M. Tofazzal Islam · Mahfuz Rahman
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Bacilli and Agrobiotechnology

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ISBN 978-3-319-44408-6 ISBN 978-3-319-44409-3 (eBook)
DOI 10.1007/978-3-319-44409-3

Library of Congress Control Number: 2016959463

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Foreword

Sustainability is not only a modern term but also is a major concept encompassing present and future activities in agriculture to minimize harmful environmental impacts and stabilize growth. In this regard 'coming back to nature' methods were developed as 'organic farming', 'biocontrol' of pathogens and 'biostimulation' of plant growth. There are many different ways to establish and practise environmentally friendly cultivation systems. Looking at the food production chain from soil to the consumer to microorganisms in particular, *Bacilli* can make a contribution to secure sustainability.

Fungi, bacteria and other microorganisms previously had rather a bad reputation because pathogens could reduce yield and quality of products or destroy parts of the plants. At the least arbuscular mycorrhizal fungi have long been known and used to improve the nutrient uptake of different crops. Moreover, bacteria, fungi and actinomycetes have been well investigated as the main contributors to the decomposition of the organic materials in the composting process. *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Pseudomonas* ssp., *Serratia* ssp. and *Flavobacterium* can easily be detected at the beginning of this process.

The ability of microorganisms to promote plant growth and reduce growth disturbance due to stress situations in the rhizosphere such as high salt concentration, inadequate pH values and soilborne diseases was less investigated. There also are fewer investigations on the role of microbes in minimizing impacts on plants that are created by unfavourable climatic conditions such as low or high temperature, drought and water stress. In intensive agriculture production, the use of biological agents may be the best way to sustain food production.

The authors of this book in 16 chapters describe different *Bacilli* with a wide range of functionalities and their interactions with plant growth as biological stimulants in fluctuating growing conditions. Authors focus on the plant growth-promoting rhizobacteria (PGPR), *Bacilli* that are able to enhance nutrient availability, e.g. mobilization of phosphorus fixed in the soil and their role in nutrient uptake. Results presented in the book from various studies also describe *Bacillus* spp. as prolific siderophore producer in order to enhance the availability of iron and ability of biological nitrogen fixation for the plants as PGPR.

Another very important effect of *Bacilli* is their antiphytopathogenic potential. *Bacillus* spp. are promising biocontrol organisms for root, foliar and postharvest diseases of plants. The use of biopesticides such as *Bacillus amyloliquefaciens* FZB42, *B. amyloliquefaciens* subsp. *plantarum* strains as well as some isolates of the genus *Paenibacillus* has several advantages over chemical products as they do not pose risk to the environment and human health and pests do not grow resistance against these products. In addition, strains of *Bacillus* ssp. can induce systemic resistance resulting in increased tolerance to harmful microbes and plant growth promotion. *Bacilli* also have importance for biotechnological and pharmacological applications varying from fermentation industry to bioremediation of recalcitrant compounds.

I believe this book will be useful for researchers and teachers in agriculture or other biological disciplines as well as students and policymakers. Likewise, farmers, farm advisory services who are interested in the subjects in the plant and agricultural sciences will be immensely benefited from this book.

February 2016 Michael H. Bohme

Preface

An ever-increasing worldwide population has raised demand for the food, which in turn has led to an increasing dependence on the use of synthetic pesticides to control major pests and plant diseases. Excessive use of agrochemicals has not only caused serious environmental and health concerns but also has resulted in pests that are resistant to pesticides making entire production systems less safe and sustainable. This situation may be exacerbated by lower soil fertility that can lead to a severe agrarian crisis on a global scale. Alternative plant production and pest control methods that involve biorational approaches may mitigate these adverse conditions. There is a critical need for increased research funding that focuses on quality food production, using biologically based methods without the use of synthetic chemicals. Compilation and publication of the results from these researches should provide directions to sustainable pest management.

Since the inception of agriculture and crop production, prokaryotes have been unsung heroes in boosting crop growth and yield by their interaction with other flora and fauna. Their involvement predominantly takes place in the plant rhizosphere, the endosphere and to some extent the phyllosphere. Among a few prominent prokaryotic genera, *Bacillus* spp. represent one of the most important unmapped pools of biodiversity. This genus contains a vast member of species with many applications in agriculture, industry and medicine. These Gram-positive spore-forming bacteria are some of the most dominant groups that exist in various ecological niches on the earth due to their survivability in adverse environmental conditions by producing endospores. Due to their survival capacity, *Bacilli* can competitively colonize plant roots and other plant organs while simultaneously acting as biofertilizers and as antagonists (biopesticides) of important plant pathogens including bacteria, fungi, peronosporomycetes and nematodes. The *Bacilli* are grabbing attention due to their ability to produce phytohormones, solubilize inorganic nutrients including phosphates, fix atmospheric nitrogen, produce antibiotics as well as lytic enzymes and induce systemic resistance in plants against pathogens. Biological systems that utilize this genus for enhanced plant growth and development may well shape the future of sustainable agriculture.

Recognizing the enormous potential of the bacteria in this genus, scientists all over the world have directed significant research towards selection and commercialization of the best organisms that may provide protection of plants from harmful microbes and/or enhance plant growth. The list of efficient disease-controlling and growth-promoting *Bacillus* species is increasing daily. Some of the most promising and widely studied microbes include *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis* and *B. firmus*.

Remarkable advancements made in understanding the biology of plant-*Bacillus* interactions have resulted in a specialized area of research in agrobiology. This book, *Bacilli and Agrobiotechnology*, was written based on those advancements. The volume is comprised of 16 chapters that cover the applications of *Bacillus* in promoting plant growth, protection and productivity. The book will be useful to students, teachers and researchers who cover subjects such as agriculture, plant physiology, plant protection, agronomy, microbiology, biotechnology and environmental sciences.

Although the book's major focus is with applied aspects in agriculture, it also highlights research findings focused on utilizing microbes that produce industrially important antibiotics, enzymes, probiotics and other biochemicals. The book also describes innovative approaches utilizing *Bacillus*-mediated bioremediation of environmental pollutants such as pesticides, explosive wastes, dyes and polycyclic aromatic hydrocarbons. However, plant growth promotion and biological control of plant pathogens remain the centrepiece of the book. Possible mechanisms and modes of action are discussed as future research directions. The most common and well-elaborated beneficial traits of *Bacilli* highlighted in the book include atmospheric nitrogen fixation, organic and inorganic phosphorus solubilization, phytohormone production, root development and nutrient uptake enhancements (e.g. siderophore production), induction of systemic resistance in plants, enhanced plant tolerance to environmental stresses and suppression of harmful microbes. Managing major diseases with *Bacillus* spp. now is within the reach of researchers who can utilize information presented in many of the chapters. A biotechnological approach including genomics, transcriptomics and metabolomics to augment beneficial biochemical production from this group of bacteria also has been suggested.

Using insecticidal toxin from *Bacillus thuringiensis* (Bt) is an approach in pest management that has been useful in the development of pest-resistant crop varieties. Agricultural biotechnology that involves a wide range of pesticidal genes from Bt has dominated the pesticide management research for the last few decades – with both favourable and unfavourable public comments. Transgenic Bt crops have gained popularity in the United States and many other countries. A unique chapter in this book illustrates the pros and cons of this technology when used to control corn rootworm. The emergence of resistant insect pests to Bt-based bioinsecticides and Bt crops has created new challenges. Readers will get some directions on how to overcome these issues by diversifying available management tools. As mosquito-borne diseases continue to threaten human health, the chapter that discusses the potential of Bt to control mosquitoes in a more environmentally friendly way is a timely addition.

This book represents a cooperative effort from all the editors and contributors representing many different countries. The editors gratefully acknowledge the authors who contributed to the project. We are grateful for the enthusiasm and collegial spirit they demonstrated. Our profound thanks also are due to Prof. Michael Böhme for writing a scholarly foreword for this book.

We would like to thank Springer Life Sciences publishing editor Dr. Elodie J. Tronche who made suggestions on making this book applicable to the audience. It has been a privilege to interact with scientists who conduct research on these fascinating microbes and compiling their findings. We believe researchers who work with the *Bacilli* will find this book an essential reference.

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Chapter 1
Role of *Bacillus* Genus in the Production of Value-Added Compounds

Milad Mohkam, Navid Nezafat, Aydin Berenjian, Manica Negahdaripour, Abdolazim Behfar, and Younes Ghasemi

Abstract Development of value-added products from renewable supplies is attracting more and more attention due to the fossil fuel resource depletion and environmental concerns. *Bacillus* species show distinctive benefits as hosts for production of industrially important enzymes and biochemical compounds. They are also improved through metabolic engineering techniques for efficient production of fuels, microbial enzymes, and fine and bulk chemicals. In this chapter, recent findings about *Bacillus* spp. and their usage as microbial factories are summarized.

1.1 Introduction

Microorganisms are progressively used as renewable sources for the manufacturing of a variety of materials and chemical compounds due to fossil supplies depletion. The microbial fermentation products derived from renewable, readily available, and low-cost crude substrates will competitively substitute some of the petroleum-based products (Keasling 2010; Kim et al. 2012; Zhang and Zhang 2012). In this regard,

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_1

Saccharomyces cerevisiae, *Escherichia coli*, and *Bacillus* species are extensively applied for the manufacturing of pharmaceuticals and industrially important biochemicals. Due to the ease of genetic manipulation and well-established systems-level strategies, *E. coli* and *S. cerevisiae* are considered suitable hosts for the production of industrially important enzymes, pharmaceuticals, and biochemicals (Ajikumar et al. 2010; de jong et al. 2011). However, *Bacillus* species are now becoming more important potential candidates. *Bacillus* species are nonpathogenic and non-toxin producers (except for *B. cereus* and *B. anthracis*) and extensively have been used in food industries. Most of *Bacillus* species products have received the status of generally recognized as safe (GRAS) by US FDA organization. Distinctive traits and remarkable advancements in genetic manipulation introduced *Bacillus* species as a favorable production system. Moreover, the attractiveness of this bacterium for application in the industrial platform is attributed to its rapid growth rates leading to a short fermentation period and its ability to excrete proteins and biochemical compounds into the medium (Schallmey et al. 2004; Wenzel et al. 2011).

Considering the recent progresses in the production of value-added products by *Bacillus* species, it is necessary to summarize the current advancements. In this chapter, different classes of produced compounds by *Bacillus* genus such as industrial enzymes, vitamins, amino acids, and antibiotics are also reviewed. Finally, the application of *Bacillus* species in foods and probiotics is discussed.

1.2 *Bacillus* Enzymes: Different Types and Applications

To date, numerous enzymes have been used for different pharmaceutical, industrial, and analytical purposes. The global market for the industrial enzyme production was estimated to be $3.3 billion/year in 2010 and is expected to rise to about $5.5 billion in 2015 (Dewan 2011). *Bacillus* species have a major role in the production of industrial enzymes. Almost 60 % of the commercial enzymes are reported to be produced by these bacteria (Dworkin 1999). Contrary to *E. coli*, the Gram-negative bacteria widely used in the enzyme production, some *Bacillus* strains, have been approved as GRAS (generally regarded as a safe); therefore, they are ideal for applying in food industry due to the absence of lipopolysaccharides (LPS), which are pyrogenic in humans (Petsch and Anspach 2000). In addition, a number of *Bacillus* spp. are broadly utilized for enzyme production in detergent, beverage, and textile industries (Table 1.1).

Nattokinase, a robust fibrinolytic serine protease, is found in Korean doenjang (Kim and Choi 2000), Korean chungkookjang (Kim et al. 1996), Japanese natto (Sumi et al. 1987), Chinese douche (Peng et al. 2003), and also in various bacteria (Peng et al. 2005). Intravenous fibrinolytic drugs, such as plasminogen activator and urokinase, have been broadly utilized in clinics as thrombolytic agents. Due to the cost-effectiveness and fewer side effects of microbial fibrinolytic enzymes over the conventional thrombolytic drugs, they have notably attracted more interest in recent

Table 1.1 Industrial applications of the important *Bacillus* spp. enzymes

Source of enzyme	Enzyme	Application
B. circulans	Transglutaminase	Texture improvement in yogurt and whipped cream
		Strength improvement of bread dough
Bacillus sp.	α-Amylase	Cleaning
		Baking
		Brewing
		Sweeteners
		Fuel ethanol
		Textile processing
Bacillus sp.	Xylanase	Baking
		Animal feed processing
		Textile processing
Bacillus sp.	Protease (subtilisin)	Cleaning
		Animal feed
		Leather production
B. licheniformis	Pullulanase	Increase fermentability of starch worts or syrups
B. cereus	β-Amylase	Mashing
		Fruit juice production
B. brevis	α-Acetolactate decarboxylase	Brewing

years (Peng et al. 2005; Dabbagh et al. 2014). Based on nattokinase high fibrinolytic activity, protective and long-lasting effects, appropriate oral administration, and stability in the gastrointestinal tract, it has attracted more attention than any similar commercially used medications (Dabbagh et al. 2014; Deepak et al. 2008). Intensive investigations have been performed on the microbial fermentation of nattokinase in order to enhance the level of the enzyme production (Berenjian et al. 2014; Vijayalakshmi et al. 2013; Cho et al. 2010; Kwon et al. 2011). Technically, nattokinase was produced by extraction of natto with physiological saline followed by addition of 75 % ethanol. The extract was then subjected to several chromatographies and gel filtration for obtaining pure nattokinase (Sandhya et al. 2005).

Proteolytic enzymes (EC 3.4) are the major subset of commercially applicable industrial enzymes. They are generally exploited in washing powders, laundry, textile manufacturing, baking, dairy, pharmaceuticals, and other applications (McAuliffe 2012). Proteases are divided to serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic acid proteases (EC 3.4.23), and metalloproteases (EC 3.4.24) (Outtrup et al. 2002). The serine proteases are broadly found in microbes. Microbial proteases are classified as acidic, neutral, and alkaline. *Bacillus* spp. proteases represent more than 95 % of the sales of all proteases (Kumar et al. 2008). The well-known serine proteases are subtilisins, a member of alkaline endoproteases family, which are mainly utilized in cleaning usages due to their wide

substrate specificity, stability under alkaline pH conditions, resistance to denaturation by surfactants, and broad temperature optima (Mukherjee et al. 2008). In addition, subtilisins are produced by different *Bacillus* strains (Vijayalakshmi et al. 2013; Maurer 2004). The main usage of *Bacillus* proteases is in detergents, in both washing powders and laundry applications. Proteinous stains, such as keratin, blood, milk, and gravy, generally coagulate on fabrics and precipitate during the usual washing. In the same way, protein dirt can be hard to clean from dishes by means of chemical-based cleaners alone. Adding protease to cleaners cleaves such proteinous dirt into soluble peptides and facilitates the dirt removal (Mukherjee et al. 2008).

The alkaline serine protease from *Bacillus licheniformis* (subtilisin Carlsberg) is the first bacterial protease that is used in detergents (Saeki et al. 2007). Since early 1980s, in order to decrease the energy waste and protect the environment, detergent companies have substituted phosphate with other detergent constructors. Thus, new proteases that were properly activated under high alkaline and low temperature conditions were sought (Aehle 2006). Presently, the subtilisins from *Bacillus* species including *B. pumilus*, *B. clausii*, *B. alcalophilus*, *B. lentus*, and *B. halodurans* are broadly applied in detergents worldwide. Commercially available proteases are Savinase®, Relase®, Kannase®, and Ovozyme® from Novozymes company; Purafect®, Purafect® Prime, and Properase® from Genencor; and also Proleather® and Protease P® from Amano Pharmaceuticals Ltd., Nagoya, Japan (Siegert et al. 2013). Recently, a protease obtained from *Bacillus pumilus* with US patent no. 8455424 B2 is employed in detergents and cleaning agents (Choudhary et al. 2004). Authors claimed that in contrast to the other proteases, their new protease has a satisfactory proteolytic activity at lower temperatures from 10 to 50 °C and ideally 10–40 °C. In addition to the abovementioned characteristics, the enzyme is stable in the presence of bleaching, surfactant agents, and a broad range of pH. Alkalophilic *Bacillus* species produce protease enzymes that are stable in high alkaline conditions (pH as high as 12), suitable for usage in leather industry and wool scouring process (Kumar and Satyanarayana 2009). The *Bacillus* spp. neutral proteases, with pH optima around 7, are applied in chill-haze removal in brewing, baking and cheese manufacturing, modification of milk proteins, and animal foods (Schallmey et al. 2004). Commercially, subtilisin is produced by submerged fermentation of *B. licheniformis* in a medium comprising of starch hydrolysate 50 g/L, soybean meal 20 g/L, casein 20 g/L, and Na_2HPO_4 3.3 g/L. In this manner, subtilisin is the only protease in the medium at the end of the fermentation. This enzyme was then marketed chiefly in the dust-free granules containing 1–5 % of subtilisin (Bano et al. 2011).

Amylase enzymes catalyze the hydrolysis of starch and have a prominent role in a broad number of applications such as cleaning, baking, brewing, sweeteners, fuel, ethanol, textile processing, mashing, and fruit juice production (Rajagopalan and Krishnan 2008). Amylases can be classified into five categories according to their fashions of cleavage: (1) α-amylase, (2) β-amylase, (3) glucoamylase (or amyloglucosidase), (4) the debranching enzymes such as pullulanase, and (5) G4-amylase. The industrial amylases are derived from microbial origins especially *Bacillus* strains such as *B. subtilis* (Hmidet et al. 2010; Gangadharan et al. 2008), *B. licheni-*

formis (Božić et al. 2011), and *B. amyloliquefaciens* (Prakash and jaiswal 2010). Thermostability is an ideal feature for most of the industrial enzymes. The liquefaction and saccharification of starch are processes performed by heat treatment at high temperatures (100–110 °C) in the presence of thermostable amylolytic enzymes. The α-amylase from *Bacillus licheniformis* retains its activity at 90 °C and pH 6.5 (Van Der Maarel et al. 2002). Similarly α-amylase from *B. amyloliquefaciens* has activity at 90 °C and pH 5.5–7. These specifications make these bacteria ideal producers of thermostable α-amylase, which is applied in a variety of commercial products (Bruinenberg et al. 1996). The main application of α-amylase is in starch industry for hydrolysis and conversion of starch to fructose and glucose syrups. Due to high sweetening ability, it is widely used in beverage industry. Nowadays, the α-amylase from *B. stearothermophilus* or *B. licheniformis* is utilized in starch industry (Saxena et al. 2007). In baking industry, the α-amylase has a crucial role in producing various high-quality products. Presently, a thermostable amylase from *B. stearothermophilus* is massively used in baking industry (Saxena et al. 2007). Many textile industries take advantage of α-amylase for textile desizing. Starch enhances the rigidity of the final products after washing out the fabrics. For a long period of time, *Bacillus* amylase has been exploited in textile industries (Van Der Maarel et al. 2002). α-Amylase is applied in paper and pulp industry to coat paper, increase the writing quality of paper, and enhance its strength and stiffness. In paper sizing process, α-amylase converts starch to glucose and fructose (Gupta et al. 2003). Ethanol is one of the most applied liquid biofuels. Starch is an ideal substrate for ethanol production because of its accessibility and low cost. Fermentable sugars are obtained from the bioconversion of starches in the presence of α-amylase; then such sugars are converted into ethanol applying ethanol-fermenting microbes (Mukherjee et al. 2009). Moreover, α-amylase is extensively used in the formulation of detergents, and currently 90 % of all liquid detergents consist of this enzyme (Demain 2007). This enzyme is employed in washing and laundry detergents in order to degrade starchy dirt (custard, chocolate, gravies, etc.) to smaller oligosaccharides, which are easier to remove (Kuninaka 1996). The industrially medium for production of α-amylase by *B. subtilis* was ground soybean meal 1.85 g/dL, autolyzed brewer yeast 1.50 g/dL, distiller dried soluble 0.76 g/dL, casein hydrolysate 0.65 g/dL, lactose 4.75 g/dL, $MgSO_4$ $7H_2O$ 0.04 g/dL, and Hodag KG-1 antifoam agent 0.05 g/dL. The fermentation was carried out at 35 °C in the fermenter vessel of 200 L at 170 rpm for 48 h. The fermentation broth was subjected to ethanol precipitation and then filtration. Finally the precipitated enzyme was vacuum dried for 8 h and then ground to obtain fine powder (Matsui et al. 2001).

1.2.1 Nucleotide Production

Primary metabolites are small molecules that are effectively involved in the growth, development, and reproduction processes of all organisms. The most commercially important primary metabolites are amino acids, nucleotides, vitamins, solvents, and

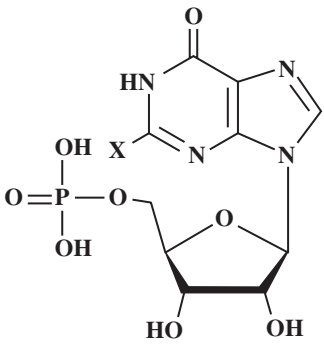

Fig. 1.1 Structure of flavor nucleotides (Kuninaka 1996). IMP: X=H, GMP: X=NH2, and XMP: (xanthosine-5′-monophosphate, 5′-xanthylate) X=O

organic acids. The main global market of nucleotides belongs to three purine nucleosides, namely guanylic acid (5′-GMP), inosinic acid (5′-IMP), and 5-xanthylic acid (XMP) (Fig. 1.1). These nucleosides are utilized in food industries as flavor enhancers and also in medicine. Three main processes are employed in the production of the abovementioned nucleosides: (1) fermentative production of the inosine and guanosine by *Bacillus subtilis* mutants through subsequent chemical phosphorylation; (2) hydrolysis of yeast RNA by fungal nuclease to AMP and GMP, by subsequent enzymatic deamination of AMP to IMP; and (3) exploiting mutants of *Corynebacterium glutamicum* for direct fermentation of sugar to IMP plus conversion of guanine to GMP via salvage pathway applying in *Brevibacterium ammoniagenes* (Asahara et al. 2010).

The achieved amount of IMP was up to 27 g/L via direct fermentation (Yang et al. 2011). The effective synthesis of GMP and IMP in *B. subtilis* is regulated by the end-product repression and feedback inhibition, and the mutants that require adenine and xanthine and are resistant to these feedback effects have been engineered genetically. In conventional methods, in order to achieve a high level of inosine, the inosine producer strains (particularly *B. subtilis*) were subjected to several random mutagenesis by chemical agents or UV irradiation. Although such conventional strategies resulted in the production of high levels of inosine, many undesired and undefined mutations occurred in these strains. Therefore, definite and selective gene-targeted mutagenesis is needed. The genes required for purine production and purine salvage pathways have been identified in *B. subtilis* and *E. coli*. Deleting *purA* (adenylosuccinate synthase), *deoD* [purine nucleoside phosphorylase (PNP)], *add* (adenosine deaminase), and *purR*, followed by replacing *purF* (glutamine PRPP amidotransferase) by a plasmid-borne mutant *purF* gene encoding an enzyme insensitive to the feedback repression by AMP and GMP in *E. coli* W3110, resulted in producing 1 g/L inosine from 40 g/L glucose in culture broth (Zhang et al. 2011a, b). It was reported that introducing seven gene-targeted mutations enhanced the fermentative inosine production in *B. subtilis* W168 (Li et al. 2011). All mutations were applied to inhibit IMP from being consumed for AMP and GMP synthesis, in order to reduce inosine degradation, and to increase the intracellular IMP levels. In the first step, *PurA*, *guaB* (IMP dehydrogenase), *punA*

(purine nucleoside phosphorylase), and *deoD* were inactivated. Subsequently, in order to increase the purine nucleotide biosynthesis, the *pur* operon repressor, PurR, and the 5′-UTR of the operon comprising the guanine riboswitch were disrupted. Ultimately, the −10 sequence of the *pur* promoter was optimized to enhance its transcription level. The *B. subtilis* W168 was able to produce 6 g/L inosine from 30 g/L glucose in the culture broth. In *Bacillus amyloliquefaciens* XH7, which is used to produce purine nucleoside in the industrial scale, the *guaB* gene was deleted, the *purA* gene was truncated, and the nonsense mutation was occurred in *guaC* gene (GMP reductase gene) (Shimaoka et al. 2007). The *Bacillus amyloliquefaciens* TA208 is utilized for the industrial production of guanosine and synthesis of ribavirin (antiviral drug) by assimilation of formamide (Zakataeva et al. 2007). Moreover, the *de novo* genetic engineering of *B. subtilis* W168 was performed to improve the fermentative production of purine nucleosides (Mandal et al. 2003). Based on the results, the maximum level of inosine biosynthesis was 7.6 ± 0.34 g/L, with a 4.7 % (w/w) conversion ratio of glucose to inosine, which was produced by the *deoD* and *purA* double mutant (Mandal et al. 2003).

Besides *deoD* and *purA*, numerous different target genes in the purine biosynthesis pathway have been identified to enhance inosine production upon deletion or overexpression. They include *purL*, *purM*, *purC*, *purR*, *pgi*, *prs*, and *purF* (Miyagawa et al. 1989); the gene encoding the purine nucleoside efflux pump *pbuE* and *pbuX* and their riboswitch containing promoter regions were targeted for further improvement of *Bacillus* strains (Zakataeva et al. 2007; Miyagawa et al. 1986; Sauer et al. 1998). Insertional inactivation of IMP dehydrogenase gene in xanthine-requiring mutant of *B. subtilis* resulted in production of 35 g/L inosine (Burkovski and Krämer 2002). Transforming of recombinant plasmid harboring IMP dehydrogenase gene into *B. subtilis* NA6128, an inosine overproducer, resulted in an increase in both the activity of IMP dehydrogenase and the yield of guanosine (Leuchtenberger et al. 2005). The level of guanosine production in *B. subtilis* NA6128 was 7 g/L, which reached 20 g/L after transforming with recombinant plasmid. Sauer et al. developed a stoichiometric model of *B. subtilis* metabolism for the quantitative analysis of the theoretical growth and production capacity of commercial biochemicals, namely inosine, guanosine, riboflavin, and folic acid. The highest productivities that can be reasonably expected by using *B. subtilis* on glucose were evaluated to be 0.343 and 0.160 (mol product/mol glucose) for purine nucleosides and riboflavin, respectively (Eggeling and Bott 2010).

1.2.2 Amino Acids

The current amino acid global market is about $6 billion and is estimated to grow around 10 % annually (Pfefferle et al. 2003). Major applications of amino acids are in flavor enhancers and feed additives such as L-lysine, L-threonine, and L-tryptophan. Microbial fermentation is the main strategy applied for the large-scale amino acid production. *Corynebacterium glutamicum*, *Brevibacterium*

flavum, and *Brevibacterium lactofermentum* utilize molasses, sucrose, and glucose as sugar sources to produce amino acids in the fermentation process (Sugimoto 2010). In this context, *C. glutamicum* is a well-studied bacterium, with a global sales of 1 million tons of L-glutamate and 0.6 million tons of L-lysine, annually (Brautaset et al. 2007). For decades, the microbial producers of different amino acids were obtained experimentally by random mutagenesis and selection. However, in the light of recent discoveries in the genetic map and metabolic pathways, particularly in *C. glutamicum*, scientists are now able to target specific genes to improve amino acid producer strains via recombinant DNA technology. Besides the improvements in genetic engineering, advances in fermentation technology also have led to prominent advantages in the industrial production of amino acids, particularly L-lysine and L-glutamic (Motoyama et al. 2001). L-Threonine, L-tryptophan, L-phenylalanine, and L-isoleucine are mainly produced by *E. coli* or *E. coli* mutant strains, and L-lysine HCl, L-tryptophan, L-histidine, and L-serine are biosynthesized by *C. glutamicum* or *C. glutamicum* mutant strains (Anakwenze et al. 2014). The economically feasible large-scale production of amino acids such as methionine has obtained a considerable interest. Due to the remarkable genetics and the physiological features of thermotolerant methylotroph *Bacillus methanolicus*, which uses methanol as a substrate, this *Bacillus* strain has been suggested as an attractive candidate for the overproduction of L-lysine and L-glutamic from methanol (Stahmann et al. 2000). Motoyama et al. showed that a *Methylobacillus glycogenes* mutant, harboring a plasmid with a mutated *dapA* gene (encoding dihydrodipicolinate synthase (DDPS)), is able to produce about 8 g/L of L-lysine and 37 g/L of L-glutamate from methanol at 37 °C (Kalingan and Krishnan 1997). Recently, the optimization of the *Bacillus thuringiensis* EC1 fermentation conditions has been performed in order to increase the methionine production (Bigelis 1989). *B. thuringiensis* EC1, which was isolated from the fermented oil bean seed, showed a methionine productivity of 1.89 mg/mL before optimization. The effects of the inoculum size, carbon and nitrogen sources, growth stimulators, vitamins, and bivalent metals on methionine production were also investigated. The highest methionine concentration that was produced by *B. thuringiensis* EC1 in the optimized culture condition was 3.18 mg/mL (Bigelis 1989).

1.2.3 Production of Vitamins by Bacillus *Species*

Microorganisms including *Bacillus* species produce many valuable small molecules such as vitamins. Many *Bacillus* species are commercially used for the production of vitamins such as biotin (vitamin H), thiamine (vitamin B_1), riboflavin (vitamin B_2), cobalamin (vitamin B_{12}), and menaquinone (vitamin K_2).

Riboflavin or vitamin B_2 is extensively present in animals and plants and has a major role in organisms' metabolism as the precursor of coenzymes like flavin adenine dinucleotide (FAD) and flavin mononucleotide (riboflavin 5′-monophosphate, FMN). These coenzymes are widely utilized in the enzymes involved in the inter-

mediate metabolism. More than 3000 tons riboflavin is reported to be produced annually, 2500 tons of which is produced via fermentation techniques (Batra 1973). Currently, riboflavin is manufactured by *Eremothecium ashbyii* and *Ashbya gossypii*, with the maximum rate of 3.3 and 20 g/L, respectively (Perkins et al. 1991; Schowen 1998). However, the long fermentation period (8 days) and instability of these organisms during fermentation and storage, especially *A. gossypii*, are not practical from an economic point of view (Melnick et al. 2004). In this regard, by means of metabolic engineering, *B. subtilis* strain is now able to produce more than 15 g/L riboflavin in a short fermentation time (Winkler et al. 2002).

Vitamin B_1 or thiamine pyrophosphate (TPP) is synthesized by various fungi, plants, and microorganisms. However, animals are not able to produce this compound and must obtain it from diet. This vitamin is a cofactor of many important enzymes in amino acid and carbohydrate metabolisms (Schyns et al. 2005). Unlike the other vitamin biosynthesis pathways in bacteria (e.g., for biotin and riboflavin), the biosynthesis of thiamine is a part of the salvage pathway but not part of the *de novo* pathway (Streit and Entcheva 2003). Therefore, the thiamine production rate in bacteria is not considerable. Hence, there is a need for seeking potent resources. One promising bacteria is *B. subtilis*, in which whole-genome sequence analysis shows no *de novo* pathway for thiamine production. This pathway was previously discovered in *Saccharomyces cerevisiae* (Sauer et al. 1998; Pirner and Stolz 2006). Recently, Schyns et al. introduced a new *B. subtilis* mutant with four mutations in thiamine biosynthesis genes that is significantly able to produce high concentrations of thiamine into the culture medium as 10 mg/L in a 20 L fermenter (Zhang et al. 2011a, b).

Biotin is comprised of an ureido and a tetrahydrothiophene ring. Biotin is a coenzyme needed for carboxylase enzymes, implicated in the synthesis of fatty acids, gluconeogenesis, and several amino acids' biosynthesis (isoleucine and valine) (Bower et al. 2001; Martens et al. 2002). Biotin deficiency is rare as the normal intestinal flora generate excess biotin needed as the recommended body's daily requirements (Moore et al. 2014). However, the most demand for biotin is in cosmetic industries and a number of metabolic disorders relating to abnormal biotin metabolism (Bower et al. 2001). Interestingly, *B. subtilis* is able to produce acceptable amounts of biotin. Pimelic acid is the precursor for biotin, although the rate of vitamin production is low in the absence of this substrate. In this regard, Zhang et al. developed an engineered *B. subtilis* capable to produce pimelic acid that would potentially diminish the cost of biotin production (Moore et al. 2014). Moreover, the manufacturing of biotin is now commercialized by Roche Vitamins Inc. with the final concentration of 1 g/L (Moore et al. 2013).

Cobalamin is a water-soluble vitamin with an important role in the normal performance of brain and nervous system as well as blood formation. This vitamin is synthesized exclusively by a number of bacteria and archaea. Cobalamin is required in animals and protists but not in plants and fungi (Berenjian et al. 2011). There are two different pathways for vitamin B_{12} synthesis in the aerobic and anaerobic mechanisms. The anaerobic pathway is utilized by *B. megaterium*. This bacterium has been previously employed for the industrial production of vitamin B_{12} in order to

treat pernicious anemia (Mahanama et al. 2012). As the production of cobalamin is controlled by riboswitch mechanism, many researchers developed *B. megaterium* mutants that are able to bypass this controlling mechanism and increase the production rate of cobalamin (Mahanama et al. 2012; Berenjian et al. 2013). These findings, therefore, provide a potential way to develop *B. megaterium* for the large-scale manufacturing of cobalamin by using inexpensive substrates.

Vitamin K is a group of fat-soluble vitamins playing a key role in blood coagulation and also in the bone and some other tissues (Mahanama et al. 2011; Sumi and Sumi 2004; Benedetti et al. 2010). This group of vitamin has two vitamers that are named as vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone). Vitamin K_1 or phylloquinone is exclusively produced by plants and, when ingested by human, is converted to vitamin K_2 form. On the other hand, vitamin K_2 or menaquinone is produced mostly by bacteria. This form of vitamin K is of interest, because it can be used directly without the need of conversion of phylloquinone to menaquinone. In this regard, there are several forms of menaquinones that are produced by bacteria, including MK-4, MK-5, MK-7, MK-8, etc. Among them, MK-7 has attracted the maximal interest due to its highest activity, better body absorption, and longer half-life in blood. Interestingly, *B. subtilis natto* is able to produce large amounts of MK-7 using inexpensive raw materials (Mahanama et al. 2011; Ashiuchi 2013). MK-7 production by this bacterium is now commercialized by various companies like Gnosis S.P.A. and Honda Trading Company (Kreyenschulte et al. 2012; Bajaj and Singhal 2011).

1.2.4 Poly-γ-glutamate and Biosurfactant Production by Bacillus *Species*

Most of the petroleum-based polymers such as silicons, glass fibers, and polyester materials are hardly degradable in the environment. Although these polymers have been used only for a short period of time, they have shown long-lasting unpleasant effects on the environmental cycles. Over the past decades, numerous biopolymers have been produced. Among them, poly-γ-glutamate (PGA), which is a water-soluble, anionic, biodegradable, and edible biopolymer, has attracted attention as an ideal substitute for the petroleum-based polymers. Due to the prominent features of PGA, it is widely applied in biodegradable plastics, fertilizers, animal feed additives, nanoparticle drugs, and pharmaceutical applications (Wei et al. 2014; Cao et al. 2011; Zeng et al. 2014). PGA consists of D- and L-glutamic acids joined by amide bonding between α-amino and γ-carboxylic groups (Fig. 1.2). Until now, three different kinds of PGA have been discovered from different microbes: (1) the homo polymer of D-glutamate (D-PGA) which is only produced by *Bacillus anthracis*; (2) the homo polymer of L-glutamate (L-PGA) obtained from *Natrialba aegyptiaca*, *Bacillus megaterium*, *Bacillus halodurans*, and *Natronococcus occultus*; and (3) a random copolymer comprising of D- and L-glutamate units (DL-PGA) which

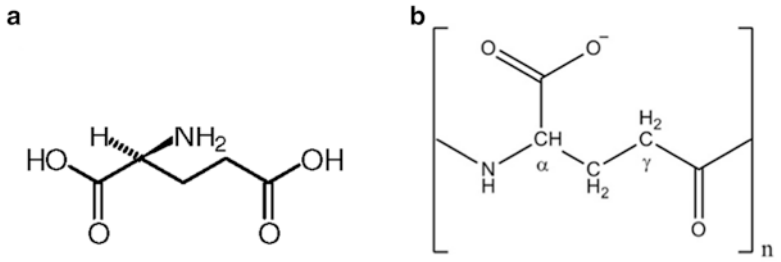

Fig. 1.2 Chemical structure of (**a**) L-glutamic acid; (**b**) poly-γ-glutamate (PGA)

is found in *Bacillus subtilis*, *Bacillus licheniformis*, and some *Staphylococcus epidermidis* strains (Wei et al. 2014). Although PGA is found in different microbes, it is mainly produced by *Bacillus* species, especially by *B. amyloliquefaciens*, *B. subtilis*, *B. megaterium*, *B. pumilus*, *B. mojavensis*, and *B. licheniformis* (Ivanovics and Bruckner 1937; Kambourova et al. 2001; Buescher and Margaritis 2007). It also can be produced either intracellularly or extracellularly throughout the fermentation process of *Bacillus* spp. PGA was first found by Ivánovics and Bruckner in the capsule of *Bacillus anthracis*, where it was considered as a crucial virulence factor of *Bacillus* (Xu et al. 2014). Because of the importance of PGA in numerous applications, many studies have been performed to increase the yield of PGA production, particularly by using *Bacillus* species in submerged fermentation (SMF) and solid-state fermentation (SSF). A number of *Bacillus* species have been investigated for the extracellular production of PGA. Generally *Bacillus* strains can be classified into two groups according to their nutrient requirement as L-glutamic acid-dependent strains and L-glutamic acid-independent strains (Jeong et al. 2013). Besides L-glutamic acid, some other important factors ranging from metal ions, carbon sources, nitrogen sources, temperature, aeration, and medium pH have significant effects on the yield of PGA (Huang et al. 2011). Intensive studies have been performed on the optimization of *Bacillus* PGA production with a great emphasize on the medium components. *B. subtilis* and *B. licheniformis* are the two species of *Bacillus* predominantly used in the large-scale production of PGA (Zeng et al. 2014). Zongqi et al. observed that immobilization of *Bacillus subtilis* NX-2 in an aerobic plant fibrous-bed bioreactor (APFB) can increase the PGA concentration to 71.21 g/L (Cao et al. 2010). A high PGA producer strain, *Bacillus subtilis* RKY3, was isolated and the highest PGA concentration (41.4 g/L) was observed under optimal culture conditions (no pH control, 1.5 vvm aeration, and 300 rpm agitation) (Manocha and Margaritis 2010). In addition, the use of fed-batch culture and the presence of glucose (3–10 g/L) significantly enhanced the concentration of PGA to 01.1 g/L; this approach resulted in achieving a large-scale cost-effective and efficient production strategy (Matsuo et al. 2013).

To date, few investigations have been performed to produce recombinant PGA. Cao et al. constructed a recombinant plasmid by pXMJ19 shuttle vector that consists of PGA biosynthesis genes (*pgsB*, *pgsC*, and *pgsA*) from *B. licheniformis*

NK-03. Consequently, the recombinant plasmid pXMJ19-PGS was transformed and expressed in *Escherichia coli* JM109 and *Corynebacterium glutamicum* (Hsieh et al. 2005). The amount of recombinant PGA expression in *C. glutamicum* was 0.69 g/L. In a similar research, the PGA biosynthesis genes (*pgsBCA*) from *B. amyloliquefaciens* LL3, a new glutamic acid-independent PGA-producing *Bacillus*, were cloned and expressed via pTrcLpgs vector in *E. coli* JM109, which led to the production of PGA without glutamate (Kambourova et al. 2001).

Since PGA is an environmental-friendly biodegradable polymer and is nontoxic to human, it is broadly utilized in numerous applications. Due to the PGA anionic nature and water solubility, it has been intensively investigated as a drug-delivery agent especially in anticancer drugs. PGA-doxorubicin conjugate (DOX/PGA) prepared by ionic interactions of native doxorubicin with PGA was designed to control the release of DOX in malignant tissues (Otani et al. 1999). In vitro, drug release studies indicate that DOX/PGA complexes were comparatively stable at neutral pH, but detach slowly under acidic pH conditions, making easier a pH-triggered release of DOX from the complex (Otani et al. 1999).

Other PGA biomedical usages include application as efficient anticancer vaccine adjuvants along with low immunogenic antigens, which results in inciting potent cellular and humoral immunity (Tanimoto et al. 2007), or as a scaffold in tissue engineering (Tanimoto 2010), and as biomedical adhesives for sealing of air and body fluid leaks in surgery (Anon. 2012).

Osteoporosis, a notable condition affecting elderly particularly aged women, originate from a dramatic reduction in the bone mass. In this context, it was suggested that PGA enhances the *biostability* of calcium (Ca^{2+}) by elevating its intestinal absorption and solubility. Tanimoto et al. reported an elevated intestinal fractional Ca^{2+} absorption (TFCA) in postmenopausal women following even a single dose of PGA (Mulligan et al. 2014). Moreover, the other applications of PGA such as antidiabetic activity, dental/oral care, prevention of high blood pressure, skin care, and enhancing taste/flavor have been comprehensively reviewed elsewhere (Sekhon et al. 2012).

Surfactants are usually organic compounds able to reduce surface tension between liquids, solids, and gases. They are utilized in different applications. It is estimated that about 54 % of the total produced surfactants are exploited in household cleaning with only 32 % destined for industrial applications (Intelligence Am. 2010). The global market of surfactants was valued about US$26.8 billion in 2012 and is expected to rise up to US$31.1 billion by 2016 (Das and Mukherjee 2007). Presently, most of the surfactants are synthesized chemically from petroleum-derived sources, which are not biodegradable and environmental friendly; for this reason, biosurfactants, which are derived from microbial sources, are ideal alternatives to chemical surfactants (Coutte et al. 2010). Biological surfactants (biosurfactants) are surface-active and amphiphilic compounds with microbial origin that comprise of lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids, and phospholipids (Barros et al. 2008). Due to biodegradability, low toxicity, and stability in high temperatures and pH values, biosurfactants have attracted considerable attention in various applications ranging from food, cosmetic, detergent, agri-

culture, and pharmaceutical to oil recovery and hydrocarbon bioremediation industries. From a biochemical point of view, biosurfactants are categorized as glycolipids, phospholipid, fatty acids, polymeric, lipopeptides, and lipoproteins (Barros et al. 2008).

Among the various microbial surfactant producers, such as *Bacillus*, *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Arthrobacter*, *Rhodococcus*, *Mycobacterium*, *Corynebacterium*, and *Candida*, and *Bacillus* species, *B. subtilis* is especially more attractive due to its high surface activity and therapeutic potential (Jung et al. 2012). Generally, *Bacillus* spp. lipopeptides are classified into three major families: surfactins, iturins, and fengycins (plipastatins); among them, surfactin, which is produced by *B. subtilis,* is well studied and utilized in different applications such as agricultural, chemical, food, and pharmaceutical industries (Abdel et al. 2008).

Many strategies including medium optimization, recombinant DNA technology, and using agro-industrial wastes for fermentation were exploited to both increase the yield and decrease the cost of surfactants production. Barros et al. reported the production of a biosurfactant by *Bacillus subtilis* LB5 in a 40-L batch bioreactor by using cassava (the starchy tuberous root of a tropical tree) wastewater as the substrate. The productivity of the semi-purified biosurfactant in foam was 2.4 g/L (Amani et al. 2010). It was indicated that the stimulation of competence-stimulating pheromones, ComX and sporulation factor (CSF), which incite the transcription of *srfA* operon in the engineered *B. subtilis* (pHT43-*comX phrC*), could enhance the surfactin production (de Faria et al. 2011). Moreover, in order to reduce the production cost of surfactin, synthetic wastewater was applied from which *B. subtilis* (pHT43-com phrC) produces 140.2 mg/L surfactin. Besides the aforementioned *B. subtilis* strains, different *Bacillus* strains such as *B. subtilis* BS5, *B. subtilis* isolate, *B. subtilis* ATCC 21332, *B. subtilis* 20B, *B. subtilis* E8, *B. subtilis* LAMI008, *B. subtilis* LSFM-05, *B. subtilis* TD7, and *B. subtilis* TD4 have been exploited in biosurfactant production (Gong et al. 2009; Liu et al. 2012; Saimmai et al. 2012; Hancock and Chapple 1999; Fischbach and Walsh 2006; Awais et al. 2010). The highest yield of surfactin was 10.26 g/L, which was produced by *B. subtilis* E8.

1.3 Different Antimicrobial Agents

Antibiotics are low-molecular-weight compounds that are mainly produced by soil microorganisms as the secondary metabolites. The total number of antibiotics produced by *Bacillus* spp. are approaching 167, among which the main antibiotic producers are *B. subtilis* (e.g., polymyxin, difficidin, subtilin, mycobacillin, and bacitracin), *B. brevis* (e.g., gramicidin, tyrothricin), *B. circulans* (e.g., circulin), *B. laterosporus* (e.g., laterosporin), *B. polymyxa* (e.g., polymyxin, colistin), *B. licheniformis* (e.g., bacitracin), *B. pumilus* (e.g., pumulin), and *B. cereus* (e.g., cerexin, zwittermicin) (Ming and Epperson 2002; Katz and Demain 1977). Gramicidin, tyrothricin, polymyxin, and especially bacitracin are the most commercially

important antibiotics produced by these bacteria. Combinations of these antibiotics is widely used in medicine as a first aid antibiotic for preventing infection in minor cut, scrape, and burned skin (Tamehiro et al. 2002).

Most of the *Bacillus* antibiotics are effectively active against Gram-positive bacteria (Riley and Wertz 2002a, b). However, antibiotics such as polymyxin, circulin, and colistin show activity toward almost all Gram-negative bacteria, while mycobacillin, bacillomycin, and fungustatin are efficient against yeasts and molds (Riley and Wertz 2002a, b).

Antibiotics that are produced by *Bacillus* spp. are categorized as ribosomal and nonribosomal. Nonribosomally derived antibiotics are circular oligopeptides including a fatty acid chain that demonstrate potent antifungal or antibacterial activity as represented by the fengycin, iturinic group, and surfactin (Héchard and Sahl 2002). The ribosomal antibiotic derivates, also commonly known as bacteriocins, are heterologous groups of peptide antimicrobial agents that are produced by bacteria (Cleveland et al. 2001; Babasaki et al. 1985). They present a high degree of target specificity against related bacteria, although some have a wider range of activity (Stöver and Driks 1999). Their proteinaceous feature offers a supposed degradation in the gastrointestinal tract of animals and humans, suggesting their usefulness in foods as natural preservatives (Paik et al. 1998). Among the bacteriocin producers, *B. subtilis* 168 is the best studied strain that is well known to generate three bacteriocins known as subtilosin (Tsuge et al. 1999), TasA (Park et al. 1998), and sublancin (Yeaman and Yount 2003). Moreover, the investigation for novel potent bacteriocins by strain 168 has also been done based on the genome sequence analysis (e.g., plipastatin) (Chatterjee et al. 2005). The ribosomal peptide antibiotics are produced during active cell multiplication, while nonribosomal ones are produced during sporulation or after growth. It is hypothesized that nonribosomal antibiotics may compete with the bacterial growth during spore germination. Moreover, most of these antibiotic peptides show their antibacterial or antifungal effects by destabilizing and interacting with the microbial membrane (Ming and Epperson 2002). Various modes of action are suggested for numerous peptides, including capturing of DNA synthesis, arresting synthesis of stress proteins or specific membrane proteins, interaction with DNA, cleavage of single-strand DNA (Mannanov and Sattarova 2001), and also generation of hydrogen peroxide. They can also act by autolysis in the bacterial targets or apoptosis in the eukaryotic cells (Smith and Hillman 2008).

The term "lantibiotics" refers to antibacterial peptides that have unique structural features and are categorized in a class of bacteriocins (Twomey et al. 2002). It should be mentioned that this term was proposed instead of an abbreviation for lanthionine-containing peptides in order to indicate their unique features (i.e., the presence of the sulfoamino acid lanthionine and antibacterial activity) (Twomey et al. 2002). In addition to lanthionine and its analog 3-methyllanthionine, all lantibiotics contain the unsaturated amino acids didehydroalanine and didehydrobutyrine. Some lantibiotics may also contain unusual amino acids such as S-aminovinylcysteine, erythro-3-hydroxyaspartic acid, and lysinoalanine (Gebhardt et al. 2002). Lantibiotics are divided into groups A and B based on their general

structure, molecular weight, and charge. Lantibiotics of group A have positive charges from 2^+ to 7^+, whereas lantibiotics of group B are globular and have no charge. Lantibiotics of group A are produced by lactococci, lactobacilli, staphylococci, streptococci, and *Bacillus* spp. They are described as elongated linear peptides with molecular weights of 2.1–3.5 KDa. This group includes nisin, lantibiotic, subtilin, epidermin, gallidermin, mersacidin, and astagardin (Stein et al. 2005). On the other hand, lantibiotics of group B (molecular weights of 1.8–2.1 KDa) are exclusively produced by streptococci. They usually share weak antibacterial activity (Abriouel et al. 2011); however, they attract scientific interest. For instance, duramycins and cinnamycin are powerful phospholipase A_2 inhibitors, though promising agents for treating allergic diseases. Moreover, they can be used to produce medicinal preparations like blood-pressure regulators (Gebhardt et al. 2002). The term "bacteriocin-like inhibitory substances" (BLIS) is used when the peptide feature of the antimicrobial substance has not been corroborated (Lawton et al. 2007; Sutyak et al. 2008a, b). Such compounds are active against bacteria, but not active against fungi or yeast. The best example of BLIS compounds are cerein, heterocycloanthracins, cereicidin, megacin, pumilicin, polyfermenticin, and thuricins, which are produced by various *Bacillus* spp. (Sutyak et al. 2008a, b).

Bacillus antimicrobial substances are used in several ways in human health, livestock, food, and some protection usages (e.g., prevention of damages caused by the activity of harmful bacteria on the industrial apparatuses). The increase in the bacterial resistance to routine antibiotics has resulted an upward interest for considering *Bacillus* antibiotics as alternative antimicrobials for the healing of human infections (Mutuş et al. 2006). More interestingly, cross-resistance between *Bacillus* antimicrobial substances and conventional antibiotics has been rarely reported, because these two groups of antimicrobials act on different cellular targets (Mutuş et al. 2006). Haloduracin is one of the most interesting *Bacillus* antimicrobial substances suitable for medical applications. This antimicrobial agent shows more endurance at physiological pH values in comparison to nisin. Subtilosin A is active against vaginal pathogens like *Gardnerella vaginalis* that are resistant to routine antibiotic treatments. In addition, this antimicrobial agent can be used as a natural contraceptive due to its spermicidal activity against human's spermatozoa (Pattnaik et al. 2001; Barboza-Corona et al. 2009). Various *Bacillus* strains are commercialized as probiotics for livestock applications, due to the functional features such as enhancing the body weight of poultry and farm animals. For instance, BioPlus2B includes a combination of *B. subtilis* and *B. licheniformis* strains that can be applied as probiotics in livestock to inhibit pathogenic bacteria and improve animal health (Gálvez et al. 2008). This feature is related to the production of "lichenin", which shows antibacterial effect against *Eubacterium ruminantium* and *Streptococcus bovis* and also has noteworthy hydrolytic activities against a variety of polysaccharides (Martirani et al. 2002). Therefore, it helps to enhance rumen fermentation as a digestive aid. Moreover, the BLIS production by *B. thuringiensis* could be helpful to control bovine mastitis as an alternative method (Bai et al. 2003). Nowadays, food application research mainly has centralized on lactic acid bacteria (LAB) bacteriocins, mainly nisin (Korenblum et al. 2005). Currently nisin is the only antimi-

crobial agent licensed as a natural preservative; however, its application is limited due to its very low activity at alkaline or neutral pH values. Therefore, the investigation for novel bacteriocins with enhanced physicochemical features (stable in a wide range of temperature and pH) and wide antimicrobial ranges has attracted attentions in food industries. In this regard, *Bacillus* bacteriocins possess a potential preservative application in various food items. The best two examples are cerein 8A and bacillocin 490. These antimicrobial peptides exhibit good bactericidal activities at high temperatures, during storage at 4 °C, and over a wide pH range (Korenblum et al. 2008). *Bacillus* spp. are naturally found in plants and soil. Therefore, the antimicrobial-producing strains could be useful as biocontrol agents. For example, ericin S is active against *Clavibacter michiganensis* (the causal agent of tomato bacterial canker), and Bac 14B (from *B. subtilis* 14B) is active against *Agrobacterium tumefaciens* infection. *Bacillus* spp. can be applied as promoting agents to enhance resistance in plants and/or plant growth. The best example is *B. thuringiensis* strain NEB17 that is shown to promote nodulation when implemented with *Bradyrhizobium japonicum* 532C. Due to this fact, antibacterial peptides provoke the root hair deformation of soybeans, which consequently leads to the nodulation increase (Araya et al. 2002). The BLIS-producing strains of *B. firmus* have high antimicrobial activity against sulfur-reducing bacteria (SRB). The mentioned features enable these bacteria as good candidates for application in petroleum industries during oil drilling (Hong et al. 2005; Cutting 2011).

1.3.1 Bacillus *Probiotics*

Probiotics are living microorganisms (bacteria or yeast) whose ingestion at sufficient amounts results in significant health benefits (Sanders et al. 2003). *Bacillus* species have been used as probiotics for at least 50 years in the Italian market known as Enterogermina®. The scientific interest in probiotics especially for *Bacillus* species has only taken place in the last 15 years. In this regard, four principal reviews have covered this field (Barbosa et al. 2005; Spinosa et al. 2000; Tuohy et al. 2007). The most extensively examined *Bacillus* species are *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus clausii*. Heat stability of the spores of these *Bacillus* species has given these species several advantages versus the other nonspore-forming bacteria such a *Lactobacillus* species. So the product can be stored at room temperature in a dried form without any detrimental influence on its viability. Another advantage is that the spore is able to survive at low pH values in the gastric barrier (Sorokulova 1996; Mazza 1994). Only a few species of *Lactobacillus* are capable to tolerate this situation (Bilev 2002). Hence, a defined amount of spores can be stored unlimitedly without refrigeration, and the whole amount of the ingested bacteria will approach the small intestine intact. Based on that, spore-forming probiotics are widely used in human diet as nutritional supplements (Table 1.2), in animals' food as competitive exclusion agents and growth enhancers (Table 1.3), and finally in aquaculture for promoting the growth and

Table 1.2 Some *Bacillus* probiotics available in the market for human use

Product	Manufacturer	Comments
Bactisubtil®	Marion Merrell (Levallois-Perret, France), Hoechst, Aventis Pharma, and Cassella-med, Cologne, Germany	Each capsule contains 1×10^9 spores of *B. cereus* strain IP5832b (ATCC 14893)
Bio-Kult®	Protexin Healthcare (UK), (http://www.bio-kult.com)	Contains *B. subtilis* (not species defined)
Biosporin®	Biofarm, Dnepropetrovsk, Ukraine	Contains a mixture of *B. subtilis* 2335 and *B. licheniformis* 2336 with the ratio of 3:1
Enterogermina®	Sanofi Winthrop SpA, Milan, Italy, www.automedicazione.it	Each vial contains 1×10^6 spores of four species of *B. clausii*
Flora-Balance	Flora-Balance, Montana, USA www.flora-balance.com	Contains *Brevibacillus laterosporus* BOD
Lactospore®	Sabinsa Corp., Piscataway, NJ, USA www.sabinsa.com	Contains *B. coagulans* $6–15 \times 10^9$ g^{-1}
Lactipan Plus	Istituto Biochimico Italiano SpA, Milan, Italy	Contains *B. coagulans* and *B. subtilis* $\times 10^9$ per capsule

assisting disease resistance of cultured shrimps, especially the black tiger shrimp (*Penaeus monodon*) (Table 1.4).

Probiotics are classified into two main groups for human consumption, those vended as health food supplements and those for preclusive use. The preventative application of probiotics is mainly for prophylaxis of gastrointestinal diseases mainly childhood diarrhea (especially rotavirus infections) or as an alternative for antibiotic use. These products are either prescribed or sold over the counter (OTC). However, their usage depends upon the local or national culture. For example, in Italy, the probiotic products are quite common. In contrast, in the UK the first probiotic product entered the market in 2011 (Spinosa et al. 2000). The best examples of probiotic products available in European market are Bactisubtil® (contain *B. cereus* termed IP5832) and Biosporin® (contain *B. subtilis* and *B. licheniformis*) (Pinchuk et al. 2001; Beliavskaia et al. 2000, 2002a, b). The *B. subtilis* constituent of Biosporin® (*B. subtilis* strain 3 or 2335) is able to generate aminocoumarin A, which is active against *Helicobacter pylori* (Smirnov et al. 1994). Moreover, this bacterium has been genetically engineered to express interferon, which has antitumor and antiviral activity (Tsukamoto et al. 2001; Hosoi et al. 2000). In Southeast Asia, there are several probiotics available in the market; however, most of them carry resistance genes to antibiotics. Besides the origin and status of these products are not well defined (Hosoi et al. 1999). A large number of *Bacillus* products are used as food or nutritional supplements with various effects on human health. Natto, the Japanese fermented cooked soybeans with *Bacillus subtilis* var. *natto*, is one of the most well-known probiotic fermented foods. The *Bacillus subtilis* var. *natto*

Table 1.3 Some *Bacillus* probiotics available in the market for veterinary use

Product	Animal	Manufacturer	Comments
Toyocerin®	Calves, poultry, rabbits, and swine. And also for aquaculture	Asahi Vet S.A., Tokyo (Head Off.), Japan http://www.asahi-kasei.co.jp	Powder contains *B. cereus* var. *toyoi*1 × 10^{10} CFU g^{-1}
Lactopure	Poultry, calves, and swine	Pharmed Medicare, Bangalore, India http://www.pharmedmedicare.com	Contains *B. coagulans*
BioGrow®	Poultry, calves, and swine	Provita Eurotech Ltd., Omagh, Northern Ireland, UK	Contains a mixture of *B. subtilis* 1.6 × 10^9 CFU $g^{-1}$and *B. licheniformis* 1.6 × 10^9 CFU g^{-1}
BioPlus® 2B	Piglets, chickens, turkeys for fattening	Christian Hansen Hørsholm, Denmark http://www.chbiosystems.com	Contains a mixture of *B. subtilis* (DSM 5750) 1.6 × 10^9 CFU $g^{-1}$and *B. licheniformis* (DSM 5749) 1.6 × 10^9 CFU g^{-1}
Esporafeed Plus®	Swine	Norel, S.A. Madrid, Spain	Contains 1 × 10^9*B. cereus* (CECT 953)
Neoferm BS 10	Poultry, calves, and swine	Sanofi Sante Nutrition Animale, France	Contains a mixture of2 strains of *B. clausii* (CNCM MA23/3V and CNCM MA66/4M)
AlCare™	Swine	Alpharma Inc., Melbourne, Australia www.alpharma.com.au/alcare.htm	Contains *B. licheniformis* (NCTC 13123) at 10^9–10^{10} spores per kg

Table 1.4 Some *Bacillus* probiotics available in the market for aquaculture use

Product	Manufacturer	Comments
Promarine	Sino-Aqua company Kaohsiung, Taiwan www.sino-aqua.com	Contains four strains of *B. subtilis*
Biostart	Microbial Solutions, Johannesburg, South Africa, and Advanced Microbial Systems, Shakopee, MN, USA	Contains a mixture of *Paenibacillus polymyxa*, *B. licheniformis*, *B. megaterium*, and two strains of *B. subtilis*
BaoZyme-Aqua	Sino-Aqua Corp., Kaohsiung, Taiwan, www.sino-aqua.com	B. subtilis strains Wu-S and Wu-T at1 × 10^8 CFU g^{-1}
Liqualife	Cargill, Animal Nutrition Division www.cargill.com	The *Bacillus* species are not defined in this product
Sanocare, Sanolife, Sanoguard	Inve Technologies nv, Dendermonde, Belgium www.inve.com	Contain various species of *Bacillus*

constituent in natto can provoke immune system and produce vitamin K_2 in addition to anticancer properties (Gatesoupe 1999; Verschuere et al. 2000).

