetrans* under field conditions, there was positive correlation between reduction percentage in nematode population and doses of tested isolates. Studies to assess the ability of *P. fluorescens* to control the cyst nematodes were conducted by Aksoy and Mennan (2004); these bacteria showed inhibitory effect on egg hatching. Moreover, *P. aeruginosa*, when used as seed or soil drench for the control of *M. javanica* in mung bean under glasshouse conditions, significantly reduced nematode population and gall formation (Nasima et al. 2002).

Biological control promises to be vast option against pathogens. Over the last decades, a great diversity of rhizospheric microorganisms has been described, characterized, and in many cases tested for activity as biocontrol agent against soil pathogens. Such microorganism can produce substances that may limit the damage caused by pathogens, e.g., by producing antibiotics, siderophores, and a variety of enzymes. These microorganisms can also function as competitors of pathogens for colonization sites and nutrients.

Table 14.2 Effect of rhizobacteria on nematode control

Bacteria	Nematode	Effect of bacteria on nematode	References
(A) *Pseudomonas* sp.			
P. aureofaciens	*Criconemella xenoplax*	One strain inhibited nematode multiplication in green house test	Westcott and Kluepfel (1993)
P. fluorescens	*Panagrellus* sp.	Bacteria cultivated in plant count broth for 24 h at 30 °C reduce nematode up to 57.4 %	Weidenborner and Kunz (1993)
P. solanacearum	*R. reniformis*	Resulted in slight inhibition of nematode activity on aubergine roots	Kermarrec et al. (1994)
P. fluorescens	*M. javanica*	Reduced nematode multiplication and morphometrics of root-knot females on tomato in different soil types	Siddiqui and Mahmood (1998)
(B) *Bacillus* sp.			
Bacillus thuringiensis	*Meloidogyne*	Prevented *M. incognita* from forming galls on tomato	Ignoffo and Dropkin (1977)
Bacillus subtilis, *B. cereus*, *B. pumilus*	*M. incognita*, *Heterodera cajani*, *H. zeae*, *H. avenae*	The most effective isolates were *Bacillus subtilis* and *B. pumilus* against all tested species. The noncellular extract exhibited a high degree of larvicidal properties	Gokte and Swarup (1988)
Bacillus subtilis	*Meloidogyne* spp. *Rotylenchulus reniformis*	Reduced nematode reproduction and galling on cotton, tomato, and peanut	Sikora (1988)
B. subtilis	*M. incognita*	Nematode multiplication was reduced on tomato in pot test	Gautam et al. (1995)
B. subtilis	*M. incognita* race 3	Greater growth in chickpea plants and reduces nematode multiplication when seeds are treated with bacteria	Siddiqui and Mahmood (1995a)
B. subtilis	*H. cajani*	Reduced multiplication of pigeon pea cyst nematode in the presence of bacteria	Siddiqui and Mahmood (1995b)
(C) *Clostridium* spp.			
Clostridium butyricum	*Tylenchorhynchus martini*	Nematicidal acids produced by the bacteria reduced nematode population	Johnston (1958)
Clostridium sp.	*T. martini*	Nematode concentrations of n-butyric acid and lesser amount of propionic acid were quickly formed in treated pots 4 days after flooding which resulted in rapid killing of nematodes	Hollis and Rodriguez-Kabana (1966)

(continued)

Table 14.2 (continued)

Bacteria	Nematode	Effect of bacteria on nematode	References
(D) *Serratia* spp.			
Serratia marcescens	*M. incognita*	Bacteria produced a volatile metabolite and were nematoxic	Zavaleta-Mejia (1985)
(E) *Agrobacterium* sp.			
Agrobacterium radiobacter	*Globodera pallida*	Resulted in reduced nematode infection by 40 % when sprayed on seed pieces of potato	Sikora et al. (1989)

Certain plant-parasitic bacteria can reduce nematode mobility while other bacteria are antagonistic and can synthesize compounds lethal to plant-parasitic nematodes (Stirling 1991; Oostendorp and Sikora 1990; Spiegel et al. 1991a, b). In a biochemical and molecular analysis of mechanisms involve in suppression of plant pathogens (fungi, bacteria, nematodes), rhizobacteria were found to produce antibiotics (phenazine, 2,4-DAPG, HCN), siderophores, and enzymes like chitinases and proteases. These bacterial metabolites were associated with inhibitory action against several pathogens and induce resistance against several pathogens, including bacteria, viruses, and nematodes (Samiyappan et al. 2003).

One of the most effective mechanisms, antagonists employ to prevent proliferation of phytopathogens, is the synthesis of antibiotics. A large number of antibiotics have been reported from different fluorescent pseudomonads including agrocin-84, agrocin-434, 2,4-diacetylphloroglucinol, herbicolin, oomycin, phenazine, pyoluteorin, pyrrolnitrin, pyrroles, etc. They have a role to play in inhibition of pathogens (Colyer and Mount 1984; Gutterson et al. 1986; James and Gutterson 1986; Kerr 1980). Fluorescent *Pseudomonas* spp. have been reported to produce 2,4-diacetylphloroglucinol (2,4-DAPG) by which they provide biological control of soil-borne pathogens and some parasites on a wide range of crops and have a key role in their suppression (Raaijmakers and Weller 2001; Weller et al. 2002; Validov et al. 2005). 2,4-DAPG has also shown to reduce juvenile mobility (Fravel 1988; Thomashow and Weller 1991; Keel 1992).

Recently, the hydrolytic enzymes have received considerable attention as these play a role in controlling diseases caused due to plant-parasitic nematodes. The enzymatic digestion or deformation of cell-wall components of nematodes occurs by chitinase and protease of several plant growth-promoting rhizobacteria (Tikhonov et al. 2002). Chitinase is the key enzyme involve in degradation of nematode cell wall since chitin is the major components of their cell wall (Andersen 1985; Mercer et al. 1992). Chitin is depolymerized by chitinase resulting in the release of ammonia, which has nematicidal activity (Spiegel et al. 1987). Addition of chitin to soil may stimulate the growth of bacteria, actinomycetes, and a limited number of fungal species with chitinolytic properties. These microorganisms may attack nematode eggs and egg masses so as to reduce nematode's

Table 14.3 The advantages and limitations of potential biological control agents with different modes of action against plant-parasitic nematodes

Type of agent	Mode of action	Comments
Rhizosphere bacteria	Toxins or modification of root exudates	Advantages: easy to culture in vitro, can be applied as seed treatments, reduce plant damage Limitations: effective for a relatively short period, activity affected by crop cultivar and nematode species, little effect on nematode multiplication
Obligate parasites *Pasteuria spp.*	Adhesive spores	Advantages: most isolates highly virulent, infective spores resistant to drying, good shelf life, reduce infectivity of nematodes, as well as fecundity Limitations: very difficult to culture in vitro, isolates are very specific, no proliferation in soil in absence of nematodes
Paecilomyces lilacinus	Hyphal penetration	Advantages: easily produced in vitro, rhizosphere competent, attacks the eggs of several nematodes species, treatment of planting material (e.g., seed tubers) can be effective Limitations: requires high soil temperature, has given variable control in range of conditions, large number of propagules (10^6/g soil) required for nematode control, some isolates are pathogenic to humans
Verticillium chlamydosporium	Hyphal penetration	Advantages: easily produced in vitro, some isolates rhizosphere competent and virulent (10^3 propagules/g soil required for nematode control), resistant resting spores produced, survives throughout growing season in soil Limitations: seed treatments ineffective; efficacy dependent on nematode species, density, and plant host
Endophytic fungi (nonpathogenic root infecting fungi and mycorrhizae)	Competition in roots and modification of root exudates	Advantages: include agents with potential to control migratory endoparasitic nematodes in roots, may improve plant growth even in absence of nematodes, reduce damage caused by wide range of nematodes and limit their multiplication, can be mass produced and formulated, could be applied to seeds or transplant material, may reduce fungal root rots Limitations: non-mycorrhizal fungi may be difficult to register as closely related to plant pathogens, efficacy affected by plant cultivar and other crops in rotation such as crucifers can reduce survival

population (Rodriguez-Kabana 1986; Spiegel et al. 1987). Tikhonov et al. (2002) also observed that the action of chitinase enzyme resulted in more severe eggshell damage. Chitin from nematode eggshells is embedded in a protein matrix and therefore, shielded from degradation by enzymes, as recently demonstrated for chitinases of entomopathogenic fungi (Wharton 1980; Andersen 1985; St. Leger et al. 1996). Tikhonov et al. (2002) observed that combined action of enzymes, protease, and chitinase resulted in more severe egg shell damage. Chitinase and

chitinolytic microorganisms play a significant role in the control of plant-parasitic nematodes following chitin amendments (Rodriguez-Kabana 1986; Spiegel et al. 1987; Mercer et al. 1992). Tian et al. (2000) isolated and characterized five chitinolytic bacterial isolates that consistently suppressed *H. glycines* when applied with a chitin substrate. Furthermore, application of both *P. lilacinus* and chitin to sterilized soil suppressed *M. incognita* population levels more than that of using the antagonists alone (Mittal et al. 1995).

Chitinase killed *Tylenchorhynchus dubius* by producing structural changes in the nematode cuticle (Miller and Sands 1977). Purified chitinase inhibited egg hatch of *Globodera rostochiensis* by up to 70% in vitro and in soil (Cronin et al. 1997). *P. chitinolytica* with strong chitinolytic activity reduced *M. incognita* infection (Spiegel et al. 1991a, b). The chitinolytic fungus, *Paecilomyces lilacinus*, destroyed nematode eggs and efficiently controlled *M. incognita* (Morgan-Jones et al. 1984). Apart from chitinases, microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes. The most compelling evidences to support microbial proteases as virulence factors have come from the studies of protease-deficient mutants (Ahman et al. 2002; Siddiqui et al. 2005; Tian et al. 2006). In nematotoxic bacteria, *Bacillus laterosporus*, the bacterium lost 57% of its nematicidal activity because of the deletion of the extracellular alkaline protease BLG4 (Tian et al. 2006).

Rhizobacteria-mediated induced systemic resistance (ISR) in plants has been shown to be active against nematode pests (Van Loon et al. 1998; Ramamoorthy and Samiyappan 2001). Plant growth-promoting rhizobacteria (PGPR) can bring about ISR by fortifying the physical and mechanical strength of the cell wall by means of cell-wall thickening, deposition of newly formed callose, and accumulation of phenolic compounds. They also change the physiological and biochemical ability of the host to promote the synthesis of defense chemicals against the challenge pathogen (e.g., by the accumulation of pathogenesis-related proteins, increased chitinase and peroxidase activity, and synthesis of phytoalexin and other secondary metabolites) (Van Loon et al. 1998; Siddiqui and Mahmood 1999; Ramamoorthy and Samiyappan 2001). Bacterial determinants of ISR include lipopolysaccharides (LPSs), siderophores, and salicylic acid (SA) (Van Loon et al. 1998; Ramamoorthy and Samiyappan 2001). The mechanism involved in resistance development seems to be directly related to nematode recognition and penetration of the root (Reitz et al. 2000). However, Siddiqui and Shaukat (2004) found that salicylic acid-negative or salicylic acid-overproducing mutants ISR to an extent similar to that caused by the wild-type bacteria in tomato plants. They concluded that fluorescent pseudomonads ISR against nematodes by means of a signal transduction pathway, which is independent of SA accumulation in roots. Pearson et al. (1999) reported that these bacteria secrete acyl homoserine lactone (AHLS) molecules having a role to play in the root colonization through quorum sensing and indirectly affect biological control (Fray 2002). *Pseudomonas* and *Bacillus* are also known to play a role by AHLS-mediated attribute that could also have an added advantage in terms of antagonist colonization and roots of plant with the impending presence of parasites. This kind of possibilities cannot be ruled out as these are

common among plant-associated bacterial species in comparison to that of soil-borne species as also evidenced by the work of Elasri et al. (2001).

Most rhizobacteria act against plant-parasitic nematodes by producing certain toxins. The effects of these toxins include the suppression of nematode reproduction, egg hatching, and juvenile survival, as well as direct killing of nematodes (Zuckerman and Jasson 1984; Siddiqui and Mahmood 1999). Strains of *B. thuringiensis Bt* can produce toxic compounds of various chemical structures and properties. Most studies confirmed that δ-endotoxin acts selectively against the larvae of some target insects (Stepanova et al. 1996). The extensive variety of *Bt* strains and the toxins that they produce permit the production of bioinsecticides using the bacteria themselves and also allow use of the toxin genes in the development of transgenic plants (Romeis et al. 2006). Mohammed et al. (2008) reported an alternative nematicidal protein from *B. thuringiensis* (*Bt*) that could be providing an effective policy for the biological control of nematodes. No single method exists that can effectively control plant-parasitic nematodes; hence, the best approach of controlling the disease complex is the use of integration of various controlling methods as also suggested by various workers (Guetsky et al. 2001; Varshney and Dwivedi 2002; Stevens et al. 2003).

14.6 Conclusion

A large number of studies have been undertaken to investigate the use of microorganisms as biocontrol agents against nematode pests. More and more bacteria have been identified as pathogens of plant-parasitic nematodes and have shown suppression effects on nematode pest populations. Rhizobacteria hold a good promise in reducing the damage caused by phytonematodes. However, research on use of rhizobacteria for nematode control has a long way to go to decide whether or not these bacteria provide practical option for nematode management. We need to identify and select the most effective strains, study their mode of action, and demonstrate their efficiency in field trials. These microorganisms are beneficial in comparison to synthetic nematicides because they are cheaper to apply and are free from environmental pollution and restore soil fertility. An increased understanding of the molecular basis of the various bacterial pathogenic mechanisms on nematodes will not only lead to a rational nematode management decision but also could potentially lead to the development of new biological control strategies for plant-parasitic nematodes. It has been recognized that the attraction between bacteria and their hosts is governed by chemotactic factors emanating from the hosts or pathogens. Knowledge of these mechanisms could be used to attract or target nematodes intentionally by modified nematicidal bacteria or to regulate nematode populations by the chemotactic factors produced by these nematophagous bacteria.

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Chapter 15
PGPR-Induced Systemic Resistance (ISR) in Plant Disease Management

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15.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are bacteria that colonise the plant root and act as an additional source of hormones, vitamins, and growth factors that are helpful to improve plant growth and yield (Kloepper and Schroth 1978; Babalola 2010). PGPR are non-pathogenic and known to posses several mechanisms to suppress the plant pathogens like competing for fundamental niche (Elad and Baker 1985; Elad and Chet 1987), antibiosis by producing antibiotics and hydrogen cyanide (HCN) (Senthilkumar et al. 2007a, b; Pierson and Thomashow 1992) and also acting as a good source of siderophores which chelate the iron in the root vicinity to limit the availability of iron necessary for the growth of phyto-pathogens (Kloepper et al. 1980; Lemanceau et al. 1992; Compant et al. 2005).

Induced resistance is a physiological "state of enhanced defensive capacity" elicited by non-pathogenic organisms (Van Loon et al. 1998) or specific environmental stimuli, whereby the plant's innate defences are potentiated against subsequent biotic challenges (Van Loon et al. 1998). Generally, induced resistance is systemic because the defensive capacity is increased not only in the primary infected plant parts, but also in non-infected, spatially separated tissues. Thus, induced systemic resistance (ISR) is a state of increased defensive capacity developed by plants when appropriately stimulated, through activation of latent resistance induced by diverse agents including rhizobacteria (Van Loon et al. 1998; Mariutto et al. 2011). The utilisation of pathogenic organisms as inducing agents is less promising under field conditions, because the induction of ISR with pathogen inoculation will give less duration for the protection than that with PGPR-mediated ISR because prior inoculation of a pathogen might act as a good source of secondary inocula (Wei et al. 1991).

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Induced systemic resistance or ISR may become localised sometimes and is known as localised acquired resistance (LAR) when the boosting of resistance occurs to some specific tissues against a primary invader. The mode of action of both the LAR and systemic resistance seems to be similar in their effect against various types of pathogens, but in case of LAR, only localised effect of resistance develops and is not propagated throughout the plant. There are major differences in ISR when compared to other mechanisms. First, the action of ISR is based on the defence mechanism that is activated by inducing agents. Second, ISR expresses multiple potential defence mechanisms that include increase in activity of chitinase, β-1,3 glucanase and peroxidase; accumulation of antimicrobial low molecular substances such as phytolexins and formation of protective biopolymers viz., lignin, callose and hydroxyproline-rich glycoprotein (Archana et al. 2011). Third, an important aspect of ISR is the wide spectrum of pathogens that can be controlled by a single inducing agent (Dean and Kuc 1985; Hoffland et al. 1996). Thus ISR appears to be the result of several mechanisms, which together are effective against a wide range of fungal, bacterial and viral pathogens. For successful disease management, it is important to find more effective, practical and economical ways to protect plants from various pests and diseases. The utilisation of natural PGPR strains as inducers of plant defence responses may increase the chance of their applicability and offer a practical way to deliver immunisation.

15.2 Defence Mechanisms in Plants

In response to the pathogen (fungi, bacteria, viruses, nematodes and insects) attack, the plant undergoes biotic stress and develops some type of defence mechanism to cope with the situation. Plant has two types of defence mechanisms: passive or constitutive and active or inducible.

Passive or constitutive defence mechanism—attack of the pathogen on the outer layer of the plant leads to damage in the cuticle or lignin of the plant surface. Secretion of plant metabolites such as phenols, resins, tannins and alkaloids at the damaged sites of the plant surface are found to be pathogenic to some pathogens. This mode of plant defence is known as passive or constitutive defence mechanism.

Active or inducible defence mechanism—in response to the attack of the pathogen, the plant acquires some changes like thickening of the outer layer known as wall opposition so that it would be tough for the pathogen to invade through the plant surface. In addition, plants also show active defence by developing hypersensitive responses. In hypersensitive response, the cells near the site of pathogen infection become necrotic and become metabolically inert. Cells start to accumulate toxic compounds and also initiate the secretion of phytoalexins as immune response.

15.3 PGPR-Induced Systemic Resistance in Plants

The importance of PGPR was realised as an off shoot of biological control of soil-borne pathogens. Systemic resistance induced by exogenous chemical agents and pathogenic organisms is termed as systemic acquired resistance (SAR), whereas PGPR-mediated protection is generally referred to as ISR (Kloepper et al. 1992). All plants possess active defence mechanisms against pathogen attack. If defence mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease incidence can be reduced. Induced resistance is not the creation of resistance where there is none, but the activation of latent resistance mechanisms that are expressed upon subsequent, so-called challenge inoculation with a pathogen (Van Loon 1997). The terms “induced” and in some cases “acquired” systemic resistance were used interchangeably by different research groups until Ryals et al. (1996) defined the type of resistance induced by pathogenic organisms and/or chemicals involving salicylic acid as mediator of SAR as a tribute to Ross, disregarding many earlier publications describing entirely the same phenomenon using ISR as a synonym. But it was Van Loon’s research group that used ISR as the term solely to describe resistance mediated by PGPR (Pieterse et al. 1996, 1998, 2000, 2002; Van Loon et al. 1998).

Strains of the genera such as *Aeromonas*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconobacter*, *Klebsiella*, *Pseudomonas* and *Serratia* have been identified as PGPR and efforts are being made to identify more and more rhizobateria having PGP traits (Dey et al. 2004; Jaizme-Vega et al. 2004; Joo et al. 2004; Tripathi et al. 2005). The diversity of PGPR in the rhizosphere largely varies according to the plant type and nutrients available (Tilak et al. 2005). *Pseudomonas* and *Bacillus* spp. have a wide distribution among this diversity of PGPR and are the extensively studied genera for PGP and biological disease control.

In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Wei et al. 1991, 1996; Vidhyasekaran and Muthamilan 1999; Viswanathan and Samiyappan 1999). Several studies have been carried out to elicit ISR by PGPR in plants. ISR by PGPR has been achieved in a large number of crops including *Arabidopsis* (Pieterse et al. 1996), cucumber (Wei et al. 1996), tomato (Duijff et al. 1998), potato (Doke et al. 1987), radish (Leeman et al. 1996), carnation (Van Peer et al. 1991), sugarcane (Viswanathan and Samiyappan 1999), chilli (Bharathi et al. 2004), brinjal (Chakravarty and Kalita 2011), tomato and hot pepper (Ramamoorthy et al. 2002), rice (Vidhyasekaran et al. 2001; Nandakumar et al. 2001) and mango (Vivekananthan et al. 2004) against a broad spectrum of pathogens including fungi (Leeman et al. 1995; Doke et al. 1987), bacteria (Liu et al. 1995a), nematodes (Siddiqui et al. 2007), insects (Tomczyk 2006) and viruses (Khalimi and Suprapta 2011).

In carnation, application of *Pseudomonas fluorescens* induces systemic resistance against an array of plant pathogens reported through a number of studies (Wei

Table 15.1 List of some studies conducted to show PGPR-induced systemic resistance

Organism	Host plant	Pathogen	Author
P. putida (89B-61) *Serratia marcescens* (90-166) *Flavomonas oryzihabitans*(INR-5) *Bacillus pumulus* (INR-7)	*Arabidopsis*	*P. syringae* pv. Lachrymans	Wei et al. (1996)
P. fluorescens (WCS417r) *Fusarium oxysporum* (Fo47[a])	Tomato	*F. oxysporum* (lycopersici race-2)	Duijff et al. (1998)
P. fluorescens (WCS 374)	Radish	Fusarium wilt	Leeman et al. (1996)
P. fluorescens (WCS417r)	Carnation	*F. oxysporum* (dianthi)	Van Peer et al. (1991)
P. fluorescens (Pf1)	Rice	*X. oryzae*	Vidhyasekaran et al. (2001)
P. fluorescens (Pf1 and Pf7)	Rice	*R. solanii*	Nandakumar et al. (2001)
P. putida(KKMI) *P. fluorescens* (VPT4)	Sugarcane	*Colletotrichum falcatum*	Viswanathan and Samiyappan (2001)
P. fluorescens (Pf1) *P. putida* (PFATR and KKM1)	Tomato and Hot pepper	*Pythium aphanidermatum*	Ramamoorthy et al. (2002)
P. fluorescens (Pf1) *Bacillus subtilis*	Chillies	*Colletotrichum capsici*	Bharathi et al. (2004)
P. fluorescens (PFV, PFP, PSV) *Bacillus subtilis*(BSV, BSP)	Tea	*Exobasidium vexaus*	Saravanakumar et al. (2007)
P. fluorescens (PFMMP) *T. viridae* (TVUV10) *Bacillus subtilis* (BSG3)	Peppermint	*R. solani*	Kamalakaman et al. (2003)
P. fluorescens (Pfl-94)	Chickpea	*F. oxysporum* fsp *ciceri*	Saikia et al. (2006)
P. fluorescens	Brinjal	*Ralstonia solanacearum*	Chakravarty and Kalita (2011)
P. putida (MTCC no.-493)	Lentil	*M. javanica* (nematode)	Siddiqui et al. (2007)
P. aeruginosa	Soyabean	*Soyabean stunt virus*	Khalimi and Suprapta (2011)
P. fluorescens (P-112)	Cucumber	*Tetranychus urticae* (insect)	Tomczyk (2006)
P. fluorescens (FP7)	Mango	*Colletotrichum gloeosporioides*	Vivekananthan et al. (2004)

[a]Known non-pathogenic strain of *Fusarium* (Alabouvette et al. 1993)

et al. 1996; Duijff et al. 1998; Leeman et al. 1996; Van Peer et al. 1991). Duijff et al. (1998) used *P. fluorescens* wcs417r and non-pathogenic *Fusarium* strain Fo47 against the fungal pathogen *Fusarium oxysporum lycopersici* race-2 for the development of ISR in tomato. The induction of resistance in radish with *P. fluoresens*

wcs417r was strategically analysed and found that inoculation of bacteria and pathogen on alternate days gave the best result (Leeman et al. 1995) (Table 15.1).

Similarly Van Peer et al. (1991) have observed that bacterisation of the plant 1 week before the inoculation of *F. oxysporum* gave best result than simultaneous bacterisation and inoculation. PGPR strains applied as a seed treatment resulted in a significant reduction in anthracnose disease caused by *Colletotrichum orbiculare* in cucumber (Wei et al. 1991, 1996). The induction of systemic resistance by *P. putida* strain 89B-27 and *S. marcescens* strain 90-166 reduced *Fusarium* wilt of cucumber incited by *F. oxysporum* (Liu et al. 1995b).

The use of a mixture of PGPR not only developed resistance towards anthracnose disease in cucumber plants but also improved plant growth promotion by an increase in the main runner length and in leaf number (Wei et al. 1996). Seed and root treatment of rice with Pseudomonad Pf1 and FP7 enhanced the resistance for the sheath blight pathogen *Rhizoctonia solani* (Vidhyasekaran and Muthamilan 1999). Similarly, in sugarcane, Viswanathan and Samiyappan (1999) established PGPR-mediated ISR against *C. falcatum* causing red rot disease in sugarcane.

PGPR is also reported to develop systemic resistance against bacterial diseases. Alstrom (1991) treated the bean seeds with *P. fluorescens* 97 and observed development of resistance against halo blight disease caused by *Pseudomonas syringae*. He also pointed that the optimum level of the inoculum of PGPR strain Pseudomonad strain 97 lie between 4.6×10^8 cfu/ml and 4.6×10^7 cfu/ml. The treatment of cucumber seed with *P. putida 89B-61* and *S. marcescens* strain 90-166 decreased the incidence of bacterial wilt disease (Kloepper et al. 1993). Angular leaf spot of cucumber, caused by *P. syringae* pv. Lachrymans, was controlled through PGPR-mediated resistance after the inoculation of combined inoculum of *Bacillus pumilus* INR7, *Curtobacterium flaccumfaciens* MEl and *Bacillus subtilis* GB0 (Raupach et al. 2000). Similar type of systemic resistance was observed in Cucumber after seed treatment with *P. putida* strain 89B-61, *Flavomonas oryzihabitans* INR-5, *S. marcescens* strain 90-166 and *B. pumilus* INR-7 against the angular leaf spot caused by *P. syringae* by reducing total lesion diameter compared with non-treated plants (Liu et al. 1995a; Wei et al. 1996).

Development of systemic resistance against viruses by the use of PGPR has also been reported in a number of important plants. A mixture of *P. putida* strain 89B-61 and *S. marcescens* strain 90-166 treated seeds of cucumber and tomato plants respectively developed the systemic resistance against cucumber mosaic virus (CMV) wherein the virus-induced symptoms got delayed (Raupach et al. 1996). Likewise, *S. marcescens* strain 90-166 and *B. pumilus* SE34 had significantly reduced severity by CMV (Murphy et al. 2000). *P. fluorescens* CHAO-induced systemic protection reduced the incidence of leaf necrosis in tobacco after the challenge of tobacco necrosis virus (TNV) (Maurhofer et al. 1994, 1998). Application of *B. cereus* (I-35) and *Stenotrophomonas* sp (II-10) through seed treatment and soil drenching reduced the effect of TMV, and chilli veinal mottle virus (ChiVMV) in hot pepper (*Capsicum annuum*) (Damayanti and Katerina 2008). Murphy et al. (2000) observed *Bacillus amyloliquefaciens* 937a, *B. subtilis* 937b and *B. pumilus* SE34 mediated significant enhancement of the resistance in tomato against tomato mottle virus (ToMoV). Similarly, inoculation of *Pseudomonas* B-25 also enhanced plant

growth through increase in NPK uptake and reduced the effect of TMV-mediated pathogenesis in tomato (Kirankumar et al. 2008).

A number of studies reported the efficacy of PGPR-mediated ISR in the control of insect pests. Zehnder et al. (1997) observed lower level of cucurbitacin, a cucumber beetle feeding stimulant, in the PGPR-treated than non-treated plant, and the choice of feeding in the cucumber beetle (*Diabrotica undecimpunctata howardii*) also shifted from treated to non-treated plants. Similarly, Tomczyk (2006) also reported the efficacy of *P. fluorescens* in inducing resistance in cucumber against the spider mites. The relative growth rate, consumption rate and digestibility of feed by *Helicoverpa armigera* have been affected when larvae fed on cotton plants treated with *Pseudomonas gladioli* due to an increase in their polyphenol and terpenoid content (Qingwen et al. 1998). Pseudomonads are good endophytic rhizospheric colonisers. Hence, efforts have been made to transfer the insecticidal crystal protein from *Bacillus thuringiensis* to *P. fluorescens* and in some studies a positive result came out (Herrera et al. 1994). The *cry* gene transformed *P. fluorescens*, suppressed the sugarcane borer *Eldana saccharina* in a greenhouse study on sugarcane. Transgenic *P. cepacia* 526 with the crystal protein gene has consistently shown insecticidal activity against tobacco hornworm (Stock et al. 1990).

The effectiveness of PGPR-mediated ISR against nematode pests is also well documented (Oostendorp and Sikora 1990; Sikora 1992; Sikora and Hofmann-Hergarten 1992; Siddiqui and Shaukat 2004). *P. fluorescens* has ISR and inhibited early root penetration by *Heterodera schachtii*, the cyst nematode in sugar beet (Oostendorp and Sikora 1989, 1990). Similarly, *B. subtilis* induced protection against *Meloidogyne incognita* and *M. arenaria* in cotton (Oostendorp and Sikora 1989). Though attempts to use PGPR for nematode control are limited, the use of PGPR as biological control agents of plant parasitic nematodes, especially for sugar beet and potato cyst nematode, has been reported as a successful strategy in management of these nematodes (Sikora 1992). Treatment of rice seed with PGPR alone or in combination with chitin and neem cake has reduced the root and soil population of the rice root nematode *Hirschmanniella oryzae* (Swarnakumari and Lakshmanan 1999; Swarnakumari et al. 1999). The level of infestation of root-knot nematode *M. incognita* in tomato was reduced with fewer galls and egg masses in the soil following root dipping with *P. fluorescens* strain Pf1 (Santhi and Sivakumar 1995). Similarly, application of *P. chitinolytica* reduced the root-knot nematode infection in tomato crop (Spiegel et al. 1991). These experiments showed that PGPR-mediated ISR is effective in both dicotyledonous plants, viz., arabidopsis, bean, carnation, cucumber, radish, tobacco tomato, etc., and certain monocotyledonous plants, viz., rice, maize and sugarcane.

15.4 Rhizobacterial Determinants in Triggering ISR

Usually a large number of Rhizobacteria are found to be present on the root surface, where they get their nutrients from plant exudates and lysates (Lynch and Whipps 1991). Some of these rhizobacteria exhibit direct antibiosis with the soil-borne

pathogens (Wei et al. 1996). PGPR-induced systemic resistance can be proved experimentally through the spatially separated inoculation of pathogens and PGPR to avoid any antagonistic reaction between plant pathogens and PGPR. Some biochemical compounds of PGPR affect the complimentary receptors on the plant surface for the successful elicitation of systemic resistance. Treatment of tobacco roots with *P. fluorescens* CHAO triggered accumulation of SA-inducible PRs in the leaves (Maurhofer et al. 1994). He suspected that siderophore pyoverdin might be associated with the increase in the level of SA and acts as a systemic resistance elicitor against TNV. A SA-deficient mutant of *Pseudomonas aeruginosa* 7NSK2 failed to induce resistance in bean and tobacco, whereas two mutants affected in other siderophores were still capable of inducing resistance, so these studies suggested the elicitation of IRS against *B. cinerea* due to the production of bacterial SA (De Meyer and Höfte 1997).

Earlier, several structural and metabolic compounds have been detected which are associated with elicitation of rhizobacteria-mediated ISR (Van Loon et al. 1998). Purified lipopolysaccharides (LPS) and flagella of some non-pathogenic *Pseudomonas* strains have been shown to induce systemic resistance as well (Leeman et al. 1995; Van Peer and Schippers 1992; Van Wees et al. 1997). Some plants have been shown to possess a sensitive perception system for bacterial flagellins (Felix et al. 1999). N-terminal of bacterial flagellin f15 acts as a strong elicitor which led to alkalisation that initiated systemic resistance in tomato and some other plants (Felix et al. 1999; Gomez-Gomez and Boller 2000). These examples ascertain that the bacterial flagella or LPS is directly involved in elicitation of a defence-signalling pathway (Van Peer and Schippers 1992; Van Wees et al. 1997). But, Van Wees et al. (1997) contradict the finding by using bacterial mutants lacking flagella or the O-antigenic side chain of the LPS and showed that these are still able to elicit ISR in Arabidopsis. So, in addition to LPS and flagellin, some more determinants are possibly involved in the elicitation of PGPR-mediated ISR. In *P. putida* BTP1, an unknown iron-regulated metabolite casamino acid appears to be responsible for ISR in bean against *Botrytis cinerea* (Ongena et al. 2002).

Ongena et al. (2007) showed that *Bacillus subtilis* strain 168 producing lipopeptides surfactins and fengycins elicited the systemic resistance in bean. Some reports of implication of antibiotics in the elicitation of ISR are also available. Iavicoli et al. (2003) demonstrated that 10–100 μM of 2,4-diacetylphloroglucinol (DAPG) applied to roots of *Arabidopsis* mimicked the ISR against *Peronospora parasitica*. Audenaert et al. (2002) concluded that phenazine antibiotic pyocyanin in combination with SA or the SA-containing siderophore pyochelin produced by *P. aeruginosa* 7NSK2 acts as a determinant for induced resistance against *B. cinerea*. On the basis of above discussion, it can be concluded that the PGPR determinants responsible for ISR elicitation can be divided into three classes: cell surface components, such as flagella or outer membrane LPS; iron-regulated metabolites with siderophore activity like casamino acid or pyoverdine and other inhibitory metabolites like DAPG and phenazine (Table 15.2).

Table 15.2 List of reported ISR determinants of PGPR

Resistance elicitor compound	Host plant	Pathogen	Author
Siderophore, pyoverdin	Tobacco	Tobacco mosaic virus	Maurhofer et al. (1994)
Bacterial SA	Bean	*B. cinerea*	De Meyer and Höfte (1997)
Fucose and rhamnose (Lipopolysaccharide)	Radish	*Fusarium*	Leeman et al. (1995)
Flagellins	Tomato	*Pseudomonas syringae* pv *tabaci*	Felix et al. (1999)
Casamino acid	Beans	*Botrytis cinerea*	Ongena et al. (2002)
DAPG	Arabidopsis	*Peronospora parasitica*	Iavicoli et al. (2003)
Phenazine and pyocyanin	Tomato	*B. cinerea*	Audenaert et al. (2002)

15.4.1 Signalling in PGPR-Induced ISR

Plants have the ability to develop an enhanced defensive capacity upon stimulation by pathogenic and non-pathogenic microorganisms. This induced disease resistance is generally expressed as a restriction of pathogen growth and reduction of symptom development (Hammerschmidt 1999). Induced resistance can be triggered by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens, or by virulent pathogens under circumstances where infection is stalled owing to environmental conditions.

The signalling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR are relatively well studied. Pathogen-induced SAR is controlled by a signalling pathway that depends on endogenous accumulation of salicylic acid (SA), and is associated with the accumulation of pathogenesis-related (PR) proteins (Ryals et al. 1996; Sticher et al. 1997; Van Loon 1997). In some cases, rhizobacteria have been shown to activate the SAR pathway by producing SA at the root surface (Maurhofer et al. 1994, 1998; De Meyer and Höfte 1997; De Meyer et al. 1999). However, in arabidopsis, ISR is triggered by *P. fluorescens* by transcriptional activation of PR genes (Pieterse et al. 1996; Van Wees et al. 1997). However, both SAR and ISR pathways must diverge downstream of NPR1. This indicates that NPR1 differentially regulates defence responses depending on the pathway that is activated upstream of it (Pieterse et al. 1998) (Fig. 15.1). Induction of PRs is invariably linked to necrotising infections giving rise to SAR, and has been taken as a marker of the induced state (Kessmann et al. 1994; Uknes et al. 1992; Ward et al. 1991). Some of these PRs are β 1,3-glucanases and chitinases and capable of hydrolysing fungal cell walls. Other PRs have more poorly characterised antimicrobial activities or unknown functions. The association of PRs with SAR suggests an important contribution of these proteins to the increased defensive capacity of induced tissues.

Non-pathogenic, rhizosphere-colonising *Pseudomonad* trigger a form of induced resistance, phenotypically similar to SAR, called rhizobacteria-mediated ISR.

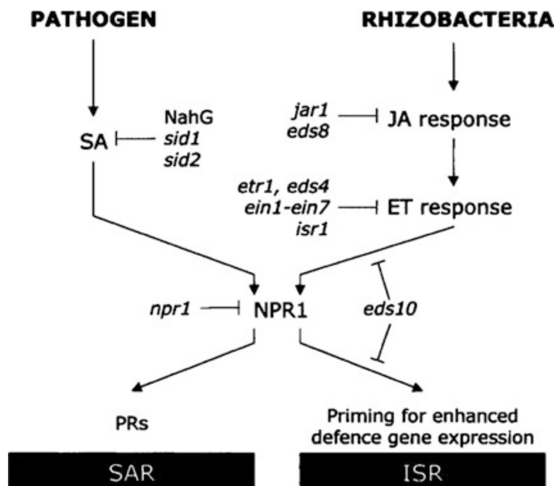

Fig. 15.1 Schematic model of pathogen-mediated SAR and rhizobacteria-mediated ISR signal transduction pathways in Arabidopsis (Courtesy: Pieterse et al. 2002)

P. fluorescens strain WCS417r (WCS417r) has been shown to activate ISR in several plant species (Duijff et al. 1998; Pieterse et al. 1996; van Peer et al. 1991). In *Arabidopsis*, WCS417r-mediated ISR is effective against different types of fungal and bacterial pathogens (Pieterse et al. 1996; van Wees et al. 1997). Interestingly, SAR and ISR are regulated by distinct signalling pathways. In contrast to SAR, WCS417r-mediated ISR functions independently of SA and PR gene activation (Pieterse et al. 1996; van Wees et al. 1997), but requires JA and ethylene signalling. The JA response mutant jar1 (Staswick et al. 1992) and the ethylene response mutant etr1 (Bleecker and Kende 1988) do not express ISR upon treatment with WCS417r, indicating that the ISR-signalling pathway requires components of the JA and ethylene response (Knoester et al. 1999; Pieterse et al. 1998). Although SAR and ISR follow distinct signalling pathways, they are both blocked in the regulatory mutant npr1 (Cao et al. 1994; Pieterse et al. 1998). Thus, NPR1 is not only required for the SA-dependent expression of PR genes during SAR, but also for the JA- and ethylene-dependent activation of unidentified defence responses resulting from rhizobacteria-mediated ISR.

The ability to develop ISR in response to rhizobacteria has been documented for many plant species (Van Loon et al. 1998) and appears to depend on the host–rhizobacterium combination (Leeman et al. 1995; Van Peer et al. 1991; Van Peer and Schippers 1992; Van Wees et al. 1997). A specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR. For instance, *P. putida* and *P. fluorescens* perform differently on different plant species: *Arabidopsis* is responsive to *P. putida*, whereas radish and carnation are not (Leeman et al. 1995; Van Peer et al. 1991; Van Peer and Schippers 1992; Van Wees et al. 1997). Conversely, radish is responsive to *P. fluorescens*, whereas Arabidopsis is not (Leeman et al. 1995; Van Wees et al. 1997). This suggests that specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR. Research on the rhizobacterial determinants

involved in the elicitation of ISR revealed several bacterial traits as potential inducers of ISR, including outer membrane LPS and iron-regulated siderophores (Leeman et al. 1995; Van Loon et al. 1998; Van Peer and Schippers 1992).