In Europe, before 1997, the major consumers of antibiotics were medical professions followed by husbandry industries. However, the usage of antibiotics for farming industries was gradually banned, and to date antibiotic usage is not allowed. As an alternative, various strategies such as using probiotics and synbiotics were applied to promote growth. Currently, there are two *Bacillus* probiotic products, namely, Toyocerin® and BioPlus® 2B licensed in Europe (Barbosa et al. 2005; Rhee et al. 2004). Another interesting market for *Bacillus* probiotics is fishing industries, which are well developed in countries with intensive fish farming particularly shellfish and shrimps (black tiger shrimp) especially in Southeast Asia (Hong et al. 2009a, b; La Rosa et al. 2003). The larva of this shrimp has a nonspecific immune response with a fair protection against pathogens. Results show that using *Bacillus* spp. as a probiotic supplement prevents catastrophic economic loss of shrimp harvesting (La Rosa et al. 2003). Three types of commercial bacterial products are used in aquaculture industries, biocontrol agents, probiotics, and bioremediation agents. *Bacillus* spp. are available as biocontrol agents and also as probiotics. In contrast, the nitrifying bacteria are used for bioremediation in order to degrade the debris produced from shellfish, fish, and shrimp in rearing ponds. For instance, Biostart® and Liqualife® carry *Bacillus* spores as both biocontrol agent and probiotic (Hong et al. 2009a, b).

Bacillus probiotics expose their beneficial effects on human health in several ways. The process of germinating spores can stimulate immune responses in the GI tract especially in gut-associated lymphoid tissue (GALT) as well as GALT development (Samanya and Yamauchi 2002; Jadamus et al. 2001). Moreover, production of antimicrobial agents such as amicoumacin, subtilisin, and coagulin provides a probiotic effect by repressing the growth of enteric pathogens and competing microbes (Samanya and Yamauchi 2002). Studies on *B. coagulans* showed that application of this bacterium as probiotic has positive effects on hyperlipidemic patients by lowering total cholesterol levels (Homma and Shinohara 2004). In animal model studies, application of *Bacillus* spp. provided a significant reduction in the colonization of pathogenic bacteria in the liver, spleen, and ceca. Moreover, application of *B. subtilis* var. *natto* showed positive influence on the feed conversion efficiency and decrease of abdominal fat in broiler chickens along with reduction in ammonia concentration (Endo et al. 1999; Sarkar et al. 1994). The latter activate gut function through affecting enterocyte cell area and villus height. Hence, the reduction in ammonia concentration enhances nutrient absorption. The use of *B. cereus* species like *toyoi* as probiotics enhances the decrease in ammonia concentration, stimulates better germination of *B. cereus* spores, and therefore helps to elicit its probiotic functions (Peng et al. 2003; Kiers et al. 2000).

The state of safety of any probiotic products for human or animal consumption should be regulated, especially when the product is consumed in large amounts on a regular basis. Concerns such as safety of probiotic bacteria on ingestion and the compliance with GMP regulations through their production should also be considered. Another main issue is about application of probiotics in animal foods, which

raises the danger of interspecies transfer of antibiotic resistance genes. Due to that reason, the Food and Drug Administration of USA has not approved any *Bacillus* probiotic product as GRAS (generally regarded as safe) for human use. However, some *Bacillus* spp. received GRAS status for application in specific industrial use, e.g., enzyme production.

1.4 *Bacillus* Fermented Food

Fermented foods are still a fundamental component of diets worldwide. In many cases, the fermentation process is applied for improvement of aroma or taste. This process results in improved nutritional composition, detoxification of anti-nutrient agents, and stabilization of the original crude materials. Based on the contribution of diverse *Bacillus* spp., such as *B. subtilis* and *Bacillus natto*, various fermented food products have been introduced to the market. Frequently, the final food products have a very traditional trait and show unique sensory properties due to their distinctive flora and processing approaches that were applied in homemade fermentations. The fermentation with *B. subtilis* and *B. natto* results in very characteristic odors in the fermented food products such as natto, douch, chungkookjang, thua nao, kinema, and dawadawa (also referred to as daddawa) (Beaumont 2002; Shrestha et al. 2010). The food material hydrolyzed by the bacteria provides a precursor-rich substrate and a suitable environment, which can lead to savor production through consequent reactions (Esteves et al. 2010). In 1995, Nestle Corporation discovered a process in which by using *Bacillus* spp. a fermented flavoring composition is produced from leguminous plant seeds (Sarkar et al. 1993). The obtained hydrolyzed protein resulted from the fermentation process with *Bacillus* species was blended with reactive flavor precursors and then was heated to provoke flavor formation and finally was converted to a powder form. This product has a basic meaty taste similar to dawadawa-like aroma (Sarkar et al. 1993).

In most of the studies and commercialized food products, fermentation systems are limited to leguminous plant seeds like soybeans and African locust beans (*Parkia biglobosa*). Among the leguminous plant seed fermented foods, natto and dawadawa are now commercialized by Nestle Corporation (Sarkar et al. 1993). Soybean (*Glycine max* L.) is one of the nutritionally richest legume seeds because of its high protein content, vitamins, and minerals. However, its high satiety level, due to its high oil content, weak digestibility, long cooking time, green beany flavor, and persistent acridness, limits its usage (Ikenebomeh et al. 1986). *Bacillus* fermentation is an approach for enhancing texture, flavor, and nutritional quality of soybeans. In addition to the physicochemical and sensory quality alterations, fermentation helps in the preservation of food via releasing some inhibitory metabolites that are unfavorable for pathogenic bacteria (Hu et al. 2010). Most of the *Bacillus* fermented soy products are still prepared by traditional methods. Usually the basic steps for the preparation of fermented soybean such as soaking, boiling, fermentation, incubation, and packing are similar; however, little differences can lead to the production

of various flavors in the fermented soybean products (Ikenebomeh et al. 1986; Lee and Chou 2006). For example, variations in salt concentration, water activity, inoculums concentration, temperature, and pH have influence on *Bacillus* activity in the fermented soybean products (Ikenebomeh et al. 1986; Sarkar et al. 1997). The use of *Bacillus* in the fermentation of soybean provides alterations in the nutritional value of fermented products. These alterations include proteolysis; amylolysis; changes in amino acids, fatty acid, vitamins, and mineral profiles; elevation in phenolic compounds, isoflavones, and other compounds; and production of aromatic compounds and flavoring agents (Ikenebomeh et al. 1986). During fermentation, *B. subtilis* secretes various types of proteases that are responsible for the flavor of soybean products. For example, in thua nao, three types of enzymes (alkaline protease, neutral proteases, and esterase) were reported to be produced during the fermentation (Ang et al. 1999). Moreover, a fibrinolytic enzyme, nattokinase, is also produced by *B. subtilis,* which has a capability of dissolving fibrin clots and can be used for curing vascular system diseases (Ang et al. 1999; Sarkar et al. 1998). The proteolysis resulted in the increase of free amino acids from 0.2 % to 12 %, which is 60-fold higher than the unfermented soybeans. These free amino acids are also much higher than egg or milk proteins (Takahashi et al. 2005; Astadi et al. 2009). Accordingly, the lipolysis activity of *B. subtilis* provides the degradation of triglycerides into assimilated fatty acids, responsible for imparting the typical flavor of fermented soybean products (Takahashi et al. 2005). Vitamins especially vitamin B group and folate are increased during *B. subtilis* fermentation as reported for kinema. In contrast, there is a decrease in minerals such as copper, calcium, potassium, phosphorus, manganese, magnesium, iron, and zinc (Azevedo et al. 2003). This phenomenon may be due to the soaking and cooking leach, which causes a significant depletion of minerals. However, fermented soybean products still contain a noticeable amount of minerals as reported by kinema and natto (Azevedo et al. 2003). It is also shown that phenolic and isoflavone compounds are increased during the fermentation. They are known to be bioactive compounds that act as inhibitors of oxidation of low-density lipoprotein (Leejeerajumnean et al. 2001) and scavengers of free radicals (Duc et al. 2006) and also decrease the occurrence of DNA damage by cyclophosphamides (Khaneja et al. 2009). Moreover, they can act as reducing agents, single oxygen quenchers, and metal chelators, which are all beneficial to the human health (Ang et al. 1999). The secretion of hydrolytic enzymes (e.g., protease, lipases, etc.) is accountable for the alteration in the volatile compounds that make the distinct flavor of this type of fermented soybean. The major volatile compounds found in *Bacillus* fermented soybean products are pyrazines, 3-hydroxybutanone(acetoin), 2-methylbutanoic acid, á-acetolactate, dimethylsulfides, hexanol, and trimethylpyrazine, along with other aromatic compounds (Hong et al. 2009).

Recently, some pigmented *Bacillus* spp. are characterized to have one or more carotenoid compounds (Yoon et al. 2005; Suresh et al. 2004). It is shown that these carotenoids have anti-oxidant activity in vitro and hence possess nutritional value (Suresh et al. 2004). Various pigments like yellow, red, pink, and orange ones can be isolated from different sources such as soil, river, and lake as well as from the gut

tracts of animals (Agnew et al. 1995; Yoon et al. 2001). Some reported pigmented *Bacillus* spp. are *B. indicus* (Li et al. 2002), *B. cibi* (Yoon et al. 2001), *B. vedderi* (Mitchell et al. 1986), *B. jeotgali* (Fritze and Pukall 2001), *B. okuhidensis* (205), *B. megaterium* (206), and *B. atrophaeus* (207) that generated a wide variety of colorful pigments. These pigments are located in both vegetative cells and spore forms that act as a protective agent against UV radiation (Suresh et al. 2004). The recommended daily amount of carotenoids usage for human is usually relatively high (e.g., 800 mg/day for β-carotene) due to their rapid degradation in the stomach (208). One solution for this problem is the usage of *Bacillus* spores containing carotenoids that are stable in the stomach acidic pH. In this regard, ANA Bio Stock Company has commercialized the pigmented *B. indicus* HU36, which is available in the market as a probiotic. In one other effort, Isabella's Healthy Bakery has produced "Activate Muffins" containing GanedenBC^{30} (*B. coagulans*) spores.

1.5 Conclusion and Future Trends

Bacillus spp. are important bacterial workhorses for the production of different chemicals, biopharmaceuticals, and enzymes. A wider spectrum of cellular phenotypes of *Bacillus* species with the ability of production of high amount of value-added products can be obtained by means of genetic engineering. On this basis, various *Bacillus* species have been developed for manufacturing of vitamins, antibiotics, amino acids, nucleotides, and industrially important enzymes. Moreover, the advent of *Bacillus* species as probiotics opens a new era for prophylaxis against enteric infections. Further advances in the application of *Bacillus* species in probiotics will enhance the food quality, flavor, and digestibility.

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Chapter 2
Bacillus: As Bioremediator Agent of Major Environmental Pollutants

Baljinder Singh and Kashmir Singh

Abstract Degradation of four major environmental pollutants, pesticides, explosive waste, dyes, and polycyclic aromatic hydrocarbons (PAHs), by *Bacillus* spp. have been reported by several workers. The use of these pollutants has resulted in severe contamination of the environment, and strategies are now being developed to clean these substances in an economical and eco-friendly manner. Microbes are among the main vehicles for remediation of these environmental pollutants, and *Bacillus* spp. are also regarded as one of the potential bioremediator agent among microbes. New discoveries, such as novel biodegradation pathways, multispecies interactions, and community-level responses to pollutants, are helping us to understand, predict, and monitor the fate of pollutants. This chapter summarizes information on the biodegradation and biotransformation pathways of four major environmental pollutants by *Bacillus* spp. Isolation, characterization, utilization, and manipulation of the major detoxifying enzymes and the molecular basis of degradation are also discussed. An attempt has been made to highlight the factors effecting four major environmental pollutants by *Bacillus* spp. This may be useful in developing safer and economically feasible microbiological methods for cleanup of soil and water contaminated with such compounds. The necessity of further investigations concerning the metabolism of these substances by *Bacillus* spp. is also discussed.

Keywords *Bacillus* spp. • Biodegradation • Pesticides • Explosive waste • Dyes • Polycyclic aromatic hydrocarbons

2.1 Introduction

The growing industrialization, testing, use of explosive and aromatic compounds, and modern agricultural practices that have spread worldwide have adversely affected the ecosystem. These practices leave persistent environmental pollutants that have resulted in severe contamination of both soils and groundwater, thus

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_2

necessitating their safe removal from the environment. The problem associated with contaminated environment now assumes increasing prominence in many countries. Contamination sources are mainly associated with their manufacture, use, loading, storage, and disposal processes. The increased awareness of the harmful effects of environmental pollution has led to a dramatic increase in research on various strategies that may be employed to clean up the environment. The methods currently used for the remediation of contaminated sites are expensive and can result in the formation of toxic products. The limitation faced by physical and chemical treatment technologies could be overcome with the help of microbes. With discovery in the 1960s that many soil microorganisms are capable of metabolizing these xenobiotic compounds, the use of biological processes to degrade hazardous materials became a viable and acceptable possibility. Microbes, the oldest inhabitants on earth, are versatile and adaptive to changing environment and will provide a cost-effective component to combat the present problems of contamination (Singh et al. 2014). Microbes and their diverse metabolic enzymes are typically employed for safe removal of environment contaminants, either through direct destruction or indirectly through transformation of the contaminant to a safer intermediate. Many indigenous microorganisms in water and soil are capable of degrading environmental pollutants. The most important classes of pollutants in the environment are pesticide residues, explosive waste, dyes, and polycyclic aromatic hydrocarbons. These environmental pollutants are degraded by several bacterial isolates. The ability of microorganisms to break down xenobiotics has been assumed to be a recent trait, selected by the presence of these compounds in the environment. Microorganisms possess a range of genetic mechanisms allowing evolutionary changes in existing metabolic pathways and therefore become easily adapted to change in environment. Van der Meer et al. (1992) proposed three mechanisms to explain the adaptation of natural bacterial communities to xenobiotic compounds. These mechanisms are (1) induction of specific catabolic enzymes, (2) outgrowth of specific populations present at low densities, and (3) selection of mutants with novel metabolic activities. In the first two mechanisms, adaptation to biodegradation has happened before exposure to xenobiotics, and the enzymes for degradation are present in the microbial community. The third mechanism requires mutations or gene rearrangements resulting in enzymatic activity that was absent from the community before exposure.

Among various bacterial isolates, *Bacillus* spp. has also reported to degrade these pollutants and can act as bioremediation agent to clean up the contaminated sites. In the present chapter, an attempt has been made to pool all the available literature on the biodegradation of four major environmental pollutants, pesticides, explosive waste, dyes, and PAHs, by *Bacillus* spp.

2.2 Pesticides

Use of pesticides and fertilizer have brought green revolution in many countries and provided global food security. Pesticides are organic compounds manufactured and used to control weeds in fields and lawns and unwanted or harmful pests, such as

insects and mites, feeding on crops. Pesticides can be divided into categories according to its target organisms, e.g., insecticides (control insects), herbicides (control specific weed in specific crops), and fungicides (control fungus). The term pesticide covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators, and others. Pesticides were introduced few decades ago. At that time their long-term health effects were not known. Because of high toxicity of organochlorine and the metal-based pesticides, agro-industries shifted toward organophosphate pesticides which were alleged to be safer because of its rapid degradation in the environment (Singh and Singh 2014).

2.2.1 Biodegradation of Pesticides by Bacillus *spp.*

Although several pesticides are biodegradable in nature, their residues are persisting in the environment. Considering their toxicity, research on biodegradation of organophosphates is being carried out all over the world. Studies on microbial biodegradation are useful in the development of strategies for detoxification of pesticides. Microbial metabolism is the primary mechanism of pesticide breakdown and detoxification in many soils and is the basis for bioremediation, as the degrading microorganisms obtain C, N, or energy from the pesticide molecules.

However, to date little data are available on the role of *Bacillus* in pesticide biodegradation. The genus *Bacillus* comprises a diverse group of bacteria widely distributed in soil and the aquatic environment. Due to their ubiquity and capability to survive under adverse conditions, they are assumed to effectively degrade pesticides and therefore play an important role in the bioremediation of pesticide contamination sites. Organophosphate pesticides can be detoxified by *Bacillus* spp. through the enzymatic activity of carboxylesterases results in hydrolysis of carboxylester bonds (Singh 2014; Nelson 1982). The potential applications of *Bacillus* members to degrade pesticides are shown in Table 2.1. Biodegradation pathways of various pesticides that are degraded by *Bacillus* spp. in the cited literature are shown in Fig. 2.1. El-Helow et al. (2013) studied biodegradation of chlorpyrifos in liquid culture under different environmental factors such as different concentrations of the insecticide, pH, and observed maximum degradation rate (16.25 mg/h) at 150 mg L−1 and pH 8, respectively. Chen et al. (2014) reported the degradation kinetics and metabolic pathway of fenpropathrin in *Bacillus* sp. DG-02. They observed the degradation rate parameters qmax, Ks, and Ki to be 0.05 h−1, 9.0 mg L−1, and 694.8 mg L−1, respectively. Singh et al. (2011a, 2012a) isolated an organophosphate-degrading bacterium, designated *Lysinibacillus* sp. strain KB1 and *Bacillus cereus* strain PU, *Brevibacillus* sp. strain KB2, from agricultural soil and studied bioremediation in soil contaminated with organophosphate (Singh et al. 2014; Rangaswamy and Venkateswaralu 1992).

Table 2.1 Pesticide degradation by *Bacillus* sp.

Microorganism	Pesticides	Isolation from or source	Degradation pathway	Degradation product (metabolite)	Percentage transformation	Technique used	Reference
Lysinibacillus sp. strain KB1	Malathion	Moist soil near Chandigarh, India	Malathion was used as sole carbon and energy source, carboxylesterase activity	Malathion monocarboxylic and dicarboxylic acids	19.91 % malathion and 46.75 % malaoxon (an analogue of malathion) of the initial malathion provided	HPLC and GCMS	Singh (2014)
Brevibacillus sp. strain KB2 and *Bacillus cereus* strain PU	Malathion	Field soil near Chandigarh, India	Yeast extract and glucose was added as cosubstrate in mineral salt medium carboxylesterase activity	Malathion monocarboxylic and dicarboxylic acids	Strain KB2 was able to degrade 72.20 % of malaoxon and 36.22 % of malathion, while strain PU degraded 87.40 % of malaoxon and 49.31 % of malathion, after 7 days of incubation	HPLC and GCMS	Sharmila et al. (1989); Singh et al. (2011a), (2012a)
B. thuringiensis MOS-5	Malathion	Agricultural wastewater near Berket El Sabaa, Egypt	Esterase activity	Malathion monocarboxylic and dicarboxylic acids	99.32 % reduction in malathion after 30 days	GCMS	Kamal et al. (2008)
Bacillus sp. strain DG-02	3-Phenoxy-benzoic acid	Soil samples	The parent compound was first metabolized by oxidization to yield 3-(2-hydroxyphenoxy) benzoic acid. Subsequently, the intermediate was further transformed by cleavage of the diaryl ether, resulting in formation of protocatechuate phenol and 3,4-dihydroxy phenol	3-(2-methoxyphenoxy) benzoic acid, protocatechuate, phenol, and 3,4-dihydroxy phenol	Degrade 95.6 % of 50 mg L^{-1}	GCMS	Chen et al. (2012)
Bacillus megaterium MCM B-423,	Monocrotophos	Soil	Biomineralization	Valeric or acetic acid and methylamine	83 % at 1000 mg L^{-1}	TLC, GC	Bhadbhade et al. (2002)

Bacillus subtilis strains	Carbendazim	Taken from culture collection center	Utilize carbendazim as the sole carbon source			HPLC, LCMS	Varsha P. Salunkhe et al. (2014)
Bacillus sp. DG-02	Fenpropathrin	Pyrethroid-manufacturing wastewater treatment system	Cleavage of its carboxylester linkage and diaryl bond, followed by degradation of the aromatic ring and subsequent metabolism	The seven compounds were characterized as 2,2,3,3-tetramethylcyclo-propanecarboxylic acid phenyl ester, 3,4-dihydroxybenzoic acid, 3-phenoxybenzoate, 3,4-dimethoxyphenol, 3-phenoxybenzaldehyde,α-hydroxy-3-phenoxybenzeneacetonitrile, and phenol	93.3 % of 50 mg L^{-1} fenpropathrin within 72 h	HPLC, GCMS	Chen et al. (2014)
Bacillus sp. N1	Metribuzin	Sludge samples	–	–	In 120 h, the degradation rate was 79.5 %	–	Zhang et al (2014)
Bacillus thuringiensis	Fipronil	Soil sample	–	Sulfide was found to be the main metabolite followed by sulfone and amide	–	–	Mandal et al. (2013)
Bacillus alkalinitrilicus	Imidacloprid	Microbial culture collection	–	6-chloronicotinic acid, nitrosimine followed by imidacloprid-NTG	97.47 % degradation after 56 days	HPLC	Smriti Sharma et al. (2014)
Bacillus subtilis strain, *Y242*	Chlorpyrifos	Agricultural wastewater	Utilize chlorpyrifos as a sole carbon and energy source	–	95.12 % pesticide decomposition within 48 h	HPLC, GCMS	Ehab R. El-Helow et al. (2013)
Bacillus pumilus strain NY97-1	Carbendazim	–	–	–	87.76 % degradation	HPLC	Zhang et al. (2009)
Bacillus sp.	Cypermethrin	Alwar marble mining site Rajasthan, India	Oxidative as well as hydrolyzing process	3-phenoxy phenyl hydroxyacetonitrile, carboxylate, 3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclo-propanecarboxylate (DCVA), and 3-phenoxybenzaldehyde	Complete degradation of cypermethrin was reported after 30 days	GCMS	Quinn et al. (1989); Sundaram et al. (2013)

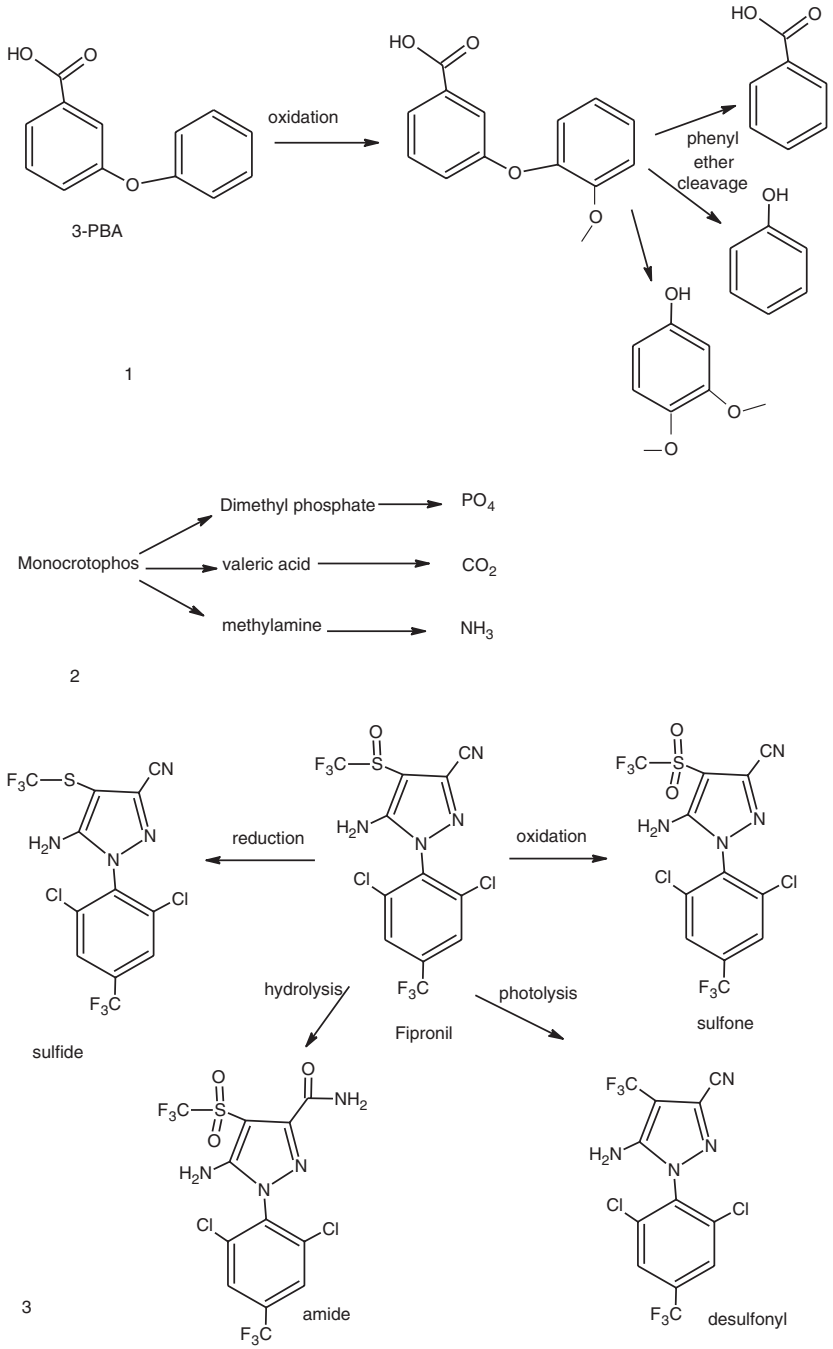

Fig. 2.1 Biodegradation pathway of pesticides by *Bacillus* sp. and metabolites formed. *1* Degradation of 3-phenoxybenzoic acid by a *Bacillus* sp. *2* Biodegradation of monocrotophos by *Bacillus megaterium* MCM B-423. *3* Degradation products of fipronil by *Bacillus thuringiensis. 4* Metabolites of imidacloprid by *Bacillus alkalinitrilicus. 5* Degradation pathway of fenpropathrin by *Bacillus* sp. *DG-02*. Biodegradation metabolites of malathion by *Bacillus cereus* strain PU, *Lysinibacillus* sp. strain KB1, and *Brevibacillus* sp. strain KB2. The scheme is based on articles cited in the text

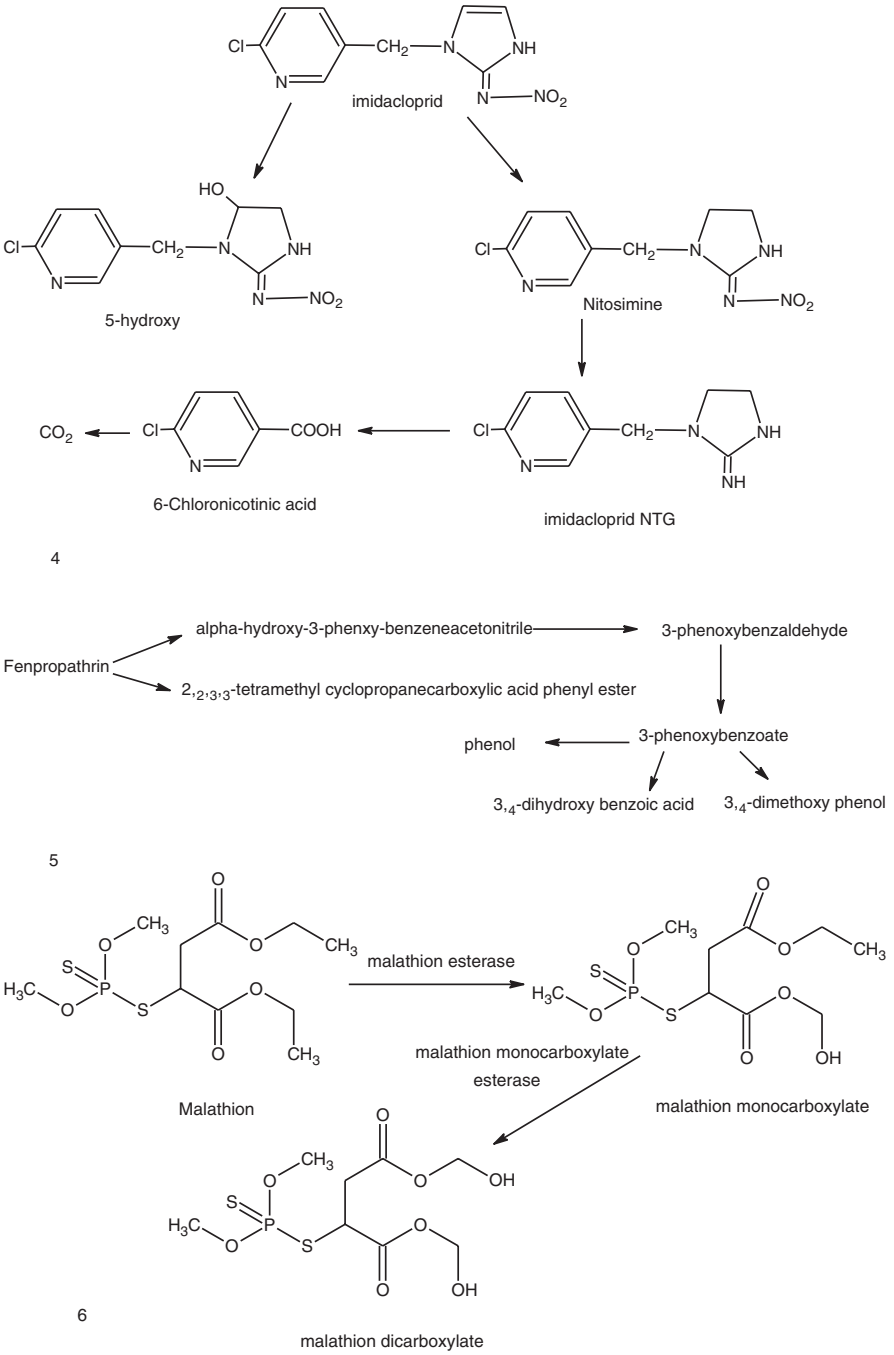

Fig. 2.1 (continued)

2.3 Explosive Waste

Explosives are materials with high nitrogen and oxygen contents on detonation expand to create a shock wave which exerts high pressures on the surroundings, causing an explosion and leaving toxic waste in the environment (Singh et al. 2014). The main classes of important explosives are nitrate esters, nitroaromatic compounds, and nitramines (Singh et al. 2012b). Explosives are used primarily for military purposes, industries, mining, and agricultural activities. A large-scale manufacturing testing, firing ranges, and destruction of ammunition stocks have created a number of environmental problems and increasing concern about their persistence in air, water, and terrestrial ecosystems.

2.3.1 Biodegradation of Explosive Waste by Bacillus *spp.*

The variety of chemical structure in different explosives used in modern day cause different types of contaminants. Due to its widespread application and subsequent exposure, the pressure to remove the explosives waste has led to the evolution of enzymatic system in bacteria to degrade explosive waste. Numerous factors can affect the biodegradation processes and depend on the nature of molecules to be degraded (e.g., molecule size, charge, number and position of functional groups, solubility, and toxicity) as well as the environmental conditions.

Biodegradation of nitrate esters occurs through successive denitrations, each nitro group reacting more slowly than the previous one. Meng et al. isolated *Bacillus thuringiensis*/*cereus* strain capable of degrading nitrate esters (Table 2.2). Pentaerythritol tetranitrate (PETN) is sequentially denitrated to produce the tri-, di-, and the metabolites pentaerythritol dinitrate, 3-hydroxy-2,2-bis-[(nitrooxy)methyl] propanal, and 2,2-bis-[(nitrooxy)methyl]-propanediol that are produced by *Bacillus* spp. (Table 2.2). An NADPH-dependent PETN reductase enzyme isolated from *Bacillus* sp. was capable of liberating nitrite from nitrate esters with oxidation of NADPH.

Nitroaromatic compounds that form the basis of some explosives are 2,4,6-trinitrotoluene (TNT) and 2, 4,6-trinitrophenol (TNP) and have been produced on a massive scale. The presence of three nitro groups makes TNP very unstable. The chemical bonding in the nitro group results in the nitrogen atom being positively charged and each oxygen atom having a partial negative charge. *Bacillus* sp. has the ability to use TNP as a sole nitrogen source under aerobic conditions. Nitro groups, due to the strong electron-withdrawing properties on the aromatic ring, are subjected to initial reductive transformation for biodegradation of TNP. TNP biodegradation by *Bacillus cereus* occurs via hydrogenation reaction (Singh et al. 2011b). TNP is hydrogenated by this mechanism forming a Meisenheimer complex, hydride σ-complex (Table 2.2).

Table 2.2 *Bacillus* spp. capable of degrading explosive wastes

Microorganisms	Explosive waste	Degradation pathway	Conditions involved (e.g., conc. of substrate, activity of enzyme, etc.)	Rate & percentage of biotransformation	Degradation Product (metabolite)	References
Bacillus thuringiensis/cereus	Glycerol trinitrates	Denitration	Denitration activities were expressed constitutively and GTN was not required for induction	Denitration activity (0.1 mmol/g of cell per h)	Ditrate and mononitrate	
Bacillus sp. ATCC 51912	Glycerol trinitrates	Denitration			Ditrate and mononitrate	Sun et al. (1996)
Bacillus cereus strain PU	Trinitrophenol	TNP used as nitrogen source	KB3 medium (yeast extract and glucose)	2.1 ± 0.15 mol nitrite/mol TNP at 539 lmol/h g dry cell wt	Hydride-Meisenheimer complex of TNP	Singh et al. (2011b)
Bacillus sp. J8A2	Pentaerythritol tetranitrate (PETN)			0.72 mmol of PETN/g of protein h		Yerson and Christian (2013)
Bacillus cereus	TNT	Source of nitrogen	–	At an initial concentration of 50 and 75 mg L, TNT was degraded, respectively, 68 % and 77 % in 96 h	2,4-dinitrotoluene and 4-aminodinitrotoluene derivates	Mercimek et al. (2013)
Bacillus sp. SF	TNT		Use NAD(P)H-dependent enzymes to transform TNT	91 % and 70 % reduction of aminodinitrotoluenes and diamninonitrotoluenes	dinitrotoluenes, 2-amino-4,6-dinitrotoluenes, different azoxy compounds, 2,6-diaminonitrotoluenes, and 2,4-diaminonitrotoluenes	Nyanhongo et al. (2008)

Degradation of TNT by *Bacillus* sp. occurs via reductive elimination of the nitro group yielding nitrite. Under aerobic conditions, *B. cereus* transformed TNT to its derivates using TNT as substrate for bacterial growth (Mercimek et al. 2013). Nyanhongo et al. (2008) isolated *Bacillus* sp. capable of degrading TNT via an initial reduction yielding aromatic amines, which may be further metabolized (Table 2.2). The reduction proceeds via a nitroso and a hydroxyamino group.

The biodegradation of nitramine explosives RDX and HMX and CL-20 in the environment occurs both aerobically and anaerobically. Biodegradation proceeds via reduction of nitro group and enzymatic cleavage of ring. Numerous investigations have documented the bacterial degradation of nitramine explosives. But, *Bacillus* spp. lack enzymatic systems required for the degradation of nitramine explosives.

2.4 Dyes

Dyes are the substances that, when applied to a substrate, provides color by forming covalent bond or complexes with compatible surfaces, by physical adsorption or by mechanical retention. The synthesis of variety of synthetic dyestuffs released by many industries, such as dyestuffs, textile, plastics, paper, food, cosmetic, and pharmaceuticals, poses a threat to environmental safety. The synthetic dyes consist of aromatic rings that contain delocated electrons and different functional groups. The chromogen-chromophore structure (acceptor of electrons) imparts color to dyes, and an auxochrome group (donor of electrons) gives the dyeing capacity.

2.4.1 Bioremediation of Dyes by Bacillus *spp.*

The color produced by minute amount of organic dyes in industrial water pollution is having possible harmful effects along with aesthetically unpleasant. Among the three methods, physical, chemical, and biological, available for dye treatment, microbial decolorization being cost-effective and environmentally friendly can be applied to a wide range of such dyes and produce less sludge than physical and chemical systems; therefore, this method is receiving much attention for treatment of textile dye wastewater. Biological treatment may involve either aerobic or anaerobic degradation of the dyes by microorganisms. Anaerobic treatment of dye wastewaters includes the use of various anaerobic bacteria producing the enzyme azoreductase. Reductive cleavage of azo bond leading to the formation of aromatic amines is the initial reaction during the bacterial metabolism of azo dyes. Deng et al. (2008) observed that malachite green was degraded into 4,4′-bis(dimethylamino) benzophenone and benzophenone by *Bacillus cereus*, DC11, due to the reduction of the azo bonds. The mechanism of microbial decoloration occurs from adsorption, enzymatic degradation, or a combination of both. Microbes are capable of

degrading dyes by the activity of both reductases and oxidases. Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. Dawkar et al. (2008) reported degradation of textile disperse dye Brown 3 REL by *Bacillus* sp. and observed product 6,8-dichloro-quinazoline-4-ol formed of demethylation mechanism of laccase (Tables 2.3 and 2.4). Decolorization of dyes takes place in two ways: adsorption on the microbial biomass and biodegradation of dyes by the cells. Decolorization of the dye involves cleavage of chromophoric center of the dye. Adsorption of dyes may occur on growing/living microbial cells as well as on the dead microbial cells. In biodegradation processes, there is breakdown of the original dye structure, and the pollutant is split into fragments by the microbial cells; ultimately complete mineralization occurred, i.e., conversion of dyes into CO2, biomass, and inorganics. Some *Bacillus* strains were found to be able to decolorize anthraquinone dye (Itoh et al. 1993), azo dye (Suzuki et al. 2001; Ooi et al. 2007), and triphenylmethane dye (Yatome et al. 1991).

2.5 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are organic chemicals that include carbon and hydrogen with a fused ring structure containing at least two benzene rings. PAHs are produced by incomplete combustion of organic materials arising, in part, from natural combustion such as forest fires and volcanic eruptions, but anthropogenic activities are the major contributors of contamination.

2.5.1 Biodegradation of PAHs by Bacillus *spp.*

Although both aerobic and anaerobic biodegradation contribute significantly, the aerobic catabolism of PAH pollutants is more prevalent in the biosphere. The associated catabolic enzymes involved in the aerobic catabolic pathway have been broadly grouped into peripheral (or upper pathway) and ring-cleavage (or lower pathway) enzymes; the aromatic ring is initially oxidized by mono- or dioxygenase, followed by the systematic breakdown of the compound to PAH metabolites and/or CO2. Many bacterial isolates, *Pseudomonas*, *Arthrobacter*, *Aeromonas*, *Sphingomonas*, *Brevibacterium*, and *Mycobacterium*, are capable of degrading PAHs. However, only few studies are available in the literature related to PAHs degradation by *Bacillus* sp. Biosurfactant secreted by *Bacillus* sp. bacteria are effective in enhancing the solubility and biodegradation of PAHs. However, production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility. Major biosurfactants secreted by *Bacillus* sp. were lipopeptide in nature containing higher amount of surfactins followed by iturins.

Table 2.3 Dye degradation by *Bacillus* sp.

Microorganism	Dyes	Isolation from or source	Degradation pathway/adsorption	Degradation product (metabolite)	Percentage transformation	Technique used	Reference
Bacillus sp.BDN2	Diazo dye – reactive blue 160 (RB160)	Naroda Industrial Area, GIDC, Naroda near Ahmedabad, Gujarat, India	Reductase enzymes symmetrically cleaved RB160 and oxidative enzymes further metabolized the degraded products	1-phenylmethanediamine, benzene sulfonate, and benzene-1,4-disulfonate, transformed products were further converted into lower molecular weight compounds, benzene, and aniline	–	HPTLC, FTIR, 1HNMRand GCMS analysis	Balapurea et al. (2014)
Bacillus megaterium NCIM 2054	Dye Disperse Red 73	Culture collection	Ionic adsorption	–	61 % decolorization in 48 h	HPTLC, FTIR, and HPLC	Kadam et al. (2014)
Bacillus cereus strain HJ-1	Azo dye reactive black B	–	–	–	Median effective concentration (EC50) of reactive black B for *B. cereus* strain HJ-1 is 48 mg L-1	–	Liao et al. (2013)
Bacillus sp. YZU1	Reactive Black 5	Soil sample collected from a textile processing factory around Suzhou city in China	Decolorization via enzyme azoreductase and small percentage of decolorization occurred via passive surface adsorption	–	95 % decolorization in 120 h	–	Wang et al. (2013)

Bacillus sp. V1DMK, *Lysinibacillus* sp. *V3DMK, Bacillus* sp. V5DMK, *Bacillus* sp. V7DMK	Reactive Violet 5R	Soil samples collected from Kharicut canal Ahmedabad, Gujarat, India	–	Four intermediatory compounds 1-diazo-2-naphthol, 4-hydroxybenze-nesulphonic acid, 2-naphthol, and benzenesulfonic acid	Decolorized 200 mg/L of Reactive Violet 5R within 18 h	FTIR, NMR, GCMS	Jain et al. (2012)
Bacillus sp. strain AK1 and *Lysinibacillus* sp. strain AK2	Sulfonated azo dye metanil yellow	Soil sample was collected in and around the Atul Dyeing Industry, Bellary, India	Azoreductase activity	p-Aminodiphenylamine	1000 mgL−1 was decolorized by strains AK2 and AK1 within 78 and 84 h, respectively	TLC, HPLC and GCMS	Anjaneya et al. (2011)
Bacillus odysseyi SUK3	Reactive Blue 59	Soil sample was collected from textile processing and dye manufacturing unit in Ichalkaranji (India)	–	Aminopyrine N-demethylase	Decolorized Reactive Blue 59 (50 mg l−1) within 60 h	FTIR, UV-VIS, HPLC	Patil et al. (2008)
Bacillus cereus strain DC11q	Anthraquinone, triphenyl-methane, and azo dyes		Basic Blue X-GRRL was probably due to the reduction of the azo bonds	Malachite Green was degraded into 4,40-bis(dimethylamino) benzophenone	High decolorization efficiency (95–98 %) was achieved within 6 h of incubation for 100 mM Acid Blue 25 (anthraquinone dye), 4 h for 55 mM Malachite Green (triphenylmethane dye), and 2 h for 750 mM Basic Blue X-GRRL (azo dye) at 20–45 °C and neutral pH under anaerobic conditions	HPLC	Deng et al. (2008)

(continued)

Table 2.3 (continued)

Microorganism	Dyes	Isolation from or source	Degradation pathway/adsorption	Degradation product (metabolite)	Percentage transformation	Technique used	Reference
Bacillus sp. VUS	Disperse textile dye Brown 3REL		A significant increase in the activities of lignin peroxidase, laccase, and NADH-DCIP reductase was observed up to complete decolorization of Brown 3REL	6,8-Dichloro-quinazoline-4-ol and cyclopentanone	Degrade (100 %) dye Brown 3REL within 8 h	FTIR, UV-VIS, HPLC, GCMS	Dawkar et al. (2008)
Bacillus vallismortis, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus megaterium*	Azo dyes Congo red, Bordeaux, Ranocid Fast Blue, and Blue BCC	Sludge samples (aerated tank) of effluent treatment plant of a Nahar Group of Textile and Dyeing Industry, Dera Bassi, Punjab, India,	–	–	80–96 % decolorization within 120 h	–	Tony et al. (2009)
Bacillus fusiformis KMK5	Disperse Blue 79 and Acid Orange 10	Textile dyes contaminated soil of Ichalkaranji, Maharashtra, India	Mineralization	–	Complete mineralization of DB79 and AO10 at the concentration of 1.5 g/l was observed within 48 h	GCMS	Kolekar et al. (2008)
Bacillus subtilis IFO 13719	Crystal Violet (BV3)			4,4′-Bis(dimethylamino) benzophenone			Yatome et al. (1991)

Table 2.4 PAH degradation by *Bacillus* spp.

Microorganism	PAHs	Isolation from or source	Degradation pathway	Degradation product (metabolite)	Percentage transformation	Reference
Bacillus sp. SBER3		Roots of *Populus deltoides*	–	–	Reduced 83.4 % and 75.1 % of anthracene and naphthalene	Bisht et al. (2014)
Bacillus spp.	Acenaphthene, fluoranthene, pyrene, benzo[e] pyrene	Hot springs, compost piles, and industrial wastewater	Utilize hydrocarbons as sole carbon and energy source		In case of hexadecane after 3 days the lower molecular weight PAH acenaphthene and fluoranthene were nearly completely metabolized (58 of the initial 60 mg/l). About half of the benzo[e]pyrene (12 of 30 mg/l) and pyrene (17 of 30 mg/l) was degraded in the same period	Feitkenhauer et al. (2003)
Bacillus subtilis DM-04		Petroleum-contaminated soil sample from Northeast India	Utilize hydrocarbons as sole carbon and energy source			
Brevibacillus sp. PDM-3	Phenanthrene	Sludge samples Hindustan Petrochemical Limited refinery (HPCL) located in Visakhapatnam, Andhra Pradesh, India	Produce biosurfactant during phenanthrene degradation		93 % of phenanthrene was degraded in 6 days	Reddy et al. (2010)
Bacillus subtilis BM1 and *Bacillus amyloliquefaciens* BR1	Fluorene	Tropical African environment	–	–	56.9 % and 46.8 % of 50 mg/L fluorene were degraded in 12 days by strains BM1 and BR1	Salam and Obayori (2014)

(continued)

Table 2.4 (continued)

Microorganism	PAHs	Isolation from or source	Degradation pathway	Degradation product (metabolite)	Percentage transformation	Reference
Bacillus subtilis BMT4i	Benzo[a]pyrene	MTTC culture collection	–	–	90 % degradation after 24 h	Lily et al. (2010)
Bacillus sp. strain 4	Pyridine		Used as sole C, N, and energy source. specific formamide amidohydrolase cleaving formamide quantitatively to formate and NH3	Formate and formamide		Watson and Cain (1975)
Bacillus pumilus	Phenanthrene (PHE), pyrene (PYR), and benzo[a] anthracene	Lab soils polluted with PAHs			90 % of PAH removal was obtained after just 3 days of cultivation at bioreactor scale	Moscoso et al. (2012)

The complete genome sequence of *Geobacillus thermodenitrificans* NG80-2, long-chain alkane degrading strain isolated from a deep oil reservoir in Northern China, consists of a 3,550,319-bp chromosome and a 57,693-bp plasmid (Feng et al. 2007).

2.6 Factors Effecting Degradation of Four Major Pollutants by *Bacillus* spp.

Bacillus spp. are sensitive to the presence of high concentration of pollutants, high salinity, high temperature, absence of oxygen, variations in pH, and high content of organic compounds. Efficient bioremediation processes require *Bacillus* spp. to be isolated from concerned pollutant-contaminated environments, including soil, effluents, and sludge from wastewater treatment plants, because they are adapted to grow in extreme conditions. The presence of carbon and nitrogen sources has an important influence on the extent of degradation using *Bacillus* spp. The carbon source gives energy for the growth and survival of the microorganisms and as electron donors, which are necessary for the breakage of the pollutant chemical structure. The uses of starch as a cosubstrate enhance decoloration of Reactive Violet 5 by the *Paenibacillus polymyxa* (Moosvi et al. 2007). The addition of yeast extract (0.05 %) and glucose (0.045 %) increased the growth rate and accelerated the degradation of TNP by *Bacillus cereus* strain PU (Singh et al. 2011b). Similarly, addition of yeast extract (0.04 %) and glucose (0.03 %) to malathion-supplemented medium increases the growth rate of *Lysinibacillus* sp. strain KB1 and *Brevibacillus* strain KB2 by 10^5-fold (Singh et al. 2011a, 2012a). The addition of glucose as a cosubstrate enhances degradation of PAHs like fluoranthene and pyrene by *Bacillus thuringiensis* strain NA2 (Maiti et al. 2012). Nitrogen sources are also important for microbial degradation of pollutants. The metabolism of organic nitrogen sources is considered essential for the regeneration of NADH. The presence of salts, CaCl2, enhances decolorization of Navy blue 2GL by *Bacillus* sp. (Dawkar et al. 2008).

2.7 Conclusion and Perspectives in Biodegradation of All Major Pollutants by *Bacillus* spp.

Although, dramatic and rapid progress has been made recently in understanding the biodegradation of pollutants by *Bacillus* spp. in the environment, the total diversity of biodegradation pathways remains still unknown. The complete detoxification of four major pollutants has not been studied; their assimilation as carbon or energy sources for growth by *Bacillus* sp. still remains an open field of study to be explored. The available information on the degradation by *Bacillus* spp. is limited by the fact that we cannot trace the final destination of all of the carbons and nitrogens from the

molecules. The biodegradation of four major pollutants by *Bacillus* spp. and their integration into existing metabolic pathways and global regulatory control networks, like catabolite repression and nitrogen regulation, have yet to be explored.

Knowing the catabolic pathways of degradation, optimization of various parameters is required for accelerating degradation. Although, recently, Hou et al. (2015) reported genome sequence of *Brevibacillus brevis* DZQ7 organic pollutant degradation strain, still, there is also a need to design *Bacillus* spp. through molecular biology tools, enabling them to degrade four major pollutants which further lead to improvements of both the qualitative and quantitative performance of bioremediation.

Cloning of genes related to degradation of four major pollutants in *Bacillus* spp. helps in improvement of enzyme activity to degrade faster. From cited literature it can be concluded that recombination, transposition, and gene transfer can accelerate the evolution of catabolic pathways by recruiting and combining new catabolic activities.

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Chapter 3
Growth Promotion of Nonlegumes by the Inoculation of *Bacillus* Species

M.A. Baset Mia, Umme Aminun Naher, Qurban Ali Panhwar, and M. Tofazzal Islam

Abstract Plant growth-promoting rhizobacteria (PGPR) exert beneficial effects on plants through fixing atmospheric nitrogen, solubilizing organic and inorganic phosphorus, producing phytohormone, enhancing root development and nutrient uptake, and acting as biocontrol agents. Bacteria of the genus *Bacillus* has created tremendous interest in researchers as some strains showed high potential for biofertilization and plant growth promotion contributing to better yield of diverse field and horticultural crops. The genus *Bacillus* that belongs to the family *Bacillaceae* is generally heterotrophic, rod-shaped, and Gram-positive bacteria, and some of them can produce endospores. Some members of the genus *Bacillus* are aerobic, while others are facultative or anaerobic. Among them, *B. sphaericus* UPMB10 was found to exert beneficial effects on several nonlegume plants such as rice, soybean, sweet potato, oil palm, and bananas. They colonize the root externally and internally and able to produce phytohormone in association with the roots of host plant. In this chapter, the isolation, identification, beneficial effects, and mode of actions of these *Bacillus* spp. have been comprehensively reviewed and discussed with special reference to *B. sphaericus*.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_3

Keywords Association • Beneficial effects • N_2-fixing bacteria • Phosphate-solubilizing bacteria • Phytohormone

3.1 Introduction

Application of plant growth-promoting bacterial inocula exerts beneficial effects on host plants through various mechanisms including fixing atmospheric N_2, producing phytohormone, and promoting uptake of essential nutrient elements by the roots (Mia et al. 2013). Interactions between bacteria and plant can occur in rhizosphere, endosphere, and phyllosphere (Sekar and Kandavel 2010). Rhizosphere interactions include root-root, root-insect, and root-microbe contacts. Rhizosphere, the thin layer of soil influenced by the root, is much richer in bacteria than the surrounding bulk soil (Hiltner 1904). Majority of the plant-beneficial bacteria belong to the rhizobacteria that inhabit in roots externally and/or internally and perform beneficial effects on the host plants. They are popularly known as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1980). Rhizobacteria that are living on the surface of the roots are known as rhizoplane bacteria. On the other hand, bacteria that are living inside the root tissues are called endophytic bacteria. Considering the potential for plant growth promotion, application of the PGPR is gaining great interests in sustainable crop production. Bacteria from diverse taxonomic genera have been shown to improve plant growth and act as PGPR; among them *Bacillus* is gaining importance as a candidate for plant growth promotion in low-input, eco-friendly, sustainable agriculture.

Bacteria of the genus *Bacillus* are generally heterotrophic, rod-shaped. They have the ability to produce endospore under special circumstances. Some genus of Bacillaceae is aerobic, while others are facultative or strictly anaerobic. The taxonomic position of *Bacillus* includes kingdom, *Bacteria*; division, *Firmicutes*; class, *Bacilli*; order, *Bacillales*; family, *Bacillacea*; and genus, *Bacillus* (Fritze 2004). A very small portion of the *Bacilli* are also harmful to human and animals as well as crop plants. However, beneficial *Bacilli* have been documented under diversified field and horticultural crops. A good number of plant-associated *Bacilli* have been commercialized as bioinoculants for protection and growth of plants (Islam and Hossain 2013). *Bacillus* spp. are ubiquitous in nature and have the ability to produce diverse classes of antimicrobial secondary metabolites, enzymes, and rare carotenoids. The chemistry, biosynthesis, and bioactivities of the antibiotics produced by marine *Bacillus* spp. have recently been reviewed (Mondol et al. 2013). One of the plant-associated members *B. sphaericus* strain UPMB10 fixes substantial amount of atmospheric nitrogen in association with various nonlegume plants including rice, banana, oil palms, and tomatoes (Mia et al. 2010a; Mia and Shamsuddin 2010; Mia et al. 2013). The strain UPMB10 has been evaluated for their N_2-fixing capacities in association with oil palm seedlings, banana, rice, and vegetable soybean through acetylene reduction assay (ARA) and ^{15}N isotopic dilution technique

(Mia et al. 2007). Although several reviews on PGPB have been published (Borris 2011; Islam and Hossain 2012, 2013), however, no reviews have so far been published on plant growth promotion by the application of *B. sphaericus* strain UPMB10.

3.1.1 *Isolation of Free-Living Nitrogen-Fixing* Bacillus *Species*

Isolation followed by molecular identification of beneficial bacteria is a primary step for the development of bioinoculant for sustainable crop production. The process of the development of a commercial formulation involves several sequential steps, which should be maintained very carefully in order to obtain higher benefits. Isolation of free-living nitrogen-fixing *Bacillus* species can be done by most-probable-number (MPN) technique or direct plate counting method. However, in both cases, nitrogen-free any media should be used instead of common nutrient agar media. A simple nitrogen-free media contains (g L^{-1}) 5 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_47H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, and 0.5 % bromothymol blue in 0.2 N KOH (2 mL), 1.64 % Fe-EDTA solution (4 mL), 0.02 g yeast extract, and agar. The pH of the media should be adjusted at 7.0. Yeast extract should be added as it is known that a trace amount of nitrogen is required for the isolation of most free-living nitrogen-fixing bacteria from the soil and rhizosphere (Watanable and Barraquio 1979). For the isolation of endophytic bacteria, plant samples should be washed, blotted, weighed (2–5 g), and surface sterilized properly before crushing by the aid of a sterilized mortar and pestle. When culturing the diluted meshed materials in the culture media, the presence of a pellicle in the semisolid medium or blue color in solid medium plate confirms the existence of N_2 fixers. Acetylene reduction assay and ^{15}N dilution technique should be used to detect the N_2-fixing ability. The nitrogen fixers are finally identified through 16S rRNA gene sequencing followed by detailed phylogenetic analyses.

3.1.2 *Isolation of Phosphate-Solubilizing* Bacillus *Species*

A large group of *Bacillus* species are able to solubilize fixed and insoluble phosphorus present in the soil. The isolation of these bacteria needs any type of media containing phosphate source such as iron phosphate, tricalcium phosphate, or aluminum phosphate. The phosphorus-solubilizing activity can be confirmed by spotting pure cultures on top of the plate of specific media. A common media which is used for phosphate-solubilizing activity is the National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal 1999). The composition of this medium is (L^{-1}) 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 5 g $Ca_3(PO_4)_2$, and 20 g of bacterial agar. After inoculation and incubation at 30 °C for

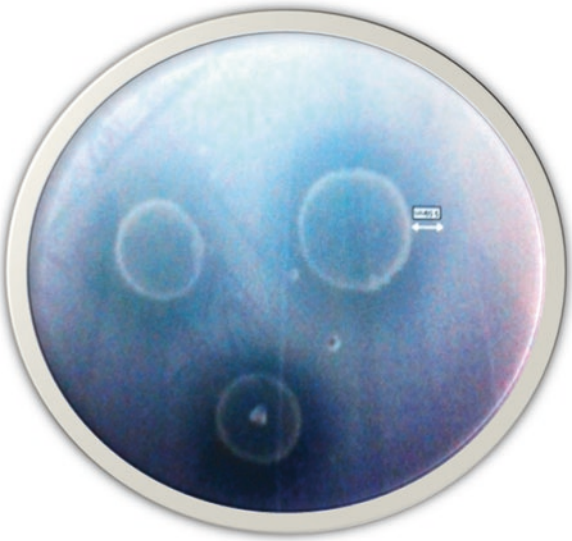

Fig. 3.1 Solubilization of tricalcium phosphate in NBRIP medium by bacterial strain *Bacillus* sp.

5–7 days, appearance of a visible halo zone confirms the activity of phosphate solubilization by the tested bacteria in the agar plate (Fig. 3.1) (Islam and Hossain 2012).

3.1.3 Identification of Strain

Usually pure culture of a strain is subjected to molecular identification. There are several techniques, namely, biochemical, physiological, and molecular, used for the identification of a bacterium. Immunogold, Biolog, and enzyme-linked immunosorbent assay (ELISA) techniques (Bashan et al. 1993) are also utilized for the identification of *Bacillus*. Biolog identifies strain diversity and depends upon the ability of individual species to catabolize specific substrates (Miller and Rhoden 1991). However, the most common practical method for strain identification is the molecular technique by using 16S rRNA gene sequencing (Bashan et al. 1993). The DNA was extracted from pure isolates using the QIAamp Genomic DNA Mini Kit UK AS. The rep-PCR was performed using the forward primer *8F*,5′-AGA GTT TGA TCC TGG CTC AG-3' and the reverse primer *1492R*,5′-GGT TAC CTT ACG ACT T-3′. The primers selected for this study amplify sequences from both symbiotic and free-living nitrogen-fixing bacteria. The PCR products were purified using the GeneJET PCR purification kit (Fermentas, Thermo Scientific, EU) and sequenced by First BASE Laboratories, Malaysia.

The partial gene sequences were aligned using the Clustal W package (Thompson et al. 1994). An unrooted phylogeny tree was constructed using the neighbor-

joining method (Saitou and Nei 1987). Partial sequences for the strains (*Bacillus* sp., GenBankJN695718.1; *Burkholderia* sp., GenBankAF219125.1; and *Stenotrophomonas maltophilia*, GenBankHQ219979.1) were obtained as references. The topology of the distance tree was tested by resampling the data using 1,000 bootstrap replicates. The phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The sequences obtained were deposited in the European Molecular Biology Laboratory data bank.

3.2 Mode of Beneficial Effects

Bacillus spp. perform beneficial effects on inoculated plants through various ways, namely, fixation of N_2, production of phytohormones, enhancement of uptaking nutrient and water, and serving as a biocontrol agent. The mechanisms of beneficial effects of *Bacillus* spp. to inoculated plants are described in the following subsections.