One of the parallels between rhizobacteria-mediated ISR and pathogen-induced SAR is that both types of induced resistance are effective against a broad spectrum of plant pathogens (Kuc 1982; Van Loon et al. 1998). To compare the spectrum of effectiveness of ISR and SAR, a range of viral, bacterial, fungal and oomycete pathogens of *Arabidopsis* was tested. Both *P. fluorescens*-mediated ISR and SAR induced by an avirulent strain of the pathogen *P. syringae* in tomato appeared to be effective against bacterial speck and black rot disease caused by the bacterial pathogens *P. syringae* and *X. campestris* respectively (Pieterse et al. 1996; Ton et al. 2002). Also fusarium wilt disease caused by the fungus *F. oxysporum* was equally affected by defence responses expressed during ISR and SAR (Pieterse et al. 1996; Van Wees et al. 1997). Moreover, disease caused by the downy mildew pathogen *P. parasitica* was reduced in both cases, although SAR was significantly more effective than ISR (Ton et al. 2002). Besides these similarities in effectiveness, there are also clear differences. For instance, ISR-expressing plants showed enhanced resistance against infection by the fungus *A. brassicicola*, whereas SAR is not effective against this pathogen. Conversely, expression of SAR inhibits multiplication of turnip crinkle virus and strongly reduces disease symptoms caused by this virus, whereas ISR has no effect at all (Ton et al. 2002). Thus, the spectrum of effectiveness of ISR and SAR partly overlaps but is clearly divergent, suggesting that the defence responses activated during both types of induced resistance are, at least partly, dissimilar.

15.4.2 SAR Signal Transduction Pathway

Early research on molecular mechanisms involved in induced disease resistance was mainly focussed on pathogen-induced SAR in tobacco, cucumber and bean plants. It was demonstrated that the onset of SAR is accompanied by a local and systemic increase in the endogenous levels of SA (Malamy et al. 1990; Metraux et al. 1990) and the concomitant up-regulation of a large set of genes (Ward et al. 1991), including ones encoding pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999). Several PR proteins possess antimicrobial activity and are thought to contribute to the state of resistance attained. Exogenous application of SA, or functional SA analogues, such as 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH), induced SAR and activates PR genes (Ryals et al. 1996). Conversely, transgenic NahG plants expressing the bacterial salicylate hydroxylase gene *nahG* were unable to accumulate SA and were compromised in SAR (Gaffney et al. 1993), demonstrating that SA is both necessary and sufficient for induction of SAR.

15.4.3 ISR Signal Transduction Pathway

Research on the molecular mechanism of rhizobacteria-mediated ISR was initially focussed on the role of PR proteins, as the accumulation of these proteins was considered to be strictly correlated with induced disease resistance. However, radish plants of which the roots were treated with ISR-inducing *P. fluorescens* did not accumulate PR proteins, although these plants clearly showed enhanced resistance against fusarium wilt disease (Hoffland et al. 1995). Similarly, *Arabidopsis* plants expressing *P. fluorescens*-mediated ISR showed enhanced resistance against *F. oxysporum* and *P. syringae*, but this did not coincide with the activation of the SAR marker genes *PR-1, PR-2* and *PR-5* (Pieterse et al. 1996; Van Wees et al. 1997). After refuting the dogma that systemically induced disease resistance strictly coincides with accumulation of PR proteins, Pieterse et al. (2002) reviewed ISR signalling pathway in more detail in *Arabidopsis*. The data regarding the role of SA in ISR are available in SA non-accumulating Arabidopsis NahG plants. In contrast to pathogen-induced SAR, *P. fluorescens*-mediated ISR against *P. syringae* was normally expressed in these plants (Pieterse et al. 1996; Van Wees et al. 1997). Likewise, the SA induction deficient mutants sid1-1 and sid2-1 (Nawrath and Metraux 1999) expressed normal levels of ISR. Moreover, determination of SA levels in ISR-expressing Arabidopsis plants revealed that, in contrast to SAR, ISR is not associated with increased accumulation of SA (Pieterse et al. 2000). This led to the conclusion that *P. fluorescens*-mediated ISR is an SA-independent resistance response, and that ISR and SAR are regulated by distinct signalling pathways. Apart from *P. fluorescens*, *P. putida* induced the SA-independent ISR pathway in *Arabidopsis* (Van Wees et al. 1997).

In addition, the biological control strain *S. marcescens* 90-166 has been shown to induce protection in both wild-type and transgenic NahG tobacco plants against *P. syringae* (Press et al. 1997), indicating that the ability to trigger an SA-independent pathway controlling systemic resistance is not uncommon among ISR-inducing rhizobacteria. However, not all resistance-inducing rhizobacteria trigger an SA-independent resistance. For instance, an SA-overproducing mutant of *P. aeruginosa* and a genetically modified, SA-overproducing *P. fluorescens* strain have been shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer and Höfte 1997; Maurhofer et al. 1998).

Besides SA, jasmonic acid (JA) and ethylene (ET) have repeatedly been implicated in the regulation of primary resistance responses in plants (Pieterse and Van Loon 1999; Pieterse et al. 2001). In many cases, infection by microbial pathogens and attack by herbivorous insects are associated with enhanced production of these hormones and a concomitant activation of distinct sets of defence-related genes. Moreover, exogenous application of these compounds often results in an enhanced level of resistance. To investigate the role of JA and ET in rhizobacteria-mediated ISR, the Arabidopsis JA response mutant jar1-1 and the ET response mutant etr1-1 were tested for their ability to express ISR. Both mutants were unable to mount resistance against *P. syringae* pv. tomato after colonisation of the roots by

P. fluorescens WCS417r (Pieterse et al. 1998), indicating that ISR requires responsiveness to both JA and ET. In addition to etr1-1, a set of other well-characterised *Arabidopsis* mutants that are affected at different steps of the ET signalling pathway were tested for their ability to express ISR. None of the mutants developed ISR against *P. syringae* (Knoester et al. 1999), indicating that an intact ET signalling pathway is required for the expression of ISR. To elucidate the sequence of the signalling events, the resistance-inducing ability of methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylate (ACC), the natural precursor of ET, was tested in wild-type, NahG, jar1-1 and etr1-1 plants. Like *P. fluorescens*, MeJA and ACC were effective in inducing resistance against *P. syringae* in SA non-accumulating NahG plants, suggesting that both inducers activate the SA-independent ISR pathway. Moreover, MeJA-induced protection was blocked in both jar1-1 and etr1-1, whereas ACC-induced protection was affected in etr1-1, but not in jar1-1 plants. Hence, it was postulated that *P. fluorescens*-mediated ISR follows a signalling pathway in which components from the JA and ET response are successively engaged (Pieterse et al. 1998). ISR is dependent on NPR1, and NPR1 has been shown to be an important regulatory factor in the SA-dependent SAR response (Cao et al. 1994). To know whether NPR1 is also involved in the SA-independent ISR response, *Arabidopsis* mutant npr1 was tested for the induction of ISR. Surprisingly, mutant npr1 plants were blocked in their ability to express *P. fluorescens*-mediated ISR, indicating that, like pathogen-induced SAR, rhizobacteria-mediated ISR is an NPR1-dependent defence response (Pieterse et al. 1998). Elucidation of the sequence of ISR signalling events revealed that NPR1 functions downstream of JA and ET in the ISR signalling pathway. Evidently, NPR1 is not only required for the SA-dependent expression of PR genes that are activated during SAR, but also for the JA- and ET-dependent activation of defence responses resulting from rhizobacteria-mediated ISR. This demonstrates that NPR1 is able to differentially regulate defence gene expression, depending on the signalling pathway that is activated upstream of it.

15.4.4 Expression of PGPR-Induced ISR

A large number of defence enzymes that have been associated with ISR include phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX) and proteinase inhibitors (Koch et al. 1992; Schneider and Ullrich 1994; Van Loon 1997). These enzymes also bring about liberation of molecules that elicit the initial steps in induction of resistance, phytoalexins and phenolic compounds (Keen and Yoshikawa 1983; Van Loon et al. 1998). The state of pathogen-induced SAR is characterised by the concomitant activation of a set of PR genes. In SAR-expressing plants, PR-gene products accumulated systemically to levels from 0.3 % to 1 % of the total mRNA and protein contents (Lawton et al. 1995). Although some PRs possess anti-microbial activity, a relationship between accumulation of PRs and the broad-spectrum

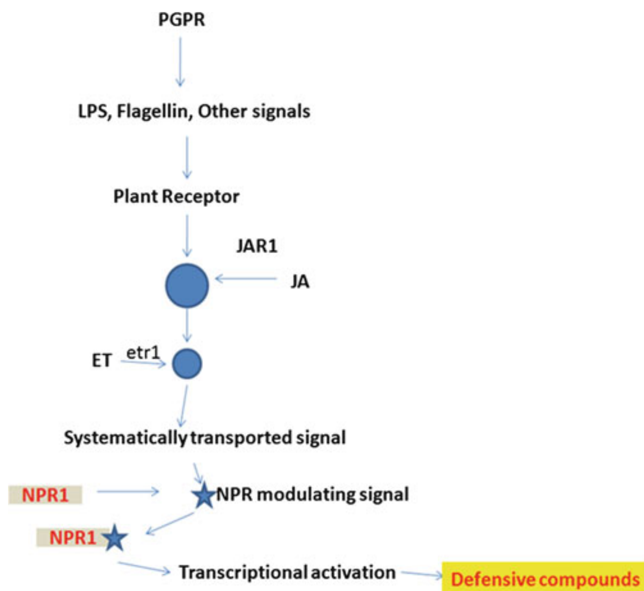

Fig. 15.2 Schematic model of PGPR-mediated pathway for production of defensive compounds in plants

resistance characteristic of SAR has never been convincingly demonstrated (Van Loon 1997) (Fig. 15.2).

Of many defence-related genes tested in Arabidopsis (e.g. the SA-inducible genes PR-1, PR-2 and PR-5 and the ethylene- and/or JA-inducible genes Hel, ChiB, Pdf1.2, Atvsp, Lox1, Lox2 and Pal1), none were found to be up-regulated in plants expressing ISR (Van Wees et al. 1999). Moreover, neither standard differential screening of a cDNA library of WCS417r-induced plants, nor 2D-gel analysis of proteins from induced and non-induced plants yielded significant differences (Van Wees et al. 1999). Thus, in contrast to SAR, the onset of ISR is not associated with major changes in gene expression. Nevertheless, ISR-expressing plants are clearly more resistant to different types of pathogens. Therefore, plants must possess as yet undiscovered defence-related gene products that contribute to broad-spectrum disease resistance.

15.4.5 PGPR-Mediated ISR: Molecular Approach

In general, induced resistance can be triggered in three ways: (1) by a predisposing infection with a necrotising pathogen (Ross 1961a, b; Kuc 1982); (2) by treatment with certain chemicals, such as salicylic acid (White 1979; Malamy and Klessig 1992) and dichloroisonicotinic acid (Metraux et al. 1991) or (3) by colonisation of

the rhizosphere with selected PGPR (Alstrom 1991; van Peer et al. 1991; Wei et al. 1991). Selected PGPR, mainly fluorescent *Pseudomonas* spp, have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, or antibiosis (Thomas et al. 2004; Thomashow and Weller 1995).

The studies related on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves. Recently, a flagellin receptor of *Arabidopsis* was characterised as a receptor kinase sharing structural and functional homology with known plant resistance genes (Gomez-Gomez and Boller 2000). Alstrom (1991) demonstrated *P. fluorescens*-mediated ISR in bean against halo blight caused by *P syringae pv phaseolicola*, van Peer et al. (1991) in carnation against *Fusarium* wilt and Wei et al. (1991) in cucumber against *Colletotrichum orbiculare* infection. Maurhofer et al. (1994) showed that ISR induced by strain CHAO of *P. fluorescens* in tobacco against TNV was accompanied by an increase in PR protein accumulation, suggesting that PGPR-mediated ISR and pathogen-induced SAR are manifestations of a similar defence mechanism. However, Hoffland et al. (1995) were unable to establish an accumulation of PR proteins in radish displaying substantial ISR against *Fusarium oxysporum* when plants were treated with strain WCS417r of *P. fluorescens*. Therefore, it is unclear whether PGPR-mediated ISR and pathogen-induced SAR share a common signal transduction pathway. With the goal of addressing whether a common pathway is shared, two bioassays for PGPR-mediated ISR were developed by using Arabidopsis as the host plant and a rifampicin-resistant mutant of the non-pathogenic, root-colonising PGPR strain WCS417 of *P. fluorescens* (*P. fluorescens* WCS417r) as an inducer. *P. fluorescens* WCS417 is an effective biocontrol agent of the take-all disease in wheat caused by *Gaeumannomyces graminis* pv tritici (Lamers et al. 1988) and has been demonstrated to be a strong inducer of ISR against vascular wilt caused by *F. oxysporum* in carnation and radish (van Peer et al. 1991; Leeman et al. 1995). It has been proved that, in contrast to classic SAR, induction of *P. fluorescens* WCS417r-mediated ISR is independent of both endogenous SA accumulation and PR gene activation.

Ward et al. (1991) found a set of plant genes expressed during the onset of SAR in tobacco; they have pronounced those genes as SAR markers which consist of at least nine families comprising acidic forms of PR-1 (PR-1a, PR-1b and PR-1c), β-1,3-glucanase (PR-2a, PR-2b and PR-2c) resistance, class II chitinase (PR-3a and PR-3b, also called PR-Q), hevein-like protein (PR-4a and PR-4b), thaumatin-like protein (PR-5a and PR-5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase (PR-Q) and the basic isoform of PR-1. A basic protein family called SAR 8.2 that is induced during the onset of SAR but which shows a pattern of gene expression distinct from that of the other SAR genes has also been described (Ward et al. 1991). In Arabidopsis, the SAR marker genes are PR-1, PR-2 and PR-5 (Uknes et al. 1992). The genes encoding these SAR marker proteins have been cloned and characterised and have been used extensively to evaluate the onset of SAR (Ward et al. 1991; Uknes et al. 1992). In order to identify genes associated with

PGPR-induced systemic resistance, a number of microarray-based study have been performed (Cartieaux et al. 2003, 2008; Verhagen et al. 2004; Wang et al. 2005).

15.5 Conclusion

The nature has provided us with PGPR which are becoming a powerful weapon for the chemical-free protection of crops from pathogens. It is an eco-friendly strategy for crop protection against plant pathogens. Among the many defence mechanisms, the induction of resistance in plants (ISR) through the application of PGPR seems to have transgressed boundaries or limitations to any particular groups of pathogens, e.g. it is effective against a broad range of pathogens of viz., bacterial, viral, nematodes, arthropods, etc. Experiments have also shown that a consortia of PGPR strains play a synergistic role in the induction of resistance. In conclusion, the exploration for more non-pathogenic strains with plant defence/resistance inducing capacity needs to be promoted. The other major challenges in the research on induced resistance are to identify signalling components from the ISR and SAR pathway that confer this specificity in NPR1-dependent defence gene activation.

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Chapter 16
Biotic Stress Management in Agricultural Crops Using Microbial Consortium

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16.1 Introduction

The rhizosphere provides home to a large number of active microbial populations capable of exerting beneficial, neutral or detrimental effects on plant growth. Microbes in general live in a heterogeneous community bound to the root surfaces and the plant–microbe signaling permits them to live and work as a community. The majority of interactions studied so far were normally concerned with single pathogen and a single biocontrol agent in the rhizosphere (Wilson and Backman 1999). But, this may sometimes account for the inconsistent performance as a single agent is not active in all soil conditions or against all pathogens that attack the host plant. Failure of the introduction of antagonistic microorganisms seems to be due to environmental factors resulting in inadequate distribution, insufficient establishment of rhizobacterial strains, poor expression of their antagonistic activity (Schippers et al. 1987), or difficulty in attaining threshold population. Indeed, variability is likely to be increased by numerous biotic and abiotic factors, and among them fluctuations in antagonistic activity is the most important one. Combining microbes as a control strategy may prove to be more relevant in the long term to give better yield and quick results (Duffy and Weller 1995; Bashan 1998). On the other hand, mixtures of biocontrol agents (BCAs) may be useful for biocontrol of different plant pathogens via different mechanisms of disease

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suppression. The recent focus on the use of multiple microorganisms with diverse activities as consortium is thus gaining momentum. By combining microorganisms, multiple antagonistic and stress tolerant traits can be combined and one may assume that at least one biocontrol mechanism will be functional under the circumstances faced by the released BCAs. Combinations of BCAs are, therefore, expected to result in a higher level of protection (Dunne et al. 1998), through reduced variability of biological control (Guetsky et al. 2001, 2002), and potentiality to suppress multiple plant diseases (Jetiyanon and Kloepper 2002).

Enhanced disease suppression may indirectly result from the multiplicity of interactions between the introduced strains which may positively influence growth, root colonization, better substrate colonization, population, and activity of each other. Combining microbial strains is not only advantageous because it can lead to enhanced levels of disease suppression, but also beneficial when a single strain fails to suppress a disease, the others in combination still may contribute to disease suppression (de Boer et al. 2003; Jain et al., 2012 a, b; Singh et al., 2012). Mixing of BCAs belonging to taxonomically different groups requires optimization of temperature, moisture, and pH for imparting benefits to the host by aggressive root colonization. Moreover, BCAs like fungi and bacteria may provide greater benefits to the host plants by being operative under varied conditions at different times and by occupying different or complementary niches.

16.2 Mechanisms of Biocontrol

Beneficial microorganisms may contribute either directly to the growth of plants or indirectly by reducing plant disease incidence (Jetiyanon et al. 2003; Gray and Smith 2005; Hass and Defago 2005). The antagonistic microorganisms exercise various mechanisms to accomplish disease control viz., inhibition of the pathogen by antimicrobial compounds (antibiosis); competition for iron through production of siderophores; competition for colonization sites and nutrients supplied by seeds and roots; induction of plant resistance mechanisms; inactivation of pathogen germination factors present in seed or root exudates; or degradation of pathogenicity factors of the pathogen such as toxins and parasitism that may involve production of extracellular cell wall-degrading enzymes like chitinase and β-1,3 glucanase that degrades pathogen cell walls (Keel and Défago 1997; Whipps 1997). Several modes of action can be exhibited by a single BCA, whereas microbial consortium can guarantee different mechanisms or combinations of mechanisms in the suppression of different plant diseases (Fig. 16.1). Most of the effects of the individual microorganisms in mixture are additive, although synergistic effects have also been reported in some cases (Ravnskov et al. 2006; Kohler et al. 2007).

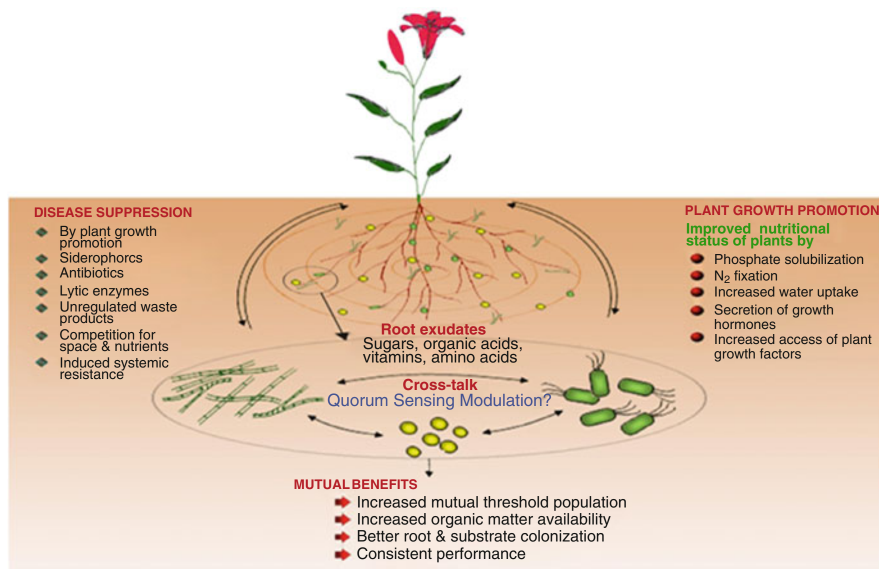

Fig. 16.1 Schematic representation of multiple interactions taking place in the rhizosphere and multifacet benefits imparted to the plants

16.2.1 Antibiosis

Antibiotics are microbial toxins which at low concentrations poison or kill other microorganisms. One of the important mode of disease suppression by BCAs include production of one or more antibiotics (e.g., 2,4-diacetylphloroglucinol (DAPG), viscosinamide, tensin, pyoluteorin (Plt), zwittermicin A, kanosamine, phenazine-1-carboxylic acid (PCA), butyrolactones, oligomycin A, oomycin A, pyrrolnitrin (Pln), xanthobaccin, or toxic substances like cyanide) (Milner et al. 1996; Keel and Défago 1997; Whipps 1997; Nielsen et al. 1998; Kang et al. 1998; Kim et al. 1999; Nakayama et al. 1999; Thrane et al. 2000). Antibiotic synthesis is very closely linked to the overall metabolic profile of the cell, which in turn is governed by nutrient availability and other environmental factors like major and minor minerals, type of carbon source and supply, pH, temperature, and other parameters (Thomashaw and Weller 1996).

Antibiotic production by bacteria, particularly by pseudomonads, seems to be closely regulated by a two-component system involving an environmental sensor and a cytoplasmic response factor (Keel and Défago 1997). By using *P. aeruginosa* PAO1 mutant unable to produce HCN, it was confirmed that cyanide poisoning is responsible for killing the nematode *Caenorhabditis elegans* (Gallagher and Manoil 2001). *Pseudomonas* species can synthesize enzymes which may also modulate the plant hormone levels and limit the available iron by production of siderophores apart from killing the pathogen by producing antibiotics (Siddiqui 2006).

The extent of mechanisms for biocontrol and the effectiveness of many BCAs are reported to depend on metabolites with antimicrobial activity in combination

with other mechanisms (Raaijmakers et al. 2002). For example, *Bacillus megaterium* KL39, a BCA of red-pepper Phytophthora blight disease, produces an antifungal antibiotic active against a broad range of plant pathogenic fungi (Jung and Kim 2003). Similarly, *B. subtilis* also synthesizes an antifungal antibiotic that inhibits *Fusarium oxysporum* f. sp. *ciceri*, the causal agent of wilt of chickpea (Kumar 1999), and a strain of *B. subtilis* RB14 produces the cyclic lipopeptides antibiotics iturin A and surfactin active against several phytopathogens. *Rhizobium* spp. have also been reported to produce extracellular compounds (such as trifolitoxin) with direct antimicrobial activities (Breil et al. 1996). Raaijmakers et al. (2002) reviewed antibiotics produced by bacterial BCAs and their role in microbial interaction. In the *Phytophthora oligandrum*/*Trichoderma harzianum* interactions disintegration of cytoplasm and cell components and loss of turgidity resulted when fungal cells were exposed to antibiotics (Floch et al. 2009). Enhanced suppression of take-all of wheat by the consortium of a nonpathogenic isolate of *Gaeumannomyces graminis* var. *graminis* and a mixture of pseudomonads was reported to be the result of direct competition for substrates at favored sites in combination with antibiotic production by the pseudomonads (Duffy and Weller 1995). The wide array of mode of action of these antibiotics obviously calls for designing consortium with different sites of action.

16.2.2 Siderophore Production

Almost all the microorganisms require iron as an essential element in a variety of metabolic and cellular pathways, and in most microbial habitats, Fe (II) is oxidized to Fe (III) which forms stable complexes. When concentration of iron is too low (10^{-6} M) to support the growth of the microorganisms, some organisms secrete iron-binding ligands called siderophores which have high affinity to sequester iron from the micro-environment. Antoun et al. (1998) determined that out of 196 *Rhizobium* spp. tested, 181 produced siderophores. Several evidences also indicate that siderophore production under iron limiting condition is responsible for the antagonism by some strains of *P. aeruginosa* against *Pythium* sp., the causal agent of damping-off and root rot of many crops (Buyens et al. 1996; Charest et al. 2005). The pseudobactin siderophore of *P. putida* WCS358 was found to increase the intensity of antagonism of the *F. oxysporum* strain Fo47 against the pathogenic *F. oxysporum* by making the pathogen more sensitive to competition for carbohydrates by Fo47 (Lemanceau et al. 1993). The involvement of siderophores in disease suppression by *P. putida* RE8 and WCS358 was investigated by de Boer et al. (2003). The pseudobactin siderophore of strain WCS358 was found to inhibit in vitro growth of RE8, whereas RE8 does not affect growth of WCS358. The improved control of Fusarium wilt of carnation by the combination of a nonpathogenic *F. oxysporum* strain Fo47 with *P. putida* WCS358 had indirect effect on competition for iron that made the pathogenic *F. oxysporum* more sensitive to competition for substrate with the nonpathogenic strain (Lemanceau et al. 1992, 1993).

16.2.3 Root Colonization

Root colonization is important being the first step in both infection by soil-borne pathogens and beneficial associations with microorganisms. Rhizosphere competence by BCAs is acquired by effective root colonization along with the ability to survive, outcompete, and proliferate on growing plant roots over a considerable time period, in the presence of indigenous microflora (Whipps 1997; Lugtenberg and Dekkers 1999). The competitive exclusion of deleterious rhizosphere organisms is also directly linked to the ability of BCAs to successfully colonize a root surface. Moreover, the O-antigen chain of bacterial lipopolysaccharides contributes to root colonization, but in a strain-dependent fashion. The O-antigenic side chain of *P. fluorescens* WCS374 does not contribute to potato root adhesion (de Weger et al. 1989), whereas the O-antigen chain of *P. fluorescens* PCL1205 is involved in tomato root colonization (Dekkers et al. 1998). Similarly, *Enterobacter cloacae* suppresses the infection of *Pythium ultimum* by effective catabolism of the available nutrients in the spermosphere (van Dijk and Nelson 2000; Kageyama and Nelson 2003).

The population dynamics of *P. putida* strains RE8 and WCS358 in the rhizosphere were investigated by applying them either singly or in combination to assess their ability to colonize roots. The population density of RE8 when combined with WCS358r was significantly higher compared to its single inoculation (de Boer et al. 2003). These findings are of considerable significance and give proper insights into the complementary/synergistic effect of the microbes in consortium for better colonization of roots.

16.2.4 Influence of Organic and Inorganic Matter

Root exudates offer a carbon-rich food to the rhizospheric microorganisms and provide them large amount of organic acids and sugars as well as variable amounts of amino acids, nucleobases and vitamins. Sometimes exudates have a major share of antimicrobial agents which give ecological niche advantage to organisms that have ability and adequate enzymatic machinery to detoxify them (Bais et al. 2004). Endophytic bacteria are shown to be selectively attracted more towards the rice exudates compared to other microbes (Bacilio-Jiméne et al. 2003). Another recent report says that root-secreted malic acid attracts beneficial soil bacteria like *B. subtilis* FB17 towards the root (Rudrappa et al. 2008).

Similarly, studies on the influence of organic matter on AM fungi and soil microbiota interactions are very limited (Green et al. 1999; Albertsen et al. 2006). AM fungi are obligate biotrophic and they receive carbon from their host plant only, whereas *Clonostachys rosea* relies on organic matter for carbon supply. The content of organic matter in the environment may therefore influence the interaction between these fungi. Thus, different types of organic matter affect the interactions between AM fungi and other soil microorganisms differently (Ravnskov et al. 1999), and the benefits derived by the host plants are highly dependent on these factors.

16.2.5 Lytic Enzymes

Diverse microorganisms having biocontrol potential secrete and excrete various metabolites that can interfere with pathogen growth or activities. Individual enzyme activities involved in the antagonism can be exploited as indicators in microbial screening to assess the antagonistic potential of strains for their precise use. Chitinase produced by *Serratia plymuthica* C48 inhibited spore germination and germ-tube elongation in *Botrytis cinerea* (Frankowski et al. 2001). Similarly, extracellular chitinases are considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii* (Ordentlich et al. 1988), and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 against *Fusarium oxysporum* f. sp. *cucumerinum* (Lim et al. 1991). An endophytic strain of *P. fluorescens* transformed with the chiA gene encoding the major chitinase of the *S. marcescens* provided effective control of *Rhizoctonia solani* on bean seedlings under growth chamber conditions (Downing and Thomson 2000). Similarly, β-1,3-glucanase contributes significantly to the biocontrol abilities of *Lysobacter enzymogenes* strain C3 (Palumbo et al. 2005). Bacterial chitinases and β-glucanases are involved in biological control of various plant pathogenic or wood deteriorating fungi (Pleban et al. 1995; Podile and Prakash 1996; Arora et al. 2007). Efforts are being made to identify cell wall-degrading enzymes produced by bacterial biocontrol strains, even though relatively little direct evidence for their presence and activity in the rhizosphere has been recorded. *Micromonospora carbonacea*, a cellulose producing isolate, was found to control root rot caused by *Phytophthora cinnamomi* (El-Tarabily et al. 1996) and actinomycete isolates that suppressed *Phytophthora fragariae* were shown to produce β-1,3-, β-1,4- and β-1,6-glucanases (Valois et al. 1996). Lytic enzyme regulation, especially proteases and chitinases particularly, involves the GacA/GacS (Gaffney et al. 1994; Natsch et al. 1994; Sacherer et al. 1994; Corbell and Loper 1995) or GrrA/GrrS regulatory systems (Ovadis et al. 2004) and colony phase variation (Lugtenberg et al. 2001).

16.2.6 Plant Growth Promotion

The plant–microbe interactions can significantly influence plant growth and crop yields. Plant growth-promoting rhizobacteria (PGPR) competitively colonize plant roots, and stimulate plant growth and/or reduce the incidence of plant diseases (Kloepper and Schroth 1978). Mechanisms of plant growth promotion include the production of volatile compounds and phytohormones, lowering of the ethylene level in plant, improvement of the plant mineral uptake, reduction of diseases, encouragement of other beneficial symbiosis, protection against degrading xenobiotics, and stimulation of disease-resistance mechanisms (ISR) (Jacobsen 1997). These PGPRs mostly belong to *Pseudomonas* and *Bacillus* spp., and are antagonists of recognized root pathogens.

Plant growth promoting microorganisms (PGPMs) such as *Rhizobium* and *Glomus* spp. can promote plant growth and productivity as their primary effect, but have also been shown to play a role in reducing disease as a secondary effect. Conversely, BCAs, such as *Trichoderma* and *Pseudomonas* spp., can control disease as their primary effect but have recently demonstrated to be a stimulator to plant growth additionally (Avis et al. 2008). Several microbes that have been studied extensively as BCAs against various phytopathogens also showed plant growth promotion activities (Singh et al. 2003; Srinivasan et al. 2009). The increase in plant growth is mostly attributed to synthesis of phytohormones such as IAA, cytokinins, and GA_3 (Shanmugaiah et al. 2006; Srinivasan et al. 2009). Many strains of fluorescent pseudomonads have shown to possess the ability to stimulate germination of seeds as well as development of shoot and root in different crops (Kloepper et al. 1988). The improvement in nutritional status of the plant has also been recognized as a possible mode of resistance to various pathogens (Karagiannidis et al. 2002; Sahni et al. 2008). Similarly, the combination of *P. fluorescens* strains EBC 5 and EBC 6 was found to increase the germination percentage, shoot length, and root length of chilli plants significantly (Muthukumar et al. 2010).

In a study on peas, three strains of *T. harzianum* increased fresh shoot weight, root weight, and/or root length (Naseby et al. 2000). Tripartite interactions among *Paenibacillus lentimorbus* NRRL B-30488 (B-30488), *Piriformospora indica* DSM 11827 (DSM 11827), and their consortia with native rhizobial population in the rhizosphere of *Cicer arietinum* L. was found to enhance nodulation and increase plant growth (Nautiyal et al. 2010). In a separate study, Seneviratne (2003) demonstrated that co-inoculation and coculture of microbes performed the tasks better than the individual microbes. When microbes are mixed into an inoculum consortium, each member of the consortium not only out-competed others for root colonization, but also complemented functionally for plant growth promotion (Shenoy and Kalagudi 2003).

16.2.7 Induced Systemic Resistance

PGPM and BCAs primarily affect plant productivity and health, but in addition to this more recently discovered effects like ISR has sparked an interest among the plant growers to use these beneficial microbes in the field (Vassilev et al. 2006). Plants have evolved a number of inducible defense mechanisms against pathogen attack (Durrant and Dong 2004). Use of microbial consortium would indirectly increase the stimulation in the plant to activate its defense mechanisms when challenged by a pathogen through strengthening of cell walls; deposition of callose and lignin (Singh et al., 2012); and the production of plant defense compounds such as phenolics, phytoalexins, and flavonoids, with simultaneous enhancement of enzyme activities such as chitinase, peroxidase, polyphenol oxidase, ascorbate peroxidase and phenylalanine ammonia lyase (Jain et al., 2012 a, b).

Bacillus sp. strain mixtures IN937b + SE49 and T4 + INR7 suppressed mosaic and anthracnose diseases in both winter and rainy seasons when compared with the nonbacterized control (Jetiyanon et al. 2003). Plants treated with the bacterial mixture of *B. amyloliquefaciens* strain IN937a and *B. pumilus* strain IN937b had superoxide dismutase (SOD) and peroxidase (PO) activity levels 25–30 % greater than the nonbacterized pathogen control. Additionally, significant disease protection in each plant pathosystem was observed with the bacterial mixture (Jetiyanon 2007). An increased level of defense-related enzymes viz., L-phenylalanine ammonia lyase (PAL), peroxidases, and polyphenol oxidase (PPO), was recorded on co-inoculation of rhizobia with *Bacillus cereus* strain BS 03 and a *P. aeruginosa* strain RRLJ 04 under the stress generated by Fusarium wilt of pigeon pea (Dutta et al. 2008). The expression of pathogenesis-related proteins (PR-proteins) can be used as a marker of ISR (van Loon 1997). Whipps (2004) also indicated that the plant defense responses occurring during *Glomus* spp. mycorrhization include phenolic and phytoalexin production, formation of structural barriers, and production of (PR) proteins and enzymes associated with plant defense mechanisms, showing the ability of some useful microbes in inducing ISR (van Loon et al. 1998).

16.3 Development of a Microbial Consortium

A preliminary step before development of a consortium requires gaining insight into the compatibility of the microorganisms used in vitro and to be used in the rhizosphere of the concerned host plant. The combination of antagonists used should be evaluated for their capacity to coexist in the rhizosphere. A successful and consistent biocontrol requires compatibility among co-inoculated microorganisms, their co-establishment in the rhizosphere, and the lack of competition among them. Evaluation is arguably, therefore, the most important phase during development of microbial consortium because it provides an understanding of its contribution in decreasing disease severity and increasing plant growth. Attempts are being made to develop microbial consortium for disease suppression and plant growth promotion (Nautiyal et al. 2005, 2006; Singh et al. 2006).

16.4 Microbial Consortium Comprising only Bacterial Strains

The use of combinations of antagonistic organisms may provide improved disease control over the use of single bioinoculant (Srivastava et al. 2010). Biocontrol by bacteria is mainly achieved through antibiosis, competition for space or nutrients in the rhizosphere, and ISR. Successful application of *Bacillus*, *Pseudomonas*, and *Streptomyces* spp. has already been reported for the control of various plant diseases in different crops (Emmert and Handelsman 1999; Anjaiah et al. 2003; Chung et al. 2005; Hass and Defago 2005). Chilli seeds treated with endophytic strains of

P. fluorescens in combination (EBC 5 and EBC 6) showed the lowest incidence of pre- and postemergence damping-off caused by *Pythium aphanidermatum* compared to individual treatment (Muthukumar et al. 2010). The combination of *P. putida* strains WCS358 and RE8 also enhanced suppression of Fusarium wilt of radish (de Boer et al. 2003). Combination of *Paenibacillus* sp. and a *Streptomyces* sp. suppressed Fusarium wilt of cucumber effectively than when used alone (Singh et al. 1999), and a combination of *P. fluorescens* and *Stenotrophomonas maltophila* improved protection against *Pythium*-mediated damping-off in sugar beet compared to when they were applied singly (Dunne et al. 1998). *P. putida* strains WCS358 and RE8 have different disease-suppressive mechanisms: pseudobactin-mediated competition for iron and/or another yet unknown disease suppressive trait for WCS358, and ISR for RE8. Combining these mechanisms by applying a mixture of the biocontrol strains leads to more effective, or at least more reliable, biocontrol of Fusarium wilt of radish (de Boer et al. 2003). Another possible reason of enhanced disease suppression may be induction in mutual population of the microbes. The population densities of *P. putida* WCS358 that developed in the presence of *P. putida* RE8 were above the threshold level required for disease suppression (Raaijmakers et al. 1995a). Similarly, *P. fluorescens* NBRI-N6 and *P. fluorescens* NBRI-N in a consortium controlled collar rot in betelvine caused by *S. rolfsii* more than either of the strains did individually (Singh et al. 2003). *Rhizobium* and *P. striata* when inoculated together improved growth and reduced nematode multiplication more than each inoculated alone. This may be due to increased availability of nitrogen (N) and phosphorus (P), as these nutrients have been reported to have adverse effect on nematode multiplication (Pant et al. 1983). Use of *Rhizobium* with *P. striata* has also been reported to reduce the multiplication of *Meloidogyne incognita* on pea (Siddiqui and Singh 2005; Kumar et al. 2005).

Under severe disease pressure of dry bean root rot caused by *Fusarium solani* f. sp. *phaseoli*, only co-inoculation with *B. subtilis* MBI600 (Epic) and *Rhizobium tropici* significantly reduced disease severity and enhanced yield compared to control and standard seed treatment (de Jensen et al. 2002). Some combinations of fluorescent pseudomonad strains increased wheat yield compared to the same strains used singly, and interestingly the best combinations did not always produce the same results in different crops (Pierson and Weller 1994). In contrast *Bacillus* strain mixture of IN937a and IN937b improved yield of all plants compared with that of plants treated with individual strain IN937a, suggesting that the combination could be useful on tomato, long cayenne pepper, and cucumber (Jetiyanon et al. 2003). Two species microbial consortium of *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 were found to promote growth of pigeon pea because of increased IAA production and ability to solubilize phosphate (Pandey and Maheshwari 2007). Similarly, Dutta et al. (2008) observed promising results on combined use of *B. cereus* strain BS 03 and a *P. aeruginosa* strain RRLJ 04 with rhizobia for induction of systemic resistance against fusarial wilt in pigeon pea.

A mixture of *B. amyloliquefaciens* strain IN937a and *B. pumilus* strain IN937b has previously shown to consistently provide a broad spectrum of disease protection against both soil- and air-borne pathogens, like *Colletotrichum gloeosporioides*,

Ralstonia solanacearum, *R. solani*, *S. rolfsii*, and cucumber mosaic virus (Jetiyanon and Kloepper 2002; Jetiyanon et al. 2003). The mixture was found to induce SOD and PO activities and significant disease protection in four plant/pathosystems, viz., tomato with *S. rolfsii* and *Ralstonia solanacearum* and pepper with *S. rolfsii* and *C. gloeosporioides* (Jetiyanon 2007). Similarly, combining proteolytic and phloroglucinol-producing bacteria can improve biocontrol of *Pythium*-mediated damping-off of sugar beet (Dunne et al. 1998). A mixture of bacteria producing chitinases and antibiotics was found to effectively suppress rice sheath blight caused by *R. solani* (Sung and Chung 1997). Thus to enhance biocontrol efficacy and consistency in performance, use of several strains from the same antagonistic microorganism, or combination of different biocontrol species should be emphasized (Alabouvette and Lemanceau 1998; Guetsky et al. 2002).