3.2.1 Root Colonization of Bacillus

Root colonization is a precondition for beneficial effects by the rhizobacteria. Without a successful colonization, no bacterium can perform any interaction with host roots. *Bacillus* spp. colonize the root both externally and internally. The entry of bacteria into the cortex through epiblema performs via degradation of middle lamella and cell wall, i.e., the apoplastic area by the activity of several enzymes, namely, cellulase and pectinase (Mutalib et al. 2012). Jensen-CMC plate assay was used to determine cellulase activity, and the plates spotted with *Bacillus* sp. (Sb26) broth showed a clear halo zone after stained with 0.1 % Congo red indicating positive response (Fig. 3.2). By the aid of scanning and transmission electron microscopies, Naher and coworkers (2009) observed an extensive colonization of *Bacillus* spp. in the intercellular spaces which was extended into cortex and vascular system in the lateral roots of rice plant (Fig. 3.3a, b). The cortex region of roots and the parenchymatous tissues of rice are the site of colonization for endophytic bacteria, which might provide the suitable niche for N_2 fixation with low concentration of oxygen and subsequently provide the fixed N_2 to the host cell. *Bacillus* sp. may enter into the cell through junction of lateral root and by crevices. Study on rice root tips showed the zone of elongation and differentiation and the junction between the primary and the lateral roots were the primary sites for entry of bacteria (Liu et al. 2006). Lateral roots originate from the pericycle and grow through the cortex of the main root; therefore, entry through the lateral roots led to the bacteria advance into the inner cortex. Emergence points of lateral roots might be other primary site of colonization. In bananas, the *B. sphaericus* strain UPMB10 colonizes the root in both externally and internally (Mia et al. 1999, 2010a). The entry of bacteria through

Fig. 3.2 Cellulase activity assayed after 24 h incubation in Jensen-CMC plate; the clear halo zone indicates cellulose degradation by *Bacillus* spp. strain SB 26

apoplastic region is mediated by dissolving the cementing material in the middle lamella, i.e., the pectate of Ca and Mg, and the cellulose of the cell wall.

Bacillus sp. produces cellulose-degrading enzyme and is competent to penetrate the root cells and grow endophytically.

The production of extracellular polysaccharide and cell aggregation might help in colonization process. Our study showed that *Bacillus* spp. can successfully colonize the roots both externally and internally (Fig. 3.3a, b). *Bacillus* sp. produces extracellular polysaccharides and shows cluster of cell aggregation in the root hairs and elongation zones. This might have positive effect on root colonization. During the colonization process, larger cell aggregation has also been found in *B. megaterium* strain C_4 and *Azospirillum brasilense* (Bahat-Samet et al. 2004; Liu et al. 2006). Bacterial surface components, such as extracellular polysaccharides and proteins, were involved in plant colonization, and extrapolysaccharide was used as a sole carbon source for its own development (Burdman et al. 2001). Moreover, bacteria with flagella might play significant role in root surface attachment.

3.2.2 *Role of* Bacillus *spp. in Nitrogen Fixation and Plant Growth Promotion*

Nitrogen-fixing environmental bacteria are popularly known as diazotrophs either associative or free living. Diazotrophic *Bacillus* spp. form colonies in association with roots as ectorhizospheric or endorhizospheric, i.e., endophytic, and fix substantial amount of atmospheric N_2. The fixed N_2 is thought to be shared by the host

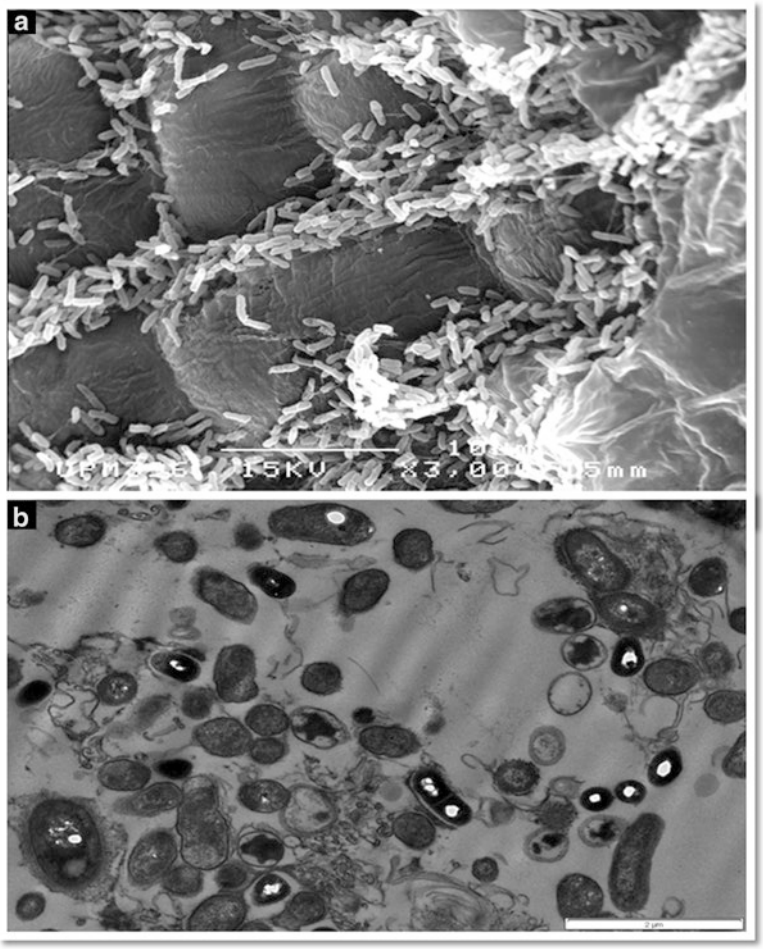

Fig. 3.3 Electron micrographs show root colonization of rice by *Bacillus* sp. (**a**) Scanning electron micrography (SEM) shows massive colonization of bacteria on the root surface. (**b**) Transmission electron micrograph (TEM) shows bacterial colonization in the internal structure, i.e., in the cortex of young rice root

plant for its growth and development. However, the mechanism of utilization of the fixed N_2 by host plant is still not fully understood. It may be performed through various ways like mineralization of decomposed bacterial dead body or excretion of fixed N_2 as NH_4^+ to host plant, where the later one is more beneficial than earlier (Mia and Shamsuddin 2010). Recently, existence of NH_4^+-excreting endophytic bacteria is drawing greater interest to the microbiologist. Application of *B. sphaericus* strain UPMB 10 supports 38 % of the total N requirement of banana tissue-cultured seedlings grown for 6 weeks and 20 % of the N requirement of the young oil palm seedlings (Mia et al. 2010a, b; Amir et al. 2001). Naher and coworkers

Table 3.1 Nitrogen fixation and indoleacetic acid production by *Bacillus* spp.

Isolates of *Bacillus* spp.	ARA (μmol C_2H_4cfu^{-1}h^{-1})	IAA (mgL^{-1})
Sb2	1.9×10^{-8}	68
Sb3	2.3×10^{-10}	46
Sb4	Nd	35
Sb6	1.3×10^{-7}	55
Sb7	Nd	65
Sb10	2.7×10^{-10}	48
Sb18	2.7×10^{-10}	51
Sb20	3.9×10^{-9}	53
Sb26	2.9×10^{-10}	12
Sb32	5.4×10^{-10}	65
Sb33	1.8×10^{-10}	24
Sb34	4.9×10^{-7}	51
Sb35	1.36×10^{-6}	29
Sb37	2.5×10^{-10}	53
Sb38	6.1×10^{-11}	32
Sb42	9.1×10^{-10}	69

Nd represents not done, *ARA* acetylene reduction assay

Table 3.2 Fixation of atmospheric N_2 by *Bacillus* sp. in two rice genotypes measured by ^{15}N isotopic dilution technique

	Mayang Segumpal rice		MR219 rice	
N_2	Control	*Bacillus* sp.	Control	*Bacillus* sp.
^{15}N (a.e.)	0.801	0.601	0.764	0.594
Ndfa (%)	–	25	–	26
Tissue N (%)	2.2	3.9	2.3	3.6
N_2 fixed (mg plant^{-1})	–	1.0	–	0.89
N_2 fixed (kg ha^{-1})	–	12.04	–	7.85

demonstrated that various strains of *Bacillus* spp. can fix appreciable amounts of atmospheric N_2 and produce IAA (Table 3.1) (Naher et al. 2009).

The N_2 fixation by *Bacillus* spp. in association with rice roots was further confirmed by ^{15}N isotopic dilution technique by Naher et al. (2013) (Table 3.2). The dilution of atomic excess (a.e.) in the *Bacillus*-inoculated tissues indicated that nitrogen is derived from atmosphere (Ndfa) through biological N_2 fixation (BNF) process. They found higher BNF in traditional rice variety Mayang Segumpal than the modern rice variety MR219 under Malaysian conditions. This discrimination of BNF capacity might be due to the specificity of root exudates. It is well known that bacteria utilize root exudates as their carbon source and fix N_2 from the atmosphere (Naher et al. 2013).

3.2.3 Enhanced Root Growth and Nutrient Uptake

The increased root growth followed by enhanced water and nutrient absorptions in inoculated plants are considered to be the possible mechanisms of the beneficial effects of *Bacillus*. These types of effects were documented in field and horticultural crops under diverse environmental condition (Mia et al. 2009b). The stimulation of root growth was likely influenced by the production of plant growth-regulating hormones especially the indole acetic acid (IAA). *Bacillus* spp. can produce IAA either in association with roots or in the culture media in vitro (Table 3.1) (Naher et al. 2009). The increased root growth (formation of root hair and lateral roots) in rice is likely due to the production of IAA by the applied bacteria as IAA is known to stimulate the formation of root hair and lateral roots by increasing the production of meristem in epiblema and pericycle (Hofer 1991). Therefore, increased surface area of roots helps plant to absorb more nutrient and water from the surroundings. The phytohormone produced by *Bacillus*-colonized seeds stimulates the seed germination and seedling vigor (Biswas et al. 2000; Mia and Shamsuddin 2009; Mia et al. 2012). However, the effects of IAA on seedling growth seem depended on the concentration as lower range of IAA concentration stimulated growth but higher concentration of IAA inhibited seedling growth (Arshad and Frankenberger 1991).

3.2.4 Increased Photosynthesis and Biomass Production

Inoculation of PGPR increases the physiological activities especially photosynthesis of the host plant. The possible explanation of the greater physiological activities might be due to enhanced water and nutrient uptake and the incorporation of the N through BNF process. The greater nutrient accumulation in the inoculated plants resulted in the higher metabolic activities, which enhanced the photosynthetic and other physiological activities. Inoculation of *A. brasilense* strain Sp7 and *B. sphaericus* strain UPMB10 increased the photosynthetic activity and biomass production in tissue-cultured banana plantlets (Mia et al. 2010a) (Fig. 3.4). Similarly, inoculation of *Bacillus* spp. in rice plant showed 48–83 % increased biomass production compared to fertilized plant (Table 3.3). Inoculation process also increased the root water conductivity of the host plant (Sarig et al. 1990).

3.2.5 *Role of* Bacillus *spp. on the Mobilization of Phosphorus*

Phosphorus is one of the most important macronutrient elements for plant, which is required for energy transfer process and synthesis of various biomolecules including nucleic acids and membrane phospholipids. Its deficiency is common in many soils. Globally, about 5.7 billion ha of land have been noted to contain too little available P

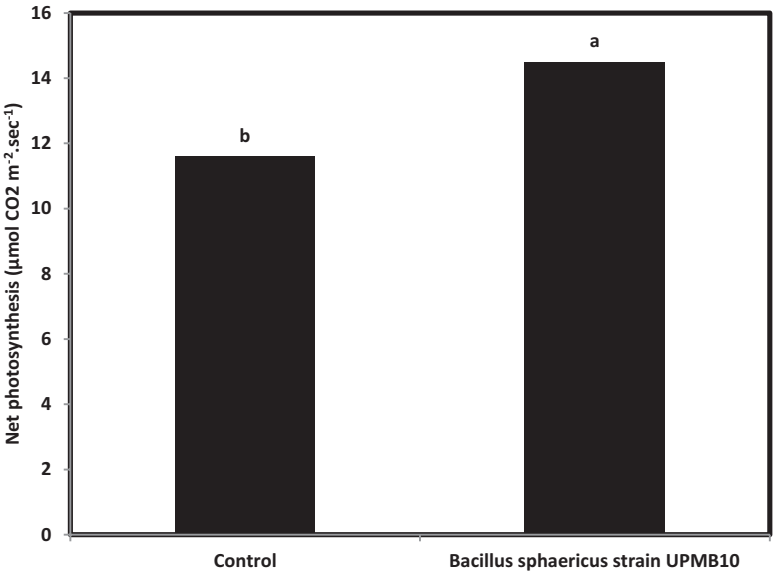

Fig. 3.4 Effect of *B. sphaericus* strain UPMB10 inoculation on the net photosynthesis of tissue-cultured banana plantlets (60 days old) (modified from Mia et al. 2010a)

Table 3.3 Growth promotion of plant biomass in rice due to inoculation with different strains of *Bacillus* spp. under pot culture condition

Rice cultivar	Diazotroph inoculum	Biomass increment over control (%)	References
Mayang Segumpal	*Bacillus* sp. (Sb26)	48	Naher et al. (2009)
MR219	*Bacillus* sp. (Sb26)	87	Naher et al. (2009)
MR219	*Bacillus* sp. (Sb42)	83	Asilah et al. (2009)

for sustaining optimal crop production. Phosphorus ion concentration ranges from 0.1 to 10 µM in most of the soils and P in soil should range from 1 to 5 µM for the optimal growth of grasses and 5–60 µM for high P-demanding crops such as tomato and pea (Hinsinger 2001). Most of the soils contain considerable reserves of total P, but a major portion of it remains comparatively immobile and only less than 10 % of soil P enters into the plant-animal cycle (Kucey et al. 1989). In agricultural soils, available P source is a chemical P fertilizer from which about 90 % is precipitated by forming complexes in soil with iron, aluminum, and calcium depending on the soil pH (Gyaneshwar et al. 2002). In many soils of the world, P deficiencies limiting crop production and application of chemical P fertilizers is insufficient. There is only 1–5 % soluble soil P available for the plants (Molla and Chowdhury 1984).

Microorganisms play an important role in phosphorus mobilization in soils. Among them, a number of *Bacillus* spp. has been shown to solubilize insoluble soil organic and inorganic phosphorus through production of organic acids and enzymes

(Panhwar et al. 2009). Generally, P solubilization correlated with the production of organic acids with the direct oxidation that happens on the outer face of the cytoplasmic membrane, and it is connected with the drop of pH (<7) (Maliha et al. 2004; Pradhan and Sukla 2005). There are different possible mechanisms by which bacteria solubilize P. Among them, organic and inorganic acid production, release of H^+ accompanying NH^+_4, and production of exo-polysaccharides, acid phosphatase, phytases, and H_2CO_3 are important (Zaidi et al. 2009).

3.2.6 *Production of Organic Acids by* Bacillus *spp.*

Low-molecular-weight organic acids play multiple roles in the soil processes, such as root nutrient acquisition, mineral weathering, microbial chemotaxis, and metal detoxification (Jones et al. 2003). The phosphorus-solubilizing bacteria can release numerous organic acids including oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glycolic, fumaric and 2-ketogluconic acid (Leyval and Berthelin 1989; Panhwar et al. 2012). Among organic acids, the amount of oxalic and malic acids were higher than the others (Zeng et al. 2008). Bacterial strains from the genera *Pseudomonas*, *Bacillus*, and *Rhizobium* are among the most powerful phosphate solubilizers than others (Rodriguez et al. 1999).

The mechanism of P solubilization is due to the production and excretion of organic acids (e.g., oxalic acids), which act as chemical agents for mobilizing insoluble phosphates. Effective microorganisms include the bacteria *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, and *Pseudomonas striata* that are able to produce organic acids (Jana 2003). A number of organic acids, namely, lactic, glycolic, citric, 2-ketogluconic, malic, oxalic, malonic, tartaric, and succinic acids, identified from bacterial culture filtrates were shown to possess chelating properties (Kucey et al. 1989). Panhwar et al. (2012) found seven *Bacillus* spp. which were able to produce organic acids in the broth culture containing insoluble P sources (tricalcium phosphate) during 48 h incubation period (Fig. 3.5). Four organic acids, oxalic, malic, succinic, and propionic acids, were identified. Among the organic acids, succinic acid production was the highest (0.25 mgL^{-1}) by PSB16 isolate followed by PSB9 (0.24 mgL^{-1}) in NBRIP broth.

3.2.7 *Production of Enzymes by* Bacillus *spp.*

In most soils, 30–50 % of P remains in organic form, while in other soils, it may be as low as 5 % and as high as 95 %. Organic P in the soil is predominantly present in the form of inositol phosphate (soil phytate). The degradability of organic P compounds mostly depends on the physicochemical and biochemical properties of their molecules, e.g., nucleic acids, phospholipids, and sugar phosphates that are easy to break down, but phytic acid, polyphosphates, and phosphonates decayed more slowly (McGrath et al. 1998). Panhwar et al. (2012) isolated seven strains of *Bacillus*

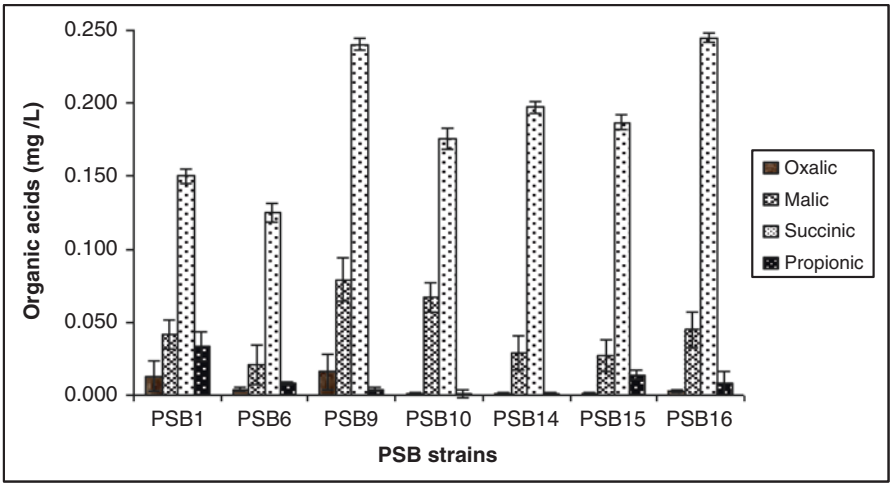

Fig. 3.5 In vitro production of organic acids by PSB isolates of *Bacillus* spp. grown in NBRIP broth culture containing inorganic P after 48 h of incubation; the vertical bar indicates standard error ($n = 5$)

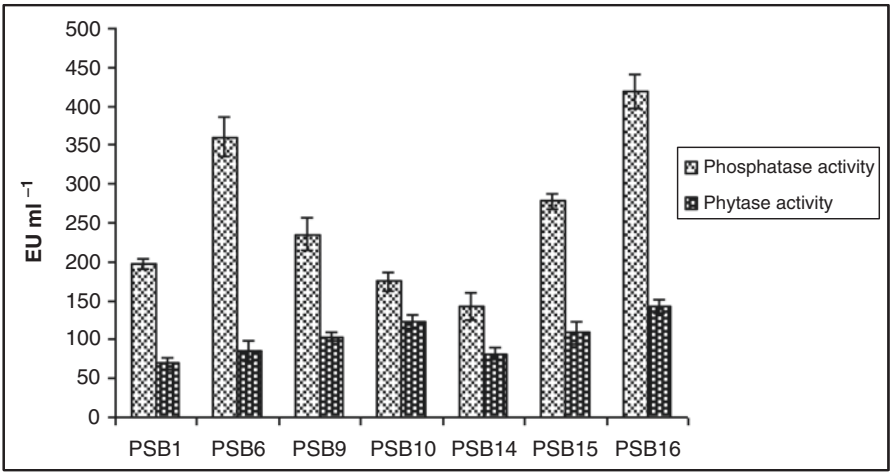

Fig. 3.6 Activities of enzymes phosphatase and phytase by phosphate-solubilizing bacteria (PSB) *Bacillus* spp. under in vitro broth culture after 48 h of incubation; vertical bar indicates standard error ($n = 5$)

spp. which were able to produce phosphatase and phytase enzymes in the broth culture during 48 h of incubation. Production of phosphatase was higher than the phytase (Fig. 3.6) in the study. Among the isolates evaluated, the highest phosphatase (417.89 EU mL^{-1}) and phytase (142.25 EU mL^{-1}) activities were produced by PSB16 isolate.

3.2.8 *Antagonistic Effect Against Pathogens*

A large body of literature revealed that several strains of *Bacillus* suppress growth of plant pathogens, and thus they are suitable candidates for biocontrol of fungal and peronosporomycetal phytopathogens (Islam and Hossain 2013). *Bacillus* spp. that can act as biological control agents (BCA) which suppress the diseases of various types of crop plants have been reported by several researchers, and *Bacillus*-based BCA has great potential in disease pest management in sustainable crop production system (Jacobsen et al. 2004).

The early root colonization by PGPB through making a barrier for pathogenic microorganisms invasion (Bais et al. 2006), antibiosis with biosurfactant activity and finally development of induced systemic resistance in the host plants are the possible mechanisms (Mia and Shamsuddin 2013; Islam and Hossain 2013). In bananas, *Fusarium* wilt can be controlled by the inoculation of *Bacillus sphaericus* strain UPMB10 and *Bacillus amyloliquefaciens* NJN-6 which also increased the fungal diseases resistance in bananas (Yuan et al. 2013). On the other hand, sheath blight is a common rice disease, which is caused by *Rhizoctonia solani*. The *Bacillus* spp. were tested for their ability to inhibit the mycelia growth of *R. solani* in vitro by dual culture technique on PDA plates. The dual culture plates showed that all PSB strains were able to inhibit the fungal growth. The highest antagonistic effect was found in PSB16 (71.14 %) followed by PSB10 (59.31 %) and PSB9 (58.33 %) (Panhwar et al. 2012). After 7 days of incubation of dual culture, fungal hyphae of *R. solani* were unable to reach the bacterial culture, and inhibition zone was established with the dimension of inhibition circle ranging from 4 to 10 mm. It has been reported that *Bacillus* species were found to colonize the root surface, increase the plant growth, and cause the lysis of fungal mycelia (Turner and Backman 1991; Podile and Prakash 1996; Takayanagi et al. 1991). Application of *B. subtilis* significantly decreases the anthracnose of chilli caused by *Colletotrichum gloeosporioides* OGC1 (Ashwini and Srividya 2014).

3.2.9 *Evidences of P Solubilization by* Bacillus *sp. using* ^{32}P *Technique*

Inoculation of plants with *Bacillus* sp. increased 24–38 % and 108–146 % of grain yield and total plant biomass of rice (Panhwar et al. 2013). Higher grain is the contribution of *Bacillus* sp. for P nutrient uptake and indoleacetic acid production. The result of ^{32}P study showed that *Bacillus* sp. inoculation with phosphate rock in aerobic rice produced lower specific activity (^{32}P) than their comparable non-inoculated treatments (Table 3.4). The PSB inoculation also showed significantly ($p < 0.05$) the higher values in plant P uptake and amount of P derived from the unavailable sources.

Table 3.4 Effect of *Bacillus* sp. inoculation on the specific activity of ^{32}P and content of P in plant tissue of aerobic rice

Treatments	Specific activity Bq mg P^{-1}	Pdfl (%)	PdfPR (%)	P uptake (mg P Pot^{-1})
Control	152.96	91.66	8.34	0.34
Rock phosphate	84.86	55.48	44.52	0.84
Bacillus sp.	54.1	73.37	26.63	0.75
Bacillus sp. + rock phosphate	32.1	30.99	69.01	1.09

3.3 Molecular Aspects of Mode of Beneficial Interactions

One of the mechanisms involving beneficial interaction of *Bacillus* sp. with plant is the nitrogen fixation. The biological N_2 fixation is restricted to prokaryotes. A genomic survey reported approximately 5 % of prokaryotes carry genes for N_2 fixation (Raymond et al. 2004). When ammonia is derived from atmospheric N_2, the process is called nitrogen fixation. In this processes, 2 mol of ammonia are produced from 1 mol of nitrogen gas. The diazotrophic bacteria contain an enzyme complex for the reduction of molecular nitrogen to ammonia called nitrogenase enzyme. The nitrogenase is a complex of dinitrogenase (Mo-Fe protein) and dinitrogenase reductase (Fe- protein) (Burris 1991).

The nitrogenases are commonly referred to as molybdenum nitrogenases (*nif*-encoded). The nitrogenases with vanadium as a cofactor-encoded *vnf* are found in *Azotobacter vinelandii* (Benemann et al. 1972). An alternative heterometal-free nitrogenases (*anf*-encoded) has been reported in *A. vinelandii* and *Rhodobacter capsulatus* (Masepohl et al. 2002). The study of *A. vinelandii* found that nitrogenase complex is stabilized by ADP-tetrafluoroaluminate and that the chain is composed of A, B, C, and D. Chains A and C are α-chain and B and D are the β-chain. Nitrogenase iron protein chains are E, F, G, and H. The process of nitrogen reduction is extremely energy dependent. The nitrogenases require 16 mol of ATP for the reduction of 1 mol of N_2 to ammonium:

$$N_2 + 8e^- + 8H^+ + 16ATP = 2NH_3 + H_2 + 16ADP + 16P_i$$

This reaction is catalyzed by the hetero-oligomeric protein complex composed of a reductase and a nitrogenase part. The reductase is a homodimer containing a 4Fe-4S cluster and an ATP-binding site at the subunit interface. The αβ-interface contains P cluster (containing two 4Fe-4S clusters) which oxidizes the reductase and is oxidized by the Mo-Fe cofactor which contains two Mo-3Fe-3S clusters comprising the N_2-binding site.

The biological N_2 fixation as well as the pathways of nitrogen assimilation is well coordinated and regulated in organisms. In the prokaryotes, the level of tran-

scription is controlled by *nif* genes or N-assimilation genes (*glnA*). In *Rhodospirillum*, regulation can occur posttranslationally by covalent modification and consequent inactivation of the nitrogenases Fe protein (Fitzmaurice et al. 1989). Molecular oxygen is a strong inhibitor of the nitrogenases.

In symbiotic N_2 fixation system, dinitrogen-fixing microbe forms a partnership in the form of nodules. Nodulation begins with secretion of flavonoids from the roots that trigger *nod* gene expression in diazotroph. Nodules take sucrose from the phloem, convert it to succinate, and through bacterial respiration generate the ATP and reduced ferredoxin required for conversion of N_2 to ammonia. The plant takes up the ammonia and assimilates it into glutamine and asparagines in temperate legumes or into ureides, allantoic acid, and allantoin in tropical legumes. However, in nonlegume *Bacillus* enter into the roots through epidermis or crakes created at the site of emerging lateral roots. In legumes, the early nodulation (ENOD) genes accomplish the processes of initiation of nodule induction. In rice homologues sequences to ENOD genes have been detected (Barraquieo et al. 1997). Nod factors are not essential for rhizobial infection in rice. Root exudates of a few rice genotypes could induce nod gene expression to a very low extent in few rhizobial strains and deformation of root hair in few rice genotypes (Plazinscki and Rolfe 1985). Nitrogen fixation performed in *nif H* gene and transcriptional fusions of the *nifH* gene of diazotroph that established the roots of rice seedlings can support nitrogenase gene expression (Engelhard et al. 2000).

Oxygen, mineral nitrogen, level of ammonia, soil temperature, and pH interfered nitrogenase activity. Dobereiner (1979) studied the details of nitrogenase enzyme activity of *Azospirillum* sp. Her study revealed that if the oxygen supply exceeds bacterial consumption, then NH_3, NO_3, and NO_2 are assimilated at maximal rates and no nitrogenase enzyme activity occurs. When the consumption of oxygen corresponds exactly to the amount transported to the site, nitrogenase synthesis reached maximum. If oxygen is totally removed from the system, ATP generation stops and nitrogen fixation is also impeded. Another study of *Azospirillum* tolerance to oxygen carried out during fermentation found that the level of tolerance is dependent on the age of the culture and optical density and the rate of oxygen tolerance during shaking is greater in *A. lipoferum* when lactate and glucose concentration of the medium is less than 0.5 % (Volpon et al. 1981).

Generally, rice genotypes show high nitrogen-fixing activity in the presence of indigenous diazotroph. Ueda et al. (1995) retrieved a *nifH* fragment in DNA from field-grown Japanese rice, which clustered with *nifH* sequences of *Azoarcus* spp. A diverse group of *nifH* sequences represent many lineages of diazotrophic members of the bacteria in rice plant (Zehr et al. 2003). Rediers et al. (2003) found rice plant colonization by *Pseudomonas stutzeri* A15, to be able to express *miaA* gene. This gene involved in the production of cytokinin was *trans*-zeatin. Expression of an auxin-responsive promoter in *Arabidopsis* indicated that the plants were able to detect bacterial synthesized IAA (O'Callaghan et al. 2001). Nitrogen nutrition in plants by diazotrophic bacteria and their mode of actions have recently been reviewed (Mia et al. 2013).

3.4 Conclusions and Future Perspective

Plant growth-promoting *Bacillus* spp. are now one of the most widely used bacteria on biopesticide and biofertilizer market in North America and other parts of the world. The use of spore-forming bacilli is gaining interest of research and commercial attention due to higher diversity among the biofunctional bacteria. Whole-genome sequences of several *Bacillus* spp. including *B. amyloliquefaciens* have been completed. The inocula of some *Bacillus* spp. have been widely used for increasing the productivity of banana, rice, oil palm, wheat, maize, and other field and horticultural crops. The potential value of *Bacillus*-based PGPR to improve agricultural productivity has been validated in some commercial agricultural system. Recent advances on genomics and post-genomics of several biofunctional *Bacillus* spp. and major crop plants will provide future basis for better understanding of *Bacillus*-plant interactions and development of improved strains as effective biofertilizer and/or biopesticide for eco-friendly, low-input, sustainable crop production. Future research is required in several key areas to support the agricultural use of PGPR-based inoculants for crop protection and growth promotion which might be included into the integrated crop management, where bacilli will be one aspect of the multidimensional approaches.

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Chapter 4
Management of the Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte, Using Transgenic Bt Maize

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Abstract The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is an important pest of maize, *Zea mays* L., in the United States and Europe. In the United States, transgenic maize hybrids that express Cry endotoxin proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) have been developed to limit feeding damage caused by corn rootworm larvae.

Transgenic maize expressing the Cry3Bb1 protein was first registered for commercial sale in 2003, and two additional products expressing different proteins were registered for commercial sale in 2005 (Cry34/35Ab1) and 2006 (mCry3A). More recently a fourth Cry protein, eCry3.1Ab, was registered as a pyramid with mCry3A in 2013. Although the United States Environmental Protection Agency has mandated that all registrants of Bt crops submit an insect resistance management (IRM) plan prior to registration, the long-term viability of transgenic Bt maize for control of western corn rootworm remains uncertain. Under laboratory conditions, selected western corn rootworm populations have developed resistance to all commercially available Bt products as well as the eCry3.1Ab protein. In addition, field resistance to transgenic Bt maize has been documented in certain fields in the United States. To extend the lifetime of this management option for the future, additional improvements in resistance management plans will be needed.

4.1 Introduction

Maize, *Zea mays* (L.), is an economically important crop grown for a variety of purposes (e.g., food, animal feed, biofuel) throughout the world. Although maize plantings can be severely impacted by a diverse assemblage of major agricultural pests, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_4

considered one of the most economically damaging. Yield losses and control costs due to this pest are estimated at over $1 billion annually in the United States (Metcalf 1986) and €0.472 billion in Europe (Wesseler and Fall 2010).

Native to North America, the western corn rootworm was first recorded in Kansas, USA, in 1867 (LeConte 1868), though it was not recognized as a pest of maize until 1909 (Gillette 1912). It is thought that this pest species originated in Guatemala and reached the southwestern United States approximately 3,000 years ago with the spread of maize production (Melhus et al. 1954; Krysan and Smith 1987). Its range expansion and importance as a pest in the Unites States increased dramatically within the last half century largely because of continuous maize production and improving irrigation systems throughout its native range (Chiang 1973). By 2005, western corn rootworm could be found infesting maize plantings in much of the eastern United States (Gray et al. 2009). In 1992, the first detection of western corn rootworm in Europe occurred near the Surcin International Airport in Belgrade, Serbia (Baca 1994). Within 15 years it had been recorded in 20 European countries (Gray et al. 2009) through at least five independent introductions across the continent (Ciosi et al. 2008).

The ability of western corn rootworm to invade new areas, and its capacity to adapt to various control measures, has necessitated development of additional pest management tactics that can serve as stand-alone practices or be readily allied with other pest management approaches. In the United States, transgenic maize that express Cry endotoxin proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) have been increasingly used to mitigate the economic damage caused by this pest. The benefits of transgenic Bt maize include effective management of the target pest (Storer et al. 2006; Hibbard et al. 2011; Clark et al. 2012), decreased use of broad-spectrum insecticides (Phipps and Park 2002), and reduced impact to nontarget species (Siegel 2001; Al-Deeb and Wilde 2003; Ahmad et al. 2005; Lundgren and Wiedenmann 2002; Mullin et al. 2005; Hönemann et al. 2008). To understand why transgenic Bt maize is effective at managing western corn rootworm, we must first understand the biology and behavior of this insect. We must then consider other management practices that are associated with controlling this pest. Efficient control of western corn rootworm requires a broad understanding of current management tactics and the limitations involved with each.

4.2 Western Corn Rootworm

The western corn rootworm is a univoltine beetle species in the family Chrysomelidae (Fig. 4.1). The insect overwinters as eggs, which are typically laid in the soil of maize fields from mid- to late summer. Three larval instars characterize western corn rootworm development following egg hatch in the spring. Larvae are the most economically damaging life stage, feeding almost exclusively on the roots of maize plants, though a limited number of other closely related plant species in the family Poaceae may also serve as hosts (Branson and Ortman 1967, 1970; Clark

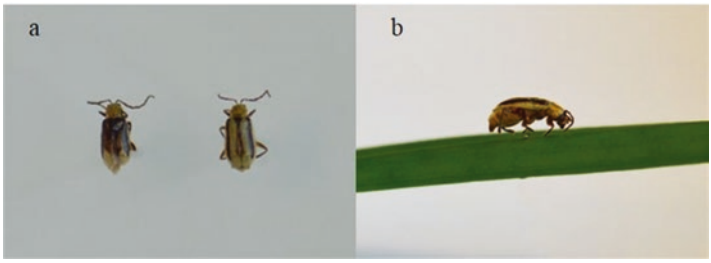

Fig. 4.1 (**a**) Adult male (*left*) and female (*right*) western corn rootworm, (**b**) Adult western corn rootworm on maize leaf

Fig. 4.2 Undamaged maize root system (*left*) and maize root systems damaged by western corn rootworm larval feeding (*right*)

and Hibbard 2004; Oyediran et al. 2004). Root injury from larval feeding (Fig. 4.2) can reduce the uptake of water and nutrients by maize plants (Kahler et al. 1985; Sutter et al. 1990; Godfrey et al. 1993) and increase susceptibility to root diseases (Palmer and Kommedahl 1969; Godfrey et al. 1993; Kurtz et al. 2010). In addition, larval feeding can result in lodging of plants because of reduced brace root support, which can lead to direct yield losses because of problems associated with mechanically harvesting fallen plants (Spike and Tollefson 1991).

Host location by western corn rootworm larvae is facilitated by carbon dioxide gradients formed in the soil by respiring roots (Strnad et al. 1986). Once larvae locate root tissue, additional primary and secondary plant metabolites are likely used during the host selection process (Hibbard and Bjostad 1988; Robert et al. 2012a, b). When exposed to maize roots, larvae exhibit a localized foraging behavior consisting of decreased speed of movement and increased turning frequency within the soil, which facilitates larval establishment by allowing a more restricted area of search for food resources (Hibbard and Bjostad 1988; Strnad and Dunn 1990). Conversely, in the absence of proper host stimuli, larvae will exhibit ranging behavior, which involves relatively quick and straight movement through the soil to allow a greater area of search (Strnad and Dunn 1990). When exhibiting ranging behavior, larvae can travel distances of at least 100 cm through the soil to locate host

tissue (Suttle et al. 1967; Short and Luedtke 1970), though factors such as soil type, soil moisture, and soil bulk density can influence distances traveled (Gustin and Schumacher 1989; Turpin and Peters 1971; MacDonald and Ellis 1990). Quickly locating and recognizing suitable host tissue is important for survival of western corn rootworm larvae because starvation for 72 h can significantly increase the rate of larval mortality (Branson 1989; Oloumi-Sadeghi and Levine 1989).

During initial establishment, western corn rootworm larvae feed in the cortex of seminal and nodal maize roots that are 2 mm or less in diameter (Strnad and Bergman 1987). Over the course of a growing season, older larvae move throughout the root zone to feed on newly developed nodal roots of larger diameter, which are required to complete their development (Hibbard et al. 2008). Western corn rootworm larvae may also move between plants after initial establishment if available food resources are lacking (Hibbard et al. 2003, 2004).

After development through the larval and pupal stages, western corn rootworm adults emerge from the soil to feed on the leaf tissue, silks, and pollen of maize plants (Bryson et al. 1953; Ball 1957). Several flowering weed species are also attractive to foraging beetles (Hill and Mayo 1980), which may help to facilitate their spread and survival when preferred maize resources are insufficient (Moeser and Vidal 2005). Although adults are not generally considered as economically important as larvae, injury from adult feeding can significantly affect maize seed production at high population densities (Culy et al. 1992; Capinera et al. 1986). Longevity of adults is typically between 45 and 57 days and females lay on average 289–356 eggs (Ball 1957). Female beetles often mate shortly after emergence, but do not typically begin laying eggs until they are approximately 20–23 days old (Short and Hill 1972).

4.3 Management of Western Corn Rootworm

Major options for the management of western corn rootworm involve insecticides, crop rotation, or planting of transgenic Bt maize.

4.3.1 Insecticides

Since its emergence as a major pest of maize in the mid-1900s, insecticides have played a large role in the management of western corn rootworm (Metcalf 2005). The application of persistent, broad-spectrum soil insecticides at planting has traditionally been used to protect maize roots from larval feeding injury in continuous maize production systems (Levine and Oloumi-Sadeghi 1991). Alternative strategies involving the use of insecticidal seed treatments (Gray et al. 2006; Cox et al. 2007) and application of foliar insecticides to suppress emerging adults and reduce egg laying (Pruess et al. 1974) have also been used with variable success. However,

the widespread adoption and overuse of several primary insecticides has led to the rapid spread of resistance in western corn rootworm populations (Ball and Weekman 1963; Ball 1968; Meinke et al. 1998; Wright et al. 2000; Parimi et al. 2006).

4.3.2 Crop Rotation

Crop rotation has been used as an effective alternative to insecticides throughout much of the United States and is the primary pest management option practiced throughout Europe (Kiss et al. 2005). Because the western corn rootworm has a close association with maize, planting a substitute nonhost crop in maize fields in alternating years can disrupt the life cycle of this pest species and decrease its presence (Shaw et al. 1978; Levine and Oloumi-Sadeghi 1991). In the United States, maize is typically rotated with soybean, *Glycine max* (L.), to control corn rootworm species and provide yield advantages. However, in certain regions of the Midwestern United States, a rotation-resistant variant of the western corn rootworm has developed where females lay eggs outside of maize fields and in soybeans (Levine et al. 2002; Gray et al. 2009). It is thought that this new strain of western corn rootworm is not necessarily attracted to soybeans, but instead lays eggs in a more general fashion outside of maize (Gray et al. 1998). Therefore if maize is planted in these areas the following year, or other alternate host plants are present, opportunities exist for larval establishment and survival to the adult stage.

4.3.3 Transgenic Bt Maize

Transgenic maize that express *Bacillus thuringiensis* Berliner (Bt) proteins toxic to western corn rootworm have been developed by several seed companies as an additional management tactic to limit damage caused by this pest (Moellenbeck et al. 2001; Ellis et al. 2002; Schnepf et al. 2005; Vaughn et al. 2005; Walters et al. 2008). Various Bt strains naturally produce insecticidal crystal (Cry) proteins known as δ-endotoxins, which are highly specific to certain insect species. When Cry proteins are ingested and solubilized in the midgut of susceptible insects, an active toxin is produced that binds to epithelial cells causing perforations, which results in cell lysis and ultimately death of the insect (Gill et al. 1992; Bravo et al. 2007). Genetic engineering has allowed the ability to incorporate novel genes encoding these insecticidal Cry proteins into specific locations in the plant genome (a process collectively termed a transformation event). These genetically modified, or transgenic, plants are then able to express Bt proteins within their tissue. Transgenic Bt maize can offer protection from aboveground lepidopteran (caterpillar) pests and/or belowground corn rootworm species. Bt maize that offer protection against one of the above pest complexes are termed single-trait hybrids, while those that target both pest complexes (in addition to providing herbicide tolerance) are termed

stacked hybrids. Pyramided Bt maize hybrids contain genes expressing more than one Cry protein that targets the same pest complex.

In 1995, the United States Environmental Protection Agency (USEPA) approved the first registration of transgenic Bt maize for the management of lepidopteran pests. In the years following, the USEPA registered several other Bt maize events for lepidopteran pests and in 2003 approved the first registration of transgenic Bt maize for the management of western corn rootworm. The first rootworm-active Bt maize hybrids contained event MON863 (developed by Monsanto under the trade name YieldGard® RW) and expressed the Cry3Bb1 protein. Additional Bt maize hybrids expressing genes for different Cry proteins were subsequently commercialized for control of western corn rootworm. The binary Cry34/35Ab1 proteins expressed in maize event DAS59122-7 (co-developed by Dow AgroSciences and Pioneer Hi-Bred under the trade name Herculex® RW) was registered in 2005, and the mCry3A protein expressed in maize event MIR604 (developed by Syngenta under the trade name Agrisure® RW) was registered in 2007. Several stacked and pyramided maize hybrids expressing one or more of these proteins have since been developed. More recently, a new Bt protein, eCry3.1Ab expressed in maize event 5307, was registered as a pyramid with mCry3A in 2013 (developed by Syngenta under the trade name Agrisure Duracade™). No additional Bt proteins targeting western corn rootworm have been registered, although the Cry3Bb1 protein began transitioning from event MON863 to event MON88017 after its registration in 2005.

Adoption of transgenic Bt maize has increased dramatically in the United States since its commercialization. However, the ability of western corn rootworm to evolve resistance to various control measures has made the long-term viability of this technology uncertain. Under laboratory and greenhouse conditions, selected western corn rootworm populations have developed resistance to all four commercially available Bt proteins: Cry3Bb1 (Meihls et al. 2008; Oswald et al. 2011), Cry34/35Ab1 (Lefko et al. 2008), mCry3A (Meihls et al. 2011), and eCry3.1ab (Frank et al. 2013). In addition, field resistance to Cry3Bb1 and mCry3A has been documented in specific fields in the Midwestern United States (Gassmann et al. 2011, 2014).

To delay resistance development to transgenic Bt maize, attention must be focused on optimizing current resistance management plans for this pest.

4.4 Insect Resistance Management in Bt Maize

In the United States, the USEPA has required that all companies registering transgenic Bt crops submit an insect resistance management (IRM) plan prior to registration. The main goal of these IRM plans is to delay the evolution of resistance to this technology. IRM plans for transgenic Bt maize have involved the use of the "high-dose refuge strategy," which involves planting Bt crops that produce a high concentration of toxin (a dose 25 times the amount needed to kill 99 % of susceptible

insects) combined with the planting of a non-Bt refuge. Essentially, non-Bt refuge plants promote survival of homozygous susceptible insects, which are available to mate with rare homozygous resistant insects that emerge from Bt plants. The high dose of toxin thus ensures that the hybrid progeny (i.e., heterozygous for resistance) do not survive, making resistance functionally recessive (Tabashnik et al. 2004).

Currently, the USEPA requires a 20 % structured refuge or 10 % blended refuge for single-trait maize hybrids targeting western corn rootworm. Structured refuges can be planted as blocks or strips within or adjacent to the 80 % Bt maize, whereas blended refuges involve seed mixtures that intersperse a 10 % blend of non-Bt seed with 90 % Bt seed in a bag sold to growers. These blended seed mixtures are called "refuge in a bag" and have been developed to help growers better comply with refuge requirements as well as allow for more efficient mixing of beetles emerging from Bt and refuge plants within maize fields. Unlike single-trait maize hybrids, pyramided hybrids only require a 5 % refuge, which can be structured or blended. Reduced refuge size for pyramided maize hybrids is predicated on simulation models that have shown that two independently acting toxins that kill the same pest can be more effective at delaying the evolution of resistance than each toxin by itself (Zhao et al. 2003; Onstad and Meinke 2010; Ives et al. 2011), though mortality of susceptible insects should be "nearly 100 %" for this to occur (Roush 1998).

Several assumptions must be met if the high-dose refuge strategy for IRM is to be successful including the following: frequency of resistant alleles is rare, mating is random between resistant and susceptible insects, and the dose of Bt toxin in plants is high enough to kill nearly all heterozygous progeny (Gould 1998; Tabashnik et al. 2004; Carrière et al. 2010). In the case of western corn rootworm, many of these assumptions are not necessarily valid. Currently, all individual Bt proteins targeting the western corn rootworm are not considered high dose as defined by the USEPA for single-trait events (Storer et al. 2006; Hibbard et al. 2010a, b; Clark et al. 2012). In addition, nonsynchronous emergence of beetles from refuge fields and Bt fields has regularly been documented (Storer et al. 2006; Murphy et al. 2010; Hibbard et al. 2011; Clark et al. 2012), which may contribute to assortative mating among Bt-resistant and susceptible insects. Furthermore, the frequency of resistant alleles is likely much higher than generally assumed (Tabashnik and Gould 2012). Current debate concerning IRM plans for western corn rootworm has centered on the appropriate size and placement of refuges needed to reduce the selection pressure of transgenic Bt maize (Murphy et al. 2010; Pan et al. 2011; Tabashnik and Gould 2012; Onstad et al. 2014). Consequently, additional research is needed to determine optimal refuge configurations for sustainable control of this pest.

4.5 Conclusion

The economic gains and environmental benefits associated with using transgenic Bt maize to manage western corn rootworm as well as its ease of incorporation have made this technology attractive to many growers. Although none of the registered

individual Bt proteins targeted toward the western corn rootworm express a concentration of Cry proteins considered high dose, root protection from larval feeding due to Bt maize is frequently comparable or better than is possible with insecticides. Nevertheless, the durability of this technology can only be ensured with effective IRM plans. Continued research on western corn rootworm biology and IRM strategies is needed to determine an acceptable long-term approach to mitigate resistance development. In addition, Integrated Pest Management (IPM) can further delay the evolution of resistance and must also be incorporated into future IRM plans. IPM practices such as scouting, crop rotation, rotation of Bt maize hybrids that produce different Cry proteins, and judicious use of insecticides are all highly compatible with the goals of IRM for western corn rootworm. Although transgenic Bt maize is a valuable tool in insect control, it is important to remember that it is not the panacea of pest management.

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Chapter 5
Bacillus spp.: A Potential Plant Growth Stimulator and Biocontrol Agent Under Hostile Environmental Conditions

Dilfuza Egamberdieva

Abstract The genus *Bacillus* is one of the ecologically significant groups of bacteria found in diverse environments. The *Bacillus* species have been shown to improve plant growth and nutrient uptake and decrease the incidence of plant disease. They also enhance resistance of plants to adverse environmental stresses such as drought, salt, heavy metals, and nutrient deficiency. Synergistic interaction of *Bacillus*, with other microbes in the plant root, has been demonstrated to promote plant growth, mineral nutrition, and stress tolerance. This chapter discusses recent developments on the potential of *Bacillus* species as plant growth-promoting rhizobacteria (PGPR) and biocontrol agent for better plant growth, nutrient uptake, and enhanced plant tolerance to environmental stresses. Mechanisms involved in *Bacillus* eliciting plant growth promotion and abiotic stress tolerance have been also discussed.

Keywords Plant nutrients • Biological control • Salinity • Drought • Heavy metals

5.1 Introduction

The world population will exceed nine billion by 2050 and it is estimated that food production needs to increase by 70 % (Charles et al. 2010). However, food security in many countries is under threat from climate change such as extreme temperature, drought, and salinity (Othman et al. 2006). For example, soil salinization affects almost 1 billion hectares worldwide (Munns and Tester 2008), and due to drought and desertification each year, 12 million ha is lost. As a result nearly one billion people worldwide are affected by severe hunger and poverty (Ondrasek et al. 2009). Another important consequence of climate change (especially temperature) and abiotic stresses increased infection/infestation from pathogens and pests (Korus et al.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_5

2015) that are along responsible for the loss of at least 10 % of global food production (Triky-Dotan et al. 2005; Egamberdieva et al. 2016). Limiting crop losses due to salinity, drought, and diseases is a major area of concern to cope with the background of increasing food requirements (Parvaiz and Satyawati 2008; Shanker and Venkateswarlu 2011). Abiotic stresses such as salinity, drought, and heavy metals are considered to be the main factors affecting plant growth and yield (Mantri et al. 2012; Egamberdieva et al. 2014). Some of the impacts of these stresses on plant growth include disturbance of the hormonal balance, alteration of protein metabolism, inhibition of the activity of enzymes involved in nucleic acid metabolism, the loss of control on nutrient uptake, and susceptibility to diseases (Arbona et al. 2005; Egamberdieva et al. 2011). Increased salt levels in the soil solution or irrigation waters cause osmotic and ionic stress in plants resulting in adverse negative impact on plant growth by hampering the several important physiological and biochemical processes (Ahmad et al. 2015; Hashem et al. 2015a). This may in turn alter the availability of nutrients for plant growth and effect on microbial associations living within the plant vicinity.

The sustainable use of natural resources is one of the approaches in preventing global climate change and improves food security. The improving soil quality and managing its fertility by novel technologies are of fundamental importance for agricultural production and environmental management. The environment-friendly agriculture such as biofertilizers and biopesticides has become the most important modern technology for many countries in the world to achieve food security (Shoresh et al. 2010; Adesemoye and Egamberdieva 2013). Biofertilizers are carrier-based preparations containing plant beneficial microorganisms, which hold enormous prospects in improved and sustainable plant production. Plants are colonized by microbes, including endophytes, nitrogen-fixing bacteria, and mycorrhizal fungi, that closely cooperate with each other and can mediate important physiological processes, especially nutrient acquisition and plant fitness to abiotic stresses (Egamberdieva et al. 2013, 2015; Berg et al. 2013; Ahanger et al. 2014). Among them plant growth-promoting rhizobacteria (PGPR) play an important role in crop production as they (i) stimulate root growth, (ii) make soil nutrients available to the plant root, (iii) fix nitrogen from air and improve soil fertility, (iv) suppress soilborne pathogens and protect plants from various diseases, and (v) enhance plant tolerance to various environmental stresses including drought, salinity, high temperature, and heavy metals (Adesemoye and Kloepper 2009; Egamberdiyeva and Höflich 2003, 2004). They may also effect positively on physiological parameters of plants and increase the photosynthetic pigments, total free amino acids, and proteins compared to uninoculated control plants under saline condition (Han and Lee 2005; Berg et al. 2013; Hashem et al. 2015b).

Frequent root-associated plant beneficial bacteria include species such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Klebsiella*, and *Rhizobium*, as they improved plant growth and nutrient uptake and also protected various plants from plant pathogens (Sharma and Sharma 2008; Cho et al. 2015). Among those species *Bacillus* are considered as an ecologically significant group of bacteria, which are also well adapted to the arid and salt-affected environment

(Egamberdiyeva 2005; Malfanova et al. 2011). They form endospores and thus can tolerate high temperature, pH, and osmotic conditions (Ashwini and Srividya 2014). This enables these bacteria to be effective as plant growth stimulator and biocontrol agent in many environmental conditions including saline, drought, heavy metal contamination, etc. The used mechanisms in improvement of plant growth include production of growth-stimulating phytohormones, osmoprotectants, siderophores and antibiotics, solubilization and mobilization of phosphate, inhibition of plant ethylene synthesis, antibiosis against soilborne fungal plant pathogens, and competition for nutrient and niches (Sessitsch et al. 2012; Prashanth and Mathivanan 2010). Notably, ecological factors may affect the different mechanisms and limit the interactions between plant and beneficial bacteria, resulting in less than acceptable performance in plant growth promotion and management of diseases (Adesemoye and Egamberdieva 2013). This chapter discusses diversity of *Bacillus* species associated with plant and their potential role in improving plant growth and nutrient uptake and reducing plant diseases under various stressed conditions.

5.2 Overview of Plant-Associated *Bacillus* Species

Members of the genus *Bacillus* are gram-positive, endospore-forming bacteria and include thermophilic, halophilic, acidophilic, and alkalophilic bacteria that can withstand extreme climatic conditions. They inhabit diverse ecological niches such as soil, plant, rock, aquatic environments, and food, and some species are pathogens of humans and livestock (Delbrassinne and Mahillon 2016). Cihan et al. (2012) isolated thermophilic and mesophilic endospore-forming bacilli from the geothermal regions of Turkey and identified as *Bacillus pumilus*, *B. licheniformis*, *B. subtilis*, and *B. smithii*. However, extreme conditions cause a disturbance of plant-microbe interactions, where spore-forming bacteria of the genus *Bacillus* were among the dominant microorganisms (Egamberdiyeva 2007; Vardharajula et al. 2011). It suggests a remarkable degree of physiological and genetic adaptability of *Bacillus* species to harsh environmental conditions where long periods of drought and nutrient deprivation are common (Wipat and Harwood 1999). A diverse species of Bacillus were isolated from the saline soil of Uzbekistan and identified as *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. laevolacticus*, *B. latvianus*, *B. licheniformis*, *B. megaterium*, *B. polymyxa*, and *B. subtilis* (Egamberdiyeva 2007; Egamberdiyeva and Islam 2008). Several other *Bacillus* species were isolated from various stressed environments, e.g., *Bacillus luteolus* from a salt field in Korea (Shi et al. 2011), *B. deserti* from desert soil sample collected from the desert of Xinjiang Province in northwest China (Zhang et al. 2011), and *B. korlensis*, a moderately halotolerant bacterium isolated from a sand soil sample in China (Zhang et al. 2009). *B. cereus* 80 that is associated with wheat grown in saline soil was able to grow in higher levels of salinity ranges between 3 % and 5 % NaCl (Egamberdieva and Kucharova 2009). According to Welsh (2000), majority of the salt-tolerant bacteria synthesize specific compatible organic osmolytes, such as glutamine, proline, and glycine

betaine, and also accumulate inorganic solutes, such as Na+, K+, and Mg2+, to a higher intracellular concentration over time for maintaining cell turgid pressure. Al-Ajilani and Hasnain (2010) isolated 118 *Bacillus* strains including *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, and *B. licheniformis* from the soil in Pakistan, and 54 of the strains demonstrated antagonistic activities against pathogenic microorganisms. Feng et al. (2015) identified *B. asahii* from the alkaline soil which played a key role in the increases of both crop yield and soil fertility, especially via accelerating carbon and phosphorus cycling.

The interaction of *Bacillus* species with plants grown under various ecological conditions is addressed by several authors (Egamberdiyeva 2005; Choudhary and Johri 2008; Yadav et al. 2011; Malfanova et al. 2011). Beneduzi et al. (2008a) isolated 311 putative nitrogen-fixing bacilli from seven distinct wheat production zones of Rio Grande do Sul, Brazil, and found that *Paenibacillus* species were the most frequently identified species, followed by *Bacillus* sp. In another study Madhaiyan et al. (2010) isolated plant growth-promoting bacteria from the rhizosphere of rice grown in field site and identified as *B. amyloliquefaciens*, *B. subtilis*, *B. vallismortis*, *B. atrophaeus*, *B. mojavensis*, *B. licheniformis*, and *B. methylotrophicus*. *Bacillus kyonggiensis* were isolated from soil of a lettuce field in Gyeonggi Province, South Korea (Dong and Lee 2011), and *B. megaterium*, *B. cereus*, *B. subtilis*, *B. licheniformis*, and *B. azotoformans* from the rhizosphere of rice fields in central China (Xie et al. 2003). Reva et al. (2002) isolated *Bacillus* species from the inner tissues of cotton plants (*Gossypium* sp.) and identified as *B. amyloliquefaciens*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis*. Malfanova et al. (2011) isolated and identified *B. subtilis* HC8 from the giant hogweed *Heracleum sosnowskyi* Manden. *B. xiaoxiensis* were isolated from forest soil (Chen et al. 2011), *B. amyloliquefaciens* from the rhizosphere of potato (Calvo et al. 2010), and *B. subtilis* and *B. cereus* from groundnut rhizosphere (Maheswar and Sathiyavani 2012).

El-Deeb et al. (2013) studied endophytes of *Plectranthus tenuiflorus*, a medicinal plant in Saudi Arabia desert, and found strains belonging to *Bacillus* sp., *B. megaterium*, *B. pumilus*, *B. licheniformis*, *Paenibacillus* sp., and *Acinetobacter calcoaceticus*. Rhoden et al. (2015) observed occurrence of endophytic *Bacillus* species associated with *Trichilia elegans* A. Juss. Yadav et al. (2011) studied the diversity of *Bacillus* species from the rice rhizosphere growing in acidic soil and identified as *B. megaterium*, *B. humi*, *B. drentensis*, *B. pocheonensis*, *B. aestuarii*, *B. arbutinivorans*, and *B. niacini*. The strains showed plant growth-promoting abilities and different metabolic capabilities. In recent studies Vardharajula et al. (2011) isolated drought-tolerant *Bacillus* species associated with various plants such as finger millet (*Pennisetum glaucum* L.), sunflower (*Helianthus annuus* L.), and maize (*Zea mays* L.) grown in India and identified as *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, and *B. subtilis*. *Paenibacillus* and *Bacillus* genera were also the most prominent groups in the rhizosphere of rice grown in South Brazil and exhibited a wide range of plant growth-promoting traits including IAA, siderophore production, and solubilization of phosphates (Beneduzi et al. 2008b). Liang et al. (2014) isolated Cd-tolerant bacteria associated with the roots of

Orychophragmus violaceus L. Schultz, identified as *B. subtilis*, *B. cereus*, *B. megaterium*, and *Pseudomonas aeruginosa*. Adesemoye et al. (2015) carried out a year-round collection and screening of autochthonous bacteria from wheat roots in semiarid climate where frostbite/damages sometimes occur and identified strains of *B. safensis*, *B. megaterium*, and *B. pumilus*.

5.3 Plant Growth Promotion Under Stress

Plant-associated bacteria, colonizing rhizosphere or internal tissues of their host plants, have the potential of promoting the growth, stress tolerance, and nutrient uptake of plants and protecting plants from soilborne pathogens (Sessitsch et al. 2012; Malfanova et al. 2011; Egamberdieva 2008a, b). They mutually cooperate with each other through synthesizing biologically active compounds and providing the nutrients for the survival and proliferation of partner organism (Arora et al. 2008; Egamberdieva et al. 2013). In previous studies the mutualistic relationship between *B. subtilis* and leguminous plant *Robinia pseudoacacia* L. was reported by Huang et al. (2011). In this study *B. subtilis* colonized plant root similar as rhizobia through infected root hairs and formed bacteroides inside plant cortical cells. Such inoculants contribute to the development of sustainable agriculture under stressed conditions (Dodd and Perez-Alfocea 2012; Berg et al. 2013; Egamberdieva and Lugtenberg 2014). A positive effect on plant growth and stimulation of stress tolerance by various *Bacillus* species under hostile environments has been extensively reviewed by Arora et al. (2012). The *Bacillus* species were improved plant growth, nutrient uptake of agricultural important crop plants, and also phytochemical constituents of medicinal and aromatic plants (Egamberdiyeva 2008b; Mahalakshmi and Reetha 2009; Mishra et al. 2010; Teixeira da Silva and Egamberdieva 2013). In *Ocimum basilicum* grown under water stress conditions, inoculation of PGPR *Bacillus lentus* showed increased chlorophyll synthesis as well as the photosynthetic electron transport and also mitigated the negative impact of water stress (Heidari et al. 2011). The various *Bacillus* inoculants can stimulate plant growth and enhance a plant's resistance to adverse environmental stresses, e.g., drought, salinity, and metal toxicity. An overview of the plant growth-promoting effects of *Bacillus* species is presented in Table 5.1.