16.5 Microbial Consortium Comprising Fungal and Bacterial Strains

The use of bacterial and fungal strain mixtures is a promising way to improve efficacy of biocontrol strains. *Pseudomonas* and *Trichoderma* strains are the most commonly studied BCAs for developing consortium and they have been reported to improve overall plant growth and suppress disease incidence in different crops (Mathivanan et al. 2000; Thirup et al. 2003). Also, a positive synergistic combination of *Trichoderma* spp. and bacterial antagonists *P. syringae* has been reported to control plant pathogens (Whipps 1997). Similarly, certain reports have shown that carbon from Arbuscular Mycorrhiza mycelium is rapidly incorporated into microbial biomass (Paterson et al. 2008) and therefore, these fungi have the potential to be important conduits of energy into rhizosphere bacteria like *P. fluorescens* for biocontrol. Brulé et al. (2001) selected a mycorrhiza helper bacterial strain *P. fluorescens* BBc6 to improve the efficiency of *Laccaria bicolor* S238N inoculation in French nurseries.

Similarly, synergistic effect has been obtained in controlling *F. oxysporum* f. sp. *radicis-lycopersici* by combining a fluorescent *Pseudomonas* sp. with a nonpathogenic *F. oxysporum* (Alabouvette et al. 1996) where the nonpathogenic *F. oxysporum* competes for carbon sources and the bacterial antagonist produces a siderophore to fulfill its nutritional requirement of iron (Lemanceau et al. 1993). Effective control of *F. oxysporum* f. sp. *cucumerinum* was achieved by the interactive effect of the bacterium *P. putida* with saprophytic strains of *F. oxysporum* (Park et al. 1988). Application of *P. fluorescens* and *T. viride* also significantly reduced sheath blight disease incidence compared to control (Mathivanan et al. 2005). In a similar way, root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi* was significantly reduced by the combined application of *T. harzianum* and phenazine antibiotic producing *P. fluorescens* strain 2-79RN10, compared to *T. harzianum* treatment alone (Dandurand and Knudsen 1993). The ability of *Bacillus mycoides* and *Pichia guilermondii* has also been found successful in

suppressing *Botrytis cinerea* infection on strawberry (Guetsky et al. 2001). In this case, the yeast effectively competed with *B. cinerea* for nutrients, whereas the *Bacillus* secreted inhibitory compounds and activated the defense systems of the host (Guetsky et al. 2002). Combined use of *Rhizobium* and *Glomus intraradices* had adverse effect on the pathogens by increasing the availability of N and P to plants and thus limiting it for pathogens (Akhtar and Siddiqui 2008). By combining the nonpathogenic *F. oxysporum* strain Fo47 with the bacterial strain *P. putida* WCS358, two different disease-suppressive mechanisms were noted to act together to enhance suppression of Fusarium wilt of carnation and flax (Lemanceau et al. 1992, 1993; Duijff et al. 1999). Overlap between fungi and bacteria in utilization of root exudates can result in selective competitive pressure and therefore have an additional impact in tackling different pathogens at the same time.

16.6 Consortium Having more than Two Microbial Components

For better and consistent performance of BCAs, it is a prerequisite to identify strains of BCAs with diverse activities. A consortium of four different PGPMs, namely, *Bacillus licheniformis* strain MML2501, *Bacillus* sp. strain MML2551, *P. aeruginosa* strain MML2212, and *Streptomyces fradiae* strain MML1042, was highly effective in reducing the sunflower necrosis virus disease (SNVD) and increased the plant growth (Srinivasan et al. 2009). Use of the consortium comprising of bioagents *T. harzianum*, fluorescent *Pseudomonas*, and *G. intraradices* against Fusarium wilt not only suppressed disease incidence but also helped in sustenance and growth promotion of crop through mechanisms like enhanced plant growth promotion and nutrient uptake (Srivastava et al. 2010). Similarly, reduced disease intensity in combined application of *G. intraradices* with *Rhizobium* and *P. striata* was observed in a study conducted on root disease complex of chickpea (Akhtar and Siddiqui 2008). The synergism between various bacterial genera such as *Bacillus*, *Pseudomonas*, and *Rhizobium* has also been demonstrated to promote plant growth and development (Halverson and Handelsman 1991; Vessey and Buss 2002). Kandan et al. (2005) observed increased leaf area and shoot length, in tomato plants treated with a consortium of three different *P. fluorescens* strains, CHA0, CoT1, and CPO1. In a similar way combination of three PGPRs, *B. pumilus*, *B. subtilis*, and *Curtobacterium flaccumfaciens* provided greater control of several pathogens on cucumber than they were inoculated individually (Raupach and Kloepper 1998). *Pythium oligandrum* inoculum containing three strains with different biological traits was proved to be greatly strain-dependent (Vallance et al. 2008). Floch et al. (2009) observed that close contact between the hyphae of *Fusarium oxysporum* Schltdl. strain Fo47 with *Trichoderma harzianum*, and *P. oligandrum* cells, caused the destruction to the latter. However, in the rhizosphere, hyphae are frequently separated by a certain distance and this allows the coexistence and persistence of the three microorganisms on the root systems.

Tomato and leek plants grown in the presence of *Glomus mosseae* along with genetically modified strains of *P. fluorescens* CHA96 and CHA0 pME3424 produced enhanced levels of antifungal compounds and had a significantly higher shoot dry weight than those grown in the absence of *G. mosseae*. Colonization and activity of *G. mosseae* was found to be unaltered in the presence of *P. fluorescens* isolates and presence of *G. mosseae* increased the population of *P. fluorescens* in the rhizosphere (Edwards et al. 1998). Inoculation of *G. intraradices*, *P. striata*, and *Rhizobium* caused a significant increase in plant growth, number of pods, and chlorophyll, nitrogen, phosphorus, and potassium contents of pathogen-inoculated chickpea plants (Akhtar and Siddiqui 2008).

Combined inoculation of *Rhizobium*, a phosphate-solubilizing *B. megaterium* sub sp., Phospaticum strain-PB, and a biocontrol fungus *Trichoderma* sp. showed increased germination, nutrient uptake, plant height, number of branches, nodulation, pod yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control (Rudresh et al. 2005). *B. licheniformis* MML2501, *Bacillus* spp. strain MML2551, *P. aeruginosa* MML2212, and *Streptomyces fradiae* MML1042 were evaluated against SNVD as consortia in all possible combinations under greenhouse conditions (Srinivasan 2007) and found that they effectively improved plant growth and reduced SNVD incidence under greenhouse conditions (Srinivasan et al. 2009). Apart from the positive results, there are reports on adverse effects of some isolates of *Trichoderma* and *Streptomyces griseoviridis* on arbuscular mycorrhiza formation (Wyss et al. 1992; McAllister et al. 1994) limiting the possibility of using microbes for developing consortia without thorough screening.

16.7 Shortcomings

Various reports indicate that use of beneficial microbial mixtures generally increase plant growth and/or decrease plant disease relative to inoculation with a single beneficial organism (Chandanie et al. 2006; Raimam et al. 2007). However, some authors have pointed out that this beneficial effect was not always observed. For example, a combination of *Bacillus subtilis* and nonpathogenic *Fusarium oxysporum* did not provide control over Fusarium wilt of chickpea (*F. oxysporum* f. sp. *ciceri*), whereas either applied singly did (Hervas et al. 1997). It indicates that the results of co-inoculation of these microorganisms on plant health and productivity should be determined on the basis of the case under study (Siddiqui and Shaukat 2002; Whipps 2004).

de Boer and coworkers (2003) documented that at the most, only limited competition for iron occurred between the strains of *P. putida* strains WCS358 and RE8. A possible explanation may be that WCS358 and RE8 colonize different niches, and therefore, no competition recorded for iron. In a mixture of microbes, one may negatively influence root colonization of others. Sometime interactions between an introduced microbial mixture can also negatively influence disease control. For instance, siderophore-mediated competition for iron or competition

for substrate may limit the colonization or activity of introduced biocontrol strains (Raaijmakers et al. 1995b; Kragelund and Nybroe 1996). Even strains of the same species can exhibit significant differences. Natural microbial communities are more closely mimicked through application of a microbial mixture comprising of several species, and therefore this control strategy may prove to be more relevant in the long term. A prerequisite for successful and consistent biocontrol is to identify different BCAs with diverse activities, which can collectively increase the crop performance. The compatibility of microbes used in microbial mixture, their co-establishment in the rhizosphere of plants, and the lack of competition among them are compulsory requirements for a microbial mixture to qualify the eligibility criteria. The complexity of the interactions taking place in the rhizosphere between BCA used in the mixture among themselves and the indigenous microbiota needs to be considered and studied deeply during development of a successful microbial consortium.

16.8 Future Prospects

Our increasing knowledge on the beneficial effects of microbial consortium would most likely enhance the usefulness of these microorganisms regardless of their sole function in agricultural management practices. Mixtures of microorganisms may increase the genetic diversity of biocontrol systems which may persist longer in the rhizosphere and utilize a wider array of mechanisms for disease control (Pierson and Weller 1994). Further studies require focus on their additive and synergistic mode of actions. Additional information would assist us in appropriate application of these organisms in improving agricultural management practices. More focus should be paid on assessing the added value of the microbial consortium in comparison to a single bioinoculant. Moreover, crop-based microbial consortium may be developed to meet the specific requirements. With the advent of functional genomics and proteomics studies of these microorganisms (Marra et al. 2006; Chacón et al. 2007), work on genes and gene products would provide more precise information on modes of action. Statistical procedures have been devised using which separation of direct growth promotion effects of a biocontrol agent from the effect obtained by disease control is possible, using data from factorial experiments in which BCA were applied in the presence or absence of pathogens (Larkin and Fravel 1999; Ryder et al. 1999). Mathematical modeling can also help us predict the results of interactions among consortium microbes and pathogen in the rhizosphere. Future relies on the application of modern molecular techniques and along with conventional experimental procedures to understand and utilize plant–microbe interactions occurring in soil. Its application would increase our knowledge about their combined mode of action, particularly with induced resistance in plants. Formulation also plays a significant role in determining the final efficacy of the mixture. A large number of microbial mixtures have been reported till date (Table 16.1), but further studies are needed to explore interactions between microbial agents to get maximum benefits out of them.

Table 16.1 Microbial consortium reported for the management of various plant diseases

Microbial mixture	Disease/pathogen	Mechanisms involved	Reference
Rhiozobim and *P. straita*	Nematode multiplication	Increased N and P availability	Pant et al. (1983)
P. putida, *Fusarium oxysporum* strain F047	*Fusarium oxysporum*	Siderophore-mediated competition for carbohydrate	Lemanceau et al. (1993)
T. harzianum and P. fluorescens strain 2-79RN10	*Aphanomyces euteiches* f. sp. *pisi*	Siderophore, ISR, and plant growth promotion	Dandurand and Knudsen (1993)
Gaemannomyces graminis var. *graminis* + mixture of pseudomonads	Take-all disease of wheat (*Gaemannomyces graminis* var. *tritici*)	Antibiosis	Duffy and Weller (1995)
P. fluorescens and *Stentrophomonas maltophila*	*Pythium*-mediated damping-off in sugarbeet	ISR	Dunne et al. (1998)
P. putida RE8 and WCS358	Fusarium wilt of radish	Siderophore and ISR, Mutual increase in population	de Boer et al. (2003)
Pichia guilermondii and *B. mycoies*	*B. cinera*	Competition for nutrients and secretion of inhibitory compound	Guetsky et al. (2001, 2002)
P. fluorescens NBRI-N6 and *P. fluorescens* NRI-N	*Sclerotium rolfsii*	ISR	Singh et al. (2003)
Rhizobium and *P. straita*	*M. incognita*	Plant growth promotion	Siddiqui and Singh (2005)
Bacillus sp. strain mixture IN937b + SE49 and T4 + INRN	Cucumber mosaic virus and *Colletotrichum gloeosporioides*	ISR	Jetiyanon et al. (2003)
P. fluorescens and *T. viride*	Sheath blight	ISR and plant growth promotion	Mathivanan et al. (2005)
B. amyloliquefaciens IN937a and *B. plumilus* IN937b	*S. rolfsii*, *Ralstonia solanacearum* and *Colletotrichum gloeosporioides*	Increased SOD and PO activity	Jetiyanon (2007)
Rhizobia with *B. cerus* strain BS03 and *P. aeruginosa* RRLJ04	*Fusarium udum*	Increased PAL, PO, and PPO activity	Dutta et al. (2008)
Bacillus licheniformis strain MML2501, *Bacillus* sp. strain MML2551, *P. aeruginosa* strain MML2212 and *Streptomyces fradiae* strain MML1042	SNVD	ISR and plant growth promotion	Srinivasan and Mathivanan (2009)

(continued)

Table 16.1 (continued)

Microbial mixture	Disease/pathogen	Mechanisms involved	Reference
P. fluorescens EBC5 and EBC6	*Pythium aphanidermatum*	ISR	Muthukumar et al. (2010)
T. harzianum, fluorescent *Pseudomonas* and *G. intraradices*	Fusarium wilt	Plant growth promotion and increased nutrient uptake	Srivastava et al. (2010)

16.9 Conclusions

The increased growth and reduced intensity of disease by using microbial consortium may be attributed to a combination of mechanisms acting in an additive manner. Microorganisms in a cocktail may enhance the level and consistency of performance of biocontrol by increasing effectiveness over a wide array of biotic and abiotic stresses and proving to be more stable by mimicking natural communities. In particular, combinations may provide protection at different times and under different conditions, by occupying different or complementary niches, supplementing each other's requirements. The present chapter on microbial consortium emphasizes their potential role in plant growth promotion and disease control. By using microbial cocktails, we can make sure that atleast one organism used in the mixture is functional under a particular stress and in a particular environmental niche. Additive and synergistic effects of mode of actions in combination would increase their potential as a BCA and would serve us with better disease control, higher yield, and improve soil quality results. However, precise knowledge of their mode of action and plant–microbe interactions would help us in their appropriate release and multi-faceted uses in managing plant health. The potential increase in use of these cocktail microorganisms along with their ability to impart multiple benefits may further help in reducing problems associated with the use of synthetic chemicals in agriculture and managing biotic stress in crop plant in an ecologically acceptable way.

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Chapter 17
Siderophore Producing PGPR for Crop Nutrition and Phytopathogen Suppression

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17.1 Introduction

The global necessity to increase agricultural productivity from decreasing land resources has placed a considerable strain on the fragile agro-ecosystem. While the use of mineral fertilizers is considered quickest and surest way of boosting crop production, their cost and other constraints discourage farmers from using them in recommended quantities. In recent years, concepts of Integrated Plant Nutrient Management (IPNM) and Integrated Plant Disease Management (IPDM) have been developed that emphasize maintaining and increasing soil fertility through plant growth promotion and phytopathogen suppression (Tilak et al. 2005).

PGPR that are present in close vicinity to plant roots play a vital role in plant growth for increasing crop/food yield to meet ever-increasing food demand of rapidly growing world population, which will be nearly ten billions by 2020. Therefore, application of biological inputs and bioinoculants has been seen as a sustainable approach for improving soil organic matter, enzymes, and microbial population thereby crop productivity. In this context siderophore producing microbes function as an efficient PGPR with multifunctional potential of plant growth promotion (Sayyed et al. 2004) and disease suppression (Compant et al. 2005).

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Past decade has witnessed increasing interest in the role of rhizobacteria, which can variously have positive, negative, or neutral effects on plant growth (Nehl et al. 1996). Rhizosphere is inhabited by diverse group of microbes. Some of these bacteria not only benefit from the nutrients excreted by the plant roots but also beneficially influence the plant in direct or indirect way resulting in a stimulation of its growth (Bloemberg and Lugtenberg 2001). The rhizobacteria most commonly referred to as PGPR are those with a major function in the inhibition of plant pathogens (Glick 1995; Kloepper 1993). These PGPR can be classified according to their beneficial effects as nitrogen fixers, hormone producers, and biocontrol agents (BCAs) (Bloemberg and Lugtenberg 2001).

17.2 Plant Growth Promoting Rhizobacteria

The term PGPR or "yield-increasing bacteria" (YIB) has been used since 1974 in a broad sense and includes rhizobacteria that promote plant growth directly by releasing phytohormones, fixing nitrogen in the rhizosphere, solubilizing insoluble forms of nutrients such as phosphate, promoting mycorrhizal function, and regulating ethylene production in plant roots. Besides this, some rhizobacteria have the capacity to suppress major plant pathogens (Nehl et al. 1996; Glick et al. 1994). The rhizobacteria most commonly referred to as PGPR are those with a major function in plant growth promotion (Glick. 1995). Table 17.1 represents the partial list of PGPR involved in controlling phytopathogen infestation in cash crops (Sayyed et al. 2004; Sindhu et al. 1997).

In addition to the common *Azotobacter* sp. and *Azospirillum* sp., a number of other bacteria including various species of *Pseudomonas*, *Acetobacter*, *Alcaligenes*, *Klebsiella*, *Enterobacter*, *Xanthomonas*, and *Bacillus* sp. have been considered as PGPR (Yang and Crowley 2000).

17.3 PGPR and Iron Nutrition in Crops

Iron is an essential element for growth, metabolism and survival of the majority of cell types on earth. Although it is a forth most abundant and common element present in soil, it is rarely found in free form. With the evolution of aerobic environment and activity of oxygen evolving blue green bacteria, the process of oxidation converted much of soluble iron into insoluble ferric oxides and oxyhydroxides, so the freely mobile iron became immobile and was laid down in massive mineral deposits (Page 1993).

Table 17.1 Siderophore producing PGPR for controlling diseases in cash crops

Siderophore-based BCAs	Target pathogen/disease	Crop
P. fluorescence	*Erwinia carotovora* *G. graminis*/Take all *Fusarium glycinia* *Sarocladium oryzae*	Potato Wheat Wheat Soybean Rice
P. putida	Fusarium sp. wilt *Fusarium solani* *Erwinia carotovora*	Radish, Cucumber Beans Potato
P. cepacia	*Fusarium oxysporum*	Onion
P. aureofaciens	*G. graminis*var *tritici*	Wheat
B subtilis A-13	*Rhizoctonia solani*	Wheat
B. pumilus	*Gaeumannomyce graminis* var. *tritici*	Wheat
Enterobacter aerogenes	*P. cactorum*	Apple
E. cloacae	*S. homeocarpa*	Turfgrass
Bradyrhizobium sp.	*Fusarium solani* *Rhizoctonia solani*	Sunflower Mungbean
Rhizobium meliloti	*Macrophomina phaseolina*	Groundnut

17.3.1 Physiological Significance of Iron

Iron plays an important role in microorganisms, plants, and animals (Dudeja et al. 1997). It exists in two states and, therefore, it is suitable as an electron transporter. It is a component of cell and its deficiency can cause growth inhibition, decrease in RNA and DNA synthesis, inhibition of sporulation, and changes in the cell morphology. In intermediately metabolic process, iron is required in TCA cycle, ETC, oxidative phosphorylation, nitrogen fixation, aromatic biosynthesis, and photosynthesis. It also regulates the biosynthesis of porphyrins, toxins, vitamins, antibiotics, cytochromes, pigments, siderophores, and aromatic compounds. It is required as a cofactor by different enzymes and proteins such as peroxidase, superoxide dismutase, nitrogenase, hydrogenase, glutamate synthase, ribonucleotide diphosphate reductase, aconitase, DAHP synthatase, cytochromes, ferridoxin, and flavoproteins. Iron storage proteins like ferritin in animals and bacterioferritin in microorganisms have also been discovered.