5.3.1 Salinity

Soil salinity affects the establishment, growth, and development of crops and can result in severe yield reduction (Al-Mutawa 2003). The inoculation of wheat seeds with *B. amyloliquefaciens* BcA12 and *B. laevolacticus* BcL28 isolated from saline soil resulted in increased root and shoot growth (15–50%) in nutrient-poor salt-affected soil conditions (Egamberdiyeva and Hoflich 2003b). In another study *B.*

Table 5.1 Summary of plant growth promoting properties of *Bacillus* species

Bacterial strain	Plant	Stress	References
B. amiloliguefaciens	Wheat	Salinity	Egamberdiyeva and Höflich (2003)
B. laevolactivus			
B. polymyxa	Pea	Salinity	Egamberdiyeva and Höflich (2004)
B. megaterium			
B. lentus	Basil	Salinity	Golpayegani and Tilebeni (2011)
B. pumilus	Rice	Salinity	Masood et al. (2016)
B. subtilis	Cotton	Salinity	Egamberdieva and Jabborova (2013)
B. subtilis	Indian bassia	Salinity	Hashem et al. (2015a)
B. subtilis	Radish	Salinity	Mohamed and Gomaa (2012)
B. cereus	Rice	Drought	Gusain et al. (2014)
B. megaterium	Clover	Drought	Marulanda et al. (2009)
Bacillus sp.	Lettuce	Drought	Vivas et al. (2003)
B. amyloliquefaciens *B. licheniformis*	Maize	Drought	Vardharajula et al. (2011)
Bacillus sp.	Maize	Drought	Kavamura et al. (2013)
B. pumilus	Quailbush	Heavy metal	de-Bashan et al. (2010)
B. edaphicus	Indian mustard	Heavy metal	Sheng et al. (2008)
B. subtilis	Indian mustard	Heavy metal	Zaidi et al. (2006)
B. megaterium	Indian mustard	Heavy metal	Rajkumar and Freitas (2008)
B. pumilus	Rice	Heavy metal	Masood et al. (2016)
Bacillus spp.	Chickpea	Heavy metal	Wani and Khan (2010)
*Bacillus*sp.	Rapeseed	Heavy metal	Zhang et al. (2011)
B. mucilaginosus	Indian mustard	Heavy metal	Wu et al. (2006)
B. megaterium			
B. subtilis, B. cereus, *B. megaterium*	Chinese violet cress	Heavy metal	Liang et al. (2014)

polymyxa BcP26 and *B. megaterium* BcM33 demonstrated increased root and shoot growth of pea (18 %) and maize (27 %) and also N and P uptake (55 %) under arid saline soil condition (Egamberdiyeva and Höflich 2004). These results were somewhat similar to those obtained by Golpayegani and Tilebeni (2011) in which salinity decreased plant growth, photosynthesis, and chlorophyll content of basil (*Ocimum basilicum*), whereas *B. lentus* alleviated the effects of salinity on plant growth. Bharti et al. (2013) observed that salt-tolerant *B. pumilus* and *Exiguobacterium oxidotolerans* stimulated plant growth and bacoside-A content of brahmi (*Bacopa monnieri*). *B. pumilus* stimulated plant growth and stress tolerance of rice under salt stress through enhanced activity of certain of antioxidative enzymes and lowering Na^+ accumulation in leaves (Masood et al. 2016). The inoculation of cotton with bacteria *B. subtilis* SUBTIN significantly increased the shoot dry weights from 14 % to 24 % as compared to the control plant. The increase in plant biomass translated into significantly higher N, P, and K contents. The untreated plants showed very poor growth in nutrient-deficient soil, where high pH makes nutrients less

available to plants. *B. subtilis* increased plant height, biological yield, and yield of cotton in small plot experiments (Egamberdieva and Jabborova 2013). Hashem et al. (2015a) observed a negative effect of salt stress on shoots, root growth, and physiological parameters of Indian bassia (*Bassia indica*). Inoculation of salt-stressed Indian bassia with *B. subtilis* significantly improved root, shoot growth, total lipid contents, and phospholipid fractions and increased oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids and chlorophyll a and b and carotenoid contents in plant leaves compared to uninoculated plants. Plant growth-promoting *B. megaterium* and *B. mucilaginous* strains improved nutritional assimilation of plant total NPK (Wu et al. 2005). The radish seeds inoculated with *B. subtilis* and *P. fluorescens* caused significantly an increase in fresh and dry masses of roots and leaves, photosynthetic pigments, proline, total free amino acids, crude protein, and N, P, K+, Ca_2+, and Mg_2+ uptake compared to uninoculated control plants under saline condition (Mohamed and Gomaa 2012).

The survival of PGPR species under adverse soil conditions largely depends on their capacity to compete with the better adapted native microflora in this highly competitive environment (Rekha et al. 2007). The rif-resistant mutant of *B. amyloliquefaciens* BcA12 was able to colonize the rhizosphere of wheat and cotton grown under saline arid soil condition due to their persistence and adaptation to harsh environment (Egamberdieva and Jabborova 2013).

Synergistic interaction of various bacterial species, e.g., *Bacillus*, *Pseudomonas*, *Azospirillum*, *and Rhizobium*, in the plant root has been demonstrated to promote plant growth and development (Cassan et al. 2009; Egamberdieva et al. 2013). In addition, a positive effect of dual inoculation with AMF and PGPR in plant growth and stress tolerance has been found (Ruiz-Sanchez et al. 2011; Almethyeb et al. 2013). Han and Lee (2005) observed alleviation of induced stress in plants and improvement in root and shoot growth of soybean by dual inoculation with *B. subtilis* and *B. japonicum*.

5.3.2 Drought

Drought reduces the growth and yields for crop plants by more than 50 % through affecting morphological, physiological, and metabolic changes (oxidative stress) (Mundree et al. 2002; Wang et al. 2003). Plants have a defense system to protect against oxidative injury that includes enzymes such as superoxide dismutase, peroxidase, glutathione reductase, and catalase (Agarwal and Pandey 2004). Gusain et al. (2014) studied plant growth-promoting *Bacillus cereus* for their role in enhancing plant growth and induction of stress-related enzymes in rice. They found that plant inoculated with *B. cereus* showed enhanced growth parameters and higher activity of superoxide dismutase, catalase, and peroxidase as compared to uninoculated plants under drought stress. Marulanda et al. (2009) found that the strain *B. megaterium* isolated from degraded soil of Southern Spain produce higher level of proline and indol acetic acid in vitro under osmotic stress conditions. The strain

also stimulated plant growth and development of clover (Marulanda et al. 2008). Similar observation reported by Vivas et al. (2003) showed that *Bacillus* sp. increased root, shoot growth, and nutrient uptake of lettuce (*Lactuca sativa*) under drought stress conditions compared to the control plants.

Vardharajula et al. (2011) reported that inoculation of maize with drought-tolerant strains of *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, and *B. subtilis* showed significantly higher root (33–5.3 %) and shoot length (32.2–42.5 %) and dry biomass (46.6–59.06 %) as compared to uninoculated control under drought stress. The strains could grow at minimal water potential (−0.73 Mpa) and are able to produce IAA and gibberellic acid (GA) (1.5–16.2 μg ml^{-1}) and solubilize phosphates (10–42 μg ml^{-1}). In addition they produce exopolysaccharide, total free amino acids, proline, and total soluble sugar under stress condition (Vardharajula et al. 2011). Increase in amino acids is considered to be an indication of drought tolerance (Greenway and Munns 1980). According Timmusk et al. (2003) osmolytes protect cells against fluctuations in osmotic conditions and enhance the stability of proteins and membrane under water-limiting environment. An earlier study (Potts 1994) reported that accumulation of compatible solutes by bacteria prevents degenerative processes and improves cell growth under drought conditions. Kavamura et al. (2013) observed increased plant growth and yield of maize (*Zea mays* L.) under water stress (30 % of field capacity) by two *Bacillus* spp. Sharma et al. (2013) observed increased germination, root and shoot length, fresh weight, and proline content of chickpea seedlings by *Bacillus* sp. under osmotic potential of up to 0.4 MPa over uninoculated control.

The negative effect of stress conditions such as drought and salinity on microbial functions have been reported by several authors (Liu et al. 2006). Vivas et al. (2003) observed reduced root colonization of lettuce by AM fungus under drought stress. Similar results reported by Al-Karaki et al. (2001) where mycorrhizal colonization was higher in well-watered plants colonized with AM fungi isolates than water-stressed plants. The combined inoculation of lettuce with fungus and *Bacillus* sp. caused a significant stimulatory effect on AM fungi *G. intraradices* development by enhancing the mycelium growth (Vivas et al. 2003). The AMF-*Bacillus* interaction stimulated plant growth and alleviated drought stress through reducing oxidative damage of lipids and increased the mycorrhizal development (Armada et al. 2015).

5.3.3 Heavy Metals

Contamination of soil with heavy metals such as arsenic, nickel, cadmium, copper, zinc, lead, and mercury is one of the world's major environmental problems, leading to significant consequences on ecosystems, agricultural productivity, and human health (Sheng et al. 2008). The phytoremediation technology has shown promise as an effective low-cost and economically sustainable approach to detoxify metals and organic chemicals through plant species capable of hyperaccumulating target ionic species in their shoots (Qadir and Oster 2004). Unlikely, environmental stress

condition inhibits growth and development of plants; thus, it may reduce efficiency of phytoremediation (Burd et al. 2000). In such condition plant-associated microorganisms can mitigate plant stress factors through secretion of acids, enzymes, proteins, and other chemicals and help plant to grow in heavy metal-contaminated soils (Zaidi et al. 2006; Dell'Amico et al. 2008). The interaction of plants with *Bacillus* species is considered as an efficient bioremediation process for the degradation of contaminants, potential metal bioaccumulation, and/or promoting plant growth (Liang et al. 2014). de-Bashan et al. (2010) reported that *B. pumilus* stimulated plant growth of a native plant Sonoran Desert shrub quailbush (*Atriplex lentiformis*) in high-metal-content mine tailings. Sheng et al. (2008) studied heavy metal-resistant *B. edaphicus* and their plant growth-promoting and lead (Pb) uptake properties for Indian mustard (*Brassica juncea*) in soil contaminated with 400–800 mg kg-1 soil. They found that bacterial strain increased Pb uptake of plant (from 18 % to 46 %), root dry matter (from 16 % to 22 %), and shoot growth (from 24 % to 30 %) in Pb-amended soil. In another study Zaidi et al. (2006) reported on the higher accumulation of Ni in stem, root, and leaf (736, 460, and 272 mg kg^{-1}, respectively) of Indian mustard (*Brassica juncea*) in the presence of bioinoculant *B. subtilis* strain SJ-101. Wu et al. (2006) studied soil bacteria which are resistant to metal (Zn, Cu, and Pb) and found that P solubilizer (*B. megaterium* HKP-1) and K solubilizer (*B. mucilaginosus* HKK-1) have much higher resistance to metal toxicity due to the endospore formation. *Bacillus maroccanus* ChrA21 and *Bacillus cereus* ES04 have been found to tolerate chromium (Cr) (Viti and Giovannetti 2006). In another study Rajkumar and Freitas (2008) observed that Ni-resistant strain *B. megaterium* Bm4C promoted growth of Indian mustard (*Brassica juncea*) and accumulation of Ni in root and shoot system and protected the plant from Ni toxicity. In addition the use of PGPB in phytostabilization of mine tailings is reported by several studies (Zhuang et al. 2007). Cd-tolerant bacteria *Bacillus subtilis*, *B. cereus*, and *B. megaterium*, associated with the roots of *Orychophragmus violaceus* L. Schultz, significantly enhanced the accumulation of Cd by plants. Cd concentration in the roots was significantly higher following bacterial treatments (from 52.40 to 84.79 mg kg^{-1}) than in uninoculated control plant roots (32.6 mg kg^{-1}). The addition of *B. megaterium* resulted in maximal water-extractable Cd, with a 50 % increase in its concentration compared with the uninoculated control (100 mg Cd kg^{-1} soil) (Liang et al. 2014). Masood et al. (2016) observed an increased boron concentration in rice (*Oryza sativa* L.) under combined stresses of salinity and high boron. Application of *Bacillus pumilus* enhanced the plant growth and decreased the Na^+ accumulation in rice leaves due to enhanced activity of certain antioxidative enzymes. In chromium-contaminated soil, the growth and development of chickpea drastically reduced, and *Bacillus* species PSB10 significantly improved growth, nodulation, chlorophyll, leghemoglobin, and seed yield of chickpea and reduced chromium uptake in plant tissue grown in the presence of chromium compared to the plants grown in the absence of bacterial strain (Wani and Khan 2010). Those reports suggested that the *Bacillus* species in soil-root interface has significance to assist plant establishment on heavy metal-contaminated soils through mediating nutrient mineralization and

uptake by plants. They stimulate root, shoot growth, and plant biomass and thus enhance phytoextraction of metals from contaminated soils.

5.4 Biological Control

The previous reports indicated that salt stress increases susceptibility of plants toward various phytopathogens (Triky-Dotan et al. 2005; Egamberdieva et al. 2011). For example, shoot and root colonization by *Macrophomina phaseolina* significantly increased by increasing salinity levels up to 1400 mg of NaCl kg^{-1} soil (Goudarzi et al. 2011). Various *Bacillus* species were able to control plant disease caused by fungal pathogens by producing antibiotic metabolites, suppressing plant pathogens, and competing for nutrients and niches (Egamberdieva et al. 2011). *Bacillus* species showed antagonistic activities against soilborne pathogens *Aspergillus flavus* (Moyne et al. 2001), *Penicillium* spp., *F. oxysporum*, and *Cercospora* spp. (Karuppiah and Rajaram 2011) and are widely used as biological control agent in various economically important crops to combat fungal diseases (Arrebola et al. 2010). Araujo et al. (2012) found an antagonistic activity of some *Bacillus* species isolated from Brazil to *Fusarium oxysporum* and *Colletotrichum truncatum*. For example, *B. subtilis* has shown promising results against several pathogens causing important crop diseases such as chir pine rot caused by *Macrophomina phaseolina* (Singh et al. 2008), powdery mildew of cucurbits caused by *Podosphaera fusca* (Romero et al. (2007), tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Baysal et al. 2008), root rot of cauliflower caused by *Pythium ultimum* (Abdelzaher 2003), and avocado root rot disease caused by *Rosellinia necatrix* (Cazorla et al. 2007). Several other *Bacillus* species showed disease control of tomato *Fusarium* wilt in vivo studies, e.g., *B. cereus* (18.75 % and 81.2 %), *B. amyloliquefaciens* (25 % and 75 %), *B. pumilus* (37.5 % and 62.5 %), and *B. subtilis* (37.5 % and 62.5 %) compared to the control (100 % and 0 %) (Ajilogba et al. 2013). *Fusarium* wilt of banana caused by *F. oxysporum cubense* was decreased after treatment of *B. pumilus* ENF24 (Figueiredo et al. 2010). Zhang et al. (2012) observed that co-inoculation of cotton with *Bacillus vallismortis* HJ-5 and *Glomus versiforme* (AM) decreased disease symptoms caused by *V. dahliae*. The plants inoculated with *Bacillus* sp. culture showed 65 % reduction in disease incidence as compared to the seed treated with pathogen alone (77.5 %). In another study *Bacillus amyloliquefaciens* inhibits the growth of *Fusarium oxysporum* and *Ralstonia solanacearum* and effectively inhibits *R. solanacearum* in the rhizosphere soil of eggplant (Chen et al. 2014). The seed treatment of sunflower seeds with *B. licheniformis* induced high reduction in percentage of infection of *R. solani* damping-off (from 60 % to 25 %) as compared with the pathogen alone (Kamil et al. 2007). *B. subtilis* (SUBTIN) was able to control cotton root rot caused by *Fusarium oxysporum* under saline soil condition (Fig. 5.1).

Salt-tolerant *B. amyloliquefaciens* BcA12 significantly reduced the damping-off of cotton caused by *R. solani* (Egamberdieva and Jabborova 2013). In other studies

Fig. 5.1 Biological control of cotton root rot caused by *Fusarium oxysporum* by *B. subtilis* SUBTIN (Plants were grown in soil infested with pathogenic *F. oxysporum under*)

Safiyazov et al. (1995) observed that *B. subtilis* 23 and *B. megaterium* 26 were able to control cotton diseases caused by *Xanthomonas malvacearum*, *Rhizoctonia solani*, *Fusarium vasinfectum*, and *Verticillium dahliae* under saline soil conditions. Kumar et al. (2014) isolated and screened soil bacterial isolates for high temperature (50 °C), salinity (7 % NaCl), and drought (−1.2 MPa) and observed that *Bacillus* strains were more stress tolerant than other microorganisms. *Bacillus* strains possessed promising antagonistic activity against *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *ricini*, and *Botrytis ricini*. These reports imply that stress-tolerant *Bacillus* species could be a useful approach for protecting plants from various soilborne pathogens under abiotic stress conditions. Bharathi et al. (2004) reported that combined inoculation of *P. fluorescens* (Pf-1) with *B. subtilis* reduced fruit rot infection in chilies caused by *Colletotrichum capsici* by 51 % over control. The strain was also able to increase shoot length (56.14 %), number of flowers (270.59 %), and fruits (133.3 %) compared to uninoculated control plants.

5.5 Mechanisms of Biocontrol and Plant Growth-Promoting Activity

The *Bacillus* species use several mechanisms for plant growth promotion and increased plant stress tolerance such as synthesis of phytohormones like indole acetic acid (IAA), gibberellic acid, and cytokinins, production of

1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the roots of developing plants, solubilization of minerals such as phosphorus and potassium, and production of exopolysaccharides (EPS) (Berg et al. 2013; Glick 2014; Hashem et al. 2015b). Auxins and cytokinins are believed to be involved in mediating environmental stresses, the signaling from root to shoots (Egamberdieva et al. 2013). The depressive effect of abiotic stresses on plant growth was explained by the decline in auxin content synthesized at endogenous levels within the cells of plant tissue (Dunleavy and Ladley 1995; Egamberdieva 2009). The application of additional natural phytohormones synthesized by *Bacillus* species may supply sufficient hormones for normal plant development and growth under water stress conditions. López-Bucio et al. (2007) observed increased lateral root number, lateral root growth, and root hair length *A. thaliana* by *B. megaterium* mediated by phytohormones. According to Ortíz-Castro et al. (2008), cytokinin receptors play an important role in plant growth stimulation by *B. megaterium*. *B. subtilis* significantly increased the root and shoot growth as well as cytokinin concentrations in lettuce plants through cytokinin-producing ability (Arkhipova et al. 2005), whereas gibberellin-producing *B. licheniformis* and *B. pumilus* increased growth and development of *Pinus pinea* plants (Probanza et al. 2002).

Colonizing rhizosphere, bacterial strains may directly affect phytoremediation process by the production of phytohormones such as auxins, gibberellins, cytokinins, that enhance the root growth and development of metal accumulating plants (Zaidi et al. 2006). The *B. subtilis* strain SJ-101 has stimulated plant growth of Indian mustard plant (*Brassica juncea*) in Ni-contaminated soil and exhibited the capability of producing indole acetic acid (IAA) (55 μg ml^{-1}) and solubilizing inorganic phosphate (90 μg ml^{-1}) in specific culture media (Zaidi et al. 2006).

Bacillus species may improve solubilization of fixed soil P and K and help improve their uptake by plants (Chen et al. 2006). *Bacillus edaphicus* NBT strain increased K content of cotton and rape plants by 30 % when the soil was treated with insoluble K sources (Sheng 2005). It has been suggested that the plant growth promotion was related to K solubilization by the strain *B. edaphicus*. The strain was able to colonize the rhizosphere of cotton and also improved N and P uptake of plants. In another study *B. megaterium* and *B. mucilaginous* improved N, P, and K assimilation of maize (Wu et al. 2005). *B. amyloliquefaciens* isolated from potato is found to solubilize tricalcium phosphate (Calvo et al. 2010; Almoneafy et al. 2012) and zinc (Ajilogba et al. 2013). Akgül and Mirik (2008) reported on the phosphate solubilization abilities of *B. thuringiensis*, *B. sphaericus*, and *B. megaterium*.

Biological control of plant disease by *Bacillus* species involves several mechanisms such as production of antifungal metabolites (bacillomycin, iturin, fengycin), cell wall-degrading enzymes (amylases, proteases, cellulase, pectinase, glucanase), and host resistance induction (Arrebola et al. 2010; Berg et al. 2013). The strain *B. subtilis* HC8 significantly promoted plant growth and protected tomato against tomato foot and root rot. In addition the strain produced gibberellin and (lipo)peptide antibiotics and possessed strong antifungal activity in vitro against *Aspergillus niger*, *F. oxysporum*, *Fusarium solani*, and *P. ultimum* (Malfanova et al. 2011). *Bacillus amyloliquefaciens* (W19) which produce antifungal lipopeptides (iturin

and bacillomycin D) were found to decrease the incidence of *Fusarium* wilt and promote the growth of banana plants (Wang et al. 2013). This strain also produces phytases, HCN, and cellulases (Ajilogba et al. 2013). Bais et al. (2004) indicated that surfactin produced by *B. subtilis* which forms extensive biofilm through root colonization plays an important role in protecting plants from pathogens. For example, antibiotic (iturin A)-producing *Bacillus* sp. CY22 strain was able to suppress root rot of balloon flower caused by *Rhizoctonia solani* (Cho et al. 2003). Ashwini and Srividya (2014) isolated *B. subtilis* from the rhizosphere of chili which showed high antagonistic activity against *Colletotrichum gloeosporioides* OGC1 and produced glucanase and cellulase enzymes. Karimi et al. (2012) reported increased plant growth and biological control of *F. oxysporum f.* sp. *ciceris* in chickpea by *B. subtilis* which produce IAA, HCN, and antifungal volatiles. Srividya et al. (2012) isolated *B. subtilis* JN032305 from chili rhizosphere and found that strain produced mucolytic enzymes—chitinase, glucanase, and cellulase—and showed broad spectrum antagonism against potent bacterial and fungal phytopathogens. In another study siderophore-producing *B. subtilis* inhibited the growth of *R. solanacearum* (Almoneafy et al. 2012). The increase in chitinase, b-1, 3 glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and phenol accumulation in plants treated with *Bacillus pumilus* which control fruit rot infection in chilies was reported by Bharathi et al. (2004). Salt-tolerant PGPR *Bacillus* strains assist plants by additional supply of plant growth-regulating hormones; induce salt stress resistance; produce antifungal metabolites, siderophores, and fungal cell wall-degrading enzymes; and indicate bacterial capacity to improve plant growth and protect plants from various fungal diseases under stress conditions.

5.6 Conclusion

From the above discussion, it is evident that *Bacillus* species stimulate plant growth and nutrient uptake, protect plants from various soilborne pathogens, and prevent the deleterious effects of stresses from the environment such as drought, salt, nutrient deficiency, heavy metals, etc. Co-inoculation techniques using *Bacillus* inoculants could be a new approach to increase the salt tolerance and the yield of agriculturally important crop and medicinal plants used for food and phytomedicine. *Bacillus* species help plant to acquire sufficient phytohormone and soluble phosphate and produce siderophores that help plants obtain sufficient iron for the optimal plant growth. Most of *Bacillus* species produce one or more secondary metabolites with antibiotic activities which are highly effective in suppression of plant pathogens. They may reduce the toxicity of the metal or increase its bioavailability and thus have some potential to improve phytoextraction efficiency in metal-contaminated soils. It is important to explore traits involved in the ability of *Bacillus* species to mitigate abiotic stress in plants and compete with the indigenous microflora for establishment into the rhizosphere and disease suppression under stress condition at plant tissue, cell, or molecular level. The effectiveness of *Bacillus*

inoculants depends upon factors like plant type, soil type, and other ecological factors; thus efforts should be directed toward identifying effective bacterial inoculants adapted to ecological conditions. In general, *Bacillus* inoculants demonstrated their potential for protecting plants against abiotic stresses and helping plants to thrive in hostile environments.

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Chapter 6
Bacillus spp.: A Promising Biocontrol Agent of Root, Foliar, and Postharvest Diseases of Plants

Mahfuz Rahman

Abstract Biological control of plant disease is gaining momentum as it offers an alternative and supplement to synthetic chemicals. Microorganisms from diverse groups have successfully been used as biocontrol agents (BCA) due to their capacity of suppressing harmful microbes with a wide array of mechanisms such as competition, antibiosis, and resistance induction in the host plant. *Bacillus* spp. is among the highly potent bacterial BCAs used for controlling principally rhizosphere and to a lesser extent foliar diseases of plants. The capacity of *Bacilli* to produce spores which are extremely resistant to high temperatures, unfavorable pH, and lack of nutrients or water are determining factors for using these organisms in a formulation. These spores are produced by the bacteria when environmental conditions are unfavorable to help these microorganisms to survive in the phytosphere and ward off the growth of harmful microbes. *Bacillus subtilis* strain QST 713 that has been used in the commercially available plant disease control product "Serenade" showed excellent disease suppression in diverse environmental conditions and crop varieties. This product along with many other similar products is now considered as an essential component of any integrated disease management effort due to their compatibility with many chemicals used for disease control. Many other strains of *Bacillus subtilis* and species of *Bacillus* have also been used for seed treatment, induction of systemic resistance, and suppression of both root and foliar disease-causing organisms. Major agrochemical companies have shown interests and diligently work in incorporating *Bacillus*-based products in their portfolio. Significant efforts have been made to unravel the genetic makeup of these beneficial *Bacilli* that encode a wide range of antimicrobial products. An appreciable number of polypeptides, polyketides, and related products have been identified and characterized by which they achieve competitive edge in the plant rhizosphere or form biofilm on root surface. Continued interest and research on this BCA in twenty-first century will make *Bacillus*-based formulations most widely used plant disease management tool.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_6

6.1 Introduction

The increasing demand for food supply for an ever-increasing population led to synthetic pesticide-based control of the major pests and plant diseases. Excessive use of agrochemicals not only caused serious environmental and health concerns (Gilden et al. 2010) but also created many resistant pest populations that could make the whole production system unsustainable. That is why sustainable crop production largely depends on sustainable pest management where integrated plant disease management is an important component and usually achieved by the use of disease-resistant hybrids, biological disease control, reduced use of chemicals based on disease prediction, and use of cultural practices that reduces the incidence and severity of diseases. Plant disease suppression by various microbes has been explored for decades due to its promise toward attaining sustainable disease management over a long period of time by maintaining microbial diversity and balance in an ecological niche. Moreover, organic crop production system warrants pest management methods that excludes synthetic pesticides and mostly relies on biological pesticides. Microbes selected for this purpose concurrently acquired multiple traits that provided superior fitness to them to thrive in an adverse environment and to outcompete harmful ones through a myriad of biological interactions. Augmenting the population of a preexisting beneficial microbial species in an environment has the highest probability to maintain its number high enough to be suppressive to harmful microbes. In most cases, introduction of single or mixtures of biocontrol agents that are not native to that microenvironment fails to sustain its population high enough for being effective. In the effort of biological control with introduced microbes, some controlled only one of several important diseases of a particular crop; others provided only partial control of the disease or simply failed to survive long enough to have any significant effect (Weller 1988). Among various microbes possessing the traits to suppress others, members of *Bacilli* such as *B. subtilis* has more potential to thrive in a less conducive environment due to the fact that they are ubiquitous naturally occurring saprophytes that is commonly recovered from soil, water, air, and decomposing plant material. In addition, they are capable of producing endospore (Piggot and Hilbert 2004) to overcome and survive through adverse environmental conditions such as ultraviolet radiation, desiccation, high temperature, extreme freezing, chemical disinfectants, and scarce of nutrients. Endospores enable *Bacilli* to lie dormant for extended periods; revival of spores millions of years old has been reported (Cano and Borucki 1995). Although several well-known species of *Bacilli* have historically been classified as an obligate aerobe, further studies on this aspect proved that in complex conditions and in the presence of nitrates, they can also grow under anaerobic conditions which add to their survivability in a wide array of unfavorable habitats (Nakano and Zuber 1998). When the environment becomes favorable, the endospore can reactivate itself to the vegetative state. Scientists were able to manipulate the use of these organisms in various formulations for extended shelf life and suitable method of transportation. Different species and strains of *Bacillus* also evolved and acquired the traits of surviving different

ecological niche providing the advantages of using different strains under different situations as biological control agents. Pathogens are typically affected by certain modes of actions and not by others according to their nature (i.e., biotrophs vs. necrotrophs). Due to possessing multiple modes of actions, *Bacillus* spp. can be used against diverse pathogen groups. For example, there are two general categories of *B. subtilis* strains: those that are applied to the foliage of a plant and those applied to the soil or transplant mix when seeding. *Bacillus* spp.-based BCAs show superior efficacy as they possess all possible biological traits to suppress pathogenic microbes and concurrently enhance plant's ability to tolerate biotic stresses. *Bacillus* BCAs compete very well with pathogenic microbes for nutrients and space if applied preventatively, antagonize plant pathogens (Ongena and Jacques 2008) through a whole host of genetic arsenal they possess, and induce host plant resistance by promoting growth and nutrient acquisition capabilities. Metabolization of phosphorous and nitrogen containing complex nutrient source into more bioavailable forms is one of the examples of multiple beneficial roles these microbes play toward plant health. As *Bacillus* can withstand thermophylic phase of composting, it has been used to enrich the effect of the compost and as a carrier of beneficial microbes during biological soil amendments. Once established in the phytosphere, they can produce bioactive compounds for a prolonged period of time obviating the need for multiple applications throughout the crop cycle. Competitive inhibition and production of natural antibiotics were documented as a dominant mode of action by these BCAs in suppressing pathogenic microbes (Chen et al. 2006). However, it is likely that several modes of action concomitantly participate in pathogen suppression although the relative importance of each one of them is not clear. Multiple modes of action are specifically required for phylloplane pathogens and may be achieved by combining *Bacillus* spp. with similar BCAs. For example, effective prevention of infection in the phyllosphere was obtained with mix application of *Bacillus mycoides* and *Pichia guilliermondii* and bacterium and yeast biocontrol agents, respectively (Guetsky et al. 2001). The extraordinary survivality in the rhizosphere, spore-forming ability, and versatility of *Bacilli* makes them highly effective as biopesticides (Losick and Kolter 2008) and have been used in multiple biocontrol products and the list is growing by days. Moreover, *Bacillus* has been used as both biofertilizer and biocontrol agents due to its multiple beneficial roles in plant production and protection (Borriss 2011). In addition with the efficacy, successful commercialization of potential BCA strains depends on formulation that preserves shelf life, aids product delivery, and enhances bioactivity. Due to the production of a resistant life stage (endospore) by *Bacillus* spp. strains, formulation is more readily achieved compared with other non-spore-forming BCAs. Formulation types that were widely used for *Bacillus* spp. included wettable powder (WP) concentrate, dry flake, aqueous suspension, and flowables. While these formulations were found efficient in preserving viability and delivery of beneficial microbes, special attention will be needed on target coverage, on target adhesion, and in enhancing biomass survival and efficacy after delivery to the target. Specifically, products for foliar applications should be fortified with UV protectants such as oxybenzone and light blockers such as lignin (Schisler et al. 2004). This chapter includes comprehensive

up-to-date information on the discovery and utilization of *Bacillus*-based biocontrol agents for managing foliar, rhizosphere, and postharvest diseases of economically important crops together with their role in integrated disease management.

6.2 Biological Basis of Disease Suppression by *Bacillus*

A biocontrol agent may operate through activation of more than one mode of action like competition, antibiosis, and induction of resistance against harmful microbes in the host. Exploiting multiple modes of action may increase efficacy of the biocontrol agent if the effects are additive.

6.2.1 Competition Through Colonization of the Rhizosphere and the Rhizoplane

In order to be effective, biocontrol agents (BCA) must have the competitive edge over pathogenic and indigenous microflora in colonizing, proliferating, and sustaining the population in the rhizosphere and rhizoplane (Whipps 1997a) of the growing root for a considerable period of time. It is an indirect interaction between the BCA and the pathogen where both groups of microbes compete for food and space from a limited amount of surface area of host and considered as one of the major modes of action of BCA to prevent pathogen from infecting plants. As some pathogens are negatively affected by lack of nutrients in the infection court, thus competition for nutrients and space was long recognized as antagonism trait (Elad and Chet 1987) as well. Prior colonization of rhizosphere by the antagonists (Lievens et al. 1989) such as *Bacillus* spp. deprive naturally occurring microorganisms especially pathogens from nutrients and essential growth factors including specific stimulus to establish on the host. Root exudates evidently have only a limited capacity to provide space and required nutrients to a certain population size and a certain species of microbe (Handelsmann and Stabb 1996). Microbes in the rhizosphere and the rhizoplane live on decaying root hair epidermal cells by utilizing metabolites excreted by the roots (Curl and Truelove 1986). A significant amount (~20%) of energy produced in the leaves of a plant may be lost via its roots as exudates (Martin 1971; Lynch 1983) in the form of carbohydrate (Griffin et al. 1976). A remarkable plant–microbe interaction can occur in border cells in the root above the root cap (Hawes et al. 1998) due to the availability of the energy source. Due to the presence of known chemical attractants such as organic acids, amino acids, and specific sugars in root exudates (Welbaum et al. 2004), *Bacillus* spp. can reach root surfaces by a combination of chemotactic responses followed by flagellar motility (Rao et al. 2008). While root exudates in most cases attract microbes, some exudates can be antimicrobial, providing ecological niche advantage to organisms that are capable of detoxifying it (Bais et al. 2004). One such detoxification module consisting of a

two-component system and an ABC transporter was reported in *Bacillus subtilis* by Staron et al. (2011). Upon the detection of a toxic compound, the two-component system of *B. subtilis* induces the expression of the ABC transporter, which in turn removes the antibiotic from its site of action in a cell.

Zimmer et al. (1998b) reported differential colonization of pea root parts by *Bacillus subtilis* with root tips preferentially colonized being the most physiologically active that released the greatest amounts of root exudates. In addition with competitive edge for root colonization and proliferation, an ideal biocontrol agent should also possess a more efficient uptake or utilizing system of nutrients than the pathogens (Parke 1991; Handelsman and Parke 1989; Harman and Nelson 1994; Nelson 1990). Thus, competition occurs to decrease the availability of a particular substance to limit the growth of the pathogen. For example, iron competition in alkaline soils may be a limiting factor for microbial growth (Leong and Expert 1989). In such a special situation, *Bacillus* spp. can out-compete other microbes by producing siderophores to acquire iron. Yu et al. (2011) reported that *Bacillus subtilis* CAS15 produced siderophores when applied for the protection of pepper plant from Fusarium wilt similar to the observation made by Leeman et al. (1996) in case of *Pseudomonas fluorescens*. Siderophore production by *Bacillus subtilis* BS 1–10 (Sivasakthi et al. 2013) and *Bacillus cereus* (Wilson et al. 2006) was also reported as means of antagonism against pathogenic microbes.

Generally, seeds are more vulnerable to infection of *Pythium* spp. during the first 6–12 h of seed germination indicating the need for the presence of *Bacillus* spp. on seeds prior to germination to facilitate root colonization as it grows. A study with tomato seeds treated with *Bacillus subtilis* FZB24® and subsequently cultivated on Murashige and Skoog medium indicated that *Bacillus subtilis* colonized the root from the treated seed and closely followed its growth in the rhizosphere region, so that a 0.4–0.8 mm thick film of bacteria was formed around the root. This study also confirmed the colonization of the roots with the aid of scanning electron microscopy (Asaka and Shoda 1996). Although colonization of root and rhizosphere by BCA can be influenced by a number of environmental factors, such as plant species, soil type, and application technique, the highest and most durable colonization rates of *Bacillus subtilis* in the rhizosphere were attained in artificial substrates or if the substrate had been sterilized before the application of the bacteria (Krebs et al. 1998; Grosch et al. 1996; Zimmer et al. 1998a). However, a clear relation between the intensity of colonization and the effects on plant health and plant productivity could have not always been demonstrated (Bull et al. 1991; Handelsmann and Stabb 1996; Tutzun and Kloepper 1994).

6.2.2 Antibiosis (Formation of Antibiotic Metabolites)

Antibiosis was reported to be involved and plays an important role in the suppression of phytopathogens by *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus*. This

process involves a low-molecular-weight compound or an antibiotic produced by *Bacilli* that directly affect other microorganisms (Handelsman and Parke 1989; Weller 1988; Weller and Thomashow 1993; Vasudeva and Chakravarthi 1954). These compounds are very diverse in structures and sizes, peptide being the predominant class produced by *Bacilli*, and established methods are now available to quantify these products in the rhizosphere (Kinsella et al. 2010). They may entirely be composed of amino acids, but some may contain other residues to form cyclic, linear, and basic aminoglycoside antibiotics (Stein 2005). Two peptide antibiotics, gramicidin S and polymyxin B, produced by *B. brevis* and *B. polymyxa*, respectively, inhibited gray mold-causing fungus *Botrytis cinerea* both in vitro and in vivo (Haggag 2008). Cyclic lipopeptides (CLPs) form another major class of *Bacillus* antibiotics that may vary widely in the type and sequence of amino acid residues and branching of the fatty acid chain depending on the species and strain (Ongena and Jacques 2008). However, surfactins, iturins, and fengycins are considered three main families of CLPs produced by different *Bacillus* spp. A recent study (Waewethongrak et al. 2015) investigating green mold (c.a., *Penicillium digitatum*) control on mandarin by *Bacillus subtilis* ABS-S14 confirmed the production of all three CLPs in culture filtrate. Efficacy study of partially purified compounds indicated that iturin A and fengycin inhibited the growth of *P. digitatum*, but surfactins had no direct effect. Antagonism of *Bacillus subtilis* strain AG1 was tested against vine wood fungal pathogens *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, and also *Verticillium dahliae* and *Botryosphaeria rhodina*. The assay indicated that metabolites of AG1 inhibited mycelial growth of all the pathogens tested. Stability of this metabolite at high temperature (60–120 °C) and its resistance to enzymatic degradation indicated it a polypeptide antibiotic (Alfonzo et al. 2009). Gong et al. (2013), in a study to investigate inhibition of *Aspergillus flavus* by *Bacillus subtilis* strain fmbJ, identified a peptide antibiotic that was similar to bacillomycin D with identical amino acid sequence, Asn-Tyr-Asn-Pro-Glu-Ser-Thr. This antibiotic component destroyed cell wall and membrane of *A. flavus* hyphae and spore and completely inhibited its growth on corn at the concentration of 200–400 μg/g. Although various antibiotics such as the abovementioned ones together with subtilisin and xanthobaccin (Milner et al. 1996) can be produced by *Bacillus subtilis* and related species, bacilysin is considered taxonomically the most relevant for the genus *Bacilli* due to the consistency and frequency of its occurrence (Loeffler et al. 1990).

Genetic analyses revealed that ~5 % and 8 % of the genomes of two most widely used *Bacillus* spp., *B. subtilis* and *B. amyloliquefaciens*, respectively, are involved with synthesis of many structurally diverse antimicrobial compounds that may have a direct correlation with their fitness as BCAs (Stein 2005). Due to the involvement of large part of the genome, antibiotics produced by *Bacilli* can also be very diverse in their modes of action to control a wide range of microbes to provide them competitive advantage over their counterparts. For example, *B. licheniformis* produced bacitracin A that inhibited cell wall synthesis (Katz and Demain 1977), and *B. circulans* produced butirosin complex that altered function of ribosome in a diverse group of microbes (De Furia and Claridge 1976). *B. subtilis* commonly produces a

class of lipopeptide antibiotics including iturins. Iturins help *B. subtilis* bacteria outcompete other microorganisms by either killing them or reducing their growth rate (Hiraoka et al. 1992). Iturins can also have direct antifungal activity on pathogens through inhibition of spore germination and disrupting germ tube growth. In some cases, however, different *Bacillus* antibiotics produced by different species of the same genera showed same mode of action. *Bacillus colistinus*, *B. polymyxa*, and *B. circulans* produced colistins, polymyxins, and circulins, respectively, all of which were reported to disrupt membrane function (Priest 1993). *Bacillus subtilis* FZB24® also produces iturin-like lipopeptides in liquid cultures that show efficacy against various phytopathogenic fungi in the range of 5–100 μg/ml, mimicking the efficacy of other fungicidal agents (Krebs et al. 1996). Culture conditions and growth phase of the culture may strongly influence the quantity and composition of these secondary metabolites by *Bacilli* such as *Bacillus subtilis* (Loeffler et al. 1990; Krebs et al. 1996, 1998; Gupta and Utkede 1987). Large-scale production of these metabolites thus requires optimization of the growth conditions and culture media composition during the fermentation process. Developmental stage of bacteria also influences the production of such metabolites. Loeffler et al. (1990) found that the lipopeptides formed by *Bacillus subtilis* were released into the medium only at the time of endogenous spore formation during the stationary phase of the culture. Silo-Suh et al. (1994) identified two fungistatic antibiotics, zwittermicin A and kanosamine produced by *B. cereus* UW85, that contributed to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis*. In general, organisms that are known to produce multiple antibiotics can also suppress one or more pathogens (Pal and Gardener 2006). Production of multiple antibiotics also enhances the capacity of BCAs to differentially suppress diverse microbial competitors including plant pathogens.

A study conducted with 184 *Bacillus* spp. strains isolated for the presence of the antimicrobial peptide (AMP) biosynthetic genes *srfAA* (surfactin), *bacA* (bacilysin), *fend* (fengycin), *bmyB* (bacillomycin), *spaS* (subtilin), and *ituC* (iturin) revealed that most strains had between two and four AMP genes and a few with five genes but none had six genes. These genes may be linked to fitness of strains in natural environments and may assist in the selection of putative biological control agents of plant pathogens (Mora et al. 2011). However, investigations conducted in vitro to determine the efficacy of *Bacillus subtilis* FZB24® from the metabolite lipopeptides against fungi could have not been clearly ascertained due to the complexity of extracting/re-extracting peptides from the growing medium. In this comparative study, maize seedlings were planted in sterile quartz sand in small pots that were either drenched with 10^7 spores/ml of substrate or lipopeptides added directly to the substrate. At the final evaluation of the experiment, no lipopeptides could be detected in the *Bacillus subtilis* FZB24® treated substrates or roots. In addition, comparison of different isolates of *Bacillus subtilis* for metabolite production and disease control did not show any strong correlation including Fusarium wilt control in greenhouse experiments with ornamentals (Grosch et al. 1999) although Koumoutsi et al. (2004) in part attributed enhancement of plant growth by *Bacillus amyloliquefaciens* FZB42 to suppression of competitive plant pathogenic microflora within rhi-

zosphere environment by secreted antifungal and antibacterial lipopeptides and polyketides. Thus far, a broad spectrum of antifungal compounds produced by *Bacillus* spp. has been identified and described (Pusey 1990), and mode of action of the well-known antagonists including *B. subtilis* has been attributed to antibiosis (Nandi and Sen 1953; Swinburne et al. 1975; Gueldner et al. 1988). However, in nature, more than one type of interactions can occur among microbial populations, depending on microbial density, diversity, and prevailing environmental conditions (Atlas and Bartha 1987). In some cases, industrial strains were also found to produce multiple compounds to suppress pathogens and promote plant growth. The *B. subtilis* industrial strain Ch-13 produced lytic enzymes, cyanide, and other antifungal metabolites and stimulated plant growth by producing phytohormones such as auxin derivatives. This strain reduced *Fusarium oxysporum* infection on tomato plants by 2.5 times compared with non-treated check. Abundance of fungi on Ch-13 inoculated roots was 6.9 times lower compared with non-treated roots (Chebotar et al. 2009). More insights on the differences and similarities among industrial or type strains and plant-associated strains of *Bacillus* have started to emerge from genome sequences of important *Bacillus* spp. (Ruckert et al. 2011).

6.2.3 Plant Resistance Induction

All plants have coevolved defense mechanisms against pathogens to survive in the natural habitats. The efficacy of these resistance reactions is modified in course of time as a function of the ontogenetic development of the plants and the influence of biotic and abiotic stress factors. Thus, interaction with less aggressive pathogen or low level of infection leads to a decrease in the overall susceptibility or increase in the resistance of plants. This increased/induced resistance can be triggered by preinoculation with nonpathogens such as *Bacillus* spp. or their metabolites (Schönbeck et al. 1993). Induced resistance is superior over competition or antibiosis as the protection of plants usually extended beyond the period when bacterial population has already decreased and the response transduced from the point of initiation to remote organ of the plant. These defense signals turn on whole host of defense reactions to produce phytochemicals such as phytoalexins, pathogenesis-related (PR) proteins (chitinases, β-1,3-glucanases, proteinase inhibitors, etc.), and lignin for reinforcement of cell walls (Van Loon 2007). Resistance inducers generally help in controlling biotrophic fungal pathogens such as powdery and downy mildews or diseases caused by *Phytophthora* spp. Rhizobacteria-mediated induced resistance generally does not confer complete protection against pathogen, and thus pathogen is not under selective pressure for development of resistance. Among a few possible resistance induction elicitors, volatile compounds such as 2,3-butanediol (Ryu et al. 2004) and lipopeptides are commonly found in *Bacillus* spp. A study conducted by Hain et al. (1995) for changes in the gene expression after application of *B. subtilis* showed that the genes for herbicide resistance were expressed within 1–5 days with the activation of three related promoters (prp1, chit2a, Vst1) in different intensities.

These three promoters prp1, chit2a, and Vst1 were involved with pathogenesis gene in potatoes, chitinase gene in peanuts, and the stilbene synthase gene in grape vines, respectively. Both soil drench and leaf spray treatments resulted in the activation of the promoters with prp1 showing the highest intensity. These experiments provided direct evidence of the involvement of resistance-inducing mechanisms in the biological efficacy of *Bacillus subtilis* FZB24® through a quick trigger of signal that can be systemically translocated within the plant. However, no systemic colonization of the plants by BCAs was observed. In a separate experiment, Podile and Lami (1998) demonstrated a systemic increase in the phenylalanine ammonium lyase (PAL) activity in pigeon pea seedlings after treatment of the seeds with the *Bacillus subtilis* strain AF1. Similar to PAL activation, Kilian et al. (2000) showed the activation of other defense genes in plants after application of *Bacillus amyloliquefaciens* plant growth promoting rhizobacteria. Many investigators also documented that upon detection of a pathogen, a plant is able to recruit bacteria to colonize the root surface, triggering induced systemic resistance (ISR) in aerial portions of the plant (Ryu et al. 2004; Lakshmanan et al. 2012) and ultimately closure of plant stomata (Kumar et al. 2012). Specifically, Rudrappa et al. (2008) found that plants can recruit specific strain of *Bacillus subtilis* to their roots via secretion of malic acid when aerial tissues come into contact with *Pseudomonas syringae* pv. *tomato* DC3000. Treatment of various plant roots with FZB24® followed by inoculation with fungal pathogens on the leaves showed distinctly less attack by *Phytophthora infestans* and *Botrytis cinerea* on tomato plants at evaluation after 5 days. Late blight severity was reduced by 50 % in a similar laboratory test. A reduction of 20 % *Botrytis cinerea* was achieved only with higher concentration of bacteria in this study. In general, control of *B. cinerea* with induced resistance is regarded as highly difficult. An experiment conducted by Kilian et al. (2000) also showed 25 % reduction of powdery mildew severity on wheat that provided further evidence of induced resistance of plants due to treatment with *Bacillus* spp. Among numerous bacterial metabolites that are considered as triggers of induced resistance, evidence to date indicates that *Bacillus subtilis* forms mainly serine-specific endopeptidases (Kula 1982). In addition with induction of resistance against pathogens, application of spore suspension of *B. subtilis* in roots of eggplant and pepper at seedling stage also increased the tolerance to salinity and increased yield significantly compared with non-treated (Bochow et al. 2001). These kinds of effects have usually been considered as induced systemic resistance (ISR), and volatile organic compounds were shown to play a key role in this process (Ryu et al. 2004). Volatiles secreted by *B. subtilis* GB03 (Brannen and Kenney 1997) and *B. amyloliquefaciens* IN937 activated ISR pathway in *Arabidopsis* seedlings challenged with the soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (Ryu et al. 2004). Culture filtrates of *Bacillus subtilis* used as a resistance inducer against powdery mildew pathogen was found to induce changes in the *sink-and-source* relationships in barley. This study proved that the application of culture filtrates had led to increased movement of assimilates from the flag leaf into the ears contributing to higher starch content in grains (Kehlenbeck et al. 1994). Fravel (1999) reported that *Bacillus subtilis*-based

product Serenade could turn on plant's natural immune system and could be used for downy mildew control on vegetables.

6.3 Effect on Plant Growth and Yield

In addition with protecting plants from various diseases, *Bacillus* spp. has also been reported to promote plant growth. Aliye et al. (2008) reported 114.4 and 105.7 % potato plant growth by applying *B. subtilis* PFMRI and *B. pumilus* BC, respectively. Similar growth promotion was reported by Schwartz et al. (2013) from *B. simplex* 30N-5. A few other reported plant growth promoting *Bacillus* spp. includes but not limited to *B. amyloliquefaciens* (Idris et al. 2007), *B. pumilus* (Benhamou et al. 1996), *B. licheniformis* (Probanza et al. 2001), *B. cereus* (Handelsman and Parke 1989), and *B. megaterium* (López-Bucio et al. 2007). A larger and healthier root system that led to improved uptake of water and nutrients has been observed in a number of greenhouse and field experiments with B*acillus subtilis* FZB24®. Drenching of soil immediately after sowing of kohlrabi and again 4 weeks later with 0.2 g FZB24®WG/L water led to a 5 % increase in the dry root weight with concurrent increase in yield up to 12 % in a greenhouse experiment. Similar increase in root weight and tuber yield was obtained with FZB24® liquid seed treatment at 10 g FZB24® WG/100 kg of seed potatoes (Kilian et al. 2000). Although the biological basis of plant growth promotion by *Bacilli* is not well understood, plant growth hormones have been implicated with such growth promotion. Kloepper et al. (1991) suggested that rhizosphere colonizing biocontrol agents could produce phytohormones and phytohormonally active metabolites such as indole-3-acetic acid (IAA), cytokinin, and gibberellic acid that are known to promote plant growth. Upregulation of these hormone-producing genes or downregulation of ethylene production via *acdS* (1-aminocyclopropane-1-carboxylic acid deaminase) activity by *Bacillus* spp. was found involved with plant growth promotion (Arkhipova et al. 2005). However, these organisms can also influence plant growth by working synergistically with nitrogen-fixing or phosphorous-acquiring symbiotic microbes (Probanza et al. 2001; Vivas et al. 2003b; Guiñazú et al. 2010). Dolej (1998) showed that the growth-promoting effect of culture filtrates of *Bacillus subtilis* FZB24® was due to a factor independent of antibiotic lipopeptides. This observation was further supported by investigations with *B. subtilis* mutants that resulted in yield increase in treated peanut plants despite the fact that BCA no longer had the ability to form antibiotics (Backmann et al. 1994). The phytohormonal activity of the metabolites formed by *B. subtilis* FZB24® showed cytokinin-like enhanced growth of radish cotyledons and auxin-like increased elongation of the cells of wheat coleoptiles indicating a mix of several proteins. However, further separation and purification of the culture filtrates led to the degradation of protein and loss of the effects (Alemayehu 1998). Tang (1994) also reported a number of *B. subtilis* isolates with potential to form phytohormones such as zeatin, gibberellic acid, and abscisic acid. Steiner (1990) also found a range of phytohormones such as cytokinins, zeatin, and zeatin riboside

in culture filtrate of a *B. subtilis* isolate that was used as a resistance inducer against biotrophic fungal plant pathogen. The senescence-delaying effect and increased yield from plants in which resistance has been induced could be in part due to these phytohormones. Since the application of *B. subtilis* leads to stronger root growth, there may also be an increased synthesis of plant cytokinins because meristems of these plants are the most important sites for the synthesis of free cytokinins (Torrey 1976).

6.4 Biological Control of Specific Plant Diseases Using *Bacillus* spp.

6.4.1 Biological Control of Foliar Diseases

Leaf spot diseases of wheat (*Triticum aestivum* L.) are one of the most destructive diseases. No single measure can effectively control leaf-spotting pathogens, and a fully integrated system of disease management including biological control is more likely to achieve a long-term solution. A stepwise screening system to select BCAs including *Bacillus* spp. showed mycelial growth inhibition, coiling, vacuolation, granulation, and plasmolysis of hyphae of leaf-spotting pathogens. Although this study included a range of fungal pathogens as potential biocontrol agents, *Bacillus* and other spore-forming bacteria revealed the most interesting possibilities for biocontrol (Analia and Cecilia 2007). Baker et al. (1985) reported 75 % reduction in bean rust severity under field condition with three applications of *B. subtilis* per week compared with unsprayed control. In some of these tests, treatments with *B. subtilis* were more or equally effective than the weekly application of the broad spectrum fungicide mancozeb. Sugar beet Cercospora leaf spot control was attempted with the isolate recovered from phyllosphere and by optimizing BCA spore concentration, application timing compared with pathogen inoculation. Although disease control was not up to acceptable level or as good as chemicals, application rate 1×10^6 CFU/ml or above and 3 days before pathogen introduction provided significant disease suppression compared to non-treated control (Collins and Jacobsen 2003). *Bacillus subtilis* reisolated from the biological control agents FZB24® and Phytovit® showed promising results against several pathogens that cause important foliar tomato diseases (late blight, early blight, powdery mildew, and leaf mold) with higher activity when applied prior to pathogen infection. Metabolites from culture filtrate showed highest efficacy against *Phytophthora infestans* restricting its developmental structures and decreasing its biomass in leaf tissue by 83 % and resulted in more than 70 % reduction in late blight severity (Sultan 2012). In a recent study, plant growth-promoting rhizobacterial strain *B. subtilis* UD1022 has been isolated at the University of Delaware, USA, that restricted the stomata-mediated pathogen entry of *Pseudomonas syringae* DC3000 in *Arabidopsis thaliana* (Kumar et al. 2012). In addition to eliciting stomatal closure

in *Arabidopsis*, UD1022 also inhibited growth of plant pathogen *P. syringae* DC3000. Preliminary studies also demonstrated that UD1022 could induce stomatal closure in commercial crops including romaine lettuce and spinach (Markland et al. 2013).

6.4.2 Biological Control of Root Diseases

Backman et al. (1997) reported that 60–75 % of the seed used for the US cotton crop was treated with *B. subtilis* strain GB03 (Kodiak ®) for suppression of *Fusarium* and *Rhizoctonia* pathogens. Although in some studies, Kodiak® gave little or no visible control of root rot pests, in one it provided 22 % control of *Fusarium* root rot in beans, and in another, its use resulted in 81 % stand increase in chickpeas. Out of 60 isolates of *B. subtilis* screened by Chen et al. (2013) against tomato vascular wilt pathogen *Ralstonia solanacearum* under greenhouse conditions, six strains exhibited above 50 % biocontrol efficacy. These wild strains formed robust biofilms both in defined medium and on tomato plant roots. Further studies revealed that the strains possessed genes for biofilm and matrix formation that allowed investigators to establish a model system for exploring *B. subtilis*–tomato plant interactions (Chen et al. 2013). Similar results were obtained by Baysal et al. (2008) in controlling *Fusarium oxysporum* f.sp. *radicis-lycopersici* that causes crown and root rot of tomato with *B. subtilis* strain EU07. This strain showed higher disease reduction (75 %) compared with *B. subtilis* QST 713 (52 %) when applied as inoculants. Molecular analysis suggested that EU07 contained YrvN protein as subunit of protease enzyme that could be used as a molecular marker for selecting effective biocontrol agent. Application of the biofungicide Serenade, a product from *Bacillus subtilis* QST713, showed high efficacy in protecting canola root infection by soilborne pathogen *Plasmodiophora brassicae*. Two applications of the biofungicide completely suppressed infection (Lahlali et al. 2013). This study also showed that the product was more effective compared with individual component as product filtrate or bacterial cell suspension. Resistance induction in canola plant by the biofungicide also contributed to upregulation of the genes encoding jasmonic acid, ethylene, and phenylpropanoid pathways by 2.2- to 23-fold relative to non-treated plants. Damping-off of tomato (a soilborne seedling disease) caused by *Rhizoctonia solani* was effectively controlled by *B. subtilis* RB14 where iturin A was involved and played a significant role (Asaka and Shoda 1996). Brewer and Larkin (2005) tested 28 potential biocontrol organisms for efficacy against *Rhizoctonia solani* that causes stem canker and black scurf on potato in a series of greenhouse trials. Although none of the treatments, including a chemical control (azoxystrobin), effectively controlled stem canker and black scurf in all trials, *B. subtilis* GB03, *Rhizoctonia zeae* LRNE17E, *Stilbella aciculosa* 112-B, and the chemical control were most effective in reducing stem canker severity (40–49 % reduction) relative to the inoculated controls over all trials. In spite of inconsistency in reproducing the results, combination of biocontrol organisms *B. subtilis and T. virens* demonstrated

somewhat better control of stem canker than each organism alone, suggesting that *B. subtilis* may provide synergistic effect when mixed with other BCAs for improved biocontrol efficacy against soilborne diseases. Rahman and Jett (2016) evaluated the efficacy of *B. subtilis* (Serenade soil) in a field trial that also included other BCA, biofumigants, and grafted tomato to control vascular wilt caused by *Fusarium* and *Verticillium* spp. Application of BCAs were made as seed and planting mix treatment, and with transplant water. While all treatments significantly reduced disease severity, all but mustard cover crop also significantly increased fruit yield compared with non-treated control. Application methods that ensure pre-colonization of root systems appear to play a critical role in minimizing pathogen infection and disease severity.

6.4.3 Biological Control of Nematodes

Plant parasitic nematodes cause an annual crop loss roughly $8 billion in the USA (Koenning et al. 1999) and $100 billion worldwide (Sasser and Freckman 1987). Since many of the effective nematicidal products including methyl bromide (bromomethane), fenamiphos (**N**emacur), and aldicarb (Temik) are being phased out, investigators have devoted additional efforts to find suitable biological alternatives. Aerobic endospore-forming bacteria, mainly *Bacillus* spp. and *Pseudomonas* spp., are among the dominant populations in the rhizosphere that are antagonistic to nematodes (Tian et al. 2007). Among the bacterial antagonists, *Bacillus firmus* strain GB-126 showed broad spectrum nematicidal effect. This bacterium was originally isolated in Israel and currently formulated as a seed treatment under the name VOTiVO or as a wettable powder under the name Nortica 5 %WP. In a field environment, it significantly reduced *Meloidogyne incognita* in tomato roots with a single application (Terefe et al. 2009). Moreover, it reduced populations of *Radopholus similis*, *Ditylenchus dipsaci*, and *Heterodera glycines* under in vitro conditions (Mendoza and Sikora 2009; Schrimsher 2013). The mode of action as revealed by in vitro studies was inhibition of egg development and root infection of *M. incognita* by bioactive secondary compounds from *B. firmus* (Mendoza and Sikora 2009; Terefe et al. 2009). Air-dried and finely grounded *Bacillus penetrans* infected *Meloidogyne javanica* females in tomato root tissues to produce a powder heavily laden with spores of *B. penetrans* (Stirling 1984). Application of this material to field soil infested with root-knot nematode at 212–600 mg/Kg soil significantly reduced galling of tomato roots and the number of nematodes in the soil at harvest, and the result was similar to application of chemical nematicide (Stirling 1984). Yap (2013) isolated four different strains of rhizobacteria with nematicidal activity. Among those, nematotoxicities of *Bacillus* strains were intensively analyzed. *Bacillus* spp. strains MPB04 and MPB93 showed remarkable nematicidal activity with 76.4 and 50.6 % kill, respectively, of tested nematodes within 2 h and complete destruction of tested nematodes within 12 h. Results also showed that nematicidal activity of *Bacillus* strains was related to their proteolytic activity. The pot trial also

revealed that the application of *Bacillus* strain MPB04 and MPB93 reduced the root population of *M. incognita* by 60.95 and 35.28 %, respectively, compared with non-treated control (Yap 2013). BioNem® prepared from *Bacillus firmus* GB-126 was proven for its efficiency in greenhouse and field trials. The numbers of nematode females, eggs, and vermiform life stages at the end of the growing season decreased in the presence of the biocontrol agent. In this field trial, cotton yields were similar to those from the chemical nematicide standard aldicarb; however, the underlying reason for this effect remained unknown (Castillo et al. 2013). Twenty isolates of unidentified *Bacillus* species were obtained from pathogen-suppressive soils of pigeon pea (*Cajanus cajan*) fields for the biocontrol of a wilt disease complex caused by *Heterodera cajani*, *Meloidogyne incognita*, and *Fusarium udum*. Five isolates (B602, B603, B605, B615, and B618) were considered to have biocontrol potential on the basis of antifungal activity, inhibitory effect on the hatching and penetration of nematodes, and colonization of pigeon pea roots by these isolates (Siddiqui and Shakeel 2007). Culture by products of *Bacillus subtilis* strain OKB105 showed promising result in controlling *Meloidogyne javanica*. Culture filtrate from OKB105 resulted in 100 % mortality of *M. javanica* within 12 h of treatment (Xia et al. 2011). Major strains of *Bacillus* spp. that showed significant suppression of plant disease or causal agents are summarized in Table 6.1.

6.4.4 Postharvest Disease Control

It is often difficult to control many post harvest diseases by applying control agent at harvest as the infections generally take place well ahead of harvest. Pesticide residue on any food item can be highly undesirable; the situation can be worse if post-harvest fruit disease control is attempted with chemicals. There is an urgent need to find nonchemical means to control postharvest diseases. Promising results were obtained from research conducted with various commodities. Pear ring rot caused by *Botryosphaeria berengeriana* is a serious disease in most pear-growing areas in the world. Liu et al. (2011) determined the efficacy of multiple *Bacillus subtilis* strains and sodium bicarbonate separately or in combination for the control of ring rot in pear during storage. All treatments significantly reduced *B. berengeriana* growth in plate or on fruit preventatively and curatively, and combination treatment significantly enhanced ring rot control. Postharvest diseases caused by multiple organisms can cause serious losses to avocado. Korsten et al. (1997) found that *B. subtilis* (isolate B246), *B. cereus* (isolate B247 and B249), and *B. licheniformis* (isolate B248) were inhibitory to avocado postharvest pathogens *Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Drechslera setariae*, *Pestalotiopsis versicolor*, and *Fusarium solani* due to antibiosis. In a recent study, plant growth-promoting rhizobacterial strain *B. subtilis* UD1022 proved inhibitory to human pathogen *Listeria monocytogenes* in culture, specifically during log phase growth. Application of the isolate as a dip treatment for cantaloupe showed significant reduction of the level of *Listeria* on cantaloupe rind after 8 h of incubation at 37 °C.

Table 6.1 *Bacillus*-based biocontrol agents (strains and commercial products) that showed promising results in suppressing major fungal, bacterial, and nematode pathogens on different crops

Organism-strain/product	Disease/pathogen suppressed[a]	Crop	Reference
Bacillus subtilis AG1	Esca trunk disease (*Phaeoacremonium aleophilum*, *Phaeomoniella Chlamydospora*)[F]	Grapevine	Alfonzo et al. (2009)
Bacillus pumilus Strain QST 2808 (Ballad Plus)	Powdery mildew, rust, *Sclerotinia*, downy mildew, leaf spots[F]	Corn, cereal crops, oilseed crops	Aliye et al. (2008)
Bacillus amyloliquefaciens PPCB004	Postharvest decay (*Penicillium* spp.)[P]	Citrus	Arrebola et al. (2009); Waewthongrak et al. (2014)
Bacillus subtilis RB14	Damping off (*Rhizoctonia solani*)[S]	Tomato seedling	Asaka and Shoda (1996)
B. subtilis FZB24 (Rhizoplus)	*Rhizoctonia*, *Pythium*, *Fusarium*[S]	Garden vegetables	Asaka and Shoda (1996)
Bacillus subtilis	Anthracnose (*Colletotrichum gloeosporioides* OGC1)[S]	Chili	Ashwini and Srividya (2014)
Bacillus amyloliquefaciens FZB24 (Taegro ECO)	*Rhizoctonia*, *Fusarium*, *Sclerotinia*, *Pythium*, *Phytophthora*, leaf spots, powdery mildew[S]	Fruiting vegetables, cucurbits, leafy vegetables	Bochow et al. (2001), Borris (2011)
Bacillus subtilis PPL-3, APPL-1	Rust (*Uromyces phaseoli*)[F]	Bean	Baker et al. (1985)
Bacillus subtilis EU07	Fusarium wilt (*Fusarium oxysporum* f.sp. *radicis-lycopersici*)[R/S]	Tomato	Baysal et al. (2008)
Bacillus subtilis Cot1	Damping off (*Pythium* spp.)[R]	*Photinia*	Berger et al. (1996)
Bacillus subtilis GB03 (Kodiak)	Stem canker and black scurf (*Rhizoctonia solani*)[R]	Potato	Brewer and Larkin (2005)
Bacillus subtilis CPA-8	Brown rot (*Monilinia laxa*)[P]	Peach and nectarine	Casals et al. (2010)
Bacillus firmus strain GB-126 (Bionem)	Root knot (*Meloidogyne incognita*)[S]	Cotton	Castillo et al. (2013)
Bacillus subtilis BacB	*Cercospora* leaf spot (*Cercospora beticola*)[F]	Sugar beet	Collins and Jacobsen (2003)
Bacillus subtilis QST 713 (Serenade)	Downy mildew(*Peronospora*, *Pseudoperonospora*)[F]	Vegetables	Fravel (1999)

(continued)

Table 6.1 (continued)

Organism-strain/product	Disease/pathogen suppressed[a]	Crop	Reference
Bacillus subtilis strain fmbJ	*Aspergillus flavus*[F]	Corn	Gong et al. (2013)
Bacillus mycoides	Gray mold (*Botrytis cinerea*)[F]	Strawberry	Guetsky et al. (2001)
Bacillus amyloliquefaciens RC-2	Anthracnose (*Colletotrichum dematium*)[F]	Mulberry	Hiradate et al. (2002)
Bacillus subtilis strain GB34	*Rhizoctonia*, *Fusarium*[S]	Soybean	Junaid et al. (2013)
Bacillus subtilis GB03 (Kodiak, Companion)	Damping-off (*Rhizoctonia*)[S], *Aspergillus*	Wheat, barley, cotton, peas	Junaid et al. (2013)
Bacillus subtilis CCTCC M 207209	*Aspergillus carbonarius* (ochratoxin A-producing strain)[P]	Table grape	Jiang et al. (2014)
Bacillus subtilis Lu144	*Bacterial wilt* (*Ralstonia solanacearum*)[S]	Mulberry	Ji et al. (2008)
Bacillus amyloliquefaciens FZB42	*Fusarium oxysporum* (Wilt)	Tomato	Koumoutsi et al. (2004)
Bacillus subtilis B246	Black spot (*Pseudocercospora purpurea*)	Avocado	Korsten et al. (1997)
Bacillus subtilis MBI 600 (Integral)	*Rhizoctonia* and *Fusarium* seedling diseases, *Fusarium* wilt[S]	Legumes, corn, alfalfa, and forage	Kumar et al. (2012)
Bacillus subtilis QST 713 (Serenade)	Clubroot (*Plasmodiophora brassicae*)[S]	Canola	Lahlali et al. (2013)
Bacillus sp. BBG100	*Pythium aphanidermatum* (Damping off)[S]	Tomato	Leclere et al. (2005)
Bacillus sp. SB10	Blight (*Phytophthora capsici*)[S]	Pepper (*Capsicum annum*)	Lee et al. (2008)
Bacillus subtilis AU195	*Aspergillus flavus* (Aflatoxin contamination)[P]	Peanut	Moyne et al. (2001)
Bacillus subtilis NJ-18	Sharp eye spot (*Rhizoctonia cerealis*)+fungicide[I]	Wheat	Peng et al. (2014)
Bacillus licheniformis SB3086 (Ecoguard)+fungicide	Dollar spot (*Sclerotinia homoeocarpa*)[I]	Turf	Probanza et al. (2001)
Bacillus subtilis QRD137	Mummy berry (*Monilinia vaccinii-corymbosi*)[F]	Blueberry	Scherm et al. (2004)
Bacillus pumilus GB34 (Yield shield)	Root rot (*R. solani* and *Fusarium*)[S]	Soybean	Schisler et al. (2004)

Bacillus amyloliquefaciens GB99 + *B. subtilis* GB122 (Bio Yield)	*Rhizoctonia*, *Pythium*, *Fusarium*[S]	Bedding plants in potting mix	Schisler et al. (2004)
Bacillus subtilis MBI600 (Subtilex)	Fungi on cotton, large-seeded legumes, soybeans[I]	Cotton, large-seeded legumes, soybeans	Schisler et al. (2004)
B. subtilis QST 713 (Serenade)	Black sigatoka (*Mycosphaerella fijiensis*)[F]	Banana	Serrano et al. (2011)
B. pumilus QST 2808 (Sonata)	*Alternaria*, downy mildew, powdery mildew, rust black sigatoka[F]	Banana and many other crops	Serrano et al. (2011)
Bacillus cereus UW85	Damping-off (*Phytophthora medicaginis*)[S]	Alfalfa	Silo-Suh et al. (1994)
Bacillus penetrans	Root gall (*Meloidogyne javanica*)[S]	Tomato	Stirling (1984)
Bacillus cereus UW85	Damping off (*Pythium aphanidermatum*)[S]	Tomato	Smith et al. (1993)
Bacillus subtilis	*Botryodiplodia theobromae*, *Fusarium oxysporum*[P]	Yam	Swain et al. (2008)
Bacillus firmus strain GB-126 (VOTiVO, Nortica)	Root knot (*Meloidogyne incognita*)[S]	Tomato	Terefe et al. (2009)
Bacillus subtilis GA1	*Botrytis cinerea*[P]	Apple gray mold	Toure et al. (2004)
Bacillus subtilis QST 713 (Rhapsody, Serenade)	Phytophthora crown rot and other soilborne diseases[S]	Strawberries, turf, and ornamentals	Warkentin (2012) http://mbao.org/2012/12Warkentin.pdf
Bacillus subtilis QST 713 (Serenade soil)	Southern blight (*Sclerotium rolfsii*) *Phytophthora capsici*, *Pythium*, *Fusarium*[S]	Tomatoes	Warkentin (2012) http://mbao.org/2012/12Warkentin.pdf
		Squash	
Bacillus subtilis ABS-S14	Green mold (*Penicillium*, *Aspergillus*)[P]	Mandarin	Waewthongrak et al. (2015)
Bacillus subtilis NJ-18	Rice sheath blight (*Rhizoctonia solani*), *Stem rot* (*Sclerotinia sclerotiorum*)[F,S]	Rice	Yang et al. (2009)
		Rape/canola	
Bacillus subtilis CAS15	Wilt (*Fusarium oxysporum*)[S]	Pepper	Yu et al. (2011)
Bacillus subtilis SB01, SB04, SB23, SB24	Root rot (*Fusarium oxysporum*, *F. graminearum*)[R/S]	Soybean	Zhang et al. (2009)

F foliar, *R/S* rhizosphere/soil, *P* postharvest

[a]Major application methods

Bacillus subtilis isolated from cow dung by Swain et al. (2008) inhibited the growth of yam postharvest pathogen *F. oxysporum* and *Botryodiplodia 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 % compared with no bacterial inoculant. These isolates could inhibit the growth of pathogens up to 83 % in wound cavities of yam tubers in vivo. Leelasuphakul et al. (2008) screened 23 strains of *Bacillus* spp. from 205 soil isolates in vitro for antagonistic activities toward *Penicillium digitatum* that cause postharvest citrus fruit rot. Culture supernatants from nine strains caused >80 % inhibition of *P. digitatum* growth with a 1:32 serial dilution indicating the abundance of *Bacillus* antagonists in soil. Volatile compounds produced by these strains also caused 30–70 % inhibition of fungal growth. Preventative application of *B. subtilis* endospore suspension on wounded citrus fruit 24 h prior to *P. digitatum* spore inoculation decreased disease incidences by 86.7 %, and disease symptoms were delayed by 6 days and decay symptoms to day 9. Similar results were obtained by Jiang et al. (2014) in controlling *Aspergillus carbonarius* on table grapes with the exception of volatiles. *Bacillus subtilis* CCTCC M 207209 cell-free culture supernatant significantly inhibited the growth of *A. carbonarius* CCTCC AF 2011004 (an ochratoxin A-producing strain) on all three grape cultivars in varying degree. Wounded apple fruits were protected by *B. subtilis* strain GA1 against gray mold disease by producing CLP antibiotics including a wide variety of fengycins. In this study, the role of fengycins was demonstrated by the effective disease control with CLP-enriched extracts and by in situ detection of fengycins in inhibitory amounts (Touré et al. 2004). Mulberry anthracnose causing fungus *Colletotrichum dematium* was inhibited by *B. amyloliquefaciens* RC-2. Further analysis of the active product revealed that iturin played dominant role in the fungal growth inhibition (Hiradate et al. 2002). Brown rot of stone fruit caused by *Monilinia* spp. can cause severe losses during postharvest processing and handling. A combination treatment of hot water (HW) and antagonists including *B. subtilis* CPA 8 and sodium bicarbonate (SBC) was tested for controlling the disease, and the effect was compared with individual component or combination of components and non-treated check. A significant additive effect to control *Monilinia laxa* was detected with the combination of HW followed by antagonist CPA-8 when fruits were incubated for 5 days at 20 °C after treatment. Only 8 % of the fruits were infected that were treated with HW and CPA-8 combination, compared to 84 %, 52 %, or 24 % among the non-treated check, CPA-8, and HW treatments, respectively (Casals et al. 2010). Combining application of two biocontrol agents *Pichia guilliermondii* yeast and *Bacillus mycoides*, PGPR bacterium significantly and consistently suppressed *Botrytis cinerea* on strawberry leaves compared with separate application of each agent. When these agents were applied in combination at different temperatures and relative humidities, *Botrytis cinerea* was controlled effectively (80–99.8 %) under all conditions, and the coefficients of variation were as low as 0.4–9 % in all cases. Control efficacy from separate application ranged between 38 % and 98 % (mean 74 %) and the coefficient of variation ranged from 9.7 % to 75 %. The results indicated that mix of compatible biocontrol agents can improve efficacy and consistency of biocontrol of plant disease (Guetsky et al. 2001).

6.5 *Bacillus* spp.: A Component in Integrated Disease Management

Biopesticides work best if it can be incorporated in plant production and protection package to ensure optimum plant health and growth. An adequate combination among healthy seeds, biopesticides, chemical pesticides, plant fertilization, and good agricultural practices (GAP) will ensure highest-quality crop with minimum pesticide use (Chakraborty et al. 2010; Kumar et al. 2010). Compatibility of biopesticides with fertilizer or chemical pesticides needs to be tested rigorously before any such decision is made especially when the applications of the biological and chemical ingredients occur simultaneously, for example, in seed treatment or in combined foliar sprays. Besides mixed application with chemicals, a microbial strain can be used together with other strains or with natural extracts possessing pesticidal property (Akila et al. 2011; Kondoh et al. 2000; Korsten et al. 1997; Liu et al. 2011) that may be more effective and reliable. Compatible strains or products are selected to provide broad spectrum pathogen control by combining different modes of actions or organisms with different ecological competences and synergistic effects (Roberts et al. 2008). Biocontrol strains have also been combined with nonliving substrate like chitin which can induce plant resistance itself against infecting pathogen and may stimulate biocontrol activity (Ahmed et al. 2003). Due to the remarkable compatibility of *Bacillus* spp. with conventional plant protection products and other biologicals, different strains of *Bacillus* spp. have been incorporated in the integrated management of various foliar and root diseases of plants to achieve enhanced disease control. *B. subtilis* has been used in conjunction with *Streptomyces gramicifaciens* for control of root rot in cucumber, corky rot of tomato, and carnation wilt. *B. firmus* suppressed *Meloidogyne* spp. on cucumber plants from 60 days after planting until the end of the season (Giannakou et al. 2004). Reduction of nematodes was similar to the soil fumigant dazomet when application of *B. firmus* was combined with soil solarization (Giannakou et al. 2007). Mendoza and Sikora (2009) reported that single application of *B. firmus* formulation Bf-125 or Bf-106 significantly reduced the number of *Radopholus similis* in banana roots, but co-applications of these formulations with banana endophytic fungus *Fusarium oxysporum* strain 162 (FO162) and the egg pathogenic fungus *Paecilomyces lilacinus* strain 251 not only decreased nematode penetration into the plant but also improved the consistency of *R. similis* control when compared to single applications of any one of the three antagonists. The authors attributed this to the endophytic presence of FO162, which inhibited nematode penetration in addition to the continued attack of the nematodes in the soil by *B. firmus*. Waewethongrak et al. (2015) co-applied *B. subtilis* ABS-S14 and chitosan to manage postharvest green mold on mandarin fruit and found that bacterial endospores, the crude extract, and chitosan showed a significant reduction of fruit decay compared to the individual effect of cyclic lipopeptide *B. subtilis* produces. In addition, *B. subtilis* ABS-S14 itself, its crude extract and chitosan each induced the activities of peroxidase (POX) and phenylalanine ammonia lyase (PAL) in the infected tissues of mandarin fruit. Most importantly,

co-application of bacteria and chitosan co-enhanced the protection of fruit from the green mold pathogen *P. digitatum* compared with individual effect from BCA and chitosan. Kiewnick et al. (2001) reported that *Rhizoctonia* crown and root rot of sugar beet caused by the fungus *Rhizoctonia solani* AG 2-2 were best controlled with a combination treatment of azoxystrobin applied at 76 g a.i./ha and the *Bacillus* isolate MSU-127 compared to separate application of fungicide and BCA, and it also provided greatest root and sucrose yield increase. It is often difficult to obtain satisfactory bacterial spot control on tomato by applying a single bactericide and such control measure has also been implicated with resistance development in the bacterial population (). Robets et al. (2008) tested diverse combination of products that also included *B. subtilis* to manage bacterial spot on tomato. In spray programs containing acibenzolar-S-methyl (ASM) or *B. subtilis* plus copper hydroxide, treated plants had significantly reduced disease compared to the untreated control plants and were not different from the plants treated with the copper-mancozeb standard. Peng et al. (2014) determined combined effect of *B. subtilis* NJ-18 with the fungicides flutolanil and difenoconazole for the control of wheat sharp eyespot caused by *Rhizoctonia cerealis*. The growth of NJ-18 was unaffected by flutolanil in a broth medium, and the survival of NJ-18 spores on wheat seed was unaffected by difenoconazole. In greenhouse experiments, disease control obtained with a combination of NJ-18 and either fungicide was better than the control obtained with the bacterium or fungicides alone. With the exception of a few study results, combination of *Bacillus* spp. strains with chemicals proved compatible and contributed to significant disease reduction with remarkably lower chemical use, which should also contribute to reduced selection pressure on the organism to become resistance against a certain product.

6.6 Conclusion and Future Perspectives

Indiscriminate use of synthetic pesticides for the last few decades has created concerns due to its detrimental effect on the environment and human health. These concerns, however, provided incentives to a continued and increased interest in searching nature for environmentally friendly pest management tools. A significant effort is underway globally to generate low-cost biorational natural pest control products. Besides crop plant resistance, various biological control methods based on natural pest-suppressing organisms are regarded as main alternatives. Thousands of microbial strains have been screened for antimicrobial properties with positive outcomes, and many of them especially those belonging to *Bacillus* spp. were found to suppress or enabled plants to resist important phytopathogens and provide plant growth-promoting effects. However, more efficient screening method that may reflect field performance of these organisms will have to be developed from simulation modeling in controlled environment. Some of these organism-based commercial preparations have been transferred successfully as natural pesticides to the conventional crop production systems. A large body of literature suggested that

integration of these products with conventional management tools could significantly reduce chemical use and make crop production more sustainable. The list of *Bacillus*-based natural pesticide products has been increasing fast due to numerous advantages such as long survival, ease of multiplication, and multiple modes of actions they possess compared with other BCAs. These products are now considered essential not only in organic production systems but also a component of integrated pest management in conventional agriculture as a resistance management tool. Judicious incorporation of these products in the integrated pest management program will substantially reduce the use of synthetic pesticides in crop fields. *Bacillus*-based products have shown efficacy against diverse plant diseases on many commodities and plant parts, but the highest potential is against soilborne diseases. Continued research and development on *Bacillus*-based pesticides against plant diseases will enable researchers finding more efficient strains given the ubiquitousness and diversity of these organisms. However, large-scale use of BCAs is still limited due to the variability and inconsistency of biocontrol activity in field conditions. Lowering the variability and increasing the consistency of disease suppressions are among the few challenges that will have to be addressed. Scientific community must determine the factors that interfere with the reproducibility of results from one location to another or controlled condition to field condition over time. Integration of BCAs with other management options or combining with other BCAs should help in reducing the variability of results. It is well known that the activity of the BCAs depends on the interactions among the BCA, the plant host, the pathogen, and the biotic and abiotic environmental factors in field conditions. As such optimization and documentation of these factors and making the information available to end users will help in achieving consistent results. In addition, genetic improvement of *Bacillus* spp. can be achieved and widened by an integration of biotechnological tool in the conventional development program to enhance production of metabolites that were found effective in controlling plant diseases. Use of metabolites from genetically improved organisms instead of using the organism itself will significantly reduce the perceived risk of releasing such an organism in the environment and unforeseen consequences it might bring. Implementation of above-suggested strategies may reduce risk of uncontrolled epidemics and increase confidence of growers in using this nonchemical control measure on a large scale (Elad 2003). The continuing need for developing sustainable crop protection tools with reliable mode of action will make discovery and commercialization of *Bacillus*-based products as an attractive and profitable pursuit in the coming days.

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Chapter 7
Bacillus spp. and Their Biotechnological Roles in Green Industry

Naser Aliye Feto

Abstract *Bacillus* is one of the genera rich in species with diverse biotechnological applications. A number of review reports addressing various aspects of different species belonging to *Bacillus* have so far been published. However, most of the up-to-date reviews addressed either details of a single species of the genus *Bacillus* or particular aspect of a given species of the genus. Thus, in this chapter comprehensive review covering most of *Bacillus* spp. and their biotechnological roles in the green industry is presented. Accordingly, assorted biotechnological roles of *Bacillus* spp. in the fermentation industry, bioremediation of recalcitrant compounds and their use as probiotic supplements are summarised. Moreover, their well-known role in bioprotecting their host plants, through either directly antagonising the pathogens or indirectly promoting growth of their host plants or as an entomopathogen against insect pests, is also dealt with. In line with the theme of the book, special emphasis was given to the part that dealt with the role of *Bacillus* spp. in phytobioprotection.

Keywords *Bacillus* spp. • Green industry • Fermentation industry • Probiotic • Bioremediation • Bioprotection

7.1 Introduction

Bacillus spp. is a large and diverse genus of bacteria in the family *Bacillaceae.* The genus *Bacillus* contains rich genetic biodiversity. *Bacillus* spp. present in an extremely diverse environments ranging from sea water to soil and are even found in extreme environments like hot springs (Hoch et al. 1993). The genus is ubiquitous because of its sophisticated ability to survive and perpetuate in a diverse and extreme environment. Thus, such an ability made bacterial species belonging to the

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_7

genus *Bacillus* to be endowed with a number biomolecules with biotechnological as well as pharmacological importance, which varies from their roles in fermentation industry, bioremediation of recalcitrant compounds and contribution to health sector as a source of antibiotics and probiotic supplements. Besides, the genus *Bacillus* has significant contribution in bioprotection of crop plants against phytopathogens and insect pests.

Bacillus species are good secretors of proteins and metabolites of industrial and pharmacological importance. Most species of *Bacillus* strains have high capacity to secrete a variety of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease and xylanase, and these enzymes play very important roles in many biotechnological processes (Sinchaikul et al. 2002; Cherry and Fidantsef 2003). *Bacillus* has also been playing central role in bioremediation of recalcitrant compounds being part of microbial consortium (Ghazali et al. 2004; Lin et al. 2011; Chen et al. 2012b). Besides, different species belonging to the genera have also been used as a critical component probiotic supplements because of their stress-resistant spores (Green et al. 1999; Hoa et al. 2000; Senesi et al. 2001). Among the most versatile uses of *Bacillus* spp. is their significant contribution in bioprotection of crop plants as well as animals from phytopathogens, insect pests and clinically important insects like mosquitoes carrying malaria parasite (Priest 1992; De Furia and Claridge 1976; Milner et al. 1996; Arsenijevic et al. 1998; Aliye et al. 2008; Aliye 2011; Sanchis 2010; Ruiu et al. 2013).

Because of its versatile use and ubiquitous nature, the genus *Bacillus* or a species belonging to the genus attracted a significant attention from the scientific community. Accordingly, a number of review articles were published. A relatively comprehensive review was presented by Schallmey et al. (2004) regarding industrial use of *Bacillus* spp. More recently a number of other reviews have also been published. For instance, Sanchis (2010) published a detailed review on roles of *Bacillus thuringiensis* as a biopesticide. Also in 2011 there was a review chapter by Cawoy et al. (2011) on *Bacillus*-Based Biological Control of Plant Diseases. Very recently an interesting and detailed review of alkaliphilic bacteria with impact on industrial applications with main focus being on extremely alkaliphilic *Bacillus* strains was also presented by Preiss et al. (2015). However, most of up-to-date reviews addressed either details of a single species of the genus *Bacillus* or a particular aspect of a given species in the genus. Thus, in this chapter comprehensive review covering major biotechnological roles of *Bacillus* spp. in the green industry is presented. Accordingly, assorted biotechnological roles of *Bacillus* spp. in the fermentation industry, bioremediation of recalcitrant compounds and their role in health sector as source of antibiotics and probiotic supplements are summarised. Moreover, in line with the theme of the book, special emphasis was given to their well-known role in bioprotection of their host plants. Under this part, different mechanisms that involve either direct antagonism of the pathogens and/or indirect mechanism, which act by promoting growth of their host plants or as an entomopathogen against insect pests, are dealt with.

7.2 Bacillus spp. Enzymes in Green Industry

Bacillus species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete stable enzymes into the extracellular medium and the GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *Bacillus subtilis* and *Bacillus licheniformis* (Schallmey et al. 2004).

The global market for industrial enzymes is estimated to be ca. 1.6 billion USD, out of which 29 %, 15 % and 56 % are divided between food and feed industry, as well as general technical enzymes for miscellaneous use, respectively (Outtrup and Jorgensen 2002). Some report estimates that *Bacillus* spp. enzymes make up about 50 % of the total enzyme market. There are three dominant enzyme suppliers, Novo Nordisk, Genencor International and DSN N.V., with a reported market shares of 41–44 %, 21 % and 8 %, respectively, while the smaller producers in North America, Europe, Japan and China made up the remaining 27–30 % (Schallmey et al. 2004).

As for the application, a number of versatile industrial applications of enzymes from *Bacillus* spp. have been reported with significant role in detergent, textile, starch processing, dairy, baking and beverage industries (Pandey et al. 2000; Schallmey et al. 2004). Sharaf and Al-Fadel (2013) very recently reported that *Bacillus circulans* L produced strong alkaline protease that effectively removed blood stain and was compatible with a commercial detergent called Tide. Such finding is impressing because the bacteria hydrolysed chicken feather and sheep wool thereby playing key role in green recycling of what otherwise would have been waste materials at poultry and sheep farming, in addition to producing industrially important enzyme. Moreover a number of other *Bacillus* spp. enzymes that have been playing critical role in the detergent industry were also reported (Schallmey et al. 2004).

There are a number of mesophilic enzymes of industrial importance; however, focus under this section will be made on alkalithermophilic *Bacillus* spp. enzymes, because of the latter's indispensable roles in green industry.

7.2.1 Alkalithermophilic Bacillus *spp. Enzymes*

Alkalithermophilic enzymes are those with pH and temperature optima of 8 or more and 50 °C or more, respectively (Horikoshi 1999; Madigan and Martino 2006). Such enzymes, unlike mesophilic enzymes, have better role in the green industry because of their stability at high pH and temperature.

Thermostable enzymes are preferred due to the fact that the intrinsic thermostability implies prolonged shelf life or storage at room temperature. Besides, it infers that increased tolerance to organic solvents reduced risk of contamination and minimal loss of activity during pre-processing at an elevated temperature often used in raw material pretreatments (Kristjansson 1989; Turner et al. 2007).

There are a number of alkaliphilic enzymes of *Bacillus* origin with wide range of industrial use. For instance, a novel highly thermostable protease was extracted from thermophilic *Bacillus* strain HUTBS62 isolated from a hot-spring located near to the Dead Sea, Jordan. The optimum pH and temperature for catalytic activity of the protease were pH 6.8 and 80 °C (Aqel et al. 2012). Other earlier reports also indicated that alkalithermophilic protease from *B. clausii* strains with a pH and temperature optima in the range of 9–12 and 50–60 °C, respectively, were registered (Horikoshi 1971; Aunstrup et al. 1972; Tsuchida et al. 1986). Moreover, Tsai et al. (1983) reported that *B. clausii* YaB has high elastase activity at pH optimum of 11. In addition, *B. clausii* AH101, closely related to *B. halodurans*, with high elastase and keratinolytic activities was reported to digest human hair and nail at pH 11–13 (Takami et al. 1992a, b).

Ara et al. (1992) reported that pullulanase enzyme from *B. halodurans* strain KSM 1876 have pH optimum of 10–10.5 making it alkaliphilic thereby making it the highest pullulanase used in detergents. Moreover, Honda et al. (1985) also reported that xylanase enzyme from *B. halodurans* c-125 have pH optima ranging from 6 to 10 and used in food processing. Besides, the starch-degrading enzyme (α-amylase) from *B. pseudofirmus* A-40-2 with optimum pH 10–10.5 had ability to scarify 70 % of starch to glucose, maltose and maltotriose, and it was also reported to be more stable in the presence of EDTA than are *B. subtilis* and *B. licheniformis* enzymes (Horikoshi 1971). Thus, these show how *Bacillus* enzymes are comprehensive in composition and dominant in their share of the industrial applications and versatile in their use and ubiquitous in abundance.

7.3 Role of Bacillus spp. in Bioremediation of Recalcitrant Compounds

Environmental contamination due to anthropogenic and natural sources is increasing from time to time because of the pressure from increase in population, industrialisation and urbanisation. The focus of a number of policies and subsequent studies is to design a tool to minimise such pollution and manage the problem using green technologies. The ideal tool to alleviate such disaster is to employ green technologies to biodegrade recalcitrant compounds into the harmless or usable form preferably complete mineralisation to CO_2 and H_2O. During degradation of complex recalcitrant compound, a number of microbes have been reported to be playing critical role at different phases of decomposition of such compound, rather than a microbe shouldering the task of degrading the whole compound on its own (Ghazali et al. 2004; Lin et al. 2011; Chen et al. 2012b). Individual microorganisms can metabolise only a limited range of hydrocarbon substrates, so clustering of mixed populations with overall broad enzymatic capacities is required to bring the rate and extent of biodegradation of recalcitrant compounds including petroleum biodegradation further (Ghazali et al. 2004).

Table 7.1 Role of *Bacillus* spp. in bioremediation environmental pollutants

Microbe	Substrate/recalcitrant compound	Reference
Bacillus spp. G-02	3-Phenoxybenzoic acid	Chen et al. (2012a)
Bacillus sp. S3.2	Crude oil, benzene, toluene, ethylbenzene, o-xylene	Ghazali et al. (2004)
Bacillus sp. 063	Benzene, toluene, ethylbenzene, o-xylene, octanol	Ghazali et al. (2004)
Bacillus sp. 113i	Crude oil, toluene, ethylbenzene, o-xylene	Ghazali et al. (2004)
Bacillus cereus ZH-3	Cypermethrin; *B. cereus* ZH-3 used as a co-culture with *Streptomyces aureus* HP-S-01	Chen et al. (2012b) and SCAN (2000)
Bacillus spp. TDS-2	Thiophanate-methyl	Logan (2004) and Cycoń et al. (2011)
Bacillus pumilus strain C2A1	Chlorpyrifos and 1,2,3-trichloropropane (TCP)	Hong et al. (2008)
Bacillus laterosporus strain DSP	Chlorpyrifos	Hong et al. (2008) and Zhang et al. (2012)
Bacillus sp. strain MW-1	4-Chloro-2-nitrophenol	Endres (2009) and Arora et al. (2012)

Different microorganisms including those belonging to *Bacillus* spp. were reported to have been playing critical role in bio-decomposition of pollutants. For instance, Ghazali and colleagues (2004) reported that different *Bacillus* spp. isolates played critical role as major component in microbial consortium to biodegrade environmental contaminants like crude oil, benzene, toluene, ethylbenzene, o-xylene and octanol.

Interestingly, Chen et al. (2012a) reported that a *Bacillus* sp. strain DG-02 biodecomposed 95.6 % of 50 mg L^{-1} of 3-phenoxybenzoic acid (3-PBA) within 72 h in mineral salt medium (MSM), a compound, which is of great environmental concern with regard to endocrine-disrupting activity and widespread occurrence in water and soil environments. Besides, Anwar et al. (2009) reported biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1.

The brief discussion and list in Table 7.1 further cement the versatile uses of the *Bacillus* spp. enzymes, making the genus a sink of one of the most indispensable microbial genera.

7.4 Bacillus spp. as Probiotic Supplements

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host (Araya et al. 2002). The potential benefits that are reported include improved nutrition and growth and prevention of various gastrointestinal disorders. One of the pioneer *Bacillus* spp. strains in probiotic is natto.

Natto is a variety of *B. subtilis* that is used in the preparation of the Japanese fermented soybean staple known as 'natto'. The natto strain has a long history of use as part of a food product and has been shown to contain more than 10^8 viable bacteria per gram (Hosoi and Kiuchi 2004).

Bacillus species have been used as probiotics for more than 50 years with the Italian product known as Enterogermina® registered in 1958 in Italy as an OTC medicinal supplement. A number of *Bacillus* spp. have continuously been investigated for their probiotic activities; among them, the most exhaustively studied are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans* and *Bacillus licheniformis* (Hong et al. 2008). The thermostability of spores made *Bacillus* spp. have a number of advantages over other non-spore formers such as *Lactobacillus* spp., which are known probiotic supplements. Such thermostability implies that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier, which is not the case for all species of *Lactobacillus*, so in principle a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact.

Probiotic-containing products are available for human nutrition, as animal feed and poultry supplements (Table 7.2). Thus, the leading and multipurpose probiotic use of different strains of *Bacillus* spp. further cement the indispensability of the genera in its versatile use for the intended purpose.

Table 7.2 *Bacillus* spp. probiotics for human, farm animals and poultry use

Bacillus spp.	Use category	Remark	Reference/URL
B. subtilis var. *natto*	Human	Basically the strain is used in the fermentation of the Japanese fermented soybean staple known as 'natto'. Natto contains more than 10^8 viable bacteria per gram of natto	Hosoi and Kiuchi (2004)
B. cereus IP 5832	Human	The spore from the strain is an active ingredient of the Vietnamese products called Biosubtyl	Hoa et al. (2000)
B. licheniformis and *B. subtilis*	Poultry, calves and swine	The strains are active ingredient of the product called BioGrow® at composition of *B. licheniformis* (1.6×10^9 CFU g^{-1}) and *B. subtilis* (1.6×10^9 CFU g^{-1}), produced by Provita Eurotech Ltd., Omagh, Northern Ireland, UK	http://www.provita.co.uk

(continued)

Table 7.2 (continued)

Bacillus spp.	Use category	Remark	Reference/URL
B. clausii (Enterogermina strains)	Human	Prevention of antibiotic associated diarrhoea in adults. Recommended dosage 2×10^9 spores, three times daily	Green et al. (1999), Hoa et al. (2000), Senesi et al. (2001), and Nista et al. (2004)
B. pumilus	Human	The species is used as an active principle of Biosubtyl I and II, a product of Biophar Company, Nha Trang, Vietnam	Green et al. (1999) and Hoa et al. (2000)
B. licheniformis DSM 5749 and *B. subtilis* DSM 5750	Piglets, chickens, turkeys for fattening	The two *Bacillus* spp. strains are active ingredients of the product called BioPlus®2B (at proportion of mixture (1/1) of *B. licheniformis* DSM 5749 and *B. subtilis* DSM 5750 at 1.6×10^9 CFU g^{-1} of each bacterium), manufactured by Christian Hansen Horsholm, Denmark	http://www.chr-hansen.com/
B. coagulans GBI-30, 6086	Human	Helps alleviate symptoms of irritable bowel syndrome	Dolin (2009)
B. cereus var. *vietnami*	Human	Capsule carrying 10^6–10^7 a strain similar to *B. cereus* spp. and designated *B. cereus* var. *vietnami*	Hoa et al. (2000)

7.5 Role of Bacillus spp. in Bioprotection of Crop Plants

The genus is one of the major sources of potential microbial biopesticides as a number of mainstream biopesticides belong to it. Besides, there are a number of reasons that make the genus special, as far as biopesticide is concerned. In one of the reports with colleagues, we detailed the antibiosis and growth promotion activity of *Bacillus* spp., such as *Bacillus subtilis* PFMRI and *B. pumilus* BC for bioprotection of *Solanum tuberosum* against bacterial wilt caused by *Ralstonia solanacearum* making the *Bacillus* strains ideal candidate for use as biopesticide against plant pathogens (Aliye et al. 2008). *B. subtilis* is known to have broad spectrum of activity and versatile use, from use as biopesticide to probiotics in human, veterinary, aquaculture, poultry and farm animal (Green et al. 1999; Hoa et al. 2000; Aliye et al. 2008; Dong et al. 2009; Aliye 2011). Particularly, *B. subtilis* was granted 'generally regarded as safe' (GRAS) status by the US Food and Drug Administration (USFDA),

thereby being recognised as non-pathogenic microbe (Harwood and Wipat 1996). On the other hand, *Bacillus thuringiensis* (Bt) and its diverse subspecies have been reported for their bioinsecticidal properties (Sanchis 2010). The stress-resistant nature of *Bacillus* spp. on the account of their spores makes them extremely resistant and capable to withstand high temperatures, unfavourable pH and lack of nutrients or water (Hoch et al. 1993; Aliye et al. 2008). Such structure has, thus, significant implication on the increased shelf lives of biopesticides of *Bacillus* origin.

7.5.1 Protection Against Phytopathogens

Bacillus spp. have been playing critical role in bioprotection of crop plants from a number of phytopathogens (Arsenijevic et al. 1998; Aliye et al. 2008; Aliye 2011). During such bioprotection process, different mechanisms mainly divided into direct and indirect ones are employed. The direct one involves competitive root colonisation (niche exclusion), antibiosis, lytic enzyme production and detoxification and degradation of virulence factors (Priest 1992; Milner et al. 1996; Aliye 2011), whereas the indirect one renders bioprotection through promotion of plant growth and induction of systemic resistance (Aliye et al. 2008; Aliye 2011).

7.5.1.1 Direct Mechanism of Action

Invasive colonisation by plant growth-promoting bacteria (PGPB) and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes and detoxification enzymes (Sturz and Christie 2003).

Competitive Root Colonisation

Despite their potential as low-input practical agents of plant protection, application of PGPB has been hampered by inconsistent performance in field tests (Thomashow 1996); this is usually attributed to their poor rhizosphere competence (Weller 1988). Rhizosphere competence of biocontrol agents comprises effective root colonisation combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (Whipps 1997). Given the importance of rhizosphere competence as a prerequisite of effective biological control, understanding root microbe communication (Ping and Boland 2004), as affected by genetic and environmental (Pettersson and Baath 2004) determinants in spatial (Bais et al. 2004) and temporal (Aliye 2011) contexts, will significantly contribute to improve the efficacy of these biocontrol agents. The root surface and surrounding rhizosphere are significant carbon sinks. Photosynthate

allocation to this zone can be as high as 40 % (Degenhardt et al. 2003). Thus, along root surfaces, there are various suitable nutrient-rich niches attracting a great diversity of microorganisms, including phytopathogens. Competition for these nutrients and, thus, niches is a fundamental mechanism by which PGPB bioprotect plants from phytopathogens (Compant et al. 2005). The PGPB reach root surfaces by active motility facilitated by flagella and are guided by chemotactic responses (Steenhoudt and Vanderleyden 2000). Known chemical attractants present in root exudates include organic acids, amino acids and specific sugars (Welbaum et al. 2004). Some exudates can also be effective as antimicrobial agents and thus give ecological niche advantage to organisms that have adequate enzymatic machinery to detoxify it (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 PGPB competence highly depends either on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions.

A number *Bacillus* spp. have been reported to have employed indirect mechanisms besides the direct ones to bioprotect their host plants (Aliye et al. 2008; Aliye 2011). Ryan et al. (2001) also reported that *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a were able to protect their host plant indirectly through induced systemic resistance (ISR), via secretion of volatiles, which in turn activate an ISR pathway in *Arabidopsis* seedlings pretreated with the antagonistic strains and challenged with the soft rot pathogen *Erwinia caratovora* sub. sp. *caratovora*.

Competition for Iron and the Role of Siderophores

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Loper and Henkels 1997). Under iron-limiting conditions, PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps 1997). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have relatively lower affinity (Loper and Henkels 1997). Some PGPB strains go one step further and draw iron from heterologous siderophores produced by cohabiting microorganisms (Whipps 1997).

Bacillus spp. are rich in production of siderophores, thus giving them competitive advantage over their counterparts. For instance, Yu et al. (2011) reported that *Bacillus subtilis* CAS15 produced siderophores during bioprotection of pepper plant against fusarium wilt. A number of other reports highlighted the siderophore-mediated antibiosis technique employed by various isolates of *Bacillus* spp., for instance, Sivasakthi et al. (2013) reported that different isolates of *Bacillus subtilis* BS 1–10 and *Bacillus cereus* (Wilson et al. 2006) produced siderophores as a means of antagonism.

Antibiosis

The basis of antibiosis as a biocontrol mechanism of bioagents has become increasingly better understood over the past three decades. A variety of antibiotics have been identified, including compounds such as subtilin and xanthobaccin, produced by *Bacillus* spp. (Milner et al. 1996). Besides, a number of other antibiotics producing *Bacillus* spp. have been reported. For instance, bacitracin-A produced by *B. licheniformis* inhibit cell wall synthesis (Katz and Demain 1977), and butirosin complex produced by *B. circulans* alters function of ribosome (De Furia and Claridge 1976). Interestingly, Priest (1992) reported other sets of *Bacillus* antibiotics produced by different species of the same genera yet with the same function. Namely, colistins by *B. colistinus*, polymyxins by *B. polymyxa* and circulins by *B. circulans*, all of them were reported to interfere with membrane function. Thus, production of such diverse antibiotics gives *Bacillus* spp. broad spectrum of activities and competitive advantage over their counterparts.

Lytic Enzyme Production

B. cepacia synthesises ß-1,3-glucanase that destroys the cell wall integrity of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Fridlender et al. 1993).

7.5.1.2 Indirect Mechanism of Action

Initiation of Induced Systemic Resistance (ISR)

The ability to act as bioprotectants via ISR has been demonstrated by both rhizobacteria and bacterial endophytes, and considerable progress has been made in elucidating the mechanisms of plant-PGPB-pathogen interaction. Several bacterial traits (i.e. flagellation and production of siderophores and lipopolysaccharides) have been proposed to trigger ISR (Van Loon et al. 1998), but there is no compelling evidence for an overall ISR signal produced by bacteria (Haas et al. 2002). It has been reported that volatile organic compounds may play a key role in this process (Ryu et al. 2004). For example, volatiles secreted by *B. subtilis* GB03, registered as Kodiak® (Gustafson, Inc, Plano, TX, USA) (Brannen et al. 1997), and *B. amyloliquefaciens* IN937a were able to activate an ISR pathway in *Arabidopsis* seedlings challenged with the soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (Ryan et al. 2001).

Plant Growth Promotion (PGP)

Plant growth-promoting bacteria (PGPB) (Bashan and Holguin 1998) are associated with many, if not all, plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting

rhizobacteria (PGPR) colonising the root surfaces and the closely adhering soil interface, the rhizosphere (Kloepper et al. 1999). As detailed by Kloepper et al. (1999) or by Gray and Smith (2005), some of these PGPR can also enter root interior and establish endophytic populations. Many of them are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers and other organs (Compant et al. 2005; Gray and Smith 2005). The extent of endophytic colonisation of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches (Hallman et al. 1997; Gray and Smith 2005). Consequently, intimate associations between bacteria and host plants can be formed (Compant et al. 2005; Kloepper et al. 1999) without harming the host plant (Hallman et al. 1997; Kloepper et al. 1999).

A number of *Bacillus* spp. have been reported to belong to the PGPB. For instance, in one of our studies, application of *B. subtilis* PFMRI and *B. pumilus* BC on potato plant promoted growth of the plant by a growth promotion efficacy (GPE) of 114.4 % and 105.7 %, respectively (Aliye et al. 2008). Very recently, Schwartz et al. (2013) reported a *Bacillus simplex* 30N-5, which was a little known PGPB with antifungal activity and a *B. subtilis* 30N-5 with growth promotion effect. Besides, there are a number of *Bacillus* species reported as having plant growth-promoting (PGP) ability to mention some *B. amyloliquefaciens* (Idris et al. 2007), *B. pumilus* (Benhamou et al. 1996), *B. licheniformis* (Probanza et al. 2001), *B. thuringiensis* (Bai et al. 2003), *B. cereus* (Handelsman et al. 1990) and *B. megaterium* (López-Bucio et al. 2007).

The mechanism of PGP activity of *Bacillus* spp. is like other PGPB, enhancing plant growth via direct and indirect means: directly by having PGP effect via production of plant growth hormones such as indole-3-acetic acid (IAA), cytokinin and gibberellic acid (GA) or by downregulating ethylene production via *acdS* (1-amino cyclopropane-1-carboxylic acid deaminase) activity (Rashid et al. 2012) and/or by acting synergistically with nitrogen-fixing or phosphorous-acquiring symbiotic microbes (Probanza et al. 2001; Vivas et al. 2003; Guiñazú et al. 2010) and indirectly by igniting the induced systemic resistance (ISR) of the crop, which gives the host plant resistance to incoming pathogen (Choudhary and Johri 2009), and by chelating heavy metals in contaminated soils (Rajkumar et al. 2008).

7.5.2 Bacillus *spp. as Entomopathogens*

Bacillus spp. have been playing critical role in bioprotecting crop plants and animals from insect pests and other insect vectors of clinical importance (Sanchis 2010; Ruiu et al. 2013). During bioprotection against insect pests, direct pathogenesis of the insect pest has been employed unlike the myriad of mechanisms employed by plant-related *Bacillus* spp. against the phytopathogens, which involve both direct and indirect means (Arsenijevic et al. 1998; Probanza et al. 2001; Aliye et al. 2008; Sanchis 2010; Guiñazú et al. 2010; Aliye 2011; Rashid et al. 2012; Schwartz et al. 2013).

Originally, the species *Paenibacillus* (former *Bacillus*) *popilliae* (Dutky 1940) was introduced for the management of the Japanese beetle *Popillia japonica* Newman (Steinhaus 1975). However, more tangible results were achieved with the discovery of new strains of *Bacillus thuringiensis* (Bt), which showed high toxicity against specific insects at competitive level compared to conventional insecticides in terms of efficacy and production costs. The strain *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (De Barjac and Lemille 1970) became the main commercial focus for the management of lepidopteran pests in agriculture and forestry. Besides it, today other *Bacillus thuringiensis* strains such as SA-11, SA-12, PB 54, ABTS-351 and EG2348 are commercially available. Isolation of novel *Bacillus thuringiensis* serovar *kurstaki* highly pathogenic against lepidopteran pests was also reported (Kati et al. 2007). All such strains were isolated either from insects and/or soil and express a range of different toxins mostly belonging to the Cry1 and Cry2 families (Ruiu et al. 2013).

Consequently, mosquito and simulid pathogenic strain of *Bacillus thuringiensis* subsp. *israelensis* (Bti) (Goldberg and Margalit 1977) serotype H14 (De Barjac 1978) was discovered and subsequently commercialised. Krieg et al. (1983) reported a new strain of *Bacillus thuringiensis* (Bt) subsp. *tenebrionis* active against coleoptera. The other strain called *B. thuringiensis* subsp. *kumamotoensis* was reported to be pathogenic against coleopterans (Rupar et al. 1991). Besides Bt bioinsecticide, a number of other non-Bt bioinsecticides have also been reported. For instance, another mosquito-pathogenic *Bacillus* spp. called *B. sphaericus* an alternative to Bti was reported. The strain is toxic against mosquitoes as a result of the production of parasporal crystals located within the exosporium and closely associated with the endospore, which is responsible for production of mosquito toxin known as Mtx protein (Charles et al. 2000).

7.5.2.1 Transgenic Bt Crops

Besides employing direct microbial spray (Bt spray) as bioprotectant against insect pests, genetic manipulation of different crops has also been in use. Genetically engineered crop is developed by inserting a gene into the plant genome that expresses a toxic Cry protein from *Bacillus thuringiensis (Bt)*. Both the leaves and stems of *Bt* maize produce the toxin which destroys the gut of any moth larvae eating the plant. The technique is effective, and unlike the wide-spectrum pesticides, it only targets larvae of moths. The genetic manipulation made the genetically modified crop produce the cry toxin itself. Such genetically engineered Bt crops have been approved and made their way in to the market in different parts of the world.

As reviewed by Sanchis (2010), in 1995 the US Environmental Protection Agency (EPA) approved the first registration of Bt potato, corn and cotton products with Bt toxin genes expressing their protein at potentially commercially viable levels. In 1996, two new Bt corn varieties, both expressing the Cry1Ab1 toxin, were commercially released by Northrup King and Monsanto. In addition, in 2001 a new corn variety, expressing the Cry1F toxin, was developed jointly by Pioneer Hi-Bred

and Dow AgroSciences, and commercialised, in 2002, a year later Monsanto released a new variety of transgenic corn resistant to the western corn rootworm, *Diabrotica virgifera virgifera* (Lec.).

7.5.2.2 Challenges Facing Bt Bioinsecticide

An all-out exclusive and intensive usage of Bt-based bioinsecticide and transgenic Bt crops exerted selection pressure on the insects, which in turn responded by developing resistance to the active ingredient, cry toxin. Consequently, a number of reports indicated that insect pests have been developing resistance to Bt spray and Bt crops (Table 7.3). Accordingly, starting from 1985, a number of reports regarding emergence of Bt-resistant insects started to surface. For instance, the presence of low-level Bt resistance in Indian meal moth *Plodia interpunctella* (Hbn) was reported by McGaughey (1985); diamondback moth *Plutella xylostella* (L.) (Liu and Tabashnik 1997) and *Helicoverpa zea* (Boddie) (Tabashnik et al. 2008) were also reported to have develop Bt resistance.

Moreover, emergence of field resistance to Bt cotton was also reported recently. Resistance of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (pink bollworm) to Bt cotton producing Cry1Ac (Bollgard) was first detected with

Table 7.3 Emergence of Bt resistance

Insect pest	Host	Type	Origin	Reference
Plodia interpunctella (Hbn.) (Indian meal moth)	Bt-treated grain	Low-level resistance in storage bins of Bt-treated grain and laboratory. The resistance was inherited as a recessive trait	Manhattan, Kansas (USA)	McGaughey (1985)
Plutella xylostella (L.) (Diamondback moth)	*Brassicaceae*	Field resistance to spray Bt pesticide containing Cry1C toxin	Hawaii (USA), Japan, China, the Philippines and Thailand	Liu and Tabashnik (1997)
Diabrotica virgifera virgifera (Western corn rootworm)	Bt corn	Field	Northwestern Illinois (USA)	Gray (2011)
Helicoverpa zea (Boddie) (corn earworm)	Bt corn	Delayed field resistance to *Bt Cry3Bb1N*	Mississippi and in North Carolina (USA)	Tabashnik et al. (2008)
Pectinophora gossypiella (pink bollworm)	Bt cotton	Field resistance to Cry1Ac Bollgard® I	India	Monsanto (2010) and Dhurua and Gujar (2011)
Busseola fusca (African caterpillar)	Bt maize	Field resistance, resistance type is dominant trait	South Africa	Campagne et al. (2013)

laboratory bioassays of the offspring of insects collected from the field in 2008 in Gujarat (Dhurua and Gujar 2011). Besides, in 2009, Monsanto entomologists detected, and ultimately confirmed, pink bollworm resistance to Monsanto's first-generation single-protein (*Bacillus thuringiensis*) Cry1Ac Bollgard® I cotton in four states in Western India (Monsanto 2010).

Yet, the most worrisome report was the one very recently reported by a group of international scientists that *Busseola fusca* developed high levels of resistance to the *Bt* toxin *Cry1Ab* expressed in *Bt* corn in South Africa (Campagne et al. 2013). Besides they indicated that the reported resistance of *B. fusca* to *Bt* corn is dominant refuting the hypothesis of recessive inheritance, which could accelerate evolution of resistance thereby posing huge challenge to manage the bug in the future. Thus, mitigation strategies as indicated under Sect. 6 should be designed, accordingly.

7.6 Conclusion and Future Prospect

The reports addressed in this review indicate how versatile, rich and diverse the *Bacillus* spp. are in terms of biological diversity and use. The review also shows the dominant role the species have been playing in white industry from biofermentation, textile and paper to detergent industry. Besides, their role in health sector as probiotic supplement has been significant. Moreover, the major roles the species have been playing in bioremediation of environmental pollutants and bioprotection of both plants and animals have been highly significant. Such genetic richness and diversified use put the species at dominant levels and make them some of the most exploited microbes in the recent human history.

However, there are still rooms to further efficiently and sustainably exploit *Bacillus* spp. as well as their enzymes of industrial, pharmacological as well as agricultural importance. As long as *Bacillus* spp. enzymes involved in fermentation, biodegradation/bioremediation, paper-pulp and detergent industry are concerned, it is very important to genetically engineer a candidate *Bacillus* sp. by transforming it with a plasmid carrying genes encoding for set of enzymes. For instance, if amylase is considered, we have α-amylase with pH optima (6.7–7), β-amylase (pH 4–5) and γ-amylase (pH 3). If genes for all three different types of amylases are fused in tandem and cloned to a *Bacillus*-compatible plasmid, then the transformed microbe shall be able to produce cocktail of enzymes that work in a range of pH (3–7). The same is true for biodegradation of recalcitrant compound, which involves a consortium of different microbes with different hydrolytic enzymes that act at different stages of degradation of such a compound. Following the same analogy as that of amylase cocktail, one can possibly develop a pure culture of genetically engineered *Bacillus* sp. with multiple enzymatic ability to act on the recalcitrant compound at different stages of degradation on its own.

The reported emergence of resistant insect pests to Bt-based bioinsecticide and/or Bt crop necessitates the need to diversify the management tools and to include other non-Bt entomopathogenic microbes, like entomopathogenic fungus *Beauveria*

bassiana (Balsamo) pathogenic to termites; thrips; whiteflies; aphids; beetles; stem borers (Pekrul and Grula 1979; Tanada and Kaya 1993; Mahr 1997); *Paenibacillus popilliae* with *P. lentimorbus*, which is the causal agents of milky disease in phytophagous scarab larvae (Zhang et al. 1997); and *Serratia entomophila* (Enterobacteriaceae) containing a specific plasmid (pADAP) encoding genes implied in the pathogenicity against the grass grub *Costelytra zealandica* (white) (Jackson et al. 1992). Besides the mentioned entomopathogens, there are a number of other natural enemies that need to be exploited further in order to diversify and increase the use and efficiency of the potential bioinsecticides at hand. In addition, increasing the population of the refuge crop (non-Bt crop) around the Bt crop more than the conventional size is also something to be integrated to other mitigation packages.

Nonetheless, *Bacillus* spp. will continue to play the significant biotechnological roles they have been playing in green industry from biofermentation, textile and paper-pulp to detergent industry and bioremediation and phytobioprotection, in addition to their under exploited role in health sector as probiotic supplements and beyond.

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Chapter 8
Phytostimulation and Biocontrol by the Plant-Associated *Bacillus amyloliquefaciens* FZB42: An Update

Rainer Borriss

Abstract *Bacillus amyloliquefaciens* FZB42, the type strain for representatives of the plant-associated subspecies *plantarum*, stimulates plant growth and suppresses soilborne plant pathogens. The strain has been sequenced in 2007 (Chen et al. (2007); *National Biotechnology 25*, 1007–1014). The *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites. In total, 11 gene clusters representing nearly 10 % of the genome are devoted to synthesizing antimicrobial metabolites and/or to confer immunity against them. Ability to synthesize non-ribosomally the antibacterial polyketides macrolactin and difficidin and the antifungal lipopeptide bacillomycin D is a unique feature of the subspecies *plantarum*. However, according to the latest research, most of the secondary metabolites are not expressed in plant rhizosphere suggesting that the antibiome expressed during the plant-associated state of PGPR *Bacilli* does not reflect the vast genetic arsenal devoted to the formation of secondary metabolites. There is now strong evidence that plant-associated *Bacilli* trigger pathways of induced systemic resistance, which protect plants against attacks of pathogenic microbes, viruses, and nematodes.

8.1 Introduction

Environmental-friendly biotechnological approaches, such as the use of microbial biopesticides, offer alternatives to chemical control of plant diseases and pests. Among these alternatives, the use of bioformulations, which are manufactured from plant growth-promoting rhizobacteria (PGPR) with biocontrol activity (BC) (Lugtenberg et al. 2013), is steadily increasing. At present, due to the long-term shelf life of their endospores, bacilli are the most widely used bacteria on the

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_8

biopesticide market. Their use in agriculture has been previously reviewed (Borriss 2011). An update of *Bacillus*-based bioformulations, currently available for the farmer interested on sustainable agriculture, is presented in Table 8.1.