17.3.2 Calcareous Soil

Calcareous soil comprise approximately a third of the land surface and are found predominantly in regions that receives less than 500-mm annual rain precipitation. Such soils do not lack iron, but its availability is just limited. They are characterized by high pH (7–9) and a significant content of free carbonates (Gildersleeve and Ocampaugh. 1989). Soluble iron may be very limited in an aerobic environment. The maximum free ferric iron concentration at pH 7.0 is 10^{-17} M. Soluble iron of 1 μM concentration is usually considered sufficient iron to sustain microbial growth.

17.3.3 Correction of Iron Deficiency

An optimum value for available iron is between 12 and 24 ppm, and there is correlation between iron available in the soil and that observed in plants. The management of soils and fertilization intended to maintain these values and correct iron deficiency includes the application of iron chelates/salts, modification of soil pH addition of organic matter to the soil deficient in available iron, and use of cultivars with the ability to take iron from soils where the element is unavailable. However, these strategies appear to be expensive and ineffective for large application. The competitive ability of microbes to sequester iron through their siderophores and make it available to plants appears as one of the possible approach to correct this deficiency (Chincholkar et al. 2000).

17.3.4 Iron Uptake and Assimilation of Plants

In general, the iron required for a typical crop during growing season is 5–10 kg ha^{-1}. In the aqueous soil solution, the minimum concentration reported for reasonable growth in different crop is 10^{-9} ppm. Under standard condition (pH 7) the concentration of iron derived from $Fe(OH)_3$ is 2×10^{-18} ppm. Thus, plant must unavoidably have means to solubilize iron (Fe^{3+}) from iron oxides and hydroxide.

Mechanisms of iron uptake in plants are more diverse than siderophore mediated iron uptake system in microbes (Romheld 1991). Plants have evolved various systems to convert iron into available form. Following three possibilities of iron assimilation have been mainly identified in plants of which strategy A and C is widespread in plant kingdom (Bienfait 1988).

17.3.4.1 Acidification of Rhizosphere

It is usually observed in nongraminaceous monocots and all dicots. Acidification of rhizosphere increases the solubility of iron by reduction of Fe^{3+} to Fe^{2+}. Reduction of iron is a necessary step in uptake of iron through iron starved dicot plants (Dudeja et al. 1997).

17.3.4.2 Phytosiderophores

This strategy has been adapted in graminaceous monocots. It involves the secretion of iron chelating substance (phytosiderophores) and uptake of Fe^{3+}-phytosiderophores by (Nomoto et al. 1987; Crowley et al. 1987).

Table 17.2 IROMPs involved in iron-siderophore uptake

IROMP	Receptor proteins
FpvA	Ferripyoverdine receptor
FptA	Ferripyochelin receptor
PupA	Pseudobactin 38 receptor
PupB	Pseudobactin BN 7 and BN 8 receptor
FhuE	Ferrioixamine E receptor

17.3.4.3 Microbial Siderophore

There are sufficient evidences available regarding iron uptake by plants through microbial siderophores, which converts the insoluble form of iron into soluble form.

Siderophore producing bacterial strains possess iron regulated outer membrane proteins (IROMPs) on their cell surface that transport ferric iron complex to the respective cognate membrane; iron thus becomes available for metabolic processes. IROMPs of various siderophore producing bacteria have been characterized (Johri et al. 2003) (Table 17.2).

17.4 Siderophores Production

Siderophores (Sid = iron, Phores = bearers) are low molecular weight (<10,000 Da), virtually ferric-specific ligands produced by microbes as scavenging agents in order to combat low iron stress (Kintu et al. 2001). The rationale for siderophore synthesis is not only to overcome the insolubility of available iron but also to regulate and control its uptake, as at high concentration it becomes toxic (Guerinot 1994).

All aerobic and facultative anaerobic microbes (except *Lactobacilli*) are known to produce siderophores, which act as iron chelates (Loper and Buyer 1991).

A wide variety of siderophores are produced by bacteria and fungi (Table 17.3) and their number is increasing as new siderophores are being identified. Siderophores have been classified based on their main chelating groups. Generally they are categorized into two groups (Neilands 1984) (1) hydroxamate, e.g., ferribactin, aerobactin, francobactin, ferrioxamine, and Schizokinen and (2) catecholates or carboxylate (Konetschny et al. 1990), e.g., enterochelin, agrobactin and parabactin (Chincholkar et al. 2000; Rane et al. 2005). Recently, Winkelmann and Dreschel (1997) have added three more classes of bacterial siderophores namely (3) peptide, (4) mycobactin, and (5) citrate hydroxamate. Fungal siderophores has been classified into five classes as (1) ferrichromes, (2) coprogens, (3) rhodotorulic acid, (4) fusarinines (fusigens), and (5) rhizoferrin (Chincholkar et al. 2000).

Siderophores are viewed as the evolutionary response to the appearance of O_2 in the atmosphere, concomitant oxidation of Fe^{2+} to Fe^{3+}, and the precipitation of latter as ferric hydroxide, $K_s = <10^{-38}$ M. The knowledge of siderophore and their cognate membrane system is crucial for understanding the basics of growth, metabolic activity, host invasion, and virulence in microbes. In all cases iron is a prerequisite (Winkelmann and Dreschel 1997).

Table 17.3 Partial list of microbial siderophore

Siderophore	Producing bacteria	Siderophore	Producing fungi
A. Catechols		Ferrichrome	*Penicillium parvum*
Agrobactin	*Agrobacterium tumefaciens*	Ferrichrome A	*Ustilago sphaerogena*
Enterobactin	*E. coli*	Ferrichrome C	*Neurospora crassa*
Chryseobactin	*Erwinia chrysanthemi*	Ferrioxamine B	*Streptomyces* sp.
Pyochelin	*Pseudomonas aeruginosa*	Ferrioxamine E	*Erwinia herbicola*
2,3 Dihydroxybenzoic acid	*Azotobacter vinelandii*	Ferricrocin	*Microsporum canis*
Azotochelin	*A. vinelandii*	Asperchrome A, B, and C	*Aspergillus ochraceus*
Aminochelin	*A. vinelandii*	Malionichrome	*Fusarium roseum*
Anguibactin	*Vibrio anguilarum 775* (*PJM*)	Rhizoferrin	*Rhizopus microsporus*, *R. arrhizus*
Cepabactin	*P. cepacia*	Canadaphore	*Helimenthosporium carbonum*
Parabactin	*Paracoccus denitrificans*	Fusarinine A and B	*Fusarium roseum*
Staphyloferrin A	*Staphylococcus hyicus*	Coprogen	*Curvularia lunata*
B. Pyoverdin	*Pseudomonas sp.*	Coprogen B	*Gliocladium virens*
	P. fluorescens	Neocoprogen I and II	*Curvularia lunata*
	P. aeruginosa	Dimerum acid	*Stemphylium botyrosum*
	P. putida	Alterobactin	*Alteromonas luteoviolaces*
C. Hydroxamate	*P. syringae*	Rhodotorulic acid	*Rhodotorula piliminae*
Acinitobactin	*Acinitobacter baumanii*		
Arthrobactin	*Arthrobacter* sp.,		
Desferrioxamine B & E	*Streptomyces viridosporus*		
Cornybactin	*Cornybacterium glutamicum*		
Aerobactin	*Erwinia carotovora*, *Enterobacter cloacae*, *Pseudomonas* sp.		
Francobactin	*Frankia* sp.		
Ferribactin	*P. fluorescens*		
Pseudobactin	*P. putida*		
Ferrioxamine E	*Erwinia herbicola*		
Schizokinen	*Bacillus megaterium*		
Alcaligin E	*Alcaligenes eutrophus*		
Alcaligin	*Bordetella purtussis*		
Protochelin	*B. bronchoseptica*		
Yersianiabactin	*Yersinia enterocolitica*		
Yersiniophore	*Yersinia enterocolitica*		

(continued)

Table 17.3 (continued)

Siderophore	Producing bacteria	Siderophore	Producing fungi
Amonabactin	*Aeromonas hydrophilla*		
Vulnibactin	*Vibrio vulnificus*		
Catechol and hydroxamate	*Azotobacter chrococcum*		
D. Other types			
Rhizobactin	*Rhizobium meliloti*		
Azotobactin	*Azotobacter vinelandii*		
Anthranilic acid	*R. leguminosarum*		
Citric acid	*Bradyrhizobium japonicum*		
E. Unknown type	*Rhizobium meliloti*		
	R. leguminosarum		
	R. trifolii		
	A. vinelandii		

Sayyed et al. (2005) have reported the production of hydroxamate type of siderophore from *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847 in modified succinic acid medium (SM). $(NH_4)_2SO_4$ and amino acids were found to stimulate bacterial growth as well as siderophore production. Increase in iron concentration up to 100 μM favored growth but drastically affected siderophore production in both the strains. Threshold level of iron ($FeCl_3$), which repressed siderophore production in both the strains, was 30 μM. Sunflower oil proved to be suitable and cost-effective defoaming agent. The results of shake flask level were reproducible at scaled up conditions in bioreactors. *P. fluorescens* NCIM 5096 inoculation enhanced seed germination, root length, and shoot length of wheat (*Triticum aestivum*) under pot culture conditions.

17.4.1 Influence of Metal Ions on Growth and Siderophore Production

Growth and siderophore production by PGPR is attributed to metal ions and several other components of root exudates (Nehl et al. 1996; Sayyed et al. 2005) and metal ions accumulating due to the liberal use of chemical pesticides and fertilizers (Sayyed and Chincholkar 2009; Sayyed et al. 2009) since siderophore synthesis occurs under low stress of iron, effect of metal ions on either the growth or siderophore production by PGPR would potential of that PGPR (Sayyed et al. 2005; Bloemberg and Lugtenberg 2001).

During stationary phase of growth under low stress of iron in succinic acid medium, *Alcaligenes feacalis* BCCM ID 2374 produced microbial iron chelators. Increase in iron concentration supported bacterial growth but suppressed siderophores production; 1 μM and 2 μM of iron was optimum for maximum siderophore yield, i.e., 354 and 360 μg/ml in untreated and deferrated medium, respectively. Threshold level of iron, which suppressed siderophores production in

A. feacalis BCCM ID 2374, was 20 µM. Ten micromoles and above concentration of $CuCl_2$ and $CoCl_2$, and 20 µM of $MgCl_2$, $MgSO_4$, $ZnCl_2$, and $ZnSO_4$ severely affected siderophores production (Sayyed and Chincholkar 2010).

Maximum metal resistance level for bacterial *Alcaligenes* sp. and *Acinetobacter* sp. were observed on nutrient agar with different concentrations of $MnCl_2$ and $NiCl_2$. Bacterial *Acinetobacter sp*. showing resistance to $MnCl_2$ salt up till 3 mg during step-by-step repeated culturing of bacterial strain on nutrient agar have been reported by Sayyed et al. (2008). Similarly *Alcaligenes* sp resistant to 1 mg $NiCl_2$ was obtained. Most bacterial strain accumulates metal by employing physicochemical mechanisms and transport system of varying specificity. However, both essential and nonessential metals in concentrations, higher than optimal level, prove toxic to organisms. Under such conditions, these organisms may activate and adapt a mechanism of detoxification to ensure survival. We in this study were successful in developing Ni- and Mn-resistant strains by step-by-step repeated culture and selection on the medium containing increasing concentration of Ni and Mn.

17.4.2 Siderophore Purification

Siderophore extraction into an organic solvent, ethyl acetate in the case of catechol type or either benzyl alcohol or chloroform:phenol (1:1) for the hydroxamate type, has been used as an effective purification step for many types of siderophores since both salts and macromolecules are removed effectively (Sayyed and Chincholkar 2006). The polystyrene resin Amberlite XAD, for the purification of neutral, ferrichrome type of siderophores and polyamide resin for chromatographic separation of catechol type have been widely used.

Sayyed and Chincholkar (2006) have reported production of two types, i.e., hydroxamate and catecholate type of siderophores from *Alcaligenes faecalis* BCCM ID 2374 in modified succinate broth, i.e., siderophore-rich cell free concentrated broth when subjected to purification on Amberlite XAD-4 column, it resulted in the separation of two fractions having absorption maxima at 264 and 224 nm. The amount of pure siderophore obtained in powdered form from Major fraction (297 µg ml^{-1}) belonged to hydroxamate type while minor fraction (50 µg ml^{-1}) belonged to catecholate type of siderophores. They reported highest recovery yield (347 µg ml^{-1}) of siderophore from XAD column while other purification methods like solvent extraction and Sep-Pak column were found ineffective for siderophore extraction and purification.

17.4.3 Chemical Characterization

Sayyed and Patel (2011) examined IEF pattern and cross feeding of siderophore of *A. faecalis* purified on XAD-4 column, IEF pattern suggested the pI value of 6.5. Cross feeding studies revealed that *A. feacalis* accepts heterologous as well as

self-(hydroxamate) siderophore in both free and iron complexed forms; howeve, the rate of siderophore uptake was more in case of siderophores complexed to iron. Siderophore iron uptake studies ruled out the possibility of similarity between siderophores of *A. feacalis* and Alc E.

17.4.4 Siderophore-Based PGPR for Plant Growth Promotion

Obviously, siderophores function to supply and store the iron in cell. The ability to use siderophores of other microbes (heterologous siderophore) is of great selective advantage during nutrient competition in soil. It can also be a means of economizing the metabolic efforts inside the microbial cell (Matzanke et al. 1987).

Most important biotechnological exploitation of siderophores in the rhizosphere region of the plant where they provide iron nutrition to the plant, serve as first defense against root invading parasites and helps in removing toxic metals from polluted soil. There are sufficient evidences available regarding iron uptake by plants through microbial siderophores, which converts the insoluble form of iron into soluble form. Iron is an essential element for growth, metabolism, and survival of the majority of cell types on earth (Dudeja et al. 1997). Although it is a forth most abundant and common element present in soil, it is rarely found in free form. Soil microbes excrete high-affinity iron chelators (siderophores) to secure the trace amounts of iron from environment to the cell interior and transport this precious metal with fidelity to the siderophore producing cell (Page 1993).

Sayyed et al. (2007a) reported that under iron deficient conditions in succinic acid medium, *A. feacalis* produced siderophores, and siderophore-rich broth of *A. feacalis* promoted growth and seed germination in *Chlorophytum borivillianum* and *Withania somnifera* both in plate assay as well as in open environment in pot assay under natural conditions in soil. 75% increase in the rate of germination was evident in the seeds of *W. somnifera* and tubers of *C. borivillianum* bacterized with siderophoregenic *A. feacalis*. In case of *W. somnifera*, 41.15% increase in root length, 26.55% increase in shoot length, and 48.66% increase in chlorophyll content was reported. While in case of *C. borivillianum* 21.17% increase in root length, 41.15%, increase in shoot length, 26.05% in chlorophyll content, 12.39% increase in number of tubers, 9.2% increase in length of tubers and 29.26% increase in weight of tubers was reported.

Sayyed et al. (2007b, 2009) reported plant growth promoting ability of *A. feacalis* and *P.* and field levels. Co-inoculation of *A. feacalis* with *P. fluorescens* NCIM 5096 showed enhanced plant growth promotion in *A. hypogea* than single inoculation with either of these two rhizobacteria. After 90 days of sowing, it resulted in 21.39% increase in shoot length, 16.30% increase in root length, 43.05% increase in chlorophyll content, 22.51% increase in number of pods, and 31.25% increase in number of branches.

17.4.5 Siderophore Producing PGPR for Phytopathogen Suppression

Siderophores producing PGPR have been implicated in the biocontrol of several plant diseases, like damping off of cotton, root rot of wheat, potato seed piece decay, vascular wilts, and stem rot of peanut (Sindhu et al. 1997). Various workers have reported the antagonistic action of *Rhizobium*, *Azotobacter*, and *Azospirillum* on phytopathogens. Saikia and Bezbaruah (1995) reported that hydroxamate type of siderophore producing *A. chrococcum* RRLJ 203 was capable of inhibiting *F. oxysporum*, *F. udum*, *F. solani*, *F. moniliforme*, *Ustulina zonata*, and *Fomes lamnensis*.

Siderophore producing PGPR function as BCAs by depriving the pathogen from iron nutrition thus resulting in increased yields of crops (Lemanceau and Albouvette 1993). Freitas and Pizzinato (1997) have reported the inhibition of *Colletotrichum gossypi* by siderophore producing rhizobacteria resulted in plant growth promotion in cotton seedlings. Sindhu et al. (1997) reviewed the role of PGPR in inhibition of phytopathogens (Table 17.4). Sindhu et al. (1997) and Johri et al. (1997, 2003) have reported the role of siderophore producing fluorescent *Pseudomonas* strains RBT 13, which exhibited antagonistic action against several bacterial and fungal plant pathogens. Many *Pseudomonads* have been reported to exhibit antifungal activity against phytopathogenic fungi (Table 17.5). Microorganisms having the ability to produce powerful siderophores become ecologically competent BCAs provided that they exhibit strong root colonizing (Chincholkar et al. 2000).

Sayyed and Chincholkar (2009) have reported the in vitro phytopathogen suppression activity of siderophoregenic preparations of *Alcaligenes feacalis* vis-a-vis the oraganochlorine fungicide, bavistin. Siderophore-rich culture broth, siderophore-rich supernatant, and purified siderophore preparation exerted antifungal activity against *Aspergillus niger* NCIM 1025, *A. flavus* NCIM 650, *Fusarium oxysporum* NCIM 1008, and *Alternaria alternata* IARI 715. Among all the preparations, siderophore-rich broth exhibited potent antifungal activity. The minimum fungicidal concentration required was 75 ll for *A. niger* and *F. oxysporum* and 50 ll for *A. flavus* and *A. alternata*.

Sayyed and Patel (2011) have shown that metal-resistant siderophore producing PGPR have also been seen as potent biocotrol agent over the chemical fungicides. They have observed potent and superior in vitro phytopathogen suppression activity of siderophoregenic preparations of Ni- and Mn-resistant *Alcaligenes* sp. STC1 and *Acinetobacter* sp. SH-94B isolated from soil over the chemical fungicides: oraganochlorine fungicide; carbistin and copper-based fungicide; and bilcop-50. Siderophore-rich culture broth and siderophore-rich supernatant exerted antifungal activity against *Aspergillus niger* NCIM 1025, *Aspergillus flavus* NCIM 650, *Fusarium oxysporum* NCIM 1281, *Alternaria alternata* ARI 715, *Cercospora arachichola*, *Metazhizium anisophilia* NCIM 1311, and *Pseudomonas solanacerum* NCIM 5103. Siderophore-rich broth and supernatant exhibited potent antifungal activity vis-à-vis chemical fungicides. The minimum fungicidal concentration

Table 17.4 Role of siderophore bearing PGPR as biocontrol agent

BCA used	Target disease	Target pathogen	Crop under application
P. fluorescens	Potato decay	*Erwinia carotovora*	Potato
	Take-all	*Gaeumannomyces graminis*	Wheat
		F. glycinia	Wheat
	Fusarium wilt	*Sarocladium oryzae*	Soybean, Rice
P. putida	Wilt	*Fusarium* sp.	Radish
	Wilt	*Fusarium* sp.	Cucumber
	Wilt	*F. solani*	Beans
	Potato decay	*Erwinia carotovora*	Potato
P. cepacia	Wilt	*F. oxysporum*	Onion
Bacillus subtilis	Wilt	*F. roseum*	Corn
Bacillus sp.	Root rot and Take-all	*Rhizoctonia*, *Pythium*	Wheat
Rhizobium sp.		*Macrophomina phaseolina*	Soybean
Bradyrhizobium sp.	Wilt	*F. solani*	Sunflower
		R. solani	Mungbean

Table 17.5 Antifungal activity of fluorescent pseudomonads

Antibiotics	Producing bacteria	Target pathogen	Target disease
DAPG	*P. fluorescens* CHAO	*G. graminins tritici*	Take-all disease
	P. fluorescens Q287 and F113	*Thielaviopsis basicola*	Black rot of tobacco
		Pythium ultimum	Damping off of sugar beet
	P. fluorescens Pf5	*Rhizoctina solani*	Sheath blight
Pyrrolnitrin	*P. cepacia*	*Bipolaris muydis*	Southern maize leaf blight
	P. fluorescens Pf5	*Sclerotina homoecarpa*	Dollar spot of turf grass
Pyoluteorin	*P. fluorescens* Pf5	*Pythium* sp.	Damping off
Phenazines	*P. fluorescens* 2-79	Various sp. of bacteria and fungi	Cucumber
	P. aureofaciens 30-84	*G. graminis tritici*	Take-all disease
	P. aureofaciens PGS 12	*G. graminis tritici*	Take-all disease

required was 25 μl for *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Cercospora arachichola*, *Metazhizium anisophilia*, and *Pseudomonas solanacerum* and 75 μl for *A. alternata*.

Siderophore producing plant growth promoting rhizhobacteria (PGPR) against common phytopathogens. PGPR showed enhanced biocontrol in comparison with chemical fungicides used singly. We observed potent and superior in vitro phytopathogen suppression activity of siderophoregenic preparations of Ni- and Mn-resistant *Alcaligenes* sp. STC1 and *Acinetobacter* sp. SH-94B isolated from soil were found superior over the oraganochlorine fungicides; carbistin and copper-based fungicide; and bilcop-50. Siderophore-rich culture broth and siderophore-rich supernatant exerted antifungal activity against *Aspergillus niger* NCIM 1025, *Aspergillus flavus* NCIM 650, *Fusarium oxysporum* NCIM 1281, *Alternaria alternata* ARI 715, *Cercospora arachichola*, *Metazhizium anisophilia* NCIM

1311, and *Pseudomonas solanacerum* NCIM 5103. Siderophore-rich broth and supernatant exhibited potent antifungal activity vis-à-vis chemical fungicides. The minimum fungicidal concentration required was 25 μl for *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Cercospora arachichola*, *Metazhizium anisophilia*, and *Pseudomonas solanacerum* and 75 μl for *A. alternata*.

17.4.6 Mechanism of Phytopathogen Suppression

Understanding the mechanism of action is important, because it gives much idea in determining the maintenance, enhancement, and implementation of (BCA. BCAs interact with phytopathogens directly or indirectly by following mechanism.

17.4.6.1 Competition for Iron

BCAs compete with the phytopathogens for available resources and thereby restrict them to colonize plant roots. Competition for Fe is one of the modes of action by which fluorescent pseudomonads limit the growth of pathogenic fungi and reduce disease incidence and severity. Under conditions of Fe stress, these bacteria produce siderophores, (Pyoverdin/pseudobactin), which show higher affinity for Fe than fungal siderophores. Number of research papers and review articles have correlated bacterial antagonism with siderophores (Bakker et al. 1991; Lemanceau and Albouvette 1993).

17.4.6.2 Antibiosis

Rhizobacteria are known to produce a variety of antibiotics including pyrrolnitrin, pyoluteorin, tropolone, pyocyanin, and 2,4-diacetylphloroglucinol (O'Sullivan and O'Gara 1992), which have been reported to be involved in suppression of different pathogens by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia, or by exerting a fungicidal effect (Sindhu et al. 1997). Phenazine, a potent antibiotic produced by *P. fluorescens*, has been used to control take-all disease of wheat caused by *Gaeumannomyces graminis*. *Agrobacterium radiobacter*, a first commercially applied BCA for controlling crown gall in dicots produce agrocin 84, which specifically inhibits *A. tumifaciens* (Sindhu et al. 1997).

17.4.6.3 Predation and Parasitism

BCA may be a predator or a parasite of the pathogen. Mycoparasites, such as *Coniothyrium minitans* and *Sporidesmium sclerotivorum*, have been tested as BCA, and some of them are efficient in controlling diseases caused by *Sclerotinia* sp. and other sclerotia forming fungi (Adams and Fravel 1993).

Table 17.6 Induction of ISR in bacterized host plants by rhizobacteria

Inducer bacteria	Target pathogen	Bacterized host plant
P. fluorescens WCS 417r	*Colletotrichum obiculare*	Cucumber
	F. oxysporum f. sp. *congulutinans*	Radish
	X. campestris	Radish
	X. campestris	Arabidopsis
P. fluorescens CHAO	Tobacco Necrosis Virus	Tobacco
P. aeruginosa 7NSK2	*B. cineria*	Bean
P. putida 89B27	*F. oxysporum* f. sp. *cucumerinum*	Cucumber
S. marscescens 90-166	*F. oxysporum* f. sp. *cucumerinum*	Cucumber
S. plymuthica 2-67	*C. obiculare*	Cucumber
B. subtilis	*E. graminis* f. sp. *hordei*	Barley
B. uniflagellatus	Tobacco Mosaic Virus	Tobacco

17.4.6.4 Hypovirulence to Reduce the Effect of Virulent Ones

Hypovirulence is a reduced virulence found in some strains of pathogens (Estrella and Chet 1998). It was first reported in *Cryphonectria parasitica* (Chestnut blight fungus) on European *Castanea sativa*; a hypovirulent strain was able to reduce the effect of natural virulent strains of *C. parasitica* ones. Hypovirulence has also been demonstrated in many other pathogens like *Rhizoctonia solani*, *Gaeumannomyces hramini* var. *tritici*, and *Ophiostoma ulmi*.

17.4.6.5 Induction of Induced Systemic Resistance

First evidence that PGPR induces defense associated changes in plant physiology directed against spatially distant pathogens was first reported in 1991 (Van Peer et al. 1991). The fluorescent pseudomonads and other PGPR have been reported to induce systemic resistance in the plants (Table 17.6) (Kloepper et al. 1996), which provides protection against a broad spectrum of phytopathogens (Jamsen 2000). Siderophores even if present in nanogram amounts induce induced systemic resistances (ISRs) (De Meyer et al. 1999; Van Wees et al. 2000; Pieterse et al. 2001).

17.4.7 Commercial Aspects of Using Siderophore-Based PGPR as BCAs

In the recent past, huge amount of work has been carried out on the biocontrol of plant diseases, which has also led to the development of commercial bioproducts. Some of the commercially available bacterial biocontrol products are listed in Table 17.7. It has been observed that biocontrol products identified to date control relatively narrow spectrum of diseases on a particular host crop. Some of the decisions that determine whether a biocontrol product is commercialized are

Table 17.7 A partial list of commercially available biocontrol formulations

Trade name	Biocontrol organism	Target pathogen/disease	Crop under application	Formulation	Mode of application
Bio-save 10[5] Biosave 11[5]	*Pseudomonas syringae*	*Botrytis cinerea*, *Mucor pyriformis*, *Geotrichum candidum*, and *Penicillium* sp.	Citrus and pome fruit	Wettable powder	Postharvest Drench, dip, and spray
BlightBan A506[6]	*Pseudomonas fluorescence*	*Erwinia amylovora*	Almond, cherry, apple, potato, and tomato	Wettable powder	Postharvest Drench, dip, and spray
Conquer[8]=Victus[26]	*Pseudomonas florescence*	*Pseudomonas tolassii*	Mushroom	Aqueous biomass suspension	Spray
Epic[11]	*Bacillus subtilis*	*Rhizoctonia solani*, *Fusarium*, *Alternaria*, and *Aspergillus* sp.	Cotton and legumes	Dry powder 5.5×10^{10} spores/g	Drip and potting mix in row
Galltrol-A[13]	*Agrobacterium radiobacter*	*Agrobacterium tumifaciens* Crown gall	Fruit, nut, and ornamental nursery plants	Bacterial suspension 1.2×10^{11} cfu	Suspension applied to seeds, seedlings, cuttings, root, stem, and as a soil drench
Intercept[14]	*Pseudomonas cepacia*	*Rhizoctonia solani*, *Fusarium*, and *Pythium* sp.	Maize, cotton, and vegetables	–	–
Kodiak[11], Kodiak HB, Kodiak AT	*Bacillus subtilis*	*Rhizoctonia solani*, *Fusarium*, *Alternaria*, and *Aspergillus* sp.	Cotton and legumes	Dry Powder 5.5×10^{10} spores/g	Slurry mixture for seed treatment
Orback 84C[17]	*Agrobacterium radiobacter*	*Agrobacterium tumifaciens* Crown gall	Fruit, nut, and ornamental nursery plants	Aqueous suspension 8×10^{10} cfu/100 ml	Root, stem, cutting, dip, or spray.

business decisions not based on science. Before approaching for commercial production, a company must assess many factors including demand for the product, potential market size, and existing competing products (Formulation). To be an ideal biocontrol product, it should satisfactorily meet important criteria such as:

(a) The biocontrol products must have a relatively wide spectrum of activity, with high, consistent, and reliable efficacy.
(b) Bioproducts also must have acceptable shelf life without special storage requirements and meet the acceptable standards for environmental and toxicological safety.
(c) Thorough understanding of mechanism(s) of action and ecological competence of the bioproducts must be made to assure an efficacious product.
(d) The application of the biocontrol products should be easy, possible with existing plant protection equipment.
(e) Bioproducts also must be highly compatible with chemical agents.

17.5 Biotechnological Exploitation of Siderophores

Most important biotechnological exploitation of siderophores in the rhizosphere region of the plant provide iron to the plant as well serve as first defense against root invading parasites. Recently, Meyer (2000) and Meyer et al. (2000), Fusch et al. (2001), and Munsch et al. (2000) have introduced a concept of "siderotyping" for classification/typing of pseudomonads on the basis of siderophores. This has opened a new era in the classification of certain microbes.

Recovery of toxic metals from industrial effluents and precious metals from mines is another important application of siderophores. High-affinity siderophores are virtually Fe^{3+} specific. However, under certain conditions like acidic, heavy metal polluted, or fertilizer-affected soil, other metal ions may be more abundant than Fe^{3+} and may bind to siderophores (Sayyed 2010; Sayyed et al. 2010). Addition of 0.5 μg ml^{-1} Al^{3+} causes a decrease in the growth rate of schizokinen negative mutants of *Bacillus megaterium* in iron-limited medium (Huyer and Page 1988, 1989). Similarly chromium (0.1 μg ml^{-1}) inhibits *B. megaterium*, suggesting that both ions interfere with low-affinity iron uptake. Thus, the cells escape aluminum or chromium toxicity by using hydroxamate ligands to mediate iron uptake by another route. In both the cases the inhibition is reversed by the addition of 1 μg ml^{-1} of iron/Desferal. Similarly, cobalt causes increased production of ferrichrome siderophores by *Ustilago sphaerogena* and other fungi. Decrease in copper toxicity has been reported in *Anabaena* culture when Cu^{2+} is complexed with Schizokinen. High level of Zinc (40 μM) promotes siderophore production in *Azotobacter vinelandii*, even in the presence of normally repressible amounts of iron (Huyer and Page 1989). This effect is caused by zinc inhibition of ferric reductase involved in low affinity iron uptake.

Siderophores may be involved in the beneficial uptake of ions other than iron. Molybdenum (VI) forms complexes with catecholate and hydroxamate, although the affinity constant is very low (10^5 to 10^{10}).

17.6 Benefits of Using Siderophore Users

Applications of PGPR as bioinoculant to crops would reduce the use of chemical fertilizers and pesticides thereby would restrict the development of pesticide resistance in pathogen. Target organisms seldom develop resistance towards BCA as happens with the use of chemicals. PGPR are safe for crops, eco-friendly, and farmer friendly as they originate from nature. PGPR-based BCAs are safer than the chemical pesticides now in use. They do not impose the problem of biomagnification. Their self-replication circumvents repeated application (Chincholkar et al. 2000; Sayyed et al. 2004).

17.7 Challenges in Using Siderophore-Based PGPR: Rhizosphere Competence

The root surface and surrounding rhizosphere are significant carbon sinks (Rovira 1965) that share 40% of photosynthates (Degenhardt et al. 2003). This nutrient-rich niche attracts a great diversity of microorganisms, including phytopathogens. Competition for these nutrients and niches is a fundamental mechanism by which PGPR protect plants from phytopathogens (Duffy 2001). PGPR reach root surfaces by flagellar movement and are guided by chemotactic responses (De Weert et al. 2002; Nelson 2004; De Weger et al. 1987; Steenhoudt and Vanderleyden 2000; Turnbull et al. 2001a, b) mediated by organic acids, amino acids, and specific sugars present in root exudates (Welbaum et al. 2004). Some of these exudates can also be effective as antimicrobial agents and thus give ecological niche advantage to organisms that have adequate enzymatic machinery to detoxify them (Bais et al. 2004). The quantity and composition of chemoattractants and antimicrobials exuded by plant roots are under genetic and environmental control (Bais et al. 2004). This implies that PGPR competence highly depends either on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions. As an example, sugars, amino acids, and organic acids induce chemotaxis in *Azospirillum*, but the degree of chemotactic response to each of these compounds differs among strains. Simons et al. (1997) reported that amino acid synthesis is required for root colonization by *P. fluorescens* WCS365, indicating that amino acid prototrophy is involved in rhizosphere competence. In addition, PGPR regulate the rate of uptake of polyamines such as putrescine, spermine, and spermidine, since their high titer could retard bacterial growth and reduce their

ability to competitively colonize roots (Kuiper et al. 2001). Root mucilage also offers utilizable carbon source for PGPR (Knee et al. 2001) to use for the competitive colonization. PGPR may be uniquely equipped to sense chemoattractants, e.g., rice exudates induce stronger chemotactic responses of endophytic bacteria than from non-PGPR present in the rice rhizosphere (Bacilio-Jiménez et al. 2003). Bacterial lipopolysaccharides (LPS), in particular the O-antigen chain, can also contribute to root colonization. The O-antigen chain of *P. fluorescens* PCL1205 is involved in tomato root colonization (Dekkers et al. 1998). It has also been recently demonstrated that the high bacterial growth rate and ability to synthesize vitamin B1 and exude NADH dehydrogenases contribute to plant colonization by PGPR (Welbaum et al. 2004). Another determinant of root colonization ability by bacteria is type IV pili, better known for its involvement in the adhesion of animal and human pathogenic bacteria to eukaryotic cells (Steenhoudt and Vanderleyden 2000). The type IV pili also play a role in plant colonization by endophytic bacteria such as *Azoarcus* sp. (Turnbull et al. 2001a).

Bioinoculants are often applied as seed coatings. After sowing, the bioinoculants must be able to establish themselves in the rhizosphere at population densities sufficient to produce a beneficial effect. Therefore, bioinoculants must survive in the rhizosphere, make use of nutrients exuded by plant roots, multiply, be able to effectively colonize the entire plant root system, and must be able to compete with the indigenous microflora. Identification of the genes and traits involved in the process of root colonization will give insight into plant–microbe interaction and will lead to the successful application of bioinoculants (Bloemberg and Lugtenberg 2001).

P. fluorescens genes that are expressed in the rhizosphere (*rhi* genes) have been identified using In Vivo Expression Technology (IVET). Many root colonization genes and traits from *Pseudomonas* sp. have been used to improve colonization by wild strains (Dekkers et al. 2000). Organic acids have been shown to form the nutritional basis of rhizosphere colonization. A defect in the utilization of organic acids, which form the major group of tomato exudates components, results in decreased competitive colonization of tomato rhizosphere.

In certain PGPB, efficient root colonization is linked to their ability to secrete a site-specific recombinase (Postma et al. 2003). Transfer of the site-specific recombinase gene from a rhizosphere-competent *P. fluorescens* into a rhizosphere-incompetent *Pseudomonas* strain enhanced its ability to colonize root tips (Sanchez-Contreras et al. 2002; Van der Broek et al. 2003).

17.8 Genetic Modifications to Improve the Performance of PGPR

The identification of genes involved in the ability of rhizobacterial strains to improve plant growth can help in improving the performance of PGPR. Complete operons, as well as single genes under the control of their own regulatory genes or

regulate by the constitutive expression of *tac* or *lac* promoters, have been transferred to rhizobacterial strains. Mini-Tn5 vector carrying genes for the synthesis of antifungal metabolite, Phenazine-1-carboxylic acid (PCA) have been transferred to *P. fluorescens*, and this genetically modified strain exhibited enhanced rhizosphere competence and ability to suppress fungal disease (Timmis-Wilson et al. 2000).

17.9 Conclusion

Application of siderophore producing PGPR is a eco-friendly alternative to chemical fertilizers and pesticides, the use of which is regulated and sometimes forbidden. Large amount of research has been devoted to Genus *Pseudomonas* because of its functional potential as PGPR and BCA (Nielsen et al. 2000; Bloemberg et al. 2000; Pieterse et al. 2001; Pal and Jalali 1998; Thrane et al. 2000) and much remains to be learned from nonsymbiotic endophytic bacteria that have more pronounced plant growth promoting effect (Bacilio-Jiménez et al. 2003; Chanway et al. 2000; Ping and Boland 2004). Biotechnology can be applied to further improve strains that have appreciated qualities, i.e., formulation ease, stability, and competent root colonization, by creating transgenic strains that combine multiple mechanisms of action (Chin-A-Woeng et al. 2001; Huang et al. 2004; Timmis-Wilson et al. 2000).

17.10 Future Perspectives

Understanding the mechanism of rhizosphere competence and root colonization provides significant information. For this purpose use of green fluorescent protein (*gfp*) and in situ monitoring based on confocal laser scanning microscope (CLSM) have become necessary (Johri et al. 1997).

Modification of the genes involved in the action of PGPR also plays a key role in improving the potential of PGPR. The rhizosphere competence, as well as antifungal activity, of *P. fluorescens* carrying phenazine-1-carboxylic acid (PCA) coding mini-Tn5 vector was enhanced by introducing carboxamide (PCN) producing *phzH* gene from *Pseudomonas chlororaphis* PCL1391 (Timmis-Wilson et al. 2000). Transforming the ACC deaminase gene, which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene (Glick et al. 1994) into *P. fluorescens* CHAO, not only increases plant growth but can also increase biocontrol properties of PGPB (Wang et al. 2000). Genome or sequence analysis of PGPR can be of great significance; the sequence data obtained facilitate the identification of genes present in PGPR that are expressed on the seed or in the rhizosphere, which are involved in the regulation of production of secondary metabolite by PGPR. The construction of Bacterial Artificial Chromosome (BAC) libraries for the study of gene expression and identification of genes of interest is of great value in the study of bacteria whose genome has not been sequenced (Bloemberg and Lugtenberg 2001).

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Chapter 18
Antifungal Substances of Bacterial Origin and Plant Disease Management

Lina Guo, Aamir Rasool, and Chun Li

18.1 Introduction

Plant diseases each year cause economical loss of billions of dollars to agriculture by reducing yields, lower product quality or decreased shelf life, decreasing esthetic or nutritional value, and contaminating food with toxic compounds. Control of plant diseases is important for providing an adequate supply of food, feed, fiber, and esthetics. But it has been almost exclusively based on the application of fungicide chemicals. Several effective fungicides have been recommended for use against pathogens, but they are not considered to be a long-term solution, due to concerns over expenses, exposure risks, fungicide residues, and other health and environmental hazards. Considering the limitations of fungicides chemical, it seems appropriate to search for a supplemental control strategy. Biological control, the use of microorganisms or their secretion and resistance plants raised genetically prevents them from being diseased and, therefore, offers an attractive alternative for the management of plant diseases, without any adverse effects of chemical control. It has, therefore, become an important aspect for sustainable agriculture.

The use of microorganisms or their products to limit the attacks and damages by phytopathogens has risen worldwide, particularly in recent decades. For example, a study proved that *Bacillus subtilis* SL-44 has a growth promoting and disease control effect on tomato plants (Tao and Li 2006a). Two bacterial mixtures CL-7 and CL-8 have demonstrated disease control and growth promoting effect on tomato plants (Tao and Li 2006b, 2009; Wu and Li 2008).