Plant rhizosphere is a highly competitive environment in which bacteria are abundantly present due to availability of nutrients actively secreted by the plant root and mucilage. Some of these bacteria which are living within or in the vicinity of plant rootsand supporting plant growth are generally referred as being "PGPR" (Kloepper et al. 1980). In many cases, their plant growth-promoting activity is linked with their ability to suppress soilborne plant pathogens (bacteria and microfungi), occurring in the competing microflora. Different mechanisms are discussed in this context. Besides production of antimicrobial ("antibiotics") and nematicidal compounds, also stimulation of plant-induced systemic resistance (ISR, Doornbos et al. 2012) and a beneficial effect on the composition of the host-plant microbiome might contribute to their suppressive effect (Erlacher et al. 2014). In other PGPR, termed "biofertilizer," plant growth promotion by hormone-like compounds and increased accessibility of nutrients dominate. The mechanisms that are involved in this process can include nitrogen fixation, phosphate and mineral solubilization, and the production of macromolecule-degrading enzymes (amylases, proteases, hemicellulases), phytohormones (auxin, cytokinin, and gibberellins), and volatile growth stimulants (such as acetoin and 2,3-butanediol) (Borriss 2011).

Bacillus amyloliquefaciens FZB42 is the type strain for a group of plant-associated *Bacillus* spp. classified as *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011). Its 3918-kb genome, containing an estimated 3693 protein-coding sequences, lacks extended phage insertions, which occur ubiquitously in the closely related but non-plant-associated *Bacillus subtilis* 168 genome. Further analysis revealed that FZB42 is a bacterium with impressive capacity to produce metabolites with antimicrobial activity (Chen et al. 2007). Its antifungal activity is due to non-ribosomal synthesis of the cyclic lipopeptides bacillomycin D and fengycin (Koumoutsi et al. 2004), while its antibacterial activity is mainly due to non-ribosomally synthesized polyketides (Chen et al. 2006), bacilysin (Chen et al. 2009a), and ribosomally synthesized bacteriocins (Scholz et al. 2011, 2014). Recent proteome and transcriptome studies revealed that plant root exudates stimulate expression of genes involved in root colonization and plant-bacteria interactions (Borriss 2015a, b; Fan et al. 2012a, b; 2015; Kierul et al. 2015). Its plant-colonizing ability was demonstrated with a GFP-labeled FZB42 strain on maize and *Arabidopsis* using confocal laser scanning microscopy (Fan et al. 2011). Beneficial effects on plant growth and disease suppression were documented for *B. amyloliquefaciens* FZB42 on tomato, cucumber, cotton, tobacco, and lettuce, for example (Grosch et al. 1999; Idriss et al. 2004; Yao et al. 2006; Guel et al. 2008; Wang et al. 2009; Chowdhury et al. 2013). Two review articles published in open access journals in 2015 (Chowdhury et al. 2015b; Wu et al. 2015b) cover the aspects stressed in this contribution in more detail and are recommended for further reading.

Table 8.1 Examples for commercial use of *Bacillus*-based bioformulations in agriculture. Note: the US governmental EPA registration does not depend on successful field trials; it is only necessary to demonstrate that no negative effects are connected with the use of the biofungicide (The table is taken from Borriss (2015b))

Trade name	*Bacillus* strain	Known properties	Company
Kodiak™	*Bacillus subtilis* GB03	EPA-registered (71065–2) biological and seed treatment fungicide	Bayer Crop Science, former Gustafson LLC
Companion	*Bacillus subtilis* GB03	EPA-registered (71065–2) biofungicide, prevent and control plant diseases. It produces a broad-spectrum iturin antibiotic that disrupts the cell wall formation of pathogens, and it triggers an advantageous induced systemic resistance (ISR) in plants, whereby a plant's natural immune system is activated to fight plant diseases	Growth Products Ltd., White Plains, NY 10603
Yield Shield	*Bacillus pumilus* GB34 (=INR7)	EPA-registered biofungicide (264–985), suppression of root diseases caused by *Rhizoctonia* and *Fusarium*	Bayer Crop Science, previously Gustafson
BioYield™	*B. amyloliquefaciens* GB99 + *Bacillus subtilis* GB122	Combination of strong ISR activity (GB99) with phytostimulation (GB122)	Bayer Crop Science, previously Gustafson
Subtilex®, INTEGRAL®	*Bacillus subtilis* MBI600	EPA-registered (71840–8) biofungicide provides protection against soilborne pathogens such as *Rhizoctonia solani*, *Pythium* spp., and *Fusarium* spp. to help prevent damping-off and other root diseases	Becker Underwood, Saskatoon, Canada acquired by BASF
VAULT®	*Bacillus subtilis* MBI600	Produced by "BioStacked®" technology, enhancing growth of soy beans and pea nuts	Becker Underwood, Saskatoon, Canada
	Bacillus pumilus BU F-33	EPA-registered (71840-RG, -RE, 2013) plant growth stimulator, induced systemic resistance	Becker Underwood, Saskatoon, Canada
SERENADE Max	*Bacillus subtilis* QST713	EPA-registered (69592–11) biofungicide, Annex 1 listing of the EU agrochemical registration directive (91/414)	Bayer Crop Science, previously AgraQuest

(continued)

Table 8.1 (continued)

Trade name	*Bacillus* strain	Known properties	Company
SERENADE SOIL(R)	*Bacillus subtilis* QST713	EPA-registered (69592-EI, 2012) biofungicide for food crops	Bayer Crop Science, previously AgraQuest
SERENADE Optimum®	*Bacillus subtilis* QST713	EPA-registered (2013) biofungicide/bactericide for prevention. It works by stopping spore germination, disrupting cell membrane and inhibiting attachment of the pathogen to leaves. For use in leafy and fruiting vegetables, strawberries, and potatoes. Active against fungal (*Botrytis*, *Sclerotinia*), and bacterial pathogens (*Xanthomonas* and *Erwinia*)	Bayer Crop Science, previously AgraQuest
CEASE(R)	*Bacillus subtilis* QST713	Aqueous suspension biofungicide, recommended for leafy and fruiting vegetables, herbs and spices, and ornamentals	BioWorks, Inc., Victor, New York, USA
SONATA®	*Bacillus pumilus* QST2808	EPA-registered (69592–13) biofungicide, powdery mildew control	Bayer Crop Science, previously AgraQuest Inc
RhizoVital®	*Bacillus amyloliquefaciens* FZB42	Biofertilizer, plant growth-promoting activity, provides protection against various soilborne diseases, stimulation of ISR	ABiTEP GmbH, Berlin
RhizoPlus®	*Bacillus subtilis*	Plant growth-promoting rhizobacterium and biocontrol agent. It can be used for potatoes, corn, vegetables, fruits and also turf	ABiTEP GmbH, Berlin
Taegro®	*Bacillus subtilis* FZB24	EPA-registered biofungicide. FZB24 has been originally isolated by FZB Berlin, the forerunner of ABiTEP GmbH. Registration as a biofungicide for the USA was performed by Taegro Inc. and then sold to Novozymes without agreement with ABiTEP GmbH where the product is still offered	Syngenta, Basel, previously Novozyme, Davis, California and Earth Biosciences

(continued)

Table 8.1 (continued)

Trade name	*Bacillus* strain	Known properties	Company
POMEX	*Bacillus subtilis* CMB26	Microbial fungicide, control and inhibition germination effect on powdery mildew, *Cladosporium fulvum* and *Botrytis cinerea*	NIN Co. Ltd.
	Bacillus subtilis CX9060	EPA-registered 71840-RG,-RE (2012) fungicide, bactericide for food crops, turf and ornamentals	Certis Columbia, MD USA
Easy Start® TE-Max	*Bacillus subtilis E4-CDX*	Rhizosphere bacterium that competes with harmful pathogens for space around the roots of the grass plant. Once established this unique strain physically protects the roots and inhibits the advance of soilborne fungi	COMPO Expert GmbH, Münster, Germany
Double Nickel 55™	*B. amyloliquefaciens* D747	EPA-registered (70051-RNI, 2011), a broad-spectrum preventive biofungicide for control or suppression of fungal and bacterial plant diseases (powdery mildew, *Sclerotinia*, *Botrytis*, *Alternaria*, bacterial leaf spot, bacterial spot and speck, fire blight, *Xanthomonas*, *Monilinia*)	Certis Columbia, MD USA
Amylo-X®	*B. amyloliquefaciens* D747	Annex 1 listing of the EU agrochemical registration directive. Launched to Italy by Intrachem Bio Italia SpA for control of *Botrytis* and other fungal diseases of grapes, strawberries, and vegetables and bacterial diseases such as fire blight in pome fruit and PSA in kiwi fruit	Certis Columbia, MD USA/Intrachem Bio Italia SpA
BmJ WG	*Bacillus mycoides* BmJ	It works entirely as a microbial SAR activator with no direct effect on the plant pathogen itself. Under development	Certis Columbia, MD USA
	Bacillus pumilus GHA 181	EPA-registered fungicide (2012), food crops, seeds, ground cover, and ornamentals	Premier Horticulture
BioNem	*Bacillus firmus* GB-126	EPA-registered (2008), suppressing plant pathogenic nematodes; *Bacillus firmus* creates a living barrier that prevents nematodes from reaching the roots	Agrogreen, Israel acquired by Bayer Crop Science

8.2 Root Colonization by FZB42 and Its Impact on the Host Plant Microbiome

The ability of FZB42 to colonize the rhizoplane is a precondition for plant growth promotion. Using a GFP-tagged derivative (Fan et al. 2011, 2012a, b), the fate of bacterial root colonization was recently studied. It ruled out that the bacterium behaves distinctly in colonizing root surfaces of different plants. In contrast to maize, FZB42 colonized preferentially root tips when colonizing *Arabidopsis thaliana* (Dietel et al. 2013). On duckweed, *Lemna minor*, FZB42 accumulated preferably along the grooves between epidermal cells of roots and in the concave spaces on ventral sides of fronds. In vitro studies performed with maize seedlings revealed that the segment within 2–8 cm distant from the basal site of the primary root was a most colonized region by FZB42. On the contrary, few bacterial cells could be observed within the range of 2 cm of root tip. In general, the green fluorescent FZB42 cells were decreasingly observed from the upper part of a root down to the root tip. Scanning electron microscopy confirmed the presence of FZB42 on root hairs, where the bacterial cells were usually associated with a wealth of presumed root exudates (Fan et al. 2012b). In lettuce, *Lactuca sativa*, seedlings, bacterial colonization occurred mainly on primary roots and root hairs as well as on root tips and adjacent border cells. Occurrence of labeled bacteria decreased toward the root tips of the lateral roots, and no colonization of the finer roots could be observed (Chowdhury et al. 2015a).

The rhizosphere competence of FZB42 was recently studied using a combination of field and greenhouse trials. FZB42 is able to effectively colonize the rhizosphere (7.45–6.61 Log_{10} CFU g^{-1} root dry mass) within the growth period of lettuce in the field. Our results demonstrated that FZB42 is able to effectively reduce the disease severity of bottom rot caused by soilborne pathogen *Rhizoctonia solani* on lettuce (Chowdhury et al. 2013).

From the practical point of view, it is interesting to note that the application mode of the biocontrol agent is a key factor for efficacy of FZB42. An effective suppression of *R. solani* was found only after two times application of FZB42 before and after transplanting. For the settlement of the inoculated strain in the rhizosphere in a sufficient high number, it might be important that the microflora in the rhizosphere of young plants is not yet stabilized (Berendsen et al. 2012).

As revealed by T-RFLP, application of FZB42, independent of its mode of application, did not shift the composition of rhizosphere bacterial community in a measurable extent – as also shown for *B. amyloliquefaciens* BNM122 on soybean (Correa et al. 2009). By contrast, inoculation with the pathogen did change the rhizosphere microbial community structure. In complementing that study, the effect of FZB42 and the pathogen *R. solani* on the microbial community of lettuce was more deeply analyzed by 454-amplicon sequencing focusing on presence of gamma-proteobacteria (Erlacher et al. 2014). Clear differences between plants infected by *R. solani* compared to non-inoculated healthy plants were found, corroborating the results obtained by T-RFLP. A significant increase in gamma-proteobacterial diver-

sity was detected in samples inoculated with the pathogen. However, together with FZB42, this increase was less distinct, suggesting a selective compensation of the impact of a pathogen on the indigenous plant-associated microbiome by FZB42. The number of DNA fragments corresponding to FZB42 in samples taken in vicinity of plant roots was steadily decreasing. After 5 weeks, still 55 % of the initial number of FZB42 DNA was traceable (Kröber et al. 2014).

8.3 Plant Growth Promotion

Although the ability of FZB42 to support growth of potatoes, maize, cotton, tobacco, leafy and fruiting vegetables, and ornamentals is well documented (Bochow et al. 2001; Yao et al. 2006; Guel et al. 2008; Burkett-Cadena et al. 2008; Chowdhury et al. 2013), the molecular reasons for the "biofertilizer" effect of beneficial plant-associated *Bacilli* are still not completely understood. However, we know that several factors are involved in the complex interplay between root-colonizing bacteria and plant:

8.3.1 Ability to Colonize and to Persist at Plant Roots

Ability to colonize and to persist at plant roots (see previous section). Their ability to suppress soilborne pathogens might positively affect the indigenous microbiome of the rhizosphere.

8.3.2 Stimulation of Plant Growth

Stimulation of plant growth by tryptophan-dependent synthesis of indole-3-acetic acid. Inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway led to reduction of both IAA concentration and plant growth-promoting activity in the respective mutant strains (Idris et al. 2007).

8.3.3 Volatiles

Volatiles, as 2,3-butanediol and 3-hydroxy-2-butanone (acetoin), released by *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a, were reported as enhancing plant growth (Ryu et al. 2003). To synthesize 2,3-butanediol, pyruvate is converted to acetolactate by the acetolactate synthase (AlsS), which is subsequently

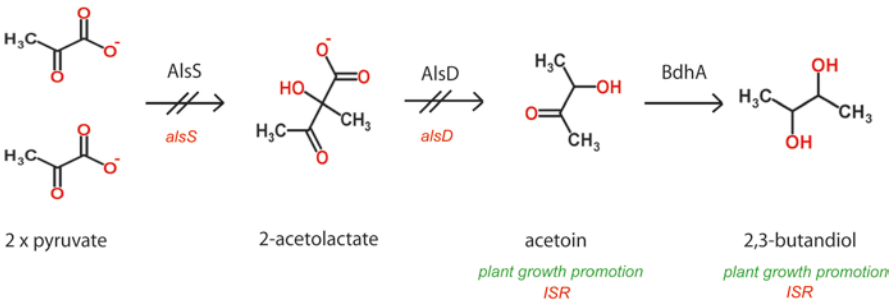

Fig. 8.1 Anaerobic and aerobic formation of 2,3-butanediol via acetoin involves acetolactate synthase and decarboxylase encoded by the *alsSD* operon. The *alsS* insertion mutation abolishes synthesis of 2,3-butandiol (Renna et al. 1993; Cruz-Ramos et al. 2000) (The figure is taken from Chowdhury et al. 2015b)

converted to acetoin by the acetolactate decarboxylase (AlsD) (Fig. 8.1). FZB42 mutant strains, deficient in synthesis of volatiles due to mutations interrupting the *alsD* and *alsS* genes, were found impaired in plant growth promotion (Borriss 2011).

8.3.4 Enhancement of Nutrient Availability

Enhancement of nutrient availability by phytase-producing bacteria. Soil phosphorous is an important macronutrient for plants. Improved phosphorous nutrition is achievable by "mobilization" of phosphorous fixed as insoluble organic phosphate in phytate (myo-inositol hexakisphosphate) by soil bacteria (Singh and Satyanarayana 2011). The extracellular 3(1)-phytase of the plant growth-promoting *B. amyloliquefaciens* FZB45 hydrolyzed phytate to D/L-Ins (1,2,4,5,6)P5 in vitro. A phytase-negative mutant strain, whose *phyA* gene was disrupted, did not stimulate plant growth under phosphate limitation (Idriss et al. 2002). Further experiments under field conditions revealed that FZB45 can only stimulate plant growth when phytate is present in soils, which are poor in soluble phosphate, suggesting that phytase acts only under certain conditions as plant growth stimulator (Ramirez and Kloepper 2010).

8.4 Biocontrol

Genome analysis revealed that nearly 10 % of the genome is devoted to synthesizing antimicrobial metabolites and their corresponding immunity genes (Chen et al. 2009b). FZB42 harbors 11 gene clusters involved in synthesis of antimicrobial compounds. Nine of them are involved in non-ribosomal synthesis of lipopeptides and

polyketides and two in conventional synthesis and modification of bacteriocin peptides. In addition, three further gene clusters contain genes mediating immunity against antimicrobial compounds produced by other related *Bacillus* strains (Table 8.2). This antibiotic arsenal makes *B. amyloliquefaciens* FZB42 and related *B. amyloliquefaciens plantarum* strains to an efficient microbial biopesticides, developed to control plant diseases (Borriss 2011).

For a long time, the plant protective activity of PGPR has been correlated with the potential to secrete a wide array of antibiotic compounds upon growth as planktonic cells in isolated cultures under laboratory conditions. We determined expression of the corresponding secondary metabolites by MALDI TOF mass spectrometry from FZB42 cultures grown in liquid Landy medium under laboratory conditions. Except the orphan *nrs* gene cluster, all expected bioactive compounds were synthesized in reasonable amounts, but the iron siderophore bacillibactin was detected only under iron-deprived conditions. In recent years, it became doubtful that synthesis of metabolites by the planktonic cells grown under laboratory conditions does correspond to their capability to produce those compounds also when grown in biofilm-related structures on the surface of plant tissues.

8.4.1 Lipopeptides, Bacillibactin, and Antifungal Activity

Five-gene cluster involved in non-ribosomal synthesis of cyclic lipopeptides and the iron siderophore bacillibactin were identified in the genome of FZB42 (Table 8.2). Three of the respective gene clusters were assigned for synthesis of surfactin, fengycin, and bacillomycin D. Bacillomycin D was identified as being the most powerful antifungal metabolite produced by FZB42 (Fig. 8.2). The heptapeptide moiety of bacillomycin D, belonging to the iturin family of cyclic lipopeptides (LP), is attached to a β-amino fatty acid chain of variable length (C_{14}–C_{17}). The peptide moiety of the heptapeptide surfactin is linked to a β-hydroxyl fatty acid (C_{12}–C_{16}), while the fengycin decapeptides are linked to a β-hydroxyl fatty acid chain (C_{14}–C_{18}). Their synthesis is performed by multimodular peptide synthetases and depends on a functional phospho-panthenyl transferase (Sfp) which transfers 4′-phosphopantheteine from coenzyme A to the carrier proteins during non-ribosomal synthesis.

Within the last few years, Ongena and coworkers performed pioneering work for elucidating antibiotic production *in planta* using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI). They investigated antibiotic production in a gnotobiotic system in which the plantlet and the associated *B. amyloliquefaciens* S499, a close relative of FZB42, were growing on a gelified medium covering the MALDI target plate. Under these conditions, S499 grows as biofilm on the surface of the plant roots, allowing exact assays of secondary metabolites in the vicinity of root surface. Surfactins were detected in the root environment in much higher relative amounts, which are representing more than 90 % of the whole LP production, and their synthesis is rapidly progressing during early biofilm formation.

Table 8.2 Genes and gene cluster encoding for secondary metabolites and immunity against bacteriocin in FZB42. The table is taken from Chowdhury et al. (2015b) with modifications

Gene cluster	From	To	Size	Metabolite	Effect against	Reference
Sfp-dependent non-ribosomal synthesis of lipopeptides						
srfABCD, aat,334,ycx,CycxD,sfp,yczE	342,618	368,776	32.0 kb	Surfactin	Virus	Koumoutsi et al. (2004)
bmyCBAD	1,871,172	1,908,422	39.7 kb	Bacillomycin D	Fungi	Koumoutsi et al. (2004)
fenABCDE	1,931,328	1,968,997	38.2 kb	Fengycin	Fungi	Koumoutsi et al. (2004)
nrsABCDEF	2,868,410	2,885,927	15.0 kb	Orphan NRP1	Unknown, siderophore?	Chen et al. (2007)
dhbABCDEF	3,021,250	3,032,970	12.8 kb	Bacillibactin	Iron deficiency, siderophore	Chen et al. (2007)
Sfp-dependent non-ribosomal synthesis of polyketides						
mlnABCDEFGHI	1,391,841	1,445,094	53.9 kb	Macrolactin	Bacteria	Schneider et al. (2007)
baeBCDE,acpK,baeGHIJLMNRS	1,700,345	1,701,022	74.3 kb	Bacillaene	Bacteria	Chen et al. (2006)
dfnAYXBCDEFGHIJKLM	2,276,743	2,346,266	71.1 kb	Difficidin	Bacteria	Chen et al. (2006)
Sfp-independent non-ribosomal synthesis						
bacABCDE,ywfG	3,593,877	3,599,784	6.9 kb	Bacilysin	Bacteria	Chen et al. (2009a)
Ribosomal synthesis of modified peptides (bacteriocins)						
pznFKGHIAJCDBEL	726,469	736,360	9.96 kb	Plantazolicin	Gram-positive bacteria	Scholz et al. (2011)
acnBACDEF	3,044,506	3,048,678	4.49 kb	Amylocyclicin	Closely related bacteria	Scholz et al. (2014)
Immunity, but no synthesis genes						
mrsK2R2FGE	3,769,734	3,774,552	4.82 kb	Mersacidin	Resistance against Y2	He et al. (2012)
bceBASR	2,856,835	2,861,322	4.49 kb	Bacitracin	Resistance against *B. cereus*	Unpubl. results
spaKREF	3,210,423	3,214,712	4.29 kb	Subtilin	Resistance against *B. subtilis*	Unpubl. results

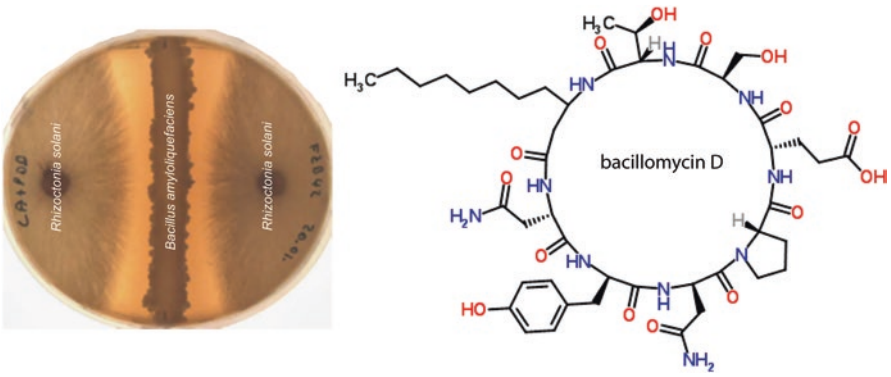

Fig. 8.2 Effect of FZB42 on *Rhizoctonia solani*. A clear inhibition zone indicating growth suppression of the fungal pathogen is visible on agar plates simultaneously inoculated with both microbes. Bacillomycin D was detected as the only prominent compound by MALDI TOF mass spectrometry of samples taken from the surface of the agar plate within the inhibition zone (The figure is taken from Chowdhury et al. 2015b with slight modifications)

By contrast, synthesis of iturin and fengycin was delayed until the end of the aggressive phase of colonization (Nihorimbere et al. 2012; Debois et al. 2014). Earlier experiments performed with FZB42 colonizing duckweed (*Lemna minor*) plantlets corroborated that surfactin is the most prominent compound which could be detected by MALDI TOF MS in the plant-bacteria system (Idris et al. 2007). Using a gnotobiotic quartz sand system consisting of lettuce plants, the beneficial bacterium FZB42, and the pathogen *R. solani*, it was demonstrated by using alternative techniques (e.g., Fourier transform ion cyclotron mass spectrometry) that lipopeptides were detectable in the order surfactin > bacillomycin D > fengycin at the plant-bacteria interface (Chowdhury et al. 2015a).

An early surfactin secretion could be of biological relevance since this lipopeptide, although less fungitoxic then iturins and fengycins, is essential for moving on tissues (Kinsinger et al. 2003) and for matrix formation in biofilms (Hofemeister et al. 2004; Lopez et al. 2009a, b). Considering the relative low amounts of the fungitoxic iturins and fengycins in vicinity of plant roots, it might be concluded that their biocontrol effect is possibly less important than expected. The same is true for the iron siderophore bacillibactin, which could not be detected under the conditions of the artificial plant-bacteria associations applied in these studies.

8.4.2 Polyketides, Bacilysin, and Bacteriocins Direct Antibacterial Activity

The polyketides, non-ribosomally synthesized by FZB42 (Chen et al. 2006; Schneider et al. 2007), have been extensively reviewed previously (Chen et al. 2009b, c; Borriss 2013). The three gene clusters encoding the modularly

organized polyketide synthases (PKS) for synthesis of bacillaene, macrolactin, and difficidin cover nearly 200 kb and are the largest ones, which are occurring in the FZB42 genome (Table 8.2). Difficidin is the most effective antibacterial compound produced by FZB42^T, but also macrolactin and bacillaene possess antibacterial activity. Difficidin is efficient in suppressing plant pathogenic bacterium *Erwinia amylovora*, which causes fire blight disease in orchard trees (Chen et al. 2009a).

Another product of non-ribosomal synthesis, the dipeptide bacilysin consisting of anticapsin and alanine moieties, was found as also being involved in suppression of *Erwinia amylovora*. By contrast to the lipopeptides and polyketides mentioned above, bacilysin synthesis is not dependent on the Sfp PP-transferase. A mutant strain CH3, with a disruption of the *sfp* gene and unable to produce any polyketide or lipopeptide, was still able to synthesize bacilysin and to suppress *E. amylovora* (Chen et al. 2009a). Recent experiments, performed by the group of Xuewen Gao, Nanjing Agriculture University, demonstrated that bacilysin, despite difficidin, is efficient in suppressing *Microcystis aeruginosa*, the main causative agent of cyanobacterial bloom in lakes and rivers (Wu et al. 2015a). However, corroborating these results in field trials has to be done. Until now, polyketides and bacilysin have not been detected in plants colonized by *B. amyloliquefaciens* (Debois et al. 2014).

Antimicrobial peptides, ribosomally synthesized as linear precursor peptides, remained unknown in *B. amyloliquefaciens plantarum* for a long time with one remarkable exception: mersacidin, a B-type lantibiotic, was detected in *Bacillus* sp HIL Y85 (Chatterjee et al. 1992). The strain HIL Y85 was later classified as being *B. amyloliquefaciens plantarum* (Herzner et al. 2011). Nowadays, mersacidin production was also detected in *B. amyloliquefaciens* B9601-Y2 (He et al. 2012). Genes involved in mersacidin self-protection reside also in the genome of FZB42. Transfer of mersacidin biosynthesis genes from HIL Y85 resulted in efficient mersacidin production by the surrogate strain constructed from the FZB42 host (Herzner et al. 2011).

Another representative of the type B lantibiotics, amylolysin from *B. amyloliquefaciens* GA1, was recently described. These lantibiotics are active on an array of Gram-positive bacteria, including *Listeria* spp. and methicillin-resistant *S. aureus* by interacting with the membrane lipid II (Arguelles Arias et al. 2013).

The driving force in our search for ribosomally synthesized peptides in FZB42 was the finding that the FZB42 mutant RS06, which is deficient in the Sfp-dependent synthesis of lipopeptides and polyketides and in the Sfp-independent bacilysin production (Chen et al. 2009a), still produced an antibacterial substance active against *Bacillus subtilis* HB0042. In fact, a metabolite (cpd1335) with a molecular mass of $[M+H]^+ = 1336$ Da was assigned by MALDI TOF MS in FZB42 and in RS06, as well. The compound was named plantazolicin, and the respective gene cluster pzn consisting of 12 genes was identified by cassette mutagenesis. Plantazolicin was characterized as a highly modified peptide undergoing several steps of modification

after synthesis. It ruled out that it is a thiazole/oxazole-modified microcin (TOMM) resembling microcin B17 and streptolysin S. Plantazolicin displayed antibacterial activity toward closely related Gram-positive bacteria. Due to its extensive degree of modification, Pzn is highly protected from premature degradation by peptidases within the plant rhizosphere (Scholz et al. 2011). Remarkably, human pathogen *Bacillus anthracis* was found sensitive against PZN and underwent massive lysis at 4 μg mL^{-1} (Molohon et al. 2011). The exact structures of plantazolicin A and B were elucidated, unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide (Kalyon et al. 2011).

By transposon mutagenesis of the FZB42 mutant strain RS06, we identified a hitherto unknown gene cluster involved in synthesis and posttranslational processing of a novel circular bacteriocin, named amylocyclicin (Fig. 8.3). It ruled out that amylocyclicin inhibits growth of bacterial strains closely related to FZB42 suggesting that this bacteriocin might have a function in competing with other *Bacillus* strains attracted to the plant rhizosphere (Scholz et al. 2014).

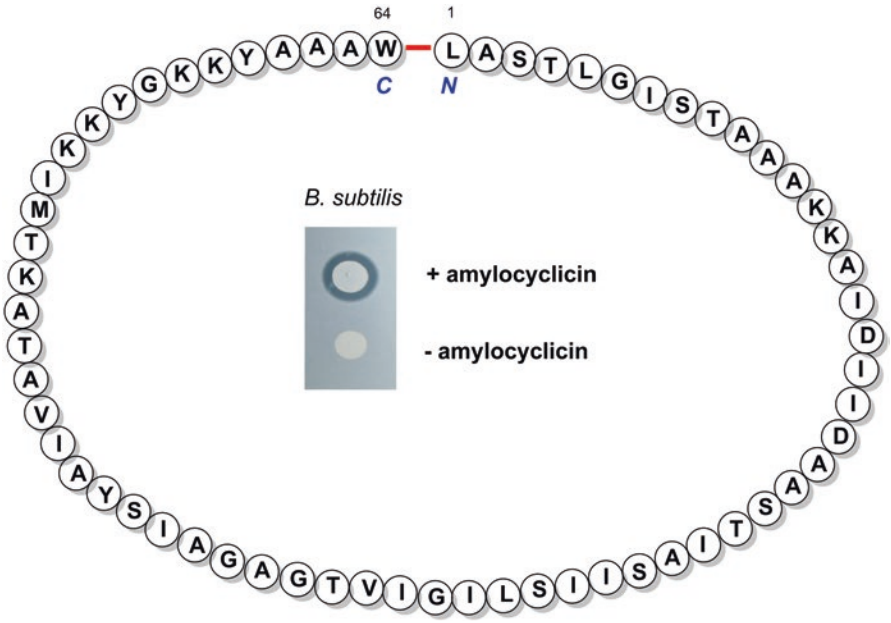

Fig. 8.3 The structure of the mature bacteriocin amylocyclicin bearing a head-to-tail cyclization of L_1 and W_{64}. Amylocyclicin effect on a related *B. subtilis* strain without immunity against the bacteriocin was demonstrated by a spot-on-lawn test performed with a amylocyclicin producing (*top*) and nonproducing strain (*bottom*) (The figure is taken from Chowdhury et al. 2015b)

8.4.2.1 Nematicidal Activity Is Directed by Plantazolicin

Parasitic nematodes of plants are important plant pathogens that represent a significant financial burden on agriculture. The annual losses in agriculture resulting from this pest amounted to $125 billion worldwide in past years (Sasser and Freckman 1987; Oka et al. 2000). Chemical insecticides of nonselective nature possessing rapid nematicidal effects are widely used as control measures against these pathogens. However, the potential negative impact on the environment and ineffectiveness after prolonged use have led to banning or restricting of the use of most chemical nematicides. Therefore, identification of safe and effective nematicides is urgently needed, and biocontrol measures have recently been given much attention as viable options (Xia et al. 2011). BioNem® prepared from *Bacillus firmus* GB-126 (Table 8.1) was proven for its efficiency in greenhouse and field trials. The numbers of nematode females, eggs, and vermiform life stages at the end of the growing season decreased in the presence of the biocontrol agent, and the cotton yields were similar to those from aldicarb, the chemical nematicide standard; however, the molecular reason for this effect remained unknown (Castillo et al. 2013).

FZB42 has been shown to reduce nematode eggs in roots, juvenile worms in soil, and plant galls on tomato (Burkett-Cadena et al. 2008). In order to identify specific nematicide-related genes, a random transposon insertion library of FZB42 was screened for relevant genes involved in nematicidal activity, and – surprisingly – a gene within the *pzn* gene cluster was identified as a pathogenic factor against nematodes. Further experiments revealed that PZN displayed a moderate nematicidal activity (Liu et al. 2013).

8.4.3 *Induced Systemic Resistance Is Triggered by Plant Growth-Promoting* Bacilli

Except surfactin, concentration of antifungal lipopeptides determined *in planta* was found relatively low. Moreover, antibacterial polyketides were not detected so far in vicinity of plant roots colonized by PGPR *Bacilli* (Debois et al. 2014). Therefore, it is tempting to speculate that induced systemic resistance (ISR) is a main factor for suppressing plant pathogens by PGPR *Bacilli*. ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon 1997). This activation is distinct from systemic acquired resistance (SAR) in which the response is triggered by pathogenic microorganisms associated with the aerial portions of the plant. Selected *Bacillus* PGPR strains emit volatiles (VOCs) that can elicit plant defenses. Exposure to VOCs consisting of 2,3-butanediol and acetoin (3-hydroxy-2-butanone) from PGPR *Bacillus amyloliquefaciens* activates ISR in *Arabidopsis* seedlings (Ryu et al. 2004). *Arabidopsis thaliana* plants exposed to *Bacillus subtilis* strain FB17 result in reduced disease severity against *Pseudomonas syringae* compared to plants without FB17 treatment. Exogenous application of

acetoin triggers ISR and protects plants against the pathogen in the aerial parts, while 2,3-butanediol did not (Rudrappa et al. 2010). In this context, it is worth to mention that expression of AlsS of FZB42 involved in synthesis of acetoin (Fig. 8.1) was triggered in presence of maize root exudate (Kierul et al. unpublished), suggesting that root exudates play a role in eliciting of acetoin biosynthesis in FZB42. It is known that some of the plant metabolites present in root exudates, such as organic acids, trigger the *alsSD* operon (Rudrappa et al. 2010). *B. amyloliquefaciens* FZB24 and FZB42 applied to tobacco roots led to a reduction of tobacco mosaic virus symptoms visible on tobacco leaves and to decreasing amounts of virus proteins present in leaf tissues. Due to spatial distance between beneficial bacterium and the pathogen, plant ISR, stimulated by the rhizobacterium, might be responsible for this effect (Wang et al. 2009).

The induction of ISR when treated with PGPRs is mediated primarily through plant signaling molecules such as jasmonic acid (JA), a lipoxygenase pathway product, and ethylene (ET). Salicylic acid (SA) appears to be a critical plant messenger of pathogen exposure and disease resistance in systemic acquired resistance (SAR) (Durner et al. 1997). ISR restricts pathogen multiplication and disease progression through a SA/ET and NPR1-dependent mechanism. In order to determine the signaling pathways triggered by FZB42, the expression of several marker genes in lettuce plants, exposed to FZB42 and the pathogenic fungus *Rhizoctonia solani*, was analyzed by quantitative real time (RT)-PCR (Chowdhury et al. 2015a). In absence of the pathogen, FZB42 increased expression of PR1 (pathogenesis protein 1, SA marker gene) and PDF1.2 (defensin, JA/ET marker gene), suggesting that SA and ET pathways are involved in upregulating defense response by ISR in lettuce. A similar result was obtained previously, when *Arabidopsis* plantlets were inoculated with *Bacillus subtilis* FB17 and acetoin (Rudrappa et al. 2010). In simultaneous presence of FZB42 and the pathogen *R. solani*, PDF1.2 expression was dramatically enhanced, suggesting a synergistic activation of the JA/ET pathway, while the SA pathway – as indicated by a decreased expression of PR-1 – was suppressed in presence of both antagonists.

It was found that the circular lipopeptides surfactin and fengycin can act as elicitors of host plant immunity and contribute to increased resistance toward further pathogenesis ingress in bean and tomato plants (Raaijmakers et al. 2010). In bean, purified fengycins and surfactins provided a significant ISR-mediated protective effect on bean plants against the fungal pathogen *Botrytis cinerea*, similar to the one induced by living cells of the producing strain *B. amyloliquefaciens* S499 (Ongena et al. 2007).

We found (Chowdhury et al. 2015a) that the dramatic increase of the defensin 1.2 gene (PDF1.2) expression in simultaneous presence of both antagonists occurred only when wild-type cells of FZB42 were applied. Mutant strains deficient in synthesis of surfactin, fengycin, or acetoin did not stimulate expression of the JA/ET pathway, suggesting that cyclic lipopeptides and acetoin contribute together to the ISR plant response triggered by FZB42.

8.5 Conclusion

An increasing amount of data has been accumulated in course of the last years, suggesting that the antibiome expressed during the plant-associated state of PGPR *Bacilli* does not necessarily reflect the vast genetic arsenal devoted to the formation of lipopeptides, polyketides, and bacteriocins, which has been elucidated, for example, in the *B. amyloliquefaciens plantarum* FZB42 genome. Obviously, there is a large discrepancy in gene expression of the planktonic cells growing in liquid laboratory cultures and cells growing as biofilms on plant tissue surfaces. Except cyclic lipopeptides, no other bioactive compounds such as polyketides were detected in samples taken from the vicinity of plant roots colonized by PGPR *B. amyloliquefaciens* (Debois et al. 2014). Interestingly, surfactin has multiple biological functions in motility, biofilm formation, and cell to cell signaling, but is less efficient in direct suppressing of other competing microbes than other lipopeptides or polyketides, and was by far the most prominent compound occurring in the plant rhizosphere, previously being inoculated by PGPR *B. amyloliquefaciens*. For this reason, I conclude that the direct effects exerted by the array of secondary metabolites encoded by the *Bacillus* genome might not be as important and that the biocontrol effects exerted by that Gram-positive bacteria are mainly due to other more indirect effects. I assume that under field conditions, the stimulating effects on plant ISR are more important than direct biocontrol by secreted secondary metabolites. In case of *Bacilli*, it is very likely that ISR stimulation is a multifactorial process dependent on several compounds produced by the rhizobacteria. Candidate compounds are surfactin, and volatiles, especially acetoin and 2,3-butanediol (Fig. 8.4), since mutants of FZB42, deficient in synthesis of these compounds, were found unable to protect plants from pathogens. Moreover, high expression of defensin, indicating the JA/ET pathway in ISR, was not found when the mutant strains were applied to the plant.

These findings are important for future strategies for screening of powerful PGPR and BC strains. It is known for long time that high efficiency in suppressing fungal or bacterial pathogens do not necessarily reflect the potential of these selected strains for their performance under field conditions. Novel screening procedures have to be developed for functional tests under more appropriate conditions, either directly on plants or at least under conditions allowing biofilm formation on artificial surfaces. However, performance under field conditions remains the most important criterion.

Taken together, the beneficial effect of *Bacillus* PGPR depends – besides their rhizosphere competence – on at least three main factors:

1. Stimulation of plant ISR by bacterial metabolites produced in vicinity of plant roots. Volatiles, such as acetoin and 2,3-butanediol, contribute not only to ISR but have a direct plant growth-promoting effect, while surfactin is important in the early stage of colonization and biofilm formation. In addition, surfactin strengthens the plant ISR response, which suppresses growth of fungal, bacterial, viral, and other plant pathogens.

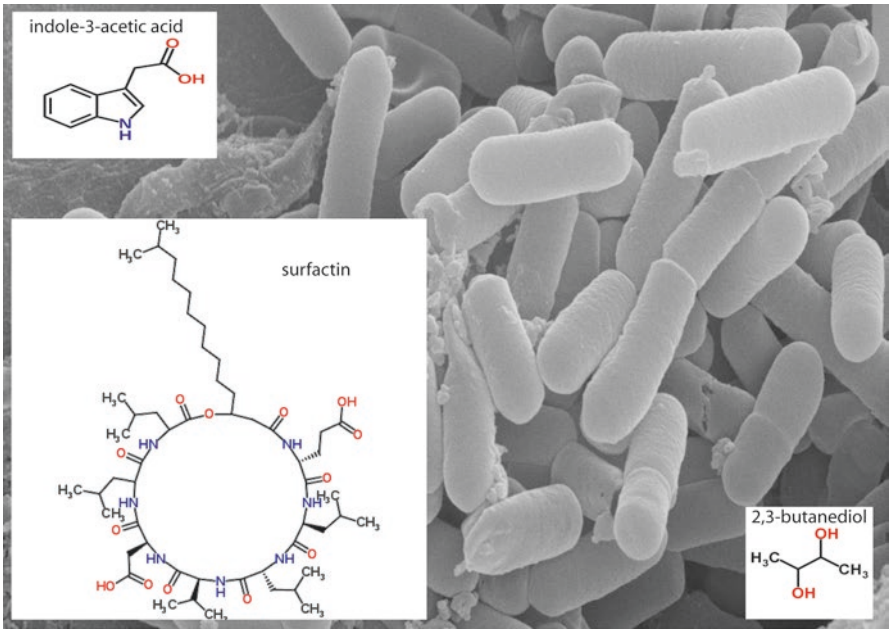

Fig. 8.4 Scanning electron microscopy of FZB42 cells colonizing *Arabidopsis thaliana* roots. Important compounds as surfactin, indole-3-acetic acid, and 2,3-butanediol, which are formed when growing on root surfaces (*in planta*), are indicated

2. Direct antifungal action by secondary metabolites, such as iturins (e.g., bacillomycin D) and fengycins, secreted into the rhizosphere. However, the suppressing effect exerted by such compounds might be relatively weak, since the amount of such compounds in vicinity of plant root was found relatively low. Until now, antibacterial compounds, such as polyketides, were not detected in this environment.
3. Application of PGPR *Bacilli*, as FZB42, might compensate, at least in part, undesired changes in the composition of the plant microbiome, caused by the presence of pathogens, as *R. solani*.

Without doubt, other features of PGPR, as production of plant hormones, and making available fixed macro- and micronutrients for plant nutrition, contribute also to the beneficial effect exerted by these microbes, but could not be appropriately treated in this review due to space limitation.

Acknowledgments Many of the recent data, reported in this review, have been obtained in close collaboration with the Helmholtz Center in Munich in frame of the PATHCONTROL project and the laboratory of Yuewen Gao, Nanjing Agricultural University, China, in frame of a Chinese Collaborative project, financially supported by the BMBF, the German Ministry of Education and Research. I thank especially Soumitra Paul Chowdhury, Anton Hartmann, Joachim Vater, Liming Wu, Xuewen Gao, and Ben Fan (Nanjing Forestry University) for fruitful collaboration during the last years.

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Chapter 9
Application of *Bacillus* spp. for Sustainable Cultivation of Potato (*Solanum tuberosum* L.) and the Benefits

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Abstract Potato is a staple crop in 130 countries worldwide, ranking fourth in production after rice, maize, and wheat. It is also an important crop which holds promise for food to millions of people especially in developing countries. But the production of potato is hindered by many phytopathogenic fungal and bacterial diseases that cause considerable loss to potato production in field. Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and induce an increase in plants growth. Among the mechanisms by which PGPR exert beneficial effects on plants are facilitating the uptake of nutrients such as phosphorus via phosphate solubilization, synthesizing stimulatory phytohormones like indole-3-acetic acid (IAA). *Bacillus* is one of the most commonly reported PGPR genera, as it has the advantage of being able to form endospores which confers them high stability as biofertilizers or biofungicides, which are resistant to heat, desiccation, organic solvents, and UV irradiation, and to produce various biologically active metabolites in addition to their abundance in soil. The ability to produce cell wall-degrading enzymes like protease, chitinase, and ß-1,3-glucanase and the production of secondary metabolites such as siderophore are other important criteria for understanding the mechanism responsible for biological control attributes of these organisms. Other mechanisms like competition for nutrients and induction of systemic resistance in plants are also involved. In spite of the benefits, application of *Bacillus* in potato cultivation is not well established. In this article, application of *Bacillus* for the management of potato diseases, and other benefits with potential for use in the future to improve potato crop, has been discussed.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_9

9.1 Introduction

Potato (*Solanum tuberosum* L.) is one of the most significant crops growing all over the world. As per the statistics provided by the Food and Agriculture Organization of the United Nations, the total world potato production is estimated at 364,808,768 tonnes in 2012 (FAOSTAT 2014), where China was the top producer followed by India. The potato was introduced in Europe and Asia at the end of the sixteenth century, and from the nineteenth century, it became one of the most important crops worldwide. It was considered a staple food for low- and middle-class families. The Food and Agricultural Organization (FAO) of the United Nations had declared 2008 as the International Year of Potato, and this raises the awareness of the importance of the potato and of agriculture in general in addressing issues of global concern including hunger, poverty, and threats to the environment. The top five potato seed-producing countries (1a) and countries with high potato yield (1b) during the last 10 years are given in Fig. 9.1. The cultivation of potato is affected by different biotic and abiotic stresses. Biotic stresses include mainly pest and disease-causing phyto-pathogens, and the estimates for actual losses due to pathogens, viruses, animal

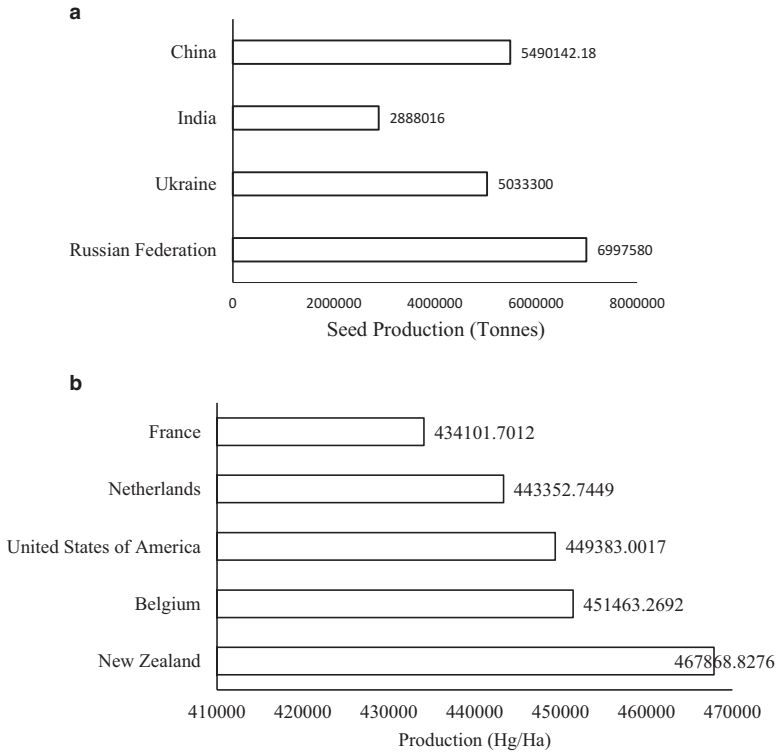

Fig. 9.1 (**a**) Top potato-seed producers during 10 years (2004–2013) (*Data source: FAOSTAT). (**b**) Values of total yield for 10 years (2004–2013) (*Data source: FAOSTAT)

pests, and weeds worldwide are approximately 14, 7, 11, and 8 %, respectively. Almost 75 % of attainable potato production could be lost to pests without crop protection (Oerke 2006).

In soil, plants interact with numerous microbes with both beneficial and harmful activities, and thus a wide range of microorganisms are present on its roots as well as on its stolon and tubers of potato (Boyd 1972; Diallo et al. 2011). Among these, fungal diseases may cause severe economic losses during potato production worldwide. Thus, cumulative annual yield loss due to viral, bacterial, fungal, and pest attack to potato tuber and potato plant can reach approximately 22 %, which translates over 65 million tonnes (Rahman et al. 2012). The susceptibility of potato plant to diseases makes it difficult to grow for commercial production without appropriate control measures (Daayf et al. 2003). It is well known that severe famines in the past caused by the plant pathogenic oomycete *Phytophthora infestans* lead to deaths of more than one million in Ireland during "the great famine" between 1845 and 1849 (O'Neill 2009).

Sustainable agriculture demands optimum disease and pest management in a successful production system that does not depend only on harmful synthetic chemical pesticides but rather use biopreparations of various rhizospheric microorganisms possessing potential characteristics of biological control of plant pathogens (Abbas et al. 2013). It has been stated that the strains of the genus *Bacillus* are among the most commonly reported plant growth-promoting rhizobacteria (PGPR) with antagonistic activity toward several phytopathogens (Gustavo Santoyo et al. 2012). *Bacillus* spp., compared with other bacterial species, offer the great advantage to form endospores which are resistant to heat, desiccation, organic solvents, and UV irradiation and to produce various biologically active metabolites in addition to their abundance in soil (Sadfi et al. 2001; Jacobsen et al. 2004). *Bacillus* species may show their antagonistic effects by antibiosis, competition, or exploitation against the fungal pathogens which is subdivided into predation and direct parasitism (Pleban et al. 1997). With the advancement of awareness and research, the direct application of microorganisms to promote plant growth and to control plant pests is gaining popularity among farmers. This chapter attempts to compile the latest available information on optimum use of *Bacillus*, for the sustainable cultivation of potato, through exploiting its biological control and PGPR activities.

9.2 Fungal Diseases of Potato

Due to high susceptibility to different pathogenic fungi, potato plants are frequently infected by several of those causing severe diseases. These diseases are blights (*Phytophthora infestans*, *Alternaria solani*, and *Phoma* spp.), wart (*Synchytrium endobioticum*), powdery scab (*Spongospora subterranea*), watery wound rot (*Pythium ultimum*), gangrene (*Phoma exigua* var. *foveata*), pink rot (*Phytophthora erythroseptica*), silver scurf (*Helminthosporium solani*), dry rot (*Fusarium* spp.),

skin spot (*Polyscytalum pustulans*), black scurf (*Rhizoctonia solani*), wilt of potato (*Verticillium* sp.), and charcoal rot (*Macrophomina phaseolina*).

Fungal diseases of potato can be grouped into two distinct categories:

1. Foliar diseases (late blight and early blight are of major importance)
2. Soil- and tuber-borne diseases (black scurf, dry rots, warts, powdery scab, tuber rot, and pink rots are important)

9.2.1 Late Blight

Late blights of potato caused by *Phytophthora infestans* (Mont.) de Bary are one of the most devastating diseases of food crops. The disease appears every year in epiphytotic form in hilly regions, but in the plains, its intensity is low (Singh et al. 2002). It's reported that *Phytophthora infestans* can cause a great devastation of the crop, with the Irish famine of the 1840 as a consequence, where over one million people died and over 1.5 million people were forced to migrate to other parts of the world (Ristaino and Johnston 1999). This oomycete, *Phytophthora infestans*, which causes the disease late blight or necrotic tissue, appears especially at high relative humidity and in low-temperature areas, causing the death of leaves, stem, and tubers of the plant. In general, *Phytophthora infestans* can develop into late blight at particular temperature ranges and behaves as a polycyclic disease in the field, originating a disease progress curve which varies with the weather (Harrison 1992).

Generally, Cu-based fungicides are applied for the control of late blight. However, as the response of *P. infestans* is highly variable, and also, awareness against chemical fungicides in developed countries, bacteria-based formulations have been commercialized and being used. One of such products is Riyoplan, which is based on *Pseudomonas putida*, while another formulation significant to the topic of discussion here is Bectofit that is based on formulations with *Bacillus subtilis* (Ke-qiang and Forrer 2001).

9.2.2 Early Blight

The causal microbe of potato early blight is *Alternaria solani*. Usually symptoms of early blight occur on the tuber and foliage. The first symptoms appear on the leaves as brown or black lesions, which develop into yellow halo under favorable environmental conditions. Later, the lesions increase in size with dark pigmented concentric rings which leads to significant defoliation. The lesions become dry, and so it is not prone to attack of secondary organisms that may cause other tuber rots (Kemmitt 2002).

9.2.3 Black Scurf

It is a fungal disease caused by *Rhizoctonia solani* Kuhn. The symptom of the diseases is dark brown to black on the surface of potato which is the sclerotia of *Rhizoctonia solani*. Usually the shapes are irregular small, flat visible blotches, and raised lumps. It cannot penetrate the tuber skin. Stem canker is the most damaging component of the diseases, and it may lead to plant wilt and death (Wharton and Wood 2013). Black surf is a serious disease in country like Japan. *Pythium oligandrum* has been used as biocontrol agent (Ikeda et al. 2012).

9.2.4 Dry Rot

Fusarium dry rot is caused by several species including *Fusarium sambucinum* Fuckel, *F. culmorum* Sacc., and *F. oxysporum* Schlecht, under field and storage conditions (Recep et al. 2009). It occurs in warm temperatures with symptoms like dark, little sunken lesions with a margin of clear defined rot (The International Potato Center, 1996). This disease is economically significant as potato dry rot disease results in severe reduction of market value of seed tubers (El-Kot 2008). Recently, biological control of dry rot such as yeast, fungi, and bacteria has been reported to be an effective control under in vitro condition. *B. cepacia* strain OSU-7 was reported to have a significant effect in controlling dry rot of potato by the mechanism of antagonism both under in vitro and in vivo conditions (Recep et al. 2009).

9.2.5 Wart

This disease is spread all over the world including India that causes massive yield loss. This disease is caused by *Synchytrium endobioticum*, which is an obligate parasite. The soil may remain contaminated, as its spores can survive for more than 20 years. Usually it is associated with powdery scab diseases, and symptoms are like development of tumors of any size up to several centimeters on tubers, stems, and stolons belowground. The infection leads to proliferation of host cells, i.e., warty gall containing sporangia. Winter sporangia are formed as the galls may remain in soil for up to 40–50 years (Frank 2007). Since chemical control is not a sustainable and practical approach to the management of this pathogen, the development of cultivars resistant to the disease seems to be the appropriate control measure (Obidiegwu et al. 2014).

9.3 Biological Control of Fungal Diseases of Potato

Several strategies and mechanisms have been used for the control of phytopathogens causing severe potato diseases. Synthetic chemical-based fungicides were extensively used to control fungal potato diseases; however, they have deleterious effects on the surrounding environment. The use of synthetic fungicides and other methods was not found to be successful in eradicating these harmful pathogens. However, the use of microorganisms to biologically control the fungal diseases in potato is becoming a potential alternative to chemical treatments (Punja and Utkhede 2003), and this biocontrol of fungal plant pathogens with microorganisms has been studied for more than 70 years (Howell 2003).

The term biological control was first coined by Harry Smith of the University of California in relation to the biological control of insects. Biological control is widely quoted and defined as "the reduction in the amount of inocula or disease-producing activity of a pathogen accomplished by or through one or more organisms." Some rhizospheric bacteria (PGPR) have a beneficial agronomic effect on plant growth as well as antagonistic activity (Compant et al. 2005). The biocontrol agents can be divided into four main groups (Thakore 2006):

(a) Microorganisms (microbial pesticides)
(b) Other organisms (nematodes, insects) used to control pests
(c) Natural substances that are derived from living organisms (biochemical pesticides)
(d) Plant-incorporated protectants

Biopesticides have several advantages over chemical products used as pesticides. The biopesticides decompose more quickly in the environment and are generally less or nontoxic toward nontarget species (Thakore 2006). Among different biopesticides, microorganism-based products represent about 30 % of total sales and have a variety of applications. They are used against different soilborne, foliar, or post-harvest pathogens of potato. These biopesticides, more specifically microbial biopesticides, have advantages as compared to most other phytosanitary products, and the multiplicity of their ways of actions is globally based on competition for nutrients and space, direct antagonism of plant pathogen growth, and host plant immunization. Thus, biopesticides have potentials to directly reduce the incidence of plant diseases. In addition, some microbial products also have other positive effects on potato plants such as promoting plant growth and nutrition (biofertilizers and phytostimulators) and facilitate the interaction between the host plant and other beneficial organisms (Antoun and Prevost 2006). Though bacterial products represent the majority of the microorganism-based biopesticides, fungal biocontrol agents were also developed as efficient products (Shoresh et al. 2010).

Bacteria have been explored as biocontrol agents for plant diseases (Gerhardson 2002) and also as plant growth promoters and inducers of disease resistance (Bargabus et al. 2002; Bais et al. 2004). The use of antagonistic bacteria to control fungal diseases of potato was reported as a powerful strategy to suppress soilborne

pathogens due to their ability to antagonize the pathogen by multiple modes and also to effectively colonize the rhizosphere. Thus, bacterial species have been used in controlling fungal diseases and subsequently increasing the growth of potato plant. These microorganisms are capable of lysing chitin, a major component of the fungal cell wall, thus playing an important role in biological control of fungal pathogens. Fungi like *Trichoderma* and bacteria like *Bacillus*, *Serratia*, and *Alteromonas* were reported to have chitinolytic activity (Ashwini and Srividya 2014). Thus, rhizospheric bacteria are excellent agents to control soilborne pathogens of potato plant.

A diverse array of microbes is present in the rhizospheric environment. Plant growth-promoting rhizobacteria (PGPR) promote growth of crops including potato plants directly by contributing to the increase in plant growth, i.e., increasing uptake of nitrogen, iron through siderophore, and phosphorus and synthesis of phytohormones. It also enhances the growth of plants by controlling plant diseases. Thus, rhizospheric bacteria reduce disease and indirectly increase health of potato plants by the antagonistic activity against the phytopathogen as such by (1) suppressing the activity of the fungal phytopathogen via the production of antagonistic metabolites, i.e., antibiotics; (2) competing for nutrients on the tuber surface, essential for growth and survival of phytopathogens; and (3) inducing the host plant's systematic and localized defense systems (Haas and Keel 2003; Haas and Defago 2005). Thus, the biocontrol agents derived from bacteria are now being developed to reduce the usage of chemical fertilizers in potato fields (Nazim et al. 2008). However, the production of biocontrol compounds depends on a number of parameters such as physiological characters, taxonomical position, geographic location, soil composition, etc. Thus, a number of bacteria have to be screened in order to maximize the chance of finding an effective biocontrol agent with broad-spectrum antifungal activity to increase yield of potato. Many researchers have reported that different bacterial genera like *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Streptomyces*, etc. have significant capacity to produce antifungal bioactive metabolites (Raaijmakers et al. 2002).

El-Kot (2008) reported black scurf and dry rot disease control of potato by certain bioagents as *Trichoderma harzianum*, *Epicoccum* sp., *S. endus*, and an isolate of actinomycetes under greenhouse conditions. The isolates showed good antagonistic activity to suppress the growth of the pathogens *R. solani* and *F. sambucinum*. These treatments significantly decreased pre- and postemergence damping-off (that kill developing potato sprouts), chlorophyll content, and potato tuber yield compared with the untreated seed pieces. It was reported that as much as 28 rhizosphere-associated bacterial isolates were found with effective antagonism against *R. solani*, causal agent of potato black scurf. The selected bacterial isolates showed antagonistic activity against *R. solani* in vitro as well as in vivo, and promoted plant growth (Tariq et al. 2010). The efficacy of *Chaetomium globosum* as a biocontrol agent against the late blight pathogen *Phytophthora infestans* was evaluated in potato plants. The application of *C. globosum* resulted in greater tuber yield by reducing late blight infection in two field trials when compared to untreated control (Shanthiyaa et al. 2013). It was reported from Egypt that treating either healthy or infected tuber seeds prior to plantation with biocontrol agent biocine S2HA as

soaking or powdering had increased the potato yield compared with the untreated (Kabeil et al. 2008). Riyoplan (*Pseudomonas putida*) and Bectofit (*Bacillus subtilis*) were used in susceptible potato cv. Lorkh under lab condition in Russia and both biological preparations suppressed development of zoospores in vitro (Ke-qiang and Forrer 2001).

As already stated, the most important bacterial genus with antagonistic activity is probably *Bacillus*. *Bacillus* species were found to increase plant growth and cause lysis of fungal mycelia by colonizing the root surface. Ranjbariyan et al. (2011) reported around 80.0 % of the isolates showed antagonistic activity against fungal pathogen of potato plant in the study. *B. subtilis* is the most important species within *Bacillus* species, and to some extent other species such as *B. amyloliquefaciens* and *B. vallismortis* have been reported to produce a wide range of structurally related antimicrobial compounds. These strains of bacteria are usually isolated from the soil, in their natural habitat (Akhavan et al. 2007).

Bacillus spp. having plant growth-promoting traits such as IAA production, phosphate solubilization, nitrogen fixation, and biocontrol attributes like production of HCN, siderophore, hydrolytic enzymes, and antibiotics have been isolated from soybean (Senthil kumar et al. 2009) and thus can promote the growth of potato crop. Several other workers have also found the biocontrol activities of *Bacillus* against many common phytopathogens of potato (Chung et al. 2008; Gajbhiye et al. 2010). However, it is likely that the most effective biological control strains act via multiple mechanisms. Isolation of *Bacillus* species from the rhizosphere of different crops has widely been studied previously. Mehta et al. (2010) have also reported the presence of almost all plant growth-promoting attributes in *Bacillus circulans* MTCC 8983. In vitro inhibition of various phytopathogens of potato by *B. subtilis* ME488 has also been reported by Chung et al. (2008). In vitro IAA production by *Bacillus* spp. in significant amount has also been reported by Singh et al. (2008) and Mehta et al. (2010).

9.4 *Bacillus* for Control of Fungal Diseases

Bacterial genus, *Bacillus*, has been proved in controlling the fungal diseases. The *Bacillus* genus encompasses a large genetic biodiversity. *Bacilli* are present in an extremely diverse environments ranging from seawater to soil and are even found in extreme environments like hot springs (Hoch 1993). Members of the *Bacillus* genus are generally found in soil, and most of these bacteria have antagonistic activity and the ability to control different plant diseases. Earlier reports showed that they are capable of lysing chitin, which is a major constituent of the fungal cell wall, and therefore play an important role in biological control of fungal pathogens (Mitchell and Alexander 1962). *Bacillus* exhibit different mechanisms to control fungal pathogens and mainly produces different metabolites and lytic enzymes. Purified forms of such metabolites and lytic enzymes inhibit the mycelial growth of certain fungi (Basha and Ulaganathan 2002; Yu et al. 2002). The mechanisms for the

suppression of pathogens by *Bacillus* have primarily known to be caused by three following mechanisms that include mycoparasitism, competition for space and resources, and antibiosis. *Bacillus* is considered as the major sources of potential microbial biopesticides because it shows several valuable traits (Ongena and Jacques 2008). Especially, *B. subtilis* is a well-studied organism which facilitates their rational use. Secondly, the US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *Bacillus subtilis*, which is thus recognized nonpathogenic (Harwood and Wipat 1996). *Bacilli* are also relatively easy to produce industrially as they are not particularly exigent regarding nutritional sources. Many species of *Bacillus* such as *B. circulans*, *B. macerans*, and *B. polymyxa* showed biocontrol of potato diseases early blight and late blight (Abbas et al. 2013). Other species such as *Bacillus* sp. (S1), *B. subtilis* isolates BS2 and BS3, reduced wilting of potato caused by bacteria (Shekhawat et al. 1993). Also reports of Aliye et al. (2008) showed control of bacterial wilt by *B. subtilis* PFMRI.

Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogen growth, such as kanosamine or zwittermicin A from *B. cereus* (Emmert and Handelsman 1999). This group of bacteria displays almost all the mechanisms of biocontrol and biostimulation/fertilization reported to date. Moreover, one strain may often act through several mechanisms. This enables these bacteria to be effective in many conditions (variety of pathogens, plants, environmental conditions) as one mechanism may act instead of another. Their spore-forming ability also makes these bacteria some of the best candidates for developing efficient biopesticide products from a technological point of view (Piggot and Hilbert 2004). One of the most commonly used and well-studied organisms, the rhizobacterium *B. subtilis*, has an average of 4–5 % of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein 2005). Others have previously reported suppression of root diseases by *Bacillus* spp. both in greenhouse and field conditions (Akhtar et al. 2010). Remadi et al. (2006) observed 46 % and 60 % inhibition of *F. solani* and *F. graminearum*, respectively, infecting potato tubers following in vivo application of *Bacillus* sp. *B. subtilis* was also found to significantly reduce root rot disease caused by *F. solani* to a level of 76 % (Kim et al. 1994). Rhizobacterial isolates such as *Bacillus circulans*, *Bacillus macerans*, and *Bacillus polymyxa* were able to suppress fungal diseases antagonistically in the bioassay on potato dextrose agar, and these isolates were included in its biopreparation along with others which have been supplied for potato cultivation by Agro Food Company, Egypt (2008–2010) and Daltex Company, Egypt (2010/2011). *B. polymyxa* produces auxins and gibberellic acid and shows significant reduction of fungal incidence in potato plant (Abbas et al. 2013). Also, potato dry rot of tuber, which is a storage disease caused by *Fusarium roseum* var. *sambucinum*, was reported to be controlled by *Bacillus* spp. It was isolated from salty soils and inhibited the mycelial growth of *F. sambucinum* in vitro. Further, even the wounded tubers in early stage of dry rot development were recovered by *B. thuringiensis* strains, when applied in the right stage of growth under in vivo conditions (Sadfi et al. 2001).

Bacillus has the ability to disintegrate proteins with proteolytic activity. It proved that *Bacillus* strain produces an antimicrobial substance related to proteases that are involved in biocontrol traits (Kamensky et al. 2003). *Bacillus pumilus* is found to produce cellulase activity which can degrade the cellulosic wall of the phytopathogens, and thus it can serve as means in disease management of potato plant (Ariffin et al. 2006).

9.5 Antagonistic Mechanism Involved in Biocontrol of Fungal Diseases of Potato by *Bacillus*

Beneficial bacterial strains efficiently colonize leaf surfaces and root systems and their surrounding soil layer by taking benefits from the nutrients constantly released from roots or leaves of host plants. In return, they beneficially influence the plant by protecting it from infection by plant pathogens via three main mechanisms: competition for ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants (Cawoy et al. 2011). This interaction is of great importance toward the potential use of biopesticides efficiently.

Bacterial colonization and antagonistic activity in the environment is enabled by the production of bacterial allelochemicals that includes lytic enzymes, iron-chelating siderophores, and antibiotics (Compant et al. 2005). Knowledge of the mechanisms involved in the *Bacillus* spp. is important for genetic enhancement (Baker 1990). However, the mode of action of *Bacillus* spp. differs depending upon the strains. The understanding of the mechanism involved in the use of biocontrol agents to exert their protective effects is a prerequisite to their effective practical application. This knowledge allows their suitable selection, production, formulation, and use and facilitates registration procedures. The action mechanisms of numerous biocontrol agents have been studied but not fully elucidated. Various mechanisms, based mainly on competition for space and nutrients, mycoparasitism, antibiosis, or elicitation of plant defenses, are reported to contribute simultaneously or sequentially to the biocontrol properties of microorganisms (Janisiewicz and Korsten 2002).

Scanning electron microscopic studies revealed the nature of antagonism involved in the interaction between the isolated *Bacillus* strains and *F. solani*. A clear distortion of fungal mycelium following lysis and bursting of the hyphae was observed at the interaction zone in dual cultures. The burst sites were surrounded by bacteria, some of which were found attached to the hyphae (Pal and Mcspadden 2006). In general, there are several reviews available that describe the mechanisms of the antagonistic activity of organisms including competition for colonization in ecological niche, and micronutrients, or due to antibiosis by the production of antibiotics, toxins, or biosurfactants (Van Loon et al. 1998) or caused due to parasitism by the production of enzymes (e.g., glucanases and chitinases) which break down the pathogen cell wall (Cherin and Chet 2002; De Souza et al. 2003). Microorganisms

are known to produce multiple antibiotics which can suppress one or more pathogens (Haas and Defago 2005; Stein 2005; Ge et al. 2007). For instance, *Bacillus subtilis* produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturins, surfactins, and zwittermicin (Asaka and Shoda 1996; Stein 2005), and it secretes also hydrolytic enzymes, i.e., protease, glucanase (Cazorla et al. 2007), chitinase (Manjula et al. 2004), lipase, and amylase (Konsoula and Liakopoulou-Kyriakides 2006).

9.5.1 Competition for Niche and Nutrients

Competition for resources such as nutrients and oxygen occurs generally in soil among soil-inhabiting organisms. For biocontrol purpose, it occurs when the antagonist directly competes against pathogens for these resources. Competition for nutrients, especially for carbon, is assumed to be responsible for the well-known phenomenon of fungistasis, characterizing the inhibition of fungal spore germination in soil (Alabouvette et al. 2006). Apart from that, the competition for trace elements like iron, copper, zinc, manganese, etc. also occurs in soils between microorganisms. Some microorganisms (biocontrol agents) produce siderophores, low molecular weight compounds with high iron affinity solubilizers, and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms including fungal pathogen which cannot grow due to lack of this element (Haas and Defago 2005).

Bacterial strains with siderophore-producing ability enhance the chelation of the available iron in the rhizosphere and thus subsequently prevent the growth of other organisms including the pathogen (O'Sullivan et al. 1992). Trivedi et al. (2008) reported that the secretion of siderophore by *Bacillus* sp. under iron-limiting condition and subsequently its antifungal activity. This antifungal activity of bacterial isolates is because of the production of siderophore (Ahmad et al. 2008) and *B. subtilis* PFMI resulted in highest inhibition in vitro against the bacterial wilt of potato (Aliye et al. 2008). Adesina et al. (2007) have suggested that the mechanism involved during in vitro inhibition of *R. solani* and *F. oxysporum* infections in potato by *Bacillus* is due to siderophore production, antibiosis, or both.

9.5.2 Direct Inhibition by Allelochemicals

9.5.2.1 Enzymatic Activity by Chitinases

Chitinases are used as potential biocontrol agents for many fungal pathogens through its chitin degradation activity. Bacterial chitinases have different morphological effects on the fungal cell wall. The visible effects (of course, under the microscope) are inhibition of spore germination, bursting of spores and hyphal tips,

and germ tube elongation (Taechowisan et al. 2003). Chitinase enzymes occur in a wide range of organisms, which include bacteria, fungi, insects, and higher organisms like plants (Park et al. 1997). Various species of *Bacillus* have been shown to secrete chitinase, including *B. circulans*, *B. licheniformis*, *B. laterosporus*, *B. amyloliquefaciens*, *B. megaterium*, *B. pabuli*, *B. stearothermophilus*, *B. subtilis*, and *B. thuringiensis* (Park et al. 2000). Three strains of *B. thuringiensis* (IT, 10 T, and 55 T) and two isolates X16 of *B. cereus* and I32 of *B. licheniformis* were able to degrade colloidal chitin and were used for the control of potato dry rot and it contribute significantly by their antagonistic activity toward *F. roseum* var. *sambucinum* (Sadfi et al. 2001).

Chitinase causes antifungal activity in in vitro (Taira et al. 2002), and this can be implicated in plant resistance against fungal pathogens. The main fungal cell wall structural component, chitin, is attacked by chitinase (Sharma et al. 2011). Chitinase lyses the fungal cell wall and the component of insect exoskeleton and thus strengthen plant immune response against a variety of pathogens. The chitin polymer is hydrolyzed into N-acetylglucosamine by either endo or exo cleavages of the 1–3 and 1–4 bond (Aalten et al. 2000). Several of the *Bacillus* strains such as *Bacillus* sp. BGII, *Bacillus* sp. NCTU, and *Bacillus* sp. 13.26 are known to produce chitinases (Yuli et al. 2004). De la Vega et al. (2006) reported that a purified chitinase from *B. thuringiensis* subsp. *aizawai* exhibited lytic activity against the cell walls of six phytopathogenic fungi and inhibited the mycelial growth of *Fusarium* sp. and *Sclerotium rolfsii*. Organisms with chitinase can utilize chitin as a source of carbon and nitrogen (Driss et al. 2005). Gomaa (2012) showed that because of the presence of chitinase from *B. thuringiensis*, the growth rate decreases up to 45 % of several fungi including *Aspergillus flavus*, *A. niger*, *A. terreus*, *F. oxysporum*, *Ralstonia solanacearum*, and *Rhizopus* sp. Also, the inhibition was better than *Bacillus licheniformis*.

The production of chitinase is often enhanced by the addition of sugar substances and dried fungal mats to the colloidal chitin media of bacterial strains. In vitro, *B. thuringiensis* and *B. licheniformis* chitinases had potential for cell wall lysis of many phytopathogenic fungi tested (Park et al. 2000).

9.5.2.2 Inhibition by Antibiosis

Several strains of *Bacillus* spp. have been reported to control plant diseases caused by fungal pathogens. Strains of *Bacillus* spp. use different mechanisms of action, including antibiosis by means of antimicrobial peptides (AMPs) which have been implicated in the biocontrol of plant pathogens. Antibiotics produced by *Bacillus* spp. strains play an important role in biocontrol of fungal diseases, and different types of metabolite (volatile and nonvolatile) production under in vitro assays (volatile and diffusible metabolite) that selected *Bacillus* spp. may use multiple modes of action against fungal pathogens (Ramarathnam et al. 2007). Aliye et al. (2008) reported that *B. subtilis* PFMRI, *P. macerans* BS-DFS and PF9, and *P. fluorescens*

PF20 antagonistic activity under in vitro condition against bacterial wilt (*Ralstonia solanacearum*) of potato plant. The mechanism involved might be due to siderophore production, antibiosis, or both which implies that it could be used for bioprotection of potato in vivo.

A large number of antifungal peptides are reported to be produced from *Bacillus* species (Ongena and Jacques 2008) including lipopeptides like iturins A–E, fengycin, surfactin, and bacillomycins D, F, and L (Kim et al. 2010). These antifungal peptides reduce the growth of a large number of fungi, such as *Rhizoctonia*, *Fusarium*, *Aspergillus*, and *Penicillium* species (Ongena and Jacques 2008).

Bacillus Lipopeptides (LPs)

The cyclic lipopeptides of *Bacillus* are of the iturin (such as bacillomycin D/F/L/Lc, iturin A/C, and mycosubtilin), fengycin (fengycin A/B and plipastatin A/B), and surfactin (halobacillin, pumilacidin, and surfactin) classes, which all share a common structure consisting of a lipid tail linked to a short cyclic peptide (Gong et al. 2015).

Bacillus lipopeptides (LPs) are synthesized non-ribosomally via large multienzymes (non-ribosomal peptide synthetases (NRPSs)) or hybrid polyketide synthases. These modular proteins are responsible for the biosynthesis of several hundred bioactive compounds (Walsh 2004). The three families of *Bacillus* lipopeptides – surfactins, iturins, and fengycins – were mostly studied for their antagonistic activity for a wide range of potential phytopathogens, including bacteria, fungi, and oomycetes.

The surfactin family demonstrates structural variants, but all members are heptapeptides interlinked with a hydroxy fatty acid to form a cyclic lactone ring structure. These compounds are powerful biosurfactants with exceptional emulsifying and foaming properties. Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers and can thus interfere with biological membrane integrity in a dose-dependent manner. Surfactins show antibacterial properties in vitro and could be involved in biocontrol functions in the rhizosphere. This disease control was associated with the production of inhibitory quantities of surfactin at the root level (Ongena and Jacques 2008). Three large open reading frames (ORFs) coding for surfactin synthetases are designated as srfA-A, srfA-B, and srfA-C (Peypoux et al. 1999).

Another lipopolypeptide family iturin has different variants like iturins A and C; bacillomycins D, F, L, and LC; and mycosubtilin which were described as the seven main variants within this family. They are heptapeptides linked to a b-amino fatty acid chain with a length of 14–17 carbons. These compounds have strong hemolytic activity, but the biological activity is different than surfactins. They result in strong antifungal action against a wide variety of yeast and fungi, but only limited antibacterial and no antiviral activities (Phae et al. 1990). This fungitoxicity of iturins certainly depends on their membrane permeabilization properties (Bonmatin et al. 2003).

However, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda et al. 2005). Iturin is also known to disturb the cytoplasmic membranes of yeast cells, causing leakage of K ions and other vital constituents in parallel with the death of yeast cells (Ongena and Jacques 2008). Mycosubtilin (the most active form in iturin family) produced by *B. subtilis* was strongly active against different yeast species but inactive against *Aspergillus* spp. (Fickers et al. 2009). Nasir and Besson (2011) reported that mycosubtilin displayed a preferential affinity to cholesterol (the main sterol in animal membranes) over ergosterol (the main fungal sterol).

The third family of LPs comprises fengycins A and B, which are also called plipastatins. Fengycins are less hemolytic than iturins and surfactins but retain a strong fungitoxic activity, specifically against filamentous fungi (Koumoutsi et al. 2004). The action of the fengycins is less well known compared with other LPs, but they also readily interact with lipid layers and to some extent retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way (Deleu et al. 2005). Although Zhao et al. (2013) reported that fengycin has no obvious effects on the morphology or cell structure of *F. oxysporum* and also have no effect on yeast as well (Vanittanakom et al. 1986); the fengycin often caused pore formation of the membranes through all-or-none mechanism; low concentrations of fengycin showed no effect on the membrane, whereas at high enough concentrations it caused large sustainable pores, allowing for the complete efflux of intercellular contents of affected cells (Falardeau et al. 2013). Tan et al. (2013) reported a novel protein with a relative molecular mass of 38708.67 Da and isoelectric point (pI) of 5.63 having the antagonistic activity against different fungal pathogens. The approach of using antifungal protein from *Bacillus subtilis* B25 to inhibit plant pathogens such as *Fusarium oxysporum* f. sp. *cubense*, *Alternaria solani*, *Corynespora cassiicola*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides* is found to be effective, and it distorted the fungal spores.

Antifungal Activity of Zwittermicin

Zwittermicin is another chemical class of antibiotic having diverse biological activities produced by *B. cereus* UW85 (Emmert et al. 2004). It is one of the molecules of interest to agricultural industry as it can suppress plant disease by protecting plants from the attack of oomycetes (Zhou et al. 2008). Potato tuber infection caused by *Fusarium roseum* var. sambucinum was found to be controlled by *Bacillus cereus* X16. Further study indicated that biologically active peptidic nature of antibiotic extracts from *Bacillus cereus* X16 might be the reason for the inhibition of mycelial growth under in vitro and in vivo conditions (Sadfi et al. 2002).

Antifungal Volatile Compounds

Different microbial antagonist strains including both bacteria and fungi capable of producing both nonvolatile compounds and volatile compounds (VOCs) exhibit strong inhibitory activity against plant pathogens. Yuan et al. (2012) reported that *B. amyloliquefaciens* NJN-6 produced around 36 volatile compounds (VOCs) that inhibit the growth and spore germination of *F. oxysporum* f. sp. *cubense*. The release of these VOCs by soil microbes has been reported to promote plant growth (Ryu et al. 2003), and display nematicidal activity (Gu et al. 2007), and induce systemic resistance in crops (Farag et al. 2006).

HCN as Antimetabolites

HCN is a volatile organic compound that is closely associated with antifungal activity, and rhizobacteria have been reported to produce HCN (Ahamad et al. 2008). However, the production of HCN by beneficial rhizobacteria is sometimes believed to be contradictory to plant growth promotion, as it may be associated with deleterious effect on potato plants (Alstrom and Burns 1989). However, there are reports that confirm HCN production helps indirectly in increasing the yield of potato (Sharma 2004). HCN-producing *Bacillus* species such as *Bacillus* (BPR1, BPR2, and BPR7) were reported to be antagonistic against phytopathogens such as *Macrophomina phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *R. solani*, and *Colletotrichum* sp. in vitro (Kumar et al. 2012). Recently, we isolated several *Bacillus* spp. from rhizosphere of potato; several of these were found to be HCN positive and showed antifungal activity against *Fusarium* spp. isolated from diseased potato plants (Fig. 9.2).

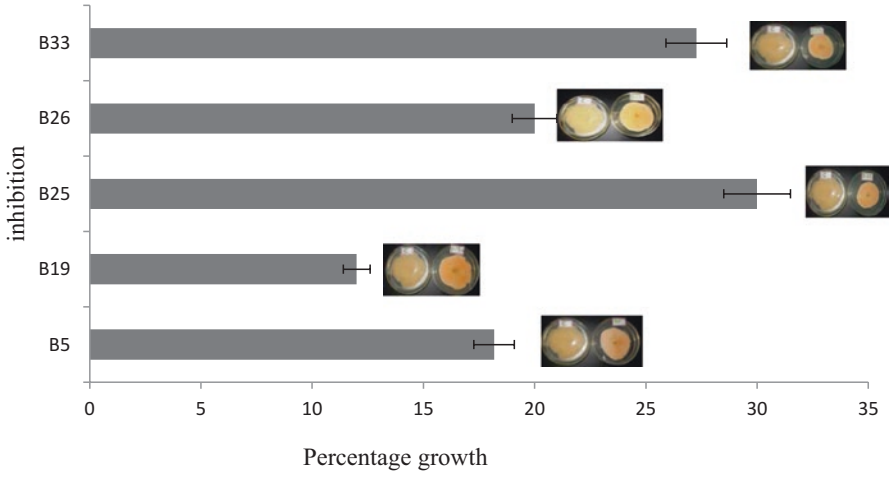

Fig. 9.2 Effect of bacterial volatile compounds on growth inhibition of *Fusarium* sp. isolated from infested potato plants (unpublished results). B5 has been identified as *Bacillus* sp.

9.5.3 Induction of Plant Resistance Mechanisms

Induced system resistance (ISR) and systemic acquired resistance are resistance mechanisms employed by many plants in response to the attack by pathogens. The stimulation of the plant immune system represents one of the most newly discovered aspects of plant-microbe interactions (Bakker et al. 2007). Some isolates are indeed able to reduce disease through the stimulation of a primed state in the host plant which allows an accelerated activation of defense responses upon pathogen attack, leading to an enhanced resistance against the attack encountered (Conrath et al. 2006). ISR is globally viewed as a three-step process involving sequentially (i) the perception by plant cells of elicitors produced by the inducing agents that initiates the phenomenon, (ii) signal transduction that is needed to propagate the induced state systemically through the plant, and (iii) expression of defense mechanisms that limit or inhibit pathogen penetration into the host tissues (Bakker et al. 2007).

Heller and Gessler first demonstrated the induction of systemic resistance against *Phytophthora infestans* (1986) in tomato and Doke et al. (1987) in potato. Cohen et al. (1993) used five kinds of unsaturated fatty acids to induce systemic resistance against *P. infestans*. Arachidonic acid and eicosapentaenoic acid applied to leaves of potato plants at a concentration of 1 mg per plant could efficiently induce up to 94–97 % protection in leaves. Jasmonic acid (JA) and jasmonic methyl ester (JME) were applied as foliar sprays to potato or tomato plants to protect them against a challenge infection with *P. infestans*. Miller and Lyong (1995) reported the effects of salicyclic acid to elicit resistance on detached potato leaves. Mucharrowah et al. (1995) demonstrated the effects of arachidonic acid (AA) on tuber disks of potato causing a hypersensitive reaction to the infection of *P. infestans*. A biogenic elicitor representing a lipoglycoprotein complex isolated from mycelium of *P. infestans* showed interesting activity after treatment of the potato tuber , as all three layers of the tuber acquired resistance to compatible races of the pathogen, and this was retained for several months. Production of phytoalexins in potato tuber was induced by phenylureas (Ke-qiang and Forrer 2001).

The list of bacteria identified as ISR inducers has grown rapidly over the last two decades and includes Gram-negative bacteria such as members of the *Pseudomonas* and *Serratia* genera but also Gram-positive bacteria and more particularly *Bacillus* spp. (Kloepper et al. 2004). ISR-based biocontrol strategies are promising, and some trials were successfully performed under field conditions. Volatile compounds such as 2,3-butanediol (Ryu et al. 2004) and lipopeptides are the sole compounds formed by *Bacillus* spp. that were identified as elicitors of ISR. The potential of *Bacillus* cLPs as plant resistance inducers was demonstrated by testing pure surfactins and fengycins that provided a significant induced protective effect similar to the one induced by living cells of the producing strain (*B. amyloliquefaciens* S499).

ISR is a mechanism of biocontrol by which the plant defense systems are induced against the pathogen infection mainly by bacteria in the group PGPR (Kloepper et al. 2004). The biotic stimuli provided by many different PGPRs and association of it with plant roots elicit a steady state of defense or ISR in plants to resist the

pathogens. Different signaling molecules such as jasmonic acid (JA), salicylic acid (SA), ethylene (EA), etc. regulate the interconnected signaling pathways which induce defense mechanisms in plants (Choudhary et al. 2007). ISR is induced by the attributes of nonpathogenic bacteria or fungi, e.g., lipopolysaccharides, siderophores, and salicylic acid (SA), which bring about changes in cell wall composition and in the production of pathogenesis-related proteins by the plant (Loon et al. 1998). For example, treatment of potato leaves by the polyunsaturated fatty arachidonic acid induces local synthesis of SA and confers systemic resistance to *P. infestans* and *Alternaria solani* (Coquoz et al. 1995). This powerful mechanism controls aerial as well as telluric ones. ISR by *Bacillus* spp. have been reported by the strains of the species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* by the elicitation of something followed by significant reductions in the severity of various diseases on a diversity of host (Kloepper et al. 2004). For example *B. subtilis* GB03 and a hypovirulent *R. solani* Rhs1A1 (HV-Rs) successfully reduced all the soilborne diseases of potato such as stem and stolon canker (20–38 %), blank scurf (30–58 %), and common scab (10–34 %) (Larkin and Tavantzis 2013). *Pectobacterium* spp. are one of the pathogens causing diseases to potato, and *Bacillus subtilis* strains were tested for controlling the diseases, and it revealed reduced maceration symptom in planta (Sharga and Lyon 1998; Aliye et al. 2008).

9.6 Application of *Bacillus* for the Sustainable Cultivation of Potato

Bacterial soft rot of potato is one of the major postharvest diseases of potato, and the disease mainly occurs in the countries where proper storage is not available. *Bacillus* spp. were utilized successfully in reducing bacterial soft rot of potato by controlling the causal organism of this disease, *E. carotovora*, before or after the infection took place (Rahman et al. 2012). The biocontrol agent *B. subtilis* was found more effective in reducing the soft rot of potato when compared with fluorescent pseudomonads (Abd-El-Khair and Karima 2007). The use of chemicals is generally not recommended to control soft rot of potato due to the high risk of the residual effect of toxic chemicals that might be hazardous to consumers' health (Rahman et al. 2012). *Bacillus* has proven to be a useful biocontrol agent in several diseases problems where high yielding or highly resistant cultivars are not available (Jacobsen et al. 2004). Rapidly growing bacteria such as *B. polymyxa* FU6 have also been found to reduce the soft rot of decaying stored potato tubers. These strains may be applied very conveniently as a spore preparation during seeding time (Aspiras and Cruz 1986).

Crop yield and tuber quality of potato is affected by fungal diseases such as black scurf and dry rot caused by *Rhizoctonia solani* and *Fusarium solani*, respectively. These diseases are among the most common potato fungal diseases, and growth of

Rhizoctonia solani was reduced by 69–91 % by antagonistic *Bacillus* strains (Calvo et al. 2010). Some of the diseases of potato and its biocontrol by the genus *Bacillus* are given in Table 9.1.

Bacillus strains which are named as Bac Ne 2C and Bac 20M1 showed high inhibition against the fungi *Rhizoctonia solani and Fusarium solani*, the two most common potato fungal pathogens. *B. amyloliquefaciens* displayed antagonistic activity against common scab of potato caused by *Streptomyces* spp. under in vitro condition (Meng et al. 2012).

Plant growth stage was the most important factor influencing the composition of the bacterial communities in roots and surface-sterilized stems of potato plants (Andreote et al. 2010). Cultural controls for soilborne diseases are very important in potato production, as other methods alone offer insufficient disease control (Sweetingham 1996). The planting of certified seed potatoes is one of the most common cultural control methods for minimizing potential disease. Studies often compare disease suppression by a variety of biocontrol organisms. In a greenhouse screening of 28 biocontrol organisms by Brewer and Larkin (2005), the best control of stem canker was achieved by *B. subtilis*, and authors also demonstrated that a combination of *B. subtilis* and *Trichoderma virens* provided better control than either strain individually.

The presence of these *Bacillus* strains in the rhizosphere of soil-grown potato was detected with a classical fluorescence microscope and a confocal laser scanning microscope (CLSM) (Krzyzanowska et al. 2012). About 40 % of the stem canker and black scurf of potato are reduced over multiple trials by using *Bacillus subtilis* GB03, and thus it is the most effective agent for control of *Rhizoctonia disease. B. subtilis* has been found to have disease-suppressive activities against several plant pathogens. About 500 ml/pot of *B. subtilis* is applied to every plant by preparing its inoculum (Larkin and Tavantzis 2013). Products such as RhizoVital 42 li and RhizoVital 42 TB, Sonata, etc. are some of the bioagents of *Bacillus amyloliquefaciens* and *Bacillus pumilus*, respectively, that are commercially available.

9.7 Conclusion

Microbial inoculants with antagonistic properties toward soilborne plant pathogens of agricultural crops have a potential to replace chemical pesticides that can be harmful to the environment and human health. The discussions here clearly emphasize that *Bacillus* spp. hold immense potential for biocontrol of fungal pathogens and sustainable cultivation of potato, as now, importance of environment-friendly plant protection methods is greatly emphasized in the modern agriculture (Rahman et al. 2012). Biocontrol may be best addressed by using combinations of successful antagonists or by applying beneficial organisms within effective crop rotations. Biocontrol organisms like *B. subtilis* in combination with other microbes demonstrated somewhat better control of stem canker of potato suggesting that this approach may provide improved biocontrol efficacy. Other species of *Bacillus* such

Table 9.1 Application of *Bacillus* species, used for biocontrol of potato diseases

S. No.	Biocontrol agent	Diseases	Causative pathogen	Mechanisms	References
1.	*Bacillus* spp.	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora Ecc P-138*	Antagonism	Rahman et al. (2012)
2.	*Bacillus* spp. such as *B. amyloliquefaciens*, *B. subtilis*	Black scurf and dry rot	*Rhizoctonia solani*, *Fusarium solani*	More than one mechanism	Pamelo Calvo et al. (2010) Somani and Arora (2010)
3.	*Bacillus subtilis GB03*(*Bsub*) and hypovirulent *Rhizoctonia solani* *Rhs* 1A1	*Rhizoctonia* canker and black scurf	*Rhizoctonia solani* Kuhn and *Streptomyces scabiei*	–	Larkin and Tavantzis (2013)
4.	*Bacillus* *polymyxa*	Bacterial wilt/brown rot	*Ralstonia solanacearum*	Pre-emptive colonization	Aspiras and de la Cruz (1986)
5.	*Bacillus subtilis* and *Paenibacillus macerans*	Bacterial wilt	*Ralstonia solanacearum*	Induced systemic resistance	Aliye et al. (2008)
6.	*Bacillus subtilis*	Brown rot	*Ralstonia solanacearum*	Antibiosis	Sharga and Lyon (1998)
7.	*Bacillus subtilis* MB 73/2	–	*Dickeya* and *Pectobacterium*	Antagonism	Krzyzanowska et al. (2012)
8.	*Bacillus* spp.	*Fusarium* dry rot	*Fusarium* spp. mainly *Fusarium roseum* var. *sambucinum*	Antagonism	Sadfi et al. (2002)
9.	*Bacillus* spp. Such as *B. cepacia* strain OSU- 7	*Fusarium* dry rot	*Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium sambucinum*	Antagonism	Recep et al. (2009)
10.	*B. thuringiensis 55T* *B. cereus*	*Fusarium* dry rot	*Fusarium roseum* var. *sambucinum*	More than one mechanism	Sadfi et al. (2002)
11.	*B. amyloliquefaciens*	*Common scab*	*Streptomyces* spp.	Antagonism	Meng et al. (2012)
12.	*Bacillus*	Late blight	*Phytophthora infestans*	Antagonism	Ke-qiang and Forrer (2001)
13.	*Bacillus circulans*, *Bacillus macerans*, and *Bacillus polymyxa*	Early blight	*Alternaria solani*	Antagonism	Abbas et al. (2013)

as *B. amyloliquefaciens*, *B. subtilis*, *B. subtilis* GB03 (Bsub), *B. polymyxa*, and *Paenibacillus macerans* are used to control potatoe diseases such as black scurf, dry rot, *Rhizoctonia* canker, brown rot, bacterial wilt, and *Fusarium* dry rot. Microbial biopesticides have great potential that is and should be even more used to make the future agriculture more sustainable. Multiple disease suppression mechanisms of *Bacillus* spp. including production of a wide range of diffusible metabolites as antibiotics, biosurfactants, and cell wall-degrading enzymes, elaborated in different studies, suggest that *Bacillus* should be exploited for better disease management in potato and made part of routine practice in potato cultivation, beyond of, looking just as an alternative strategy to chemicals (Sadfi et al. 2001).

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Chapter 10
Potential and Prospects of Aerobic Endospore-Forming Bacteria (AEFB) in Crop Production

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Abstract Members of *Bacillus* and the genera derived from it are an ubiquitous and important component of the agroecosystem. The diverse roles essayed by these bacteria in crop production range from nutrient cycling to protection of crops from various biotic and abiotic stress factors. The versatility and ecological fitness of this bacterial group have been attributed to its ability to form hardy endospores that help them tide over stress conditions and confers a survival advantage in the rhizosphere and related environmental niches, during unfavorable times. This chapter attempts to briefly explore the historical evolution of this group of bacteria from a two-species genus to the present-day *Bacillus* and the whole gamut of *Bacillus*-derived genera, both of which constitute the broader umbrella term, viz., aerobic endospore-forming bacteria (AEFB). The various functional facets of AEFBs in crop production and the ways and means to exploit them as functional bio-inoculants for the future are discussed.

10.1 Introduction

The genus *Bacillus* was established as early as 1872 by Ferdinand Cohn with two prominent and truly endospore-forming species, viz., *B. anthracis and B. subtilis*. The type strain of this genus is *B. subtilis* (Fritze 2004). The most striking original feature of this genus was its ability to form heat and desiccation-tolerant calcium dipicolinate-rich endospores that help it to tide over unfavorable growing environments. But subsequent midcourse taxonomical realignments that appeared in the Bergey's Manual of Determinative Bacteriology (Bergey et al. 1939; Buchanan and Gibbons 1974) resulted in the addition or deletion of several new species. Continuing this trend of addition/deletion, and factoring newer trends in bacterial taxonomy, at present the genus *Bacillus* has evolved a whole host of new genera known as *Bacillus*-derived genera, all of which posses the ability to form endospores.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_10

The agronomic significance of this group of bacteria is tied with its ubiquitous distribution in the rhizospheric and non-rhizospheric soils of almost all growing environments. By colonizing the immediate vicinity of plant roots and its interiors, they positively influence plant growth through a myriad of activities that includes nitrogen fixation, phosphate/zinc solubilization, secretion of plant growth-promoting substances (both volatile and nonvolatile), growth/development inhibition of several soilborne plant pathogenic fungi, and resistance induction against a variety of plant pathogens. Though several *Bacillus*-based formulations have been commercially utilized worldwide, *Bacillus* and its related genera are yet to gain the popularity of "fluorescent pseudomonads," which also colonize the same ecological niche. It would not be inappropriate to argue that considering the sheer size and distribution of this bacterial group, the quantum of effort that has gone into deciphering the beneficial role played by these organisms has been clearly disproportionate, perhaps with the exception of the insecticidal bacterium *Bacillus thuringiensis* and the plant growth-promoting bacterium *Bacillus subtilis*. In this milieu we shall discuss in brief the historical evolution of this genus and its potential in crop production systems. Since exhaustive literature is available on *Bacillus thuringiensis*, we shall exclude its insecticidal activity from the purview of this chapter.

10.2 Aerobic Endospore-Forming Bacteria (AEFB): A Historical Perspective

The origin of the genus *Bacillus* dates back to 1872, when it was first described by the German microbiologist Ferdinand Cohn. While the fifth edition of the Bergey's Manual of Determinative Bacteriology (Bergey et al. 1939) listed 146 species under the genus *Bacillus*, subsequent taxonomic developments reduced the number of species that were assigned, and the eighth edition of the Bergey's Manual of Determinative Bacteriology restricted the number of species to 22 (Buchanan and Gibbons 1974). A new paradigm in bacterial taxonomy started to emerge during 1980, when the "Approved Lists of Bacterial Names" was first published. This publication recognized endospore formation as a tool for grouping bacterial species and the 38 species of "Aerobic Endospore-Forming Bacteria" (AEFB) were recognized, of which 31 were assigned to the genus *Bacillus* and the rest to other genera (Skerman et al.1980). A subsequent important development was the creation of the genus *Paenibacillus* (*Paene* = almost), to accommodate over 30 species of facultative anaerobic endosporeforming, low G+C, Gram-positive bacilli. A defining feature of this genus is the ability of its ellipsoidal spores to swell the sporangia (Ash et al. 1993). Subsequent developments in DNA–DNA hybridization, sequencing of the 16S rRNA gene, and other molecular approaches have paved the way for a literal explosion in terms of novel endospore-forming genera and species that have since been described. The taxonomic outline to the second edition of the Bergey's Manual of Systematic Bacteriology, based on 16S rRNA gene similarities, includes under

order Bacillales three families, viz., *Caryophyllaceae*, *Listeriaceae*, and *Staphylococcaceae*, whose members do not form true endospores. Another feature of this edition is the inclusion of some species that do not form true endospores into families predominated by true endospore-forming species, prompting the authors to speculate that such bacteria would have lost their endospore-forming potential as a result of evolution (Garrity et al. 2004, 2005). In the backdrop of this dramatic reassignment of bacterial genera and species, the genus *Bacillus* and the several new genera of aerobic endospores forming bacteria that are commonly referred to as "*Bacillus*-derived genera" have acquired both a greater level of importance in both academic and application-oriented research. But in the field of agriculture, the role of AEFBs has largely remained restricted to the genus *Bacillus*, *Paenibacillus*, and to a limited extent, *Brevibacillus*. Hence, it is obvious that the utility and potential of this important group of bacteria remain to be fully elucidated and utilized in sustainable crop production.

10.3 Current Taxonomical Features of AEFBs

The volume three of the second edition of the Bergey's Manual of Systematic Bacteriology (Vos et al. 2009) accommodates true aerobic endospore-forming bacteria (AEFB), in phylum Firmicutes, class Bacilli, and order Bacillales. AEFBs are spread over the families Bacillaceae (15 genera), Alicyclobacillaceae (three genera), Paenibacillaceae (five genera), Planococcaceae (one genus), Sporolactobacillaceae (one genus), and Thermoactinomycetaceae (one genus), while the families *Caryophyllaceae*, *Listeriaceae*, and *Staphylococcaceae* do not posses any true endospore-forming bacterial genera. Subsequent to the publication of this grand treatise, several new genera and species continue to be validly published on a regular basis, thereby adding to the numerical, morphological, and physiological diversity of the AEFBs.

10.4 Plant Growth Promotion by AEFBs

Generally PGPRs affect plant growth by two mechanisms. Direct mechanisms involve secretion of phytohormones, improved nutrient availability by solubilization of nutrients, nitrogen fixation, etc. Indirect mechanisms include biological control of plant pathogens and other deleterious microorganisms by secretion of antibiotics, siderophores, hydrogen cyanide (HCN) production, and induction of systemic resistance in plants (Burdman et al. 2000). Some evidence has emerged on the utility of *Paenibacillus* strains in enhancing the porosity of soil and production of soil flocculants and biofilms on the root surface. Wheat inoculation experiments with *P. polymyxa* CF43 wild-type and a mutant strain deficient in the levansucrase *SacB* revealed that though both strains possessed equivalent root colonization

efficiency, C43 wild type increased the root adhering soil mass, while its deficient mutant failed to do so, suggesting that levan synthesis by *P. polymyxa* contributes directly to soil adherence at the plant roots (Bezzate et al. 2000). But such mechanisms require to be proved in diverse soil conditions. Table 10.1 is a non-exhaustive compilation of plant growth promotion by aerobic endospore-forming bacteria (AEFB). Individual plant growth promotion traits are discussed in detail in the ensuing sections.

10.4.1 Nutrient Solubilization/Diazotrophy

An important bacterial trait that has a direct influence on plant growth and development is bacterial mineral phosphate solubilization that results in increased phosphorus availability in the root zone. Bacterial-mediated phosphate solubilization has been mainly attributed to the activity of glucose dehydrogenase, a membrane-bound enzyme of bacterial origin that is involved in the direct oxidation of glucose to gluconic acid (Goldstein 1995). Subsequently gluconic acid is enzymatically converted to 2-ketogluconic acid and 2,5-diketogluconic acid. The 2-ketogluconic acid is more effective than gluconic acid in solubilizing phosphate (Kim et al. 2002). Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids in addition to the major organic acids (Jones 1998; Vazquez et al. 2000). Several *Bacillus* strains are reported to solubilize insoluble sources of phosphorus, the most important being *Bacillus subtilis*, *B. megaterium*, *B. circulans*, *B. coagulans*, *and Paenibacillus polymyxa* (Subbarao 1988; Kucey et al. 1989).

Since a significant portion of soil phosphorus exists in the organic form mainly as nucleotides and inositol phosphates, bacterial phosphatases and phytases play an important role in the release of such organic forms of phosphorus into the soil solution wherein they become available for plant uptake and utilization. Thermally stable phytase genes (*phy*) have been cloned from *Bacillus* sp. DS11 (Kim et al. 1998) and *B. subtilis* VTT E-68013 (Kerovuo et al. 1998). Neutral phytase genes have been cloned from *B. subtilis* and B. *licheniformis* (Tye et al. 2002). A *phyA* gene has been cloned from *B. amyloliquefaciens* strain FZB45. This strain was isolated from a group of bacilli having plant growth-promoting activity. It showed the highest extracellular phytase activity, and diluted culture filtrates of these strains stimulated growth of maize seedlings under limited phosphate in the presence of phytate. Culture filtrates obtained from a phytase-negative mutant strain, whose *phyA* gene was disrupted, did not stimulate plant growth. In addition, growth of maize seedlings was enhanced in the presence of purified phytase and the absence of culture filtrate (Idriss et al. 2002). These experiments provide strong evidence that phytase activity of AEFBs can be important for stimulating growth under limited P concentrations in soil.

Diazotrophy or the ability to fix atmospheric nitrogen is an important trait, which seems to be sporadically distributed among strains of the genera *Bacillus* and *Paenibacillus*. Another reason that can be attributed for the sporadic distribution of

Table 10.1 Plant growth promotion by AEFBs

AEFB	Role	References
Aneurinibacillus aneurinilyticus	Plant growth promotion traits and antagonistic activity	Chauhan et al. (2014)
Geobacillus sp.	Posses plant growth promotion traits under in vitro conditions	Das et al. (2014)
Oceanobacillus sp.	Growth promotion under sodic soil conditions	Kannan et al. (2014)
Bacillus amyloliquefaciens	Enhances soybean nodulation	Masciarelli et al. (2014)
Halobacillus sp.	Plant growth promotion under salinity and heavy metal stress	Desale et al. (2013)
Bacillus, *Brevibacillus*, *Lysinibacillus*, *Paenibacillus*, *Terribacillus*, and *Jeotgalibacillus*	Posses plant growth promotion traits under in vitro conditions	Kadyan et al. (2013)
B. amyloliquefaciens	Enhances the plant growth, nutrient assimilation, and yield of soybean	Sharma et al. (2013)
B. firmus strain NARS1	Promotes growth of *Cicer arietinum* at low temperatures	Khan and Patel (2007)
Bacillus spp. and *Paenibacillus* spp., *B. subtilis*, and *B. amyloliquefaciens*	Enhancement of plant growth by phytohormone production	Idris et al. (2007) Idris et al. (2004)
Brevibacillus choshinensis	IAA production under in vitro conditions	Poonguzhali et al. (2006)
B. megaterium var. phosphaticum and *B. polymyxa*	Enhanced plant growth in cotton and wheat	Khan et al. (2006), Gaur (1990), Zaidi and Khan (2005), Kundu and Gaur (1980)
Brevibacillus brevis	Enhanced plant tolerance to cadmium when co-inoculated with AM fungi	Vivas et al. (2005)
Bacillus spp.	Plant protection and growth promotion in cotton, peas, spruce and sweet corn	Berg et al. (2005b) and Shishido et al. (1999)
Brevibacillus brevis	Improvement in seedling growth parameters	Girish and Umesha (2005)
Paenibacillus polymyxa	Plant growth promotion	Timmusk (2003)
B. pumilus and B. licheniformis	Gibberellin production	Gutierrez-Manero et al. (2001)
P. azotofixans	Efficient nitrogen fixer prevalent in the rhizosphere of maize, sorghum, sugarcane, wheat, banana, and forage grasses	Rosado et al. (1998) and Seldin et al. (1998)
B. firmus	Increased seed yield in lentil (*Lens esculentus*) and black gram (*Vigna mungo*)	Tomar et al. (1993)

diazotrophs among AEFBs is the diminished interest in the discovery of novel diazotrophs among various bacterial groups. In one of the earliest reports on N fixation by *Bacillus*, three former *Bacillus* species, viz., *Paenibacillus azotofixans*, *P. macerans*, and *P. polymyxa*, were shown to fix nitrogen fixers, based on nitrogenase activities (Seldin et al. 1984). Later Xie et al. (1998) reported that *B. megaterium*, *B. cereus*, *B. pumilus*, *B. circulans*, *B. licheniformis*, *B. subtilis*, *B. brevis*, and *B. firmus* were able to fix atmospheric nitrogen. The nitrogen-fixing ability of *P. polymyxa* was demonstrated by Guemouri-Athmani et al. (2000), who measured the nitrogenase activity of some representative isolates of *P. polymyxa* recovered from Algerian soils by ARA. Their results showed that only 14 of the 23 strains tested were able to reduce acetylene. Later additions to the category of diazotrophs include *P. odorifer*, *P. graminis*, *P. peoriae*, and *P. brasilensis* (Berge et al. 2002; von der Weid et al. 2002). A fairly recent addition is the *P. riograndensis* isolated from the rhizosphere of *Triticum aestivum* (Beneduzi et al. 2010). *B. rhizosphaerae* is a novel diazotroph originally isolated from the rhizospheric soil of sugar cane (Madhaiyan et al. 2011). Though nitrogen fixation has been reported in certain strains of *Bacillus* and *Paenibacillus*, it seems unlikely that this trait is universally distributed across a species, and critical evidence attributing the observed plant growth promotion phenomena to N fixation by the bacterial inoculant is missing in most cases.

Another facet of plant nutrition that is addressed by AEFBs is siderophore-mediated iron nutrition. Chemically, siderophores are low molecular weight compounds that may be either of the catecholate or hydroxamate types, which form complexes with Fe^{2+} and render it available to crop plants (Leong 1986). Neilands (1986) reported the ability of several bacterial genera including *Bacillus* to produce siderophores at low iron levels, which was supported by the finding that a number of plants are capable of using rhizobacterial Fe–siderophore complexes as a means of obtaining Fe from soil (Hughes et al. 1992; Wang et al. 1993). *B. megaterium* ATCC 19213 produces two hydroxamate siderophores, viz., schizokinen and N-deoxyschizokinen, under iron-limited conditions, which also chelate aluminum in addition to their high affinity for ferric ions (Hu and Boyer 1996). In a study on siderophore production among the members of the *B. cereus* group, Wilson et al. (2006) reported that *B. cereus* ATCC 14579 excretes two catecholate siderophores, petrobactin (which contains 3,4-dihydroxybenzoyl moieties) and bacillibactin (which contains 2,3-dihydroxybenzoyl moieties). However, the insecticidal organism *B. thuringiensis* ATCC 33679 makes only bacillibactin. The siderophore system of *B. subtilis* has been studied in detail by Bsat et al. (1998), and it has been shown that there are three distinctly and distantly related Fur homologues (encoded by the genes *yqkL*, *yqfV*, and *ygaG*) and a DtxR homologue (encoded by *yqhN*). Paenibactin is a novel siderophore structurally similar to bacillibactin produced by *P. elgii* (Wen et al. 2011). Information on the nature and properties of siderophores produced by other AEFB species is scanty and requires to be deciphered, in the context of iron nutrition of plants.

10.4.2 Production of Plant Growth Hormones

Among the growth hormones of microbial origin that affect plant growth auxins, gibberellins and cytokinins are the most important. Auxin production has been proposed as a major means of attaining early growth promotion by Khalid et al. (2004), who observed a linear positive correlation between in vitro auxin production and increase in early growth parameters of inoculated wheat seeds. Auxin production is known to stimulate root development which results in better absorption of water and nutrients from the soil (Hoflich et al. 1994). Cytokinins are plant hormones which promote root growth and are thought to mediate environmental stresses from root to shoot and are produced by a variety of root inhabiting microbes, but a slight overproduction leads to retarded root development. Production of auxin- and cytokinin-like substances has been reported from a number of *Bacillus* and *Paenibacillus* strains (Arkhipova et al. 2005; Lal and Tabacchioni 2009; Lim and Kim 2009). Gibberellins belong to a group of plant growth hormones that are involved in the control of different physiological processes such as the stimulation of stem elongation by stimulating cell division and elongation, the stimulation of bolting/flowering in response to long days, the break of seed dormancy in some plants which require stratification or light to induce germination, the stimulation of enzyme production (α-amylase) in germinating cereal grains for mobilization of seed reserves, the induction of maleness in dioecious flowers (sex expression), the inducement of a parthenocarpic (seedless) fruit development, and the retardation of senescence in leaves and citrus fruits (Davies 1995). The production of GA_5, GA_8, GA_{34}, GA_{44}, and GA_{53} by strains of *B. cereus*, *B. macroides*, *and B. pumilus* was reported by Joo et al. (2005).This finding assumes significance since GAs were only known to be widely synthesized by fungi. Full-scan gas chromatography–mass spectrometry analyses on extracts of *B. pumilus* and *B. licheniformis*, isolated from the rhizosphere of alder (*Alnus glutinosa*), showed the presence of GA_1, GA_3, GA_4, and GA_{20}, in addition to the isomers 3-*epi*-GA_1 and *iso*-GA_3.

The revelation that AEFBs play a role in alleviating stresses on plants grown under unfavorable environments is a fairly recent development. In this realm, the role of the gaseous hormone ethylene and the enzyme ACC deaminase is crucial. Ethylene is a gaseous important hormone that is produced in the roots of most plants, while minute quantities of this hormone promote plant growth; it acquires deleterious propositions when produced in larger quantities. Certain bacterial strains which produce the enzyme ACC deaminase hydrolyze the precursor molecule of ethylene into α-ketobutyrate and ammonia, thereby pre-empting the accumulation of ethylene in root tissues. Though such bacteria are commonly encountered among pseudomonads, their distribution among AEBBs seems to be quite scarce. In one of the early reports on this aspect, *B. circulans*, *B. firmus*, and *B. globisporus* have been reported to produce ACC deaminase and promote canola growth (Ghosh et al. 2003). A later significant discovery was the isolation of a novel methylotrophic strain of *B. methylotrophicus* capable of producing ACC deaminase and promoting plant growth (Madhaiyan et al. 2010). Recently a consortium of *Bacillus cereus*,

Bacillus subtilis, and *Serratia marcescens* has been reported to induce drought tolerance in cucumber (Wang et al. 2012), while rhizospheric strains of *Bacillus* have been found to positively influence the chlorophyll and proline contents of sorghum grown under moisture stress conditions (Grover et al. 2013). The isolation of ACC deaminase producing *Bacillus* from tropical rice soils has added a new dimension in ACC deaminase production by AEFBs under flooded soil conditions (Bal et al. 2013).

10.4.3 Antibiotics and Other Antagonistic Traits

Biological control of plant pathogens by AEFBs is either mediated by the production of antibiotics; catabolic enzymes like proteases, chitinases, and glucanases (Priest 1993); volatile compounds (Chaurasia et al. 2005), and pathogen quorum quenching (Dong et al. 2001). From the available literature, it seems that siderophore production and niche exclusion which are two major biocontrol traits of fluorescent pseudomonads are not very widespread among AEFBs. But an interesting feature of the genera *Bacillus* and *Paenibacillus* is their ability to produce peptide antibiotics that may be either linear or cyclic in nature. The peptide molecules produced by different strains include bacillopeptins (Kajimura et al. 1995), fusaricidin group of peptides (Beatty and Jensen 2002), matacin (polymyxin M) (Martin et al. 2003), gavaserin and saltavalin (Pichard et al. 1995), iturin (Yoshida et al. 2001), and polymyxin B (Selim et al. 2005).

Lipopeptides are another group of antibiotic compounds most frequently produced by *Bacillus* species. Lipopeptides are amphiphilic compounds that share a common structure consisting of a lipid tail linked to a short cyclic oligopeptide. Lipopeptides are synthesized non-ribosomally by the action of non-ribosomal peptide synthetases (Finking and Marahiel 2004); hence, there is a remarkable heterogeneity in the type and sequence of amino acid residues, the nature of the peptide cyclization, and nature, length, and branching of the fatty acid chain. Depending on their amino acid sequence, they are classified into three families, viz., iturins, fengycins, and surfactins. The surfactins are powerful biosurfactants, which show antibacterial activity but posses limited fungal toxicity, while the iturins display strong antifungal action against a wide variety of yeasts and fungi and limited antibacterial activity, and the fengycins posses strong fungitoxic activity, specifically against filamentous fungi (Ongena and Jacques 2007). The biological control ability of various *Bacillus* strains has been attributed mostly to iturins and fengycins (Romero et al. 2007a; Arrebola et al. 2010). The amphiphilic structure of lipopeptides facilitates their interaction with biological membranes and induces pore formation, in the plasma membrane, leading to internal osmotic imbalance and widespread cytoplasmic disorganization in fungal cells (Romero et al. 2007b). Another interesting group of antimicrobial compounds produced by the *Bacillus* are the polyketides that have a wide range of biological activity (Chen et al. 2006). But these compounds are yet to find widespread use in agriculture.

Antibiotic production by soil bacteria is governed by several environmental factors, while physical factors such as temperature and soil moisture are known to affect antibiotic production; the pH is known to affect production as well as the activity of the produced antibiotic. Phosphate concentrations are known to affect the production of antibiotics by rhizobacteria, thereby raising questions on the adverse effects of the applied phosphate fertilizers (Raijmakers et al.2002). Besides environmental factors the plant genotype plays a major role in the proliferation and disease suppression activity of the applied biocontrol agents. An ideal biocontrol bacterium should be motile and aggressively colonize the roots, besides producing an antibiotic in sufficient quantities if the desired beneficial effects are to be realized. But AEFBs in general are thought to be less rhizocompetent than pseudomonads (Weller 2007), which is a probable reason for their limited exploitation as biological control agents. The success of antibiotic-mediated biological control by bacteria requires exhaustive screening procedures that can be both time and resource intensive. Developments in molecular biology have made available several cloned and sequenced antibiotic biosynthetic regulatory genes, which have facilitated the development of specific primers and probes that can be used for targeted detection of antibiotic producers from the rhizospheric and bulk soil. The *zmaR* gene that confers Zwittermicin resistance to the producer in strains of *B. cereus* is known to function as a reliable marker for the identification of *B. cereus* strains that produce the antibiotic zwittermicin (Raffel et al. 1996). A nonexhaustive compilation of the various antibiotics of agricultural importance produced by AEFBs is presented in Table 10.2.

The N-acylhomoserine lactone (AHL)-dependent mechanism in Gram negative bacteria helps them to sense their population densities and coordinate the expression of target genes and virulence factors, by a mechanism known as quorum sensing (QS). Certain oligopeptides along with substituted γ-butyrolactones, are the primary signal molecules found in Gram-positive bacteria (Faure et al. 2009). The universal pheromone LuxS is found in both Gram-positive and Gram-negative bacteria (Schauder et al. 2001). Quorum sensing plays a crucial role in the regulation of rhizospheric competence factors such as antibiotic production, horizontal gene transfer and control several functions that are directly or indirectly related to plant–microbe interactions (Whitehead et al. 2001). Several soil bacteria are able to interfere in this QS mechanism by the enzymatic degradation of AHLs, by a process known as quorum quenching (QQ), which involves various phenomena that lead to disruption of QS-regulated functions (Dong et al. 2007). Studies on *B. thuringiensis* have shown that many sub-species of this organism produce AHL-degrading enzymes like N-acyl homoserine lactone lactonases which open the lactone ring of AHLs, thereby attenuating bacterial virulence (Lee et al.2002). A potential AHL-degrading enzyme AiiA was identified in *B. thuringiensis* by Park et al. (2008), who observed that it could effectively attenuate the virulence factors of the Gram-negative bacterium *Erwinia carotovora* when present in the root system of pepper plant. Genetically modified plants which expressed AHL lactonase, AiiA of *Bacillus*, were found to be more resistant to *Pectobacterium carotovorum* infection compared to the wild-type plants (Dong et al. 2001). A significant reduction was observed in

Table 10.2 Antibiotics produced by AEFBs and their significance

AEFB species	Antibiotic	Significance	References
B. amyloliquefaciens	Bacillomycin D	Contributes to antifungal activity and biofilm formation	Xu et al. (2013)
B. subtilis	Fengycins, iturins, and surfactins	Growth suppression of *Aspergillus niger*, *Alternaria burnsii*, *Fusarium oxysporum*, *Cladosporium herbarum* 1112, *Candida albicans*	Pathak and Keharia (2014) and Pathak et al. (2012)
B. amyloliquefaciens CNU114001	Antifungal substance	Inhibits the mycelial growth of *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea*, and *Sclerotinia sclerotiorum*	Ji et al. (2013)
Brevibacillus brevis	Tostatidin	Strongly inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* in vitro	Song et al. (2011)
B. amyloliquefaciens strain A1Z	Produces iturin-like compounds	Active against *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, and *Fusarium oxysporum*	Kumar et al. (2011)
B. amyloliquefaciens GA1	Fengycin	Suppression of plant pathogenic fungi	Arguelles-Arias et al. (2009)
P. polymyxa E 681	Fusaricidin A	Peptide molecule with six amino acids	Choi et al. (2008)
Brevibacillus brevis	Gramicidin S	Active against *Botrytis cinerea*	Edwards and Seddon (2001)
P. polymyxa	Fusaricidin	Management of the nematode *Melodidogyne javanica*	Khan et al. (2008)
Brevibacillus brevis	Gramicidin S and Polymyxin B	Against *Botrytis* gray mold in strawberry	Haggag and Timmusk (2008)
B. licheniformis BC98	Antifungal molecule	Effective against *Magnaporthe grisea*	Tendulkar et al. (2007)
B. subtilis strains UMAF6614, UMAF6619, UMAF6639, and UMAF8561	Fengycins, Iturins	Control of the cucurbit powdery mildew pathogen *Podosphaera fusca*	Romero et al. (2007a, b)
B. subtilis BS 49	Bacillomycin D (an iturin peptide)	Suppresses head blight of wheat caused by *Fusarium graminearum* and *Sclerotinia sclerotiorum* and blackleg of canola by *Aspergillus flavus*	Ramarathnam et al. (2007)
B. subtilis ATCC 6633	Mycosubtilin	Reduction of seedling infection by *Pythium aphanidermatum*	Lecle're et al. (2005)
B. subtilis BBG 100	Mycosubtilin	Over production of mycosubtilin	Leclere et al. (2005)

(continued)

Table 10.2 (continued)

AEFB species	Antibiotic	Significance	References
B. subtilis M4	Fengycin-type lipopeptides	Suppresses bean damping-off caused by *Pythium ultimum* and apple gray mold caused by *Botrytis cinerea*	Ongena et al. (2005)
B. subtilis	Surfactin	Effective against *Pseudomonas syringae*	Bais et al. (2004)
B. subtilis strain AU195	Bacillomycin D (an iturin)	Active against *Aspergillus flavus*	Moyne et al. (2001)
B. subtilis RB14	Iturin A	Active against *Rhizoctonia solani*	Asaka and Shoda (1996)
B. cereus UW85	Kanosamine	Suppresses damping-off of alfalfa caused by *Phytophthora medicaginis*	Milner et al. (1996)
Bacillus pumilus strain CL 45	A novel nonpeptide antibiotic	Suppresses several fungi	Leifet et al. (1995)
B. cereus UW85	Zwittermicin A	Suppresses damping-off of alfalfa caused by *Phytophthora medicaginis*	Silo-Suh et al. (1994)
B. subtilis	Iturin	Suppresses peach brown rot	Gueldner et al. (1988)
B. subtilis CL 27	Two novel peptide antibiotics	Suppresses *Botrytis cinerea* and *Alternaria brassicicola*	Leifert et al. (1995)
B. subtilis ATCC 6633	Rhizoctin A	Suppression of *Rhizoctonia solani*	Kugler et al. (1990)

the severity of soft rot of potato caused by *Pectobacterium carotovorum* and crown gall of tomato caused by *A. tumefaciens*, when genetically modified strains encoding the lactonase gene AiiA of *Bacillus* sp. A24 or *P. fluorescens* P3 were applied, indicating the possible role of QQ in plant disease suppression (Molina et al. 2003). But studies conducted on the QQ mechanism have been largely carried out under in vitro conditions, and it needs to be explored as to how this mechanism can be extrapolated under field conditions where the number of variables is huge and population densities vary spatially and temporally.