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Recent efforts have focused on two prime objectives: first, to identify microorganisms with antifungal activities and isolate and characterize the specific natural products from these microorganisms, and, second, to determine their mechanisms of action. In the past few years, numerous microorganisms with antifungal activities and their activity factors have been identified (Akihiro et al. 1993; Lim et al. 1991; Lorito et al. 1993; Robert and Selitrennikoff 1986, 1988; Silo-Suh et al. 1994; Kang et al. 2004), and the mechanisms by which antifungal substances inhibit the growth of potentially pathogenic fungi have been demonstrated (Lim et al. 1991; Robert and Selitrennikoff 1988; Silo-Suh et al. 1994; Elad et al. 1982; Howell and Stipanovic 1980; Mauch et al. 1988; Phae et al. 1992). It is reported that intense research efforts have been devoted for the development of antifungal compounds for agriculture biocontrol.

So far, in many previous studies, a few Plant Growth Promoting Rhizobacteria (PGPR) strains have been shown to be effective biocontrol agents for a number of plant pathogenic bacteria and fungi (Kotan et al. 1999, 2002). Antibiotic production from bacteria plays a major role in agriculture biocontrol (Cook et al. 1995; Dowling and O'Gara 1994; Fravel 1988; Klich et al. 1994). Approximately 100 peptides have been investigated till to date for their antifungal properties. They can vary on the basis of their source: natural, semisynthetic, and totally synthetic source. The most studied peptides are natural peptides although the semisynthetic or totally synthetic peptides are increasing in number. However, only a few antibiotics from some strains have been isolated, identified, and their role in biological control has been studied (Asaka and Shoda 1996; Gueldner et al. 1988). This chapter will briefly discuss antibiotic substances from bacteria with their antifungal activity against plant pathogens associated with agriculture crops. They are quite diverse, either non-ribosomally synthesized or ribosomally synthesized.

18.2 Ribosomally Synthesized Peptides or Chemical Substances

The antimicrobial ribosomal peptide or chemical substances are biosynthesized during active bacterial growth, while non-ribosomal peptides or chemical substances are biosynthesized after bacterial growth has ceased (Tamehiro et al. 2002). It is reported that most of the antimicrobial peptides target to the biological membranes. These peptides kill cells by increasing membrane permeability (Maget-Dana and Peypoux 1994; Avrahami and Shai 2004).

18.2.1 Bacteriocin

Bacteriocin is a complex heterogeneous group of antibacterial and antifungal proteinaceous toxin produced by bacteria. Bacteriocins have been in focal research since colincin, from *Escherichia coli*, was discovered by. Bateriocins can work

against fungal pathogens in agriculture biocontrol as well, such as Echcin, a bacteriocin isolated from *Erwinia chrysanthemi*. It has an inhibitory effect on the growth of *Alternaria solani* and *Phytophthora capsici*.

The strains, which can produce bacteriocins are: *Erwinia carotovora*, *E. chrysanthemi*, *Emberizoides herbicola*, *Erwinia salicis*, *Phytophthora aptata*, *Phytophthora syringae*, *Phytophthora solanacearum*, *Clavibacter insidiosum*, *Clavibacter michiganensis*, *Phytophthora glycinea*, *Phytophthora phaseolicola*, etc.

According to the structure and composition of the bacteriocins, these are divided into three groups: high-molecular weight protein particles, low-molecular weight protein particles, and small nonprotein substances. Most bacteriocins belong to high-molecular weight group and are heat and trypsin sensitive, such as Carotovoracin, isolated from *E. carotovora* var. *earotovora* and *E. carotovora* var. *atroseptica*, of high-molecular weight compound, sedimentated at $10{,}000\times g$ centrifugation for 10 min. It is unstable in acid or alkaline conditions and can be passivated with sodium dodecyl sulfate (SDS). However, another class of bacteriocins has been isolated from *Pseudomonas solanacearum* and *Pseudomonas syringae*. They are trypsin sensitive but heat resistant and effective to control the *Agrobacterium tumefaciens*.

Subtilin is a type I bacteriocin with antibacterial and antifungal activity, produced by *B. subtilis* (Kleerebezem et al. 2004). It belongs to type-A lantibiotics: like nisin (Gross and Morell 1971; Buchman et al. 1988; Kaletta and Entian 1989; Dodd et al. 1990) from *Lactococcus lactis*, ericin from *B. subtilis* A 1/3 (Stein et al. 2002), and pep5 (Kaletta et al. 1989; Sahl and Brandis 1982) and epidermin (Allgaier et al. 1985; Schnell et al. 1988) from *Staphylococcus epidermidis*. Type-B lantibiotics showed globular structures like cinnamycin (Kaletta et al. 1991; Kessler et al. 1992; Widdick et al. 2003), ancovenin (Wakamiya et al. 1985), and duramycin (Fredenhagen et al. 1990). The action of type-A lantibiotics depends on pore formation into the cytoplasmic membrane (Driessen et al. 1995), which concomitantly accomplish with specific binding to precursor lipid II of the membrane. (Breukink et al. 1999; Wiedemann et al. 2001).

18.2.2 Enzymes

The chitinase and β-1,3-glucanase are major enzymes for the degradation of cell wall of pathogenic fungi. Chitin, a linear homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine residues, is the most abundant nitrogen containing organic compound found in the cell wall of microoganism (Henrissat and Bairoch 1993; Brunner et al. 1998).

Chitinases, secreted by bacteria, are classified as glycosyl hydrolases that catalyze the hydrolysis of β-1,4-glycosidic bonds of chitin and used for the degradation of pathogen cell wall. The antifungal effect of β-1,3-glucanase is due to the hydrolysis of β (1–3) glycosidic bond. Chitinase and β-1,3-glucanase are often induced in plants at the same time, and the antifungal effect is more stronger when they are used together (Li 2006; Lan 2000). They can completely digest the cell

wall of pathogenic fungi, inhibit their growth, and therefore achieve the goal of disease control. They are commonly found in many bacteria, which can degrade chitin (Hoster et al. 2005; Meanwell et al. 2008; Yu et al. 2008; Cohen-Kupiec and Chet 1998; Kao et al. 2009; Felse and Panda 1999).

Chitinases have received increasing attention because of their broad applications in the field of agriculture (Han 2008). Among the chitinolytic bacteria, several *Actinobacteria* and *Streptomyces* species are thought to degrade the chitinous cell wall of plant pathogens through the production of chitinases and antibiotics (Kawase et al. 2006; Yu et al. 2008). Such chitinolytic bacteria are known to be involved in the suppression of plant fungal pathogens and used for the biocontrol of soilborne fungal diseases (Hoster et al. 2005; Singh et al. 1999).

The role of chitinase and β-1,3-glucanase in the inhibition of *Rhizoctonia solani* and *P. capsici* was earlier reported by Arora et al. (2007, 2008). It was reported by Liu et al. (2011) that chitinase originated from *Bacillus* SL-13 can suppress the growth of *R. solani* and promote the sprouting, seedling, and growth of tomato. Lin (Lin and Li 2003) showed S9 strain could lyse pathogenic fungal mycelium *R. solani*, *Pythium ultimum*, and *Fusarium oxysporum* f.sp. *niveum* under confrontation culture condition. Gu et al. (2003, 2005) showed *B. subtilis* G3 strain produced chitinase that was able to inhibit sclerotium formation by *R. solani*, besides having antimicrobial activity against *Sclerotinia sclerotiorum* and *R. solani*, and used in control of tomato leaf mold.

18.2.3 Active Protein

In many bacterial metabolic processes, bacteria secrete some active protein, which could control plant diseases. *B. subtilis* BS-98 strain can strongly inhibit the apple ring rot bacteria *Physalospora piricola* and other plant pathogenic fungi. Amino acid composition analysis showed that the protein contains 11 kinds of amino acids, especially rich in Glutamate and Cysteine. Purified protein has strong inhibitory effect on apple ring rot pathogens and asparagus stem blight pathogens; its main mechanism of action is the dissolution of cell wall. It results in abnormal hyphae formation; therefore, spores either do not germinate or germinate abnormally (Hu et al. 1996).

There are some unknown proteins that also have inhibitory effect. The protein secreted by *B. subtilis* strain SO113 has a good broad-spectrum antimicrobial effect against *Xanthomonas oryzae* pv. *oryzae* including seven pathotypes of rice bacterial blight in China (Lin et al. 2001). Rice sheath blight pathogen and rice moniliforme pathogen are strongly inhibited by a variety of active proteins from *B. subtilis* strain B-916. The protein secreted by *B. subtilis* strain BS2LX04 delay the growth of mycelium and result in mycelium malformation (Liu et al. 2005). *B. subtilis* strain BS98 contains antagonistic substances named X98 III. Different kinds of antifungal proteins have been extracted from *B. subtilis* TG26 which contained antifungal protein BI and BII and antifungal cyclic peptide LP21. In fact LP21 has been confirmed as a new cyclic peptide, which contains nine amino acid residues, and its molecular weight is 105,713 Da with good thermal stability (Liu et al. 1994).

18.3 Non-ribosomally Synthesized Antibiotic Substances

In nature, bacteria can biosynthesize polypeptides with antifungal activity via non-ribosomal pathway. Non-ribosomal peptides were biosynthesized by non-ribosomal peptide synthetases, polyketide synthases, or the complex of non-ribosomal peptide synthetases and polyketide synthases.

18.3.1 Lipopeptides

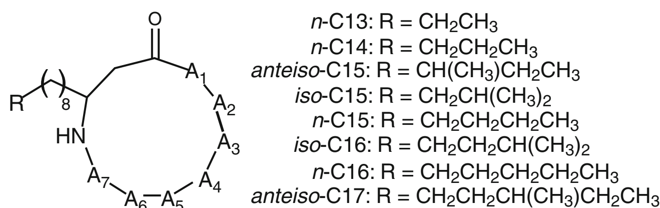
Lipopeptides strongly inhibit the growth of some soilborne fungi, especially *Pythium ultimum* Trow and *Colletotrichium* sp., but it has little effect on *Candida albicans* Robin, several other yeasts species, and some pathogenic and nonpathogenic filamentous fungi.

18.3.1.1 Iturin

The first isolated antifungal peptides are the iturin and bacillomycin families produced by *B. subtilis* (Bloquiaux and Delcambre 1956; Landy et al. 1948). The iturins produced by several strains of *B. subtilis* are cyclic lipopeptide antibiotics (Chen et al. 2008; Maget-Dana and Peypoux 1994). Iturins A–E (Besson et al. 1976; Besson and Michel 1987; Kikuchi and Hasumi 2003); bacillomycins D, F, L, and Lc (Peypoux et al. 1981, 1984, 1985; Eshita et al. 1995; Volpon et al. 2007); and mycosubtilin (Besson and Michel 1990), which have been previously described as the main variants of the iturin family. They are cyclic lipopeptides that contain a heptapeptide of α-amino acids that is cyclized with a C_{13} to C_{17} β-amino fatty acid (Table 18.1, entries 1–8) (Thasana et al. 2010). Today, several *B. subtilis* strains have been isolated to suppress the growth of plant pathogens. Such strains proved broad suppressive effect over a variety of plant pathogens (Phae and Shoda 1990; Phae et al. 1990) by producing the lipopeptide antibiotics iturin A and surfactin (Asaka et al. 1996; Hiraoka et al. 1992).

Iturin A is a cyclic lipopeptide, which contains a heptapeptide (L-Asn–D-Tyr–D-Asn–L-Gln–L-Pro–D-Asn–L-Ser) cyclized with a β-amino fatty acid. It is a small molecule yet displays strong antifungal activity (Shih et al. 2008). The strong efficacy of iturin A against various phytopathogenic fungi proved similar to the available chemical fungicides (Phae and Shoda 1990; Phae et al. 1990). It confers low toxicity effect on humans and animals (Delcambe et al. 1977) along with its wide spectrum antibiotic activity and high biodegradability. These characteristics qualify iturin A as a safe and environment friendly candidate for control of fungi (Phae et al. 1992). According to the different lengths of side chains, the eight different homologes of iturin have been designated as A1 to A8 (Fig. 18.1) (Delcambe et al. 1977; Isogai et al. 1982). Beside this, *Bacillus amyloliquefaciens* strain RC-2 produced seven antifungal compounds secreted into the culture filtrate

Table 18.1 Amino acid composition of the iturin family and compositional differences of the β-amino acid[a] (Thasana et al. 2010)



Entry	Antibiotic	Amino acid sequences L-A1–D-A2–D-A3–L-A4–L-A5–D-A6–L-A7
1.	Iturin A	Asn–Tyr–Asn–Gln–Pro–Asn–Ser
2.	Iturin C	Asp–Tyr–Asn–Gln–Pro–Asn–Ser
3.	Bacillomycin D	Asn–Tyr–Asn–Pro–Glu–Ser–Thr
4.	Bacillomycin F	Asn–Tyr–Asn–Gln–Pro–Asn–Thr
5.	Bacillomycin L	Asp–Tyr–Asn–Ser–Glu–Ser–Thr
6.	Bacillomycin Lc	Asn–Tyr–Asn–Ser–Glu–Ser–Thr
7.	SCP[b]	Asp–Tyr–Asn–Ser–Glu–Ser–Thr
8.	Mycosubtilin	Asn–Tyr–Asn–Gln–Pro–Ser–Asn
9.	Subtulene A[c]	Asn–Tyr–Asn–Gln–Pro–Asn–Ser

[a]Normal β-amino acid side chain (C_{13} to C_{17}).
[b]SCP is the synthetic cyclopeptide (β-amino acid = Ala).
[c]β-Amino acid side chain is 3-amino 13-methyltetradec-8-enoic acid (R = $(CH_2)_3CH$ CH $(CH_2)_3CH(CH_3)_2$).

and inhibit the development of mulberry anthracnose caused by *Colletotrichum dematium* (Hiradate et al. 2002).

18.3.1.2 Surfactin

In contrast to iturin A, another lipopeptide, surfactin, which is a biosurfactant consists of heptapeptide cyclized with a β-hydroxy fatty acid. It has a weak antibiotic activity (Arima et al. 1968). Its chemical structure is Glx–Leu–D-Leu–Val–Asp (Asn)–D-Leu–Leu (Ile or Val) forming a lactone at the end (Kakinuma et al. 1969).

18.3.1.3 Subtulene A

Subtulene A, a new cyclic lipopeptide, has been isolated from the cell free culture broth of *B. subtilis* SSE4. This peptide contains seven common α-amino acids, L-Asn-1, D-Tyr-2, D-Asn-3, L-Gln-4, L-Pro-5, D-Asn-6, L-Ser-7, and the unique β-amino acid-8 present in the iturin family. *B. subtilis* SSE4 exhibited antifungal activities (Thasana et al. 2010). It was reported that *B. subtilis* SSE4 can also produce iturin A.

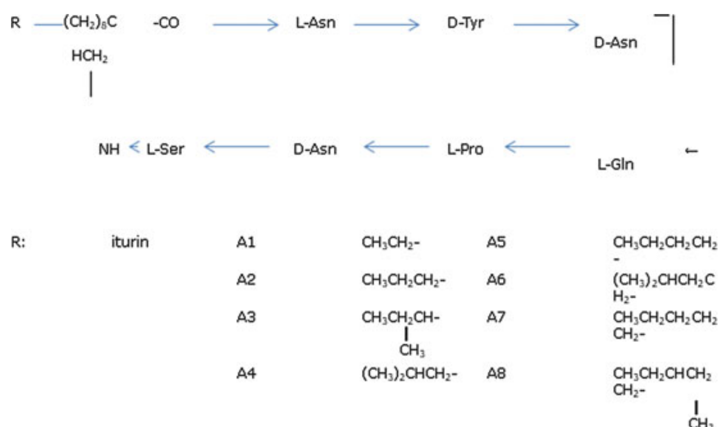


Fig. 18.1 Structure of iturin A: cyclic chain and homologues. *R* indicates the different structure of side chain of the homologues (Iwase et al. 2009)

18.3.1.4 Fengycin

Fengycin, another antifungal lipopeptide from *B. subtilis* strain F-29-3, has been found inhibitory to filamentous fungi but not to yeast. Its amino acid structure is L-Glu–D-Om–L-Tyr–D-allo–Thr–L-Glu–D-Ala (D-Val)–L-Pro–L-Glu–D-Tyr–L-Ile. And L-Tyr and L-Ile forms a lactone ring structure (Vanittanakom et al. 1986).

18.3.2 Polypeptide Substances

Antifungal peptides contain cyclic, linear, and branch structure. *B. subtilis* spores can produce a cyclic 13-peptide mycobacillin (Banerjee and Bose 1963) having composition D-Pro–D-Asp–D-Glu–Tyr–Asp–Tyr–Ser–D-Asp–Ala–D-Glu–Leu–D-Asp. The polypeptide from *B. subtilis* TG26 has molecular weight of 14.5 kDa and its N terminal sequence is Tyr–Gin–Ala–Pro–Glu–Tyr–Ile–Tyr– (Liu et al. 1994). Besides, the strain has a strong inhibition effect on a variety of plant pathogenic fungi such as rice sheath blight fungus (*R. solani*) and rice blast fungus (*Magnaporthe grisea*). Tts antimicrobial product is a cyclic peptide P1 with molecular weight 1,476.71 Da (He et al. 2002). Two novel antimicrobial homolgous peptides are produced by JM4-A and JM4-B and have seven identical amino acids (Wu et al. 2005). BS-2 strains secrete antimicrobial peptide of molecular weight 2,884.39 Da. It is heat-stable, resistant to UV radiation, and prevents many plant diseases. Effect of the certain antimicrobial peptides involve the control of pepper fruit anthracnose (He et al. 2003).

18.3.3 Other Antibiotic Substances

A novel phospholipid antibiotic named bacilysocin *B. subtilis* strain 168 was isolated from *B. subtilis* (Tamehiro et al. 2002) structure by NMR and mass spectrometry and has significant antifungal activity (Tamehiro 2002). Another novel small antifungal peptide produced by a *Bacillus strain* B-TL2 that has purified from having molecular mass of 2,500 Da and 2,237.7 Da. The purified product was obtained by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, respectively. The N-terminal amino acid sequence of BTL was determined as NH2-KQQLATEAESAGPIL. The peptide showed strong inhibitory activity against mycelial growth of *Bipolaris maydis*, *Alternaria brassicae*, *Aspergillus niger*, and *Cercospora personata* (Zhang et al. 2008).

In addition, the Cepacidines, glycopeptides from *Burkholderia cepacia* are noteworthy. The activity of pathogen suppression has enhanced both the molecules used together. A wide range of pathogen including *Candida* sp., *A. niger*, *Cryptococcus neoformans*, and *F. oxysporum* are also suppressed (Lee et al. 1994; Lim et al. 1994). Another novel antifungal antibiotic cepacidine is produced by *Pseudomonas cepacia* II. Compounds such as cepacin A and cepacin B exhibit antibacterial activity, whereas pyrrolnitrin is also effective against fungi, yeasts, and Gram-positive bacteria. Cepaciamide A and B are toxic compounds that exhibit strong activity against *Botrytis cinerea* Pers. Ex Fr. and *Penicillium expansum* Link (Quan et al. 2006).

18.4 Concluding Remarks and Future Perspectives

Nature has developed many defense systems to protect living organisms from pathogenic fungi. These antibiotics in many cases appeared to be important for crop plant disease control. Among them are the antibiotic substances produced by diverse life forms. They possess various industrial and agricultural applications such as biocontrol agents against pathogenic fungi as bio-fungicides to aggrandize the agriculture production. Over 100 natural peptides or their analogs have been found with varying activities against pathogenic fungi. Undoubtedly, many more remain to be discovered and, because analogs can be more potent than their parents, future research will certainly find novel antifungal peptides with potential pharmaceutical utility. The development of environmentally and human friendly antimicrobial agents for control of plant diseasess will substantiate the sustainable crop protection.

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