A relatively less explored aspect of AEFB is the nature and properties of different volatile compounds produced, since volatile compounds produced by *B. subtilis* GB03 and IN937a are known to play a significant role in disease suppression and growth promotion (Ryu et al. 2006). The AEFB strains, viz., *B. cereus* L254, *B. simplex* L266, and *Bacillus* sp. L272a, produce a variety of volatile compounds (Gutiérrez-Luna et al. 2010). Since many of the antimicrobial compounds are volatile in nature and are seldom detected by the diffusible compound assays that are routinely followed in the laboratory for the detection of antagonistic action, this

area continues to be less explored. Besides antagonism to plant pathogens, the volatile organic compounds also play a role in the induction of systemic resistance against a wide range of plant pathogens by playing the role of elicitors (Kloepper et al. 2004). From the above discussion, it is quite obvious that AEFB strains posses a wide arsenal of antagonistic mechanisms to combat the plant pathogens. But in vitro studies alone are not sufficient for a successful biocontrol program which largely depends on the rhizospheric and functional competence of the introduced bacterium. Hence a judicious blend of multiple antagonistic traits combined with rhizocompetence traits is the key for the exploitation of AEFBs in the biological control of plant pathogens.

10.4.4 Induction of Systemic Resistance

Induced systemic resistance (ISR) may be defined as a physiological "state of enhanced defensive capacity" elicited in response to specific environmental stimuli, and consequently the plant's innate defenses are potentiated against subsequent biotic challenges (van Loon 2000). This mechanism differs from the other plant defense mechanisms, viz., systemic acquired resistance (SAR), in the nature of elicitor and regulatory pathways involved. While ISR relies on pathways regulated by jasmonic acid and ethylene, SAR involves the accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins. Several rhizobacterial AEFB strains predominantly belonging to the genera *Bacillus* and *Paenibacillus* are known to induce systemic resistance in plants when inoculated in sufficient numbers. Apart from inducing resistance to foliar fungal/bacterial pathogens, certain strains of *B. pumilus* are also known to induce resistance to viral pathogens such as the cucumber mosaic virus (Murphy et al. 2003). Another exciting feature of *Bacillus* inoculants is the induction of systemic resistance to insect pests such as white flies and the cucurbit beetle. The distinguishing morphological features that were observed in bacterized plants include the strengthening of epidermal and cortical cell walls, callose formation and accumulation of phenolic compounds (Zehnder et al. 1997). Studies on the elicitors of ISR in bacilli revealed that volatile organic compounds such as acetoin and butanediol are primarily responsible for induction of systemic resistance. The compound 2,3 butanediol has the dual functionality of induction of systemic resistance and plant growth promotion (Ryu et al. 2003; Pare et al. 2005). Other elicitor molecules include the antibiotics surfactin and fengycin produced by *B. subtilis* (Ongena et al. 2005). Studies on the signaling mechanisms involved in the induction of systemic resistance by bacilli reveal similarities with the signaling mechanisms of fluorescent pseudomonads, but in some cases induction of *PR1a* protein is seen which is quite characteristic of the salicylic acid-dependent pathway (Choudhary and Johri 2009). The specific signal transduction pathway that is promoted during ISR by *Bacillus* spp. depends on the strain, the host plant, and in certain cases the pathogen. An additional feature of ISR induction by bacilli is the accompanying plant growth promotion effects. A compilation of recorded instances where systemic resistance has been induced by AEFBs is presented in Table 10.3.

Table 10.3 Induction of systemic resistance by AEFBs in various crops

AEFB	Significance	References
B. amyloliquefaciens, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus*	Significant reduction in the incidence or severity of various diseases on diverse hosts	Choudhary and Johri (2009) and Kloepper et al. (2004)
B. pumilus and *B. mycoides*	Reduced the severity of *Cercospora* leaf spot of sugar beet caused by *Cercospora beticola*	Bargabus et al. (2004)
Bacillus sp.	Elicits systemic protection against *Xanthomonas campestris* pv. *armoraciae*	Krause et al. (2003)
Bacillus sp.	Reduction in the severity and incidence of viral diseases (cucumber mosaic virus) under greenhouse conditions	Murphy et al. (2003) and Zehnder et al. (2000)
B. pumilus	Systemic protection of tomato against late blight, caused by *Phytophthora infestans*	Yan et al. (2003)
B. pasteurii C-9 and *B. pumilus* SE34 and T4	Significant reduction of leaf lesions caused by *Peronospora tabacina* in tobacco	Zhang et al. (2002)
Bacillus spp.	ISR against *Ralstonia solanacearum* infection in tomato	Jetiyanon and Kloepper (2002)
B. subtilis IN937b, *B. pumilus* SE34, and *B. amyloliquefaciens* IN937a	ISR against CMV in tomato, significant yield increase also seen	Zehnder et al. (2000)
B. amyloliquefaciens IN937a, *B. subtilis* IN937b, and *B. pumilus* SE34	Reduced the incidence and severity of the tomato mottle virus (ToMoV)	Murphy et al. (2000)
B. subtilis AF1	Induction of systemic resistance against *Aspergillus niger* on peanut (*Arachis hypogaea*)	Podile and Dube (1988)

10.4.5 AEFBs as Endophytes

Apart from free living forms, the occurrence of AEFB species as endophytes has been reported from different plant tissues such as cotton, grape, peas, spruce, and sweet corn (Berg et al. 2005a; Shishido et al. 1999; Bell et al. 1995). The most frequently recovered AEFB species from the inner tissues of plants include *B. cereus*, *B. insolitus*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens*, B. *licheniformis*, and *P. polymyxa* (Shishido et al. 1999; Benhamou 1996; Sturz et al. 1997; Bell et al. 1995; Reva et al. 2002). While some recovered AEFB endophytes are known to possess plant growth promotion traits, the role of several others remains to be deciphered. Bai et al. (2002) were able to isolate putative endophytic *Bacillus* from nodules of soybean plants which promoted nodulation and growth of soybean. Selvakumar et al. (2008) isolated a putative endophytic stain of *Bacillus thuringiensis* from the root nodules of the wild legume *Kudzu*. This strain possessed several

beneficial traits besides producing parasporal crystals during sporulation. The beneficial effect of this strain on the growth and development of soybean and garden pea under in vitro conditions has been subsequently established (Mishra et al. 2008, 2009). A recent development in this direction is the detection of the ability of *B. thuringiensis* strains originating from legume root nodules to migrate in an ascending fashion from the root portion to the aboveground plant tissues and promote plant growth in a wide variety of legumes (Tanuja et al. 2013). AEFBs reported to occur as endophytes with plant growth promotion traits include *Lysinibacillus* (Vendan et al. 2010; Andrade et al. 2014) and *Sporosarcina aquimarina* (Janarthine and Eganathan 2012).

10.5 Other Roles in the Agroecosystem

Apart from the abovementioned roles, AEFBs play an important role in the recycling of organic matter and crop residues. During the composting process, a gradual increase of the temperature of the composting pile is followed by sustained high temperatures (thermophilic) and a gradual cooling (maturation) of the composting mass (Halet et al. 2006). AEFBs are known to proliferate during the mesophilic and thermophilic stages, and frequently recovered genera include *Bacillus*, *Paenibacillus*, *Brevibacillus*, and *Geobacillus* (Ryckeboer et al. 2003). The compost environment has also proved to be a rich habitat for the isolation of novel AEFBs. Some novel validly published AEFBs isolated from the compost environment include *Geobacillus toebii* (Sung et al. 2002), *Paenibacillus motobuensis* (Lida et al. 2005), *Tuberibacillus calidus* (Hatayama et al. 2006), *Paenibacillus humicus* (Vaz-Moreira et al. 2007), *Thermobacillus composti* (Watanabe et al. 2007) and *Paenibacillus residui* (Vaz-Moreira et al. 2010). Another feature of AEFBs is the ability of several non-Bt species to cause diseases in insects damaging crops, and hence they have the ability to be utilized as biological control agents. Such species include entomopathogenic strains of *Bacillus cereus* against white grubs (Selvakumar et al. 2007), *Brevibacillus laterosporus* which causes diseases in a wide range of invertebrates and nematodes (Huang et al. 2005; Ruiu 2013), *Paenibacillus* spp. which infect white grubs (Redmond and Potter 2010), and *Pasteuria penetans* for nematode control (Stirling 2014). But compared to their more illustrious cousin *B. thuringiensis*, these species have only received sporadic attention in terms of commercial utilization.

10.6 Conclusion

The property of endospore formation makes AEFBs an attractive candidate for microbial inoculant production, but considering the enormous diversity among this group of bacteria, the potential of this group is yet to be exploited fully and has not

gone beyond *B. subtilis B. amyloliquefaciens* and selected *Paenibacillus* strains. Therefore it is imperative to expand the search for agriculturally important strains among the entire gamut of AEFBs rather than focus on a couple of genera alone. Compared to the Gram-negative bacterial species, AEFBs are more amenable for developing novel bioinoculant delivery systems like liquid formulations with enhanced shelf life and delivery potential, but novel inoculant delivery systems are still largely focused on traditional Gram-negative bacterial strains, and hence AEFBs urgently require the attention of the bioinoculant industry as well. The utility of AEFBs as inoculants in unfavorable growing environments especially in water- and heat-stressed environments needs to be exploited fully, considering the ability of AEFBs to produce endospores and alleviate stress effects in plants. Similarly the potential of AEFBs to degrade organic matter and recalcitrant organic compounds has not been exploited to the core. The recent developments in the discovery of novel AEFBs from the composting environments with novel enzyme systems is one area that holds a lot of promise. While focusing on the above issues, care has to be exercised to exclude the undesirable ones, since some opportunistic human pathogens are found in the genus *Bacillus* and pathogenic features are being attributed to some soil bacterial species like *Brevibacillus* (Suneeva et al. 2014). If such a careful approach is followed, it can be rightly claimed that AEFBs would be the bioagents of the future.

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Chapter 11
New Insights in Plant-Associated *Paenibacillus* Species: Biocontrol and Plant Growth-Promoting Activity

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Abstract A wide number of new species have been included in recent years in the *Paenibacillus* genus, prompting to a new ecological and biotechnological appraisal of *Paenibacillus* bacteria. Several species are involved in plant growth promotion and biocontrol, and a few of them have also been reported to cause human infections. Some isolates of the genus *Paenibacillus* are among the most efficient microbial biocontrol agents, and some strains have been included in formulations that have been granted a patent to control plant pathogens. A strain belonging to the species *Paenibacillus lentimorbus* has recently been described as a potent plant growth-promoting and bioremediation agent in Cr-contaminated rhizosphere soil. Nitrogen fixation has been described in several species, and some of these bacteria are promising candidates for crop inoculation. Fourteen complete genome sequences are publicly available so far. Five of them belong to *Paenibacillus polymyxa* strains that have been isolated from crop rhizosphere and show traits related to plant growth promotion. Recently, the draft genome sequence of *Paenibacillus riograndensis* strain SBR5^T, which in addition to nitrogen fixation has shown several plant growth-promoting traits, has been published.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_11

11.1 Introduction

The microbial world is the largest not yet fully explored reservoir of biodiversity. Exploration of microbial diversity holds great promise because of the role of microbes in nutrient cycling, environmental detoxification, and novel metabolic abilities in pharmaceuticals and industrial processes (Satyanarayana 2005).

The *Paenibacillus* genus has attracted considerable interest because of its high biotechnological potential in sustainable agriculture and in different industrial processes (Govindasamy et al. 2011; Lal and Tabacchioni 2009). The genus *Paenibacillus* was created by Ash et al. (1993) to accommodate the former "group 3" of the genus *Bacillus* which comprises aerobic or facultative anaerobic endospore-forming bacterial species. Comparative 16S rRNA sequence analyses revealed that rRNA group 3 bacilli represents a phylogenetically distinct group and exhibits high intragroup sequence relatedness and is only remotely related to *Bacillus subtilis,* the type species of the genus *Bacillus*. The number of recognized member of this genus has been increased in the last few decades. At present, 174 species of the genus *Paenibacillus* with validly published names are available in the List of Prokaryotic Names with Standing in Nomenclature (LPSN), (http://www.bacterio.cict.fr/p/paenibacillus.html) database (Parte 2014). To date, a total of 152 finished and unfinished genomes of *Paenibacillus* are publicly available on NCBI (http://www.ncbi.nlm.nih.gov/) (Tatusova et al. 2014) and IMG-JGI (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) (Markowitz et al. 2014). Species belonging to the genus *Paenibacillus* can be aerobic, anaerobic or facultatively anaerobic, mesophilic, psychrotolerant, or alkali tolerant and have the ability to degrade a variety of complex substrates such as xylan and cellulose (Rivas et al. 2005b). They have been isolated from a wide range of sources including soil, water, rhizosphere, vegetable matter, forage, and insect larvae (Daane et al. 2002), as well as from clinical samples such as blood samples (Noskin et al. 2001; Roux and Raoult 2004), cerebrospinal fluid shunt (Bosshard et al. 2002), human urine, and cerebrospinal fluid (Roux et al. 2008). *Paenibacillus polymyxa*, *Paenibacillus thiaminolyticus*, and *Paenibacillus hongkongensis* were reported as the cause of bacteremia and pseudobacteremia in a patient with cerebral infarction, in a patient on hemodialysis, and in a patient with neutropenic fever, respectively (Nasu et al. 2003; Ouyang et al. 2008; Teng et al. 2003). The highly diverse niche occupancy of *Paenibacillus* reflects the metabolic diversity of these bacteria. Indeed, fixation of atmospheric nitrogen, production of phytohormones, antibiotics, hydrolytic enzymes, and valuable chemical compounds such as 2.3-butanediol has been observed among isolates of the different *Paenibacillus* species (Govindasamy et al. 2011; Lal and Tabacchioni 2009). The majority of strains that are considered beneficial to plants have been isolated from soil and rhizosphere (McSpadden Gardener 2004; Govindasamy et al. 2011). Members of the genus *Paenibacillus* have also been recognized as important pathogens of insects. *Paenibacillus popilliae* and *Paenibacillus lentimorbus* are responsible of milky disease in the larvae of some beetles (order Coleoptera) including those that can cause crop diseases (Pettersson et al. 1999). *Paenibacillus larvae* is

the cause of the American foulbrood of honeybees (*Apis mellifera*) larvae, a disease that can significantly reduce pollination activity of fruit and vegetable crops (McSpadden Gardener 2004; Ashiralieva and Genersch 2006).

Over the past few years, sequencing of several genomes of *Paenibacillus* sp. bacteria isolated from the soil and rhizosphere has been carried out providing data on the genetic basis of determinants involved in plant growth promotion, nitrogen fixation, and biocontrol (Eastman et al. 2014).

In this chapter, highlights from recent studies on the taxonomy, genomics, ecology, plant growth promotion, and biocontrol of the different *Paenibacillus* species will be presented with the aim to evaluate their potential application in sustainable agriculture.

11.2 Taxonomy and Genomics

In the last few decades, a great number of prokaryotic genomes have been sequenced, and at present 32,525 bacterial genomes are available in the IMG database. Of these 32,525 genomes, 3998 genomes are finished, 3874 genomes are draft, and 24,753 genomes are permanent draft. As of January 2016, a total of 152 genomes belonging to species of the *Paenibacillus* genus are available in JGI-IMG (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) (Markowitz et al. 2014) and in NCBI (http://www.ncbi.nlm.nih.gov/) (Tatusova et al. 2014). Out of 152 genomes, 28 are finished, 22 are draft, and 102 are permanent draft. In Table 11.1, a comprehensive view of the *Paenibacillus* species whose genomes have been or are being sequenced is given.

Autonomously replicating plasmids that do not integrate into the host genome were observed in only five *Paenibacillus* sp.: *Paenibacillus larvae larvae* 4-309, DSM 25430, *P. polymyxa* SC2, *P. polymyxa* M1, *Paenibacillus alvei* DSM 29, and *P. larvae larvae* 08-100, DSM 25719 (Table 11.1). Annotation of the *Paenibacillus* genome sequences showed that the genome size varies from 3.83 (*P. popilliae* ATCC 14706) to 8.82 Mb (*Paenibacillus mucilaginosus* K02) and % GC varies from 43 (*Paenibacillus assamensis* DSM 18201) to 67 (*Paenibacillus* sp. HW567) (Table 11.1).

Fifty-six *Paenibacillus* sp., well known to promote plant growth and to be involved in the biosynthesis of antibiotics and hydrolytic enzymes, were used to construct a phylogenetic tree (Table 11.2). The phylogenetic tree based on 16S rRNA nucleotide sequences of the 56 *Paenibacillus* sp. isolated from various habitats (Table 11.2) grouped into 13 clusters (Fig. 11.1). The 16S rRNA phylogenetic tree constructed in this study (Fig. 11.1) has shown almost similar topology to that of previously published trees on 16S rRNA phylogeny (Hong et al. 2009; Jin et al. 2011b).

Paenibacillus cellulositrophicus KCTC 13135, *Paenibacillus lactis* 154, *Paenibacillus lautus* Y412MC10, and *Paenibacillus vortex* V453 were grouped into Cluster 1 based on 16S RNA phylogenetic analysis (Fig. 11.1). *P. cellulositrophicus* KCTC 13135 is a thermophilic (50 °C) facultative anaerobe isolated from soil (Akaracharanya et al. 2009), whereas *P. lactis* 154, *P. lautus* Y412MC10, and *P.*

Table 11.1 Metadata of finished, draft and permanent draft genome of *Paenibacillus* species

Genomes	Sources of isolation	GenBank Accession no.	IMG Taxon ID	Genome status	Genome size (Mb)	% GC	Genes	Proteins
*P. lautus*Y412MC10	Obsidian Hot Spring (Yellowstone National Park, Montana, USA)	NC_013406	646311929	F	7.12	51	6444	6238
P. larvae larvae 4–309, DSM 25430	American foulbrood infected honey bee hive	NC_023134	2523231078	F	4.06	45	4133	3928
*P. lactis*154	Bioreactor	AGIP00000000	2506783046	F	6.84	52	6234	6149
*P. mucilaginosus*K02	Soil of maize-farming fields (Guizhou, China)	NC_017672.3	2513237182	F	8.82	58	7578	7354
*P. mucilaginosus*KNP414	Soil of Tianmu Mountain (Zhejiang, China)	NC_015690	650716070	F	8.66	58	7983	7811
*P. mucilaginosus*3016	Rhizosphere soil (Shandong, China)	NC_016935	2512564039	F	8.73	58	7528	7057
P. polymyxa E681	Rhizosphere of winter barley (Chonnam, South Korea)	NC_014483	648028048	F	5.39	46	4933	4805
*P. polymyxa*CR1	Corn rhizosphere	NC_023037	–	F	6.02	46	5510	5283
*P. polymyxa*SC2	Rhizosphere of pepper (Guizhou, China)	NC_014622	649633079	F	6.24	45	6286	6032
P. polymyxa M1	Surface-sterilized wheat root tissues	NC_017542	2517572207	F	6.23	45	5516	5364

*P. polymyxa*SQR-21	Rhizosphere of healthy watermelon plants	CP006872	–	F	5.83	46	5174	5024
*P. terrae*HPL-003	Soil of forest residue, Daejeon (Republic of Korea)	NC_016641	2511231079	F	6.08	47	5642	5525
Paenibacillus sp. JDR-2	Sweet gum stem wood buried in surface soil	NC_012914	644736396	F	7.18	50	6414	6213
Paenibacillus sp. J10	–	–	2505679063	F	4.7	52	4442	4328
P. alvei A6-6i-x	Leaf (tomato growing field - Virginia Eastern Shore	ATMS00000000	2541047089	D	6.48	47	5669	5584
*P. alvei*DSM 29	European foulbrood infected honey bee hive	AMBZ00000000	2561511085	D	6.83	46	6790	6605
P. alvei TS-15	Soil (tomato growing field – Virginia Eastern Shore)	ATMT00000000	2541048009	D	6.71	47	5867	5784
*P. azotofixans*ATCC 35681	Wheat roots (Parana state, Brazil)	ASQQ00000000	–	D	5.36	51	–	–
*P. barengoltzii*G22	–	ASSZ00000000	–	D	4.78	52	4394	4307
*P. curdlanolyticus*YK9	Soil (Kobe, Japan)	AEDD00000000	648276707	D	5.45	52	4957	4824
*P. dendritiformis*C454	Soil (Tel Aviv, Israel)	AHKH00000000	2513237294	D	6.37	54	5690	5660

(continued)

Table 11.1 (continued)

Genomes	Sources of isolation	GenBank Accession no.	IMG Taxon ID	Genome status	Genome size (Mb)	% GC	Genes	Proteins
*P. elgii*B69	Soil samples (Hangzhou, China)	AFHW00000000	2547132108	D	7.96	53	7828	7777
P. forsythiae T98	Rhizosphere soil of *Forsythia mira*, (Beijing, China)	ASSC00000000	–	D	5.08	53	–	–
P. graminis RSA19	Maize rhizosphere soil (Ramonville, France)	ASSG00000000	–	D	6.99	50	–	–
*P. larvae larvae*BRL-230010	Scales collected from a single severely diseased colony (Berkeley, CA, USA)	AARF00000000	641736255	D	4.01	44	5021	4955
P. larvae larvae B-3650	Lab culture	–	649989978	D	4.35	44	3630	3558
*P. lentimorbus*NRRL B-30488	Cows' milk	ANAT00000000	2554235229	D	3.91	46	4404	4292
*P. massiliensis*T7	Willow rhizosphere (Beijing, China)	ASSE00000000	–	D	6.3	48	–	–
P. peoriae KCTC 3763	Soil (Republic of Korea)	AGFX00000000	2547132140	D	5.77	46	5165	5073
P. polymyxa ATCC 842	Rhizosphere soil	AFOX00000000	2547132099	D	5.89	45	5468	5348
P. polymyxa ATCC 12321	Spoiled starch	ARYD00000000	2554235114	D	4.13	46	4201	4120
*P. polymyxa*OSY-DF	Fermented vegetables	AIPP00000000	2548876929	D	5.69	45	5069	5003

*P. polymyxa*1–43	Corn rhizosphere, (Shanxi China)	ASRZ00000000	–	D	5.97	45	–	–
P. polymyxa TD94	*Scutellaria* rhizosphere (Liaoning, China)	ASSA00000000	–	D	6.0	45	–	–
P. popilliae ATCC 14706	From commercial spore dust (CCEB 296) made with strain DeBryne 14F-D80 (H. Tashiro).	BALG00000000	2537561634	D	3.83	51	3934	3855
P. riograndensis SBR5	Wheat field (south of Brazil)	AGBD00000000	2548876532	D	7.41	51	7567	7494
P. senegalensis JC66	Fecal flora of a healthy patient	CAES00000000	2547132139	D	5.49	48	5134	5040
*P. sonchi*X19-5	Rhizosphere soil of *Sonchus oleraceus*	AJTY00000000	2548877040	D	7.51	50	7606	7511
P. sophorae S27	Rhizosphere of *Sophora japonica* (Beijing, China)	ASSF00000000	–	D	8.42	48	–	–
P. vortex V453	Rhizosphere (Tel Aviv, Israel)	ADHJ00000000	649989979	D	6.38	49	5993	5926
P. zanthoxyli JH29	Rhizosphere soils of *Zanthoxylum Simulans* (Beijing, China)	ASSD00000000	–	D	5.05	51	–	–
*Paenibacillus*sp. HGF5	Human intestinal microflora, USA	AEXS00000000	651324080	D	6.95	51	6578	6496

(continued)

Table 11.1 (continued)

Genomes	Sources of isolation	GenBank Accession no.	IMG Taxon ID	Genome status	Genome size (Mb)	% GC	Genes	Proteins
*Paenibacillus*sp. HGH0039	Human intestinal microflora, USA	AGEN00000000	2541046994	D	6.29	53	5758	5669
*Paenibacillus*sp. HW567	–	ARFI00000000	–	D	6.32	67	6047	5944
Paenibacillus sp. PAMC 26794	Tundra grasslands (Alaska)	ANHX00000000	2551306498	D	6.65	46	5969	5879
*Paenibacillus*sp. J14	–	JADQ00000000	2528768218	D	4.86	52	4552	4435
Paenibacillus sp. oral taxon 786 str. D14	Oral swab from female patient (USA)	ACIH00000000	647533191	D	5.09	52	4529	4460
Paenibacillus sp. HGF7	–	AFDH00000000	651324081	D	6.28	53	6075	5992
Paenibacillus sp. OSY-SE	Soil	ALKF00000000	2551306117	D	6.93	49	6337	6288
*Paenibacillus*sp. ICGEB2008	Gut of *Helicoverpa armigera*	AMQU00000000	2551306396	D	5.69	46	5147	4852
*Paenibacillus*sp. 1–18	Wheat rhizosphere, (Beijing, China)	ASSB00000000	–	D	5.4	46	–	–
Paenibacillus sp. 1–49	Corn rhizosphere (Shanxi, China)	ASRY00000000	–	D	5.62	47	–	–
Paenibacillus sp. JCM 10914	Gut of a soil-feeding termite	NZ_BAUO00000000	–	D	6.11	48	6149	6080
Paenibacillus sp. FSL H7-689	Milk	ASPU00000000	–	D	6.84	46	6008	5913
Paenibacillus sp. FSL H8-237	Milk	ASPV00000000	–	D	7.32	44	6563	6477

Paenibacillus sp. FSL H8-457	Milk	ASPW00000000	–	D	7.08	51	6415	6337
Paenibacillus sp. FSL R5-192	Milk	ASPW00000000	–	D	7.08	46	6275	6163
*Paenibacillus*sp. FSL R5-808	Milk	ASPT00000000	–	D	6.45	49	5922	5847
*Paenibacillus*sp. FSL R7-269	Milk	ASPS00000000	–	D	7.6	51	6816	6726
Paenibacillus sp. FSL R7-277	Milk	ASPX00000000	–	D	7.6	52	6523	6429
Paenibacillus sp. G1	–	CBVJ000000000	–	D	6.26	48	–	–
*Paenibacillus*sp. GD11	Human gut microbiota by culturomics	CBLK000000000	–	D	5.58	49	–	–
Paenibacillus sp. MAEPY1	Malaysian landfill leachate	AWUJ00000000	–	D	7.48	46	–	–
Paenibacillus sp. MAEPY2	Malaysian landfill leachate	AWUK00000000	–	D	7.48	46	–	–
Paenibacillus sp. WLY78	Bamboo rhizosphere (Beijing, China)	ALJV00000000	–	D	5.91	45	–	–
P. alginolyticus DSM 5050	Soil	AUGY00000000	2524614879	P	8.33	45	8232	8060
P. assamensis DSM 18201	Warm spring	AULU00000000	2524023086	P	5.03	43	4520	4415
P. barengoltzii J12	Spacecraft	–	2528768208	P	4.7	52	4414	4291
P. daejeonensis DSM 15491	Soil	ARKE00000000	2519103188	P	7.46	53	6524	6446
P. fonticola DSM 21315	Warm spring	ARMT00000000	2519899635	P	6.31	48	5753	5662

(continued)

Table 11.1 (continued)

Genomes	Sources of isolation	GenBank Accession no.	IMG Taxon ID	Genome status	Genome size (Mb)	% GC	Genes	Proteins
P. ginsengihumi DSM 21568	Soil of a ginseng field	ARKW00000000	2521172661	P	5.67	57	5245	5150
P. harenae DSM 16969	Desert sand	AULV00000000	2523533617	P	6.97	51	6377	6249
P. larvae larvae 08–100, DSM 25719	–	ADFW00000000	2524023248	P	4.57	44	5020	4843
P. massiliensis DSM 16942	Human blood	ARIL00000000	2517572189	P	6.39	49	5585	5475
P. pasadenensis DSM 19293	Clean room floor	AULW00000000	2524023087	P	5.71	63	4971	4870
P. panacisoli DSM 21345	Soil of a ginseng field	AUFO00000000	2524614514	P	6.32	48	5719	5591
P. pinihumi DSM 23905	Rhizosphere of the pine trees (Daejeon, Republic of Korea)	AULX00000000	2524023129	P	6.76	49	6170	6064
P. sanguinis DSM 16941	Human blood	ARGO00000000	2517572150	P	4.8	49	4501	4411
P. taiwanensis DSM 18679	Farmland soil	AULE00000000	2524614883	P	5.24	45	4780	4679
P. terrigena DSM 21567	Coastal soil (Chiba, Japan)	ARGP00000000	2517572151	P	6.36	46	5958	5865
P. uliginis N3/975	Fen peat soil of a nitrogen fertilization long-term experiment	–	2529292568	P	6.45	45	6136	6033
Paenibacillus sp. URHA0014	Mediterranean grassland soil	–	2556921041	P	7.37	45	6641	6504
*Paenibacillus*sp. J6	Rhizosphere	–	2528768219	P	4.83	52	4519	4406

– Genomes not available in the database, F Finished, D Draft, P Permanent draft

Table 11.2 *Paenibacillus* sp. used to construct 16S rRNA phylogenetic tree

Strain	NCBI Taxon ID	Genome Status	Genome Size (Mb)	16s rRNA (gi no.)[a]	Sources of isolation	References
P. lautus Y412MC10	481743	F	7.12	646360186[b]	Obsidian Hot Spring (Yellowstone National (Park, Montana, USA)	Mead et al. (2012)
P. polymyxa M1	1052684	F	6.23	2518127049[b]	Surface-sterilized wheat root tissues	Niu et al. (2011)
*P. polymyxa*SC2	886882	F	6.24	649679891[b]	Rhizosphere of pepper (Guizhou, China)	Ma et al. (2011)
P. lactis 154	743719	F	6.84	2507047847[b]	Bioreactor	Scheldeman et al. (2004)
*P. mucilaginosus*3016	997761	F	8.73	CP003235.1	Rhizosphere soil (Shandong, China)	Ma et al. (2012)
*P. mucilaginosus*KNP414	1036673	F	8.66	CP002869.1	Soil of Tianmu Mountain (Zhejiang, China)	Lu et al. (2013)
P. mucilaginosus K02	1116391	F	8.82	CP003422.2	Soil of maize-farming fields (Guizhou, China)	–
Paenibacillus sp. JDR-2	324057	F	7.18	644856946[b]	Sweet gum stem wood buried in surface soil	Chow et al. (2012)
P. polymyxa E681	349520	F	5.39	648168277[b]	Rhizosphere of winter barley (Chonnam, South Korea)	Kim et al. (2010)
*P. terrae*HPL-003	985665	F	6.08	2511570078[b]	Soil of forest residue (Daejeon, Republic of Korea)	Shin et al. (2012), Song et al. (2014)
*P. alvei*A6-6i-x	1117109	D	6.48	528200848	Leaf (tomato growing field – Virginia Eastern Shore)	Luo et al. (2013)
P. alvei TS-15	1117108	D	6.71	2545554562[b]	Soil (tomato growing field – Virginia Eastern Shore)	Luo et al. (2013)
P. azotofixans ATCC 35681	1333534	D	5.36	11342579	Wheat roots (Paranà State, Brazil)	Xie et al. (2012a), Hong et al. (2009)

(continued)

Table 11.2 (continued)

Strain	NCBI Taxon ID	Genome Status	Genome Size (Mb)	16s rRNA (gi no.)[a]	Sources of isolation	References
*P. elgii*B69	1007103	D	7.96	–	Soil samples (Hangzhou, China)	Teng et al. (2012), Ding et al. (2011)
P. forsythiae DSM17842	1333861	D	5.08	84798579	Rhizosphere soil of *Forsythia mira* (Beijing, China)	Xie et al. (2012a), Hong et al. (2009)
*P. graminis*RSA19	1333858	D	6.99	5701910	Maize rhizosphere soil (Ramonville, France)	Berge et al. (2002), Xie et al. (2012a), Hong et al. (2009)
*P. peoriae*KCTC 3763	1087481	D	5.77	359804172	Soil (Republic of Korea)	Jeong et al. (2012)
*P. pinihumi*DSM 23905	1122924	D	6.76	256807276	Rhizosphere of the pine trees (Daejeon, Republic of Korea)	Kim et al. (2009a)
*P. polymyxa*ATCC 842	1036171	D	5.89	2547356833[b]	Rhizosphere soil	Jeong et al. (2011), Hong et al. (2009)
P. polymyxa ATCC12321	1206104	D	4.13	2554737808[b]	Spoiled starch	Tong et al. (2013)
P. polymyxa OSY-DF	1156938	D	5.69	2550365550[b]	Fermented vegetables	Huang and Yousef (2012)
P. sophorae S27	682957	D	8.42	289623203	Rhizosphere of *Sophora japonica*(Beijing, China)	Jin et al. (2011a), Xie et al. (2012a)
*P. sonchi*X19-5	1173684	D	7.51	89033260	Rhizosphere soil of *Sonchus oleraceus*	Hong et al. (2009), Xie et al. (2012a)
P. terrigena DSM 21567	1122927	D	6.36	121308845	Coastal soil (Chiba, Japan)	Xie and Yokota (2007)
P. zanthoxyli JH29	1333860	D	5.05	94183943	Rhizosphere soils of *Zanthoxylum simulans* (Beijing, China)	Ma et al. (2007a), Hong et al. (2009)
Paenibacillus sp. A9	1284352	D	5.48	2554317331[b]	Soil	Jiang et al. (2013)
*P. curdlanolyticus*YK9	717606	D	5.45	648725777[b]	Soil (Kobe City, Japan)	–

P. vortex V453	58172	D	6.38	650065237[b]	Rhizosphere	Sirota-Madi et al. (2010)
*P. riograndensis*SBR5	1073571	D	7.41	2549005511[b]	Wheat field (Brazil)	Beneduzi et al. (2011)
P. beijingensis DSM24997	1126833	–	–	363498786	Jujube rhizosphere soil (Beijing, China)	Gao et al. (2012)
*P. brasilensis*PB172	128574	–	–	219857518	Rhizosphere of maize, (Cerrado, Brazil)	von der Weid et al. (2002)
*P. borealis*DSM13188	160799	–	–	219878160	Spruce forest humus (Finland)	Elo et al. (2001)
P. castaneae DSM 19417	474957	–	–	157787625	Phyllosphere of *Castanea sativa* (Spain)	Valverde et al. (2008)
P. catalpae DSM 24714	1045775	–	–	336283389[b]	Rhizosphere soil of *Catalpa speciosa*	Zhang et al. (2013)
*P. cellulositrophicus*KCTC 13135	562959	–	–	325672522	Soil (Thailand)	Akaracharanya et al. (2009)
P. donghaensis KCTC 13049	414771	–	–	118766593[b]	Deep-sea sediment	Choi et al. (2008a)
P. filicis JCM 16417	669464	–	–	256807274	Rhizosphere of ferns (Daejeon, Republic of Korea)	Kim et al. (2009b)
*P. frigoriresistens*JCM 18141	1143711	–	–	374923057	Peat bog sample (Mohe County, Heilongjiang Province, Northern China)	Ming et al. (2012)
*P. hordei*JCM 17570	980239	–	–	340780743	Naked barley (South Korea)	Kim et al. (2013)
*P. jilunlii*Be17	682956	–	–	289623202	Rhizosphere soil of *Begonia semperflorens*, (Beijing Botanical Garden, PR China)	Jin et al. (2011b), Xie et al. (2012a)
*P. macquariensis*JCM 14954	467974	–	–	157073843	Soil samples (Oblast Magadan, Russian Far East)	Hoshino et al. (2009)
*P. marinum*strain THE22	1033264	–	–	333756450	Sea hot spring “Ain Echefa” (Tunisia)	Bouraoui et al. (2013)

(continued)

Table 11.2 (continued)

Strain	NCBI Taxon ID	Genome Status	Genome Size (Mb)	16s rRNA (gi no.)[a]	Sources of isolation	References
*P. marinisediminis*17886	1031539	–	–	333123116	Marine sediment (south coast of the Republic of Korea)	Lee et al. (2013a)
*P. odorifer*TOD45	189426	–	–	265678582	Soil, plant rhizospheres, plant roots and pasteurized pureed vegetables	Hong et al. (2009), Berge et al. (2002)
P. phyllosphaerae CECT 5862	274593	–	–	47059737	Phyllosphere of *Phoenix dactylifera*	Rivas et al. (2005a)
*P. pini*JCM 16418	1236976	–	–	256807275	Rhizosphere of pine trees	Kim et al. (2009c)
P. prosopidis DSM 22405	630520	–	–	225696302	Root nodules of *Prosopis farcta* (Tunisia)	Valverde et al. (2010)
P. sabinae T27	1268072	–	–	84798580	Rhizosphere soils of plants of the species *Sabina squamata*, *Weigela florida* and *Zanthoxylum simulans*	Ma et al. (2007b), Xie et al. (2012a), Hong et al. (2009)
P. swuensis JCM 18491	1178515	–	–	385654395	Soil (South Korea)	Lee et al. (2014)
P. taohuashanense DSM25809	1184688	–	–	386785732	Rhizosphere soil sample of Caragana kansuensis Pojark	Xie et al. (2012b)
*P. taihuensis*NBRC 108766	1156355	–	–	378947806	Decomposing algal scum (eutrophic lake)	Wu et al. (2013)
*P. triticisoli*DSM 25425	1155956	–	–	378926930	Wheat rhizosphere soil	Wang et al. (2013), Wang et al. (2014)
*P. validus*DSM 3037	1349783	–	–	1089786	Contaminant from plates with germinating spores of *Glomus intraradices*	Hildebrandt et al. (2006)

*P. wynnii*LMG22176	268407	–	–	343201518	Soil from 12 different locations (Mars Oasis, Alexander Island, Antarctica)	Rodríguez-Díaz et al. (2005), Hong et al. (2009)
*P. wooponensis*WPCB018	554310	–	–	197091737	Fresh water sample collected from Woopo wetland (Korea)	Baik et al. (2011)
*P. xylanilyticus*XIL14	248903	–	–	37624893	Xylan-containing agar plates exposed to air	Rivas et al. (2005b)

F Finished, D Draft

[a]gi Gene ID or sequence identification numbers

[b]16s rRNA nucleotide sequences retrieved from JGI-IMG

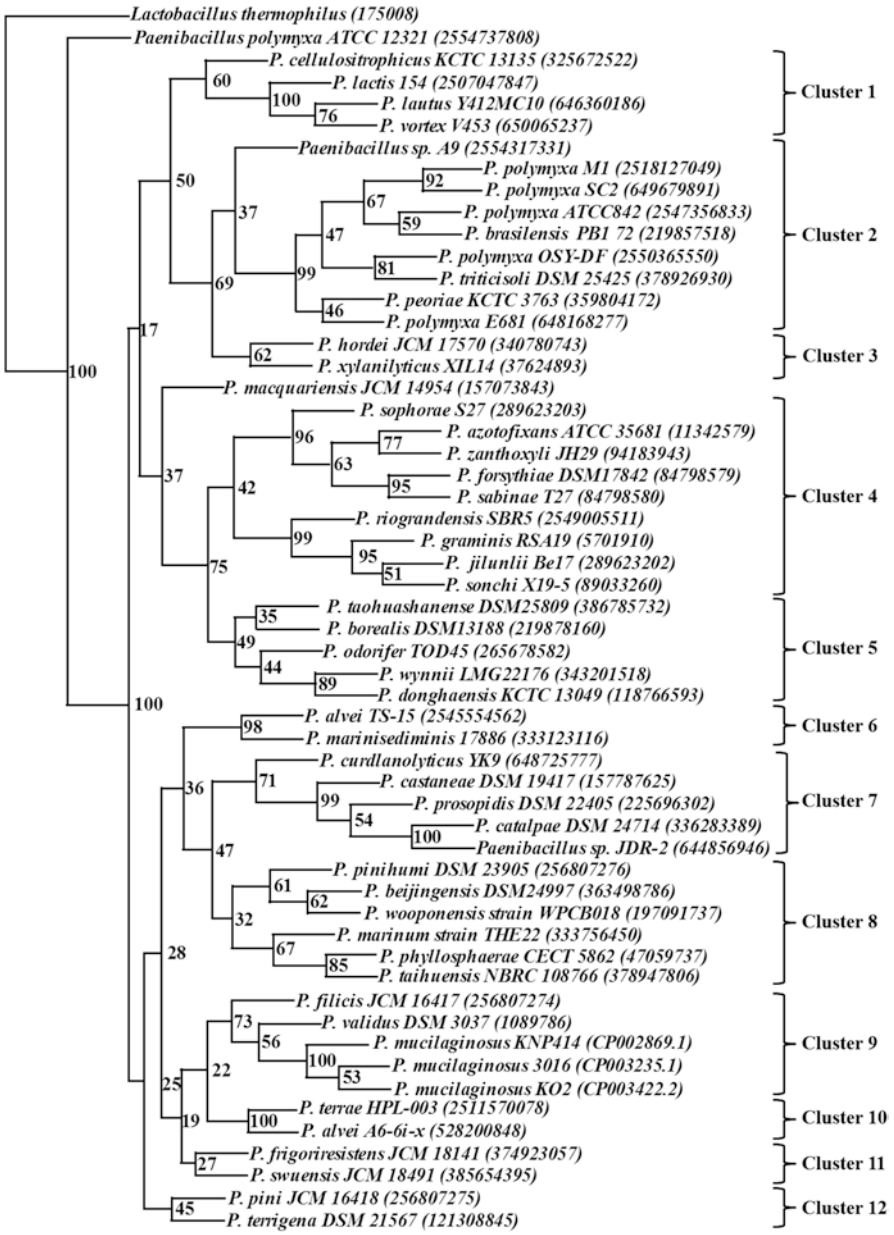

Fig. 11.1 16S rRNA phylogenetic tree of *Paenibacillus* species based on neighbour joining analysis was performed using PHYLIP software version 3.695. Bootstrap analyses were performed with 1000 cycles and values are given at nodes. Identification numbers (gi) of the respresentative sequences are given in parentheses. *Lactobacillus thermophillus* was used as an out group

vortex V453 are mesophiles isolated from a bioreactor, hot spring, and soil rhizosphere, respectively. *P. vortex* is the first sequenced genome providing the basis for the understanding of social organization and pattern formation within Gram-positive bacteria. *P. vortex* genome encodes an extensive set of transcription factors (TFs), two-component system (TCS), and defense-related genes which can support traits needed for thriving in heterogeneous, fluctuating, and highly competitive environment. Two other bacterial strains, *Paenibacillus* sp. JDR-2 and *P. lautus* Y412MC10, have more TCS genes in comparison to other Gram-positive bacteria (Sirota-Madi et al. 2010). Comparative genomic analysis of 500 complete bacterial genomes revealed that these bacteria can survive successfully in different environments if they possess extensive signal transduction and regulatory networks (Alon 2006; Galperin and Gomelsky 2005; Whitworth and Cock 2008). The analysis also revealed that *P. vortex* has many genes involved in the production of antimicrobial compounds and extracellular degrading enzymes (Sirota-Madi et al. 2010).

Except *P. polymyxa* ATCC 12321, all five *P. polymyxa* M1, *P. polymyxa* SC2, *P. polymyxa* E681, *P. polymyxa* ATCC842, and *P. polymyxa* OSY-DF are clustered together in Cluster 2 along with *Paenibacillus peoriae* KCTC 3763, *Paenibacillus brasiliensis* PB172, and *Paenibacillus triticisoli* DSM 25425 (Fig. 11.1). *P. polymyxa* M-1 produces polymyxin P encoded by the *pmxABCDE* gene cluster involved in suppressing phytopathogenic *Erwinia amylovora* and *Erwinia carotovora* (Gram-negative, facultative anaerobic, rod-shaped bacteria), the causative agents of the important plant diseases fire blight and soft rot, respectively. This finding suggested that *P. polymyxa* M-1 and antibiotic polymyxin P are a potential option to control fire blight, soft rot, and other plant diseases caused by Gram-negative bacteria (Niu et al. 2011). Polymyxin synthetase gene cluster from *P. polymyxa* M-1 consists of five open reading frames, *pmxA*, *pmxB*, *pmxE* involved in nonribosomal peptide synthesis and *pmxC* and *pmxD* and encoding ABC transporters (ATPase and permease components) (Shaheen et al. 2011). Similarly, *P. polymyxa* SC2 has also been widely used in biological control of soilborne plant diseases. It is known as an important plant growth-promoting rhizobacterium (PGPR) isolated from the rhizosphere of pepper in Guizhou, China (Zhu et al. 2008). Sequencing of the complete genome of *P. polymyxa* SC2 revealed that it consists of one circular chromosome (5.73 Mb) which possess many genes involved in antibiotic biosynthesis and one plasmid (510 kb) which carries many essential genes required for purine, pyrimidine, and lipid metabolism, as well as gene encoding for ribosomal proteins, translation elongation factors, and different types of DNA methyltransferase (Konz et al. 1997; Altena et al. 2000; Ma et al. 2011).

Complete genome sequence of *P. polymyxa* M-1 contains 41 kb gene cluster showing 96.41 % identity with polymyxin synthetase gene cluster of *P. polymyxa* E681 (Choi et al. 2009; Niu et al. 2013). Polymyxin produced by *P. polymyxa* E681 is a potent antimicrobial agent and can be used for the treatment of multidrug-resistant Gram-negative bacteria (Landman et al. 2008; Giamarellou and Poulakou 2009; Velkov et al. 2010). Genome analysis of *P. polymyxa* E681 revealed various genes encoding enzymes such as xylanases, pectic enzymes, cellulases, and amy-

lases that degrade plant-derived polysaccharides. It also has a gene cluster for lantibiotic production, but genes for nitrogen fixation were not detected (Kim et al. 2010). Genes cluster for lipopeptide antibiotics such as tridecaptin (58.5 kb) and fusaricidin (Li and Jensen 2008; Choi et al. 2008b) and the genes for biosynthesis of polymyxin (Choi et al. 2009), polyketides, lantibiotic, homoserine lactonase, and several extracellular carbohydrolases have been identified in the genome sequence of *P. polymyxa* ATCC 842. Comparative analysis of *P. polymyxa* genomes revealed the close relatedness between *P. polymyxa* ATCC 842 and *P. polymyxa* SC2 strains (Jeong et al. 2011) (Fig. 11.1). *P. polymyxa* OSY-DF also produces lantibiotic (paenibacillin) and polymyxin E1 (He et al. 2007). Draft genome sequence of *P. polymyxa* OSY-DF revealed two complete gene cluster of lantibiotic. One gene cluster (11.7 kb) was responsible for paenibacillin biosynthesis, and the other may encode a new kind of lantibiotic (de Jong et al. 2010). The analysis also identified many nonribosomal peptide synthetase (NRPS) genes (Huang and Yousef 2012). Phylogenetically, *P. peoriae* is closely related to *P. polymyxa* E681. Analysis of draft genome sequence of *P. peoriae* revealed gene clusters for the biosynthesis of tridecaptin, a heptapeptide antibiotic that is similar to hexapeptide fusaricidin antibiotics, and several NRPS genes encoding unknown products on different contigs. Many genes have been shown to encode for extracellular carbohydrases such as amylase, xylanase, cellulase, and several glucanases that may be responsible for the utilization of plant-derived polysaccharide in the rhizosphere (Jeong et al. 2012).

Phylogenetic analysis of 16S rRNA gene sequences of a novel nitrogen-fixing bacterium, BJ-18, isolated from wheat rhizosphere soil showed a close relation with *P. peoriae* DSM 8320 (99.05 %) and *P. brasiliensis* DSM13188 (98.55 %) (Wang et al. 2013). On the basis of phenotypic and genotypic characteristics, the BJ-18 strain was named *Paenibacillus beijingensis* BJ-18 (Wang et al. 2013). This name with a different type strain has been effectively published previously (Gao et al. 2012). Thus, *P. beijingensis* BJ-18 was renamed as *P. triticisoli* (triticum wheat; solum soil) (Wang et al. 2013, 2014). *Paenibacillus hordei* is closely related to *Paenibacillus xylanilyticus* XIL14T and grouped together in Cluster 3 (Fig. 11.1) (Kim et al. 2013).

Cluster 4 includes two distinct groups of *Paenibacillus* sp. In the first group, *Paenibacillus sophorae* S27 showed a close relation with *Paenibacillus azotofixans* ATCC35681, *Paenibacillus zanthoxyli* JH9, *Paenibacillus forsythiae* DSM 17842, and *Paenibacillus sabinae* T27 (Fig. 11.1). This was supported by phylogenetic analysis based on *nifH* gene sequences revealing that *P. sophorae* S27 is clustered with *P. forsythiae* (96.9 %), *P. zanthoxyli* (96.3 %), *Paenibacillus durus* (95.1 %), and *P. sabinae* (79.6 %) (Jin et al. 2011a).

In the second group, *P. riograndensis* SBR5 was grouped with *Paenibacillus graminis* RSA19, *Paenibacillus jilunlii* Be17, and *Paenibacillus sonchi* X19-5 (Fig. 11.1). Comparative analysis of N_2-fixing *Paenibacillus* strains revealed that a *nif* gene cluster (10.5–12 kb) comprising *nifB*, *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, *nifX*, *hesA*, and *nifV* encoding Mo-nitrogenase is highly conserved. This analysis also identified two homologous alternative nitrogenases: V- and Fe-nitrogenase encoded by the *vnf* and *anf* genes, respectively, in some *Paenibacillus* species (Xie et al. 2014). The

draft genome of nitrogen-fixing bacteria *P. riograndensis* SBR5 showed 23 genes for nitrogen fixation and complete *nif* operon comprising the *nifBHDKENXV* genes. Several other copies of *nif* genes were identified along with the operon coding for alternative nitrogenase (*Anf*). Genome analysis also showed 46 sigma factors suggesting that it has a versatile transcriptional regulation and genes involved in antibiotics resistance such as ampicillin, tetracycline, erythromycin, fosfomycin, gentamicin, and bleomycin (Beneduzi et al. 2011). *Paenibacillus taohuashanense*, *Paenibacillus borealis* DSM 13188, *Paenibacillus odorifer* TDO45, *Paenibacillus wynnii* LMG22176, and *Paenibacillus donghaensis* KCTC 13049 were grouped together in Cluster 5 (Fig. 11.1). This topology was supported by 16S rRNA phylogenetic analysis performed by Xie et al. (2012b) revealing that nitrogen-fixing *P. taohuashanense* DSM25809 has close relation with *P. borealis* DSM 13188 (97.5 %), *P. odorifer* ATCC BAA-93 (97.3 %), *P. durus* DSM 1735 (97.0 %), and *P. sophorae* DSM23020T (96.9 %).

P. alvei and *Paenibacillus marinisediminis* were grouped together in Cluster 6 (Fig. 11.1). *P. alvei* is known to produce peptide antibiotics that affect a wide spectrum of Gram-positive and Gram-negative bacteria (Anandaraj et al. 2009). The genomes of the two strains, *P. alvei* A6-6i and TS-15, isolated from plant material and soil, respectively, were sequenced, and several genes involved in antimicrobial biosynthetic pathways were identified (Luo et al. 2013).

Paenibacillus curdlanolyticus YK9, *Paenibacillus castaneae* DSM 19417, *Paenibacillus prosopidis* DSM 22405, *Paenibacillus catalpae* DSM 24714, and *Paenibacillus* sp. JDR-2 are grouped together in Cluster 7. Only *Paenibacillus* sp. JDR-2 (finished) and *P. curdlanolyticus* YK9 (draft) genomes are sequenced.

Cluster 8 includes six *Paenibacillus* sp., out of which only *Paenibacillus pinihumi* DSM 23905 genome was sequenced. The remaining five strains, *P. beijingensis* DSM24997, *Paenibacillus wooponensis* WPCB018, *Paenibacillus marinum* strain THE22, *Paenibacillus phyllosphaerae* CECT 5862, and *Paenibacillus taihuensis* NBRC 108766, were identified on the basis of 16S RNA phylogenetic analysis (Fig. 11.1). Bouraoui et al. (2013) described in detail the xylanolytic, thermophilic, and facultatively halophilic species *P. marinum* (strain THE22) isolated from the water of thermal spring in the sea of Korbous, northeastern of Tunisia. 16S rRNA gene sequence analysis showed that strain THE22 is clustered with *P. phyllosphaerae* PALXIL04T (95.8 %).

Cluster 9 includes three species, *P. mucilaginosus* 3016, *P. mucilaginosus* KNP414, and *P. mucilaginosus* K02, whose genomes have been already sequenced, and two species, *P. filicis* JCM 16417 and *Paenibacillus validus* DSM 3037 which are not sequenced yet (Fig. 11.1).

P. mucilaginosus 3016 has been used as a microbial fertilizer in agricultural applications due to its growth-promoting properties (Liu et al. 2006; Hu et al. 2008). Many genes involved in the metabolism of nitrogen, phosphorus, and potassium, as well as nitrogen-fixing NifU domain-containing protein, potassium channel protein, and potassium-transporting ATPase subunit A were detected in the genome (Ma et al. 2012). In particular, the genome analysis of *P. mucilaginosus* KNP414 revealed eight genes related to nitrogen assimilation which help strain KNP414 to fix the

nitrogen and allow it to grow in a nitrogen-free environment even though the mechanisms to do so are still unknown (Lu et al. 2013).

Paenibacillus terrae HPL-003 and *P. alvei*A6-6i-x are clustered together in Cluster 10. *Paenibacillus terrae* HPL-003 is considered a very good xylanase producer (Hwang et al. 2011). *P. alvei* A6-6i-x is known to produce peptide antibiotics (Anandaraj et al. 2009; Luo et al. 2013).

Cluster 11 includes *Paenibacillus frigoriresistens* JCM 18141 and *Paenibacillus swuensis* JCM 18491. Genomes of these strains have not been sequenced yet, but strains were identified on the basis of 16S rRNA (Fig. 11.1).

Paenibacillus pini JCM 16418 is closely related to *Paenibacillus terrigena* DSM 21567 and they are clustered together in Cluster 12. Permanent draft genome sequence of *P. terrigena* DSM 21567 is available in NCBI and IMG database. 16S rRNA phylogenetic analysis of *Paenibacillus* sp. revealed that *P. terrigena* DSM 21567 exhibited very low levels of similarity (not more than 94 %) which is sufficient to indicate that strain DSM21567 represents a novel member of the genus (Stackebrandt and Goebel 1994; Xie and Yokota 2007).

11.3 Ecology of *Paenibacillus* Species

Endospore-forming *Paenibacillus* bacteria are essentially ubiquitous. They have been isolated from a wide range of extremely diverse habitats (McSpadden Gardener 2004; Lal and Tabacchioni 2009). Cold and warm springs, glaciers, sea sediments, desert soil, animal feces, plant nodules, industrial wastewater, etc. are some of the habitats where different *Paenibacillus* species can thrive. *Paenibacillus* bacteria have been also isolated from human specimen, namely blood, urine, cerebrospinal fluid, and sputum and have been associated with some human health disorders, such as bacteremia, endocarditis, meningitis, prosthetic osteoarticular infection, and chronic prostatitis (Reboli et al. 1989; Noskin et al. 2001; Roux and Raoult 2004; Ko et al. 2008; Ouyang et al. 2008; Rieg et al. 2010).

The spread of *Paenibacillus* sp. over so different habitats reflects a tremendous degree of ecologically relevant diversity at the species level in this genus, as revealed by the phenotypic characterization of culture isolates. For example, although most species are mesophile, several psychro- or thermo-tolerant species are able to colonize or to survive in extreme environments, such as hot springs and Antarctic soil (Mead et al. 2012; Van Houdt et al. 2013). Diversity can also be observed as far as nitrogen fixation is concerned. In fact, nitrogen fixation occurs in *P. azotofixans*, *P. macerans*, *P. polymyxa*, *Paenibacillus massiliensis*, *Paenibacillus stellifer*, *P. forsythiae*, *P. sophorae*, and in several other species, but not in the majority of *Paenibacillus* species (Xie et al. 2012a, b). Another example of ecological diversity at the species level concerns *Paenibacillus* invertebrate pathogens: *P. larvae*, known to cause American foulbrood in honeybees, and *P. popilliae* and *P. lentimorbus*, causative agents of type A and type B milky disease in Japanese beetle and related scarab larvae (Pettersson et al. 1999; Ashiralieva and Genersch 2006). On the other

hand, niche specificity and important ecological activities appear to span phylogenetic boundaries. Most species can survive as saprophytes in soils, which are considered the primary reservoirs of these bacteria; however, most viable cells probably occur as inactive spores at any given time. Furthermore, multiple species can be recovered as epiphytes and endophytes of plants and animals, as well as foodstuffs and composts derived from them. The rich variety of organic substrates and micro-niches present in those environments supports a complex milieu of microbial species, so it is perhaps not surprising that multiple species of *Paenibacillus* inhabit them. Moreover, it is well known that most *Paenibacillus* species produce a wide array of hydrolytic enzymes, such as chitinases, amylases, xylanases, cellobiohydrolases, proteases, etc., which can confer an advantage in the colonization of the abovementioned environments (Mavingui and Heulin 1994; Budi et al. 2000; Lee and Lim 2004). Thus, it is reasonable to infer that functionally distinct isolates occur within and among the phylogenetically distinct species of this genus. This is clearly the case for isolates functionally defined as "beneficial" to plant health because only a fraction of isolates of *P. polymyxa*, for example, can be shown to inhibit the activity of a pathogen under a given set of conditions (McSpadden Gardener 2004).

Spore formation and metabolic versatility associated with other characteristics as, for example, the ability to fix nitrogen, to solubilize phosphorus, to degrade polyaromatic hydrocarbons, and to produce antimicrobial substances and phytohormones may well explain the impressive display of colonization ability of the genus *Paenibacillus*.

Beside soil, the major isolation source of *Paenibacillus* spp. is represented by plants; indeed, around 40 % of *Paenibacillus* species have been isolated from different soil types, such as volcanic, desert, antarctic, and alkaline soil, but more than 20 % can be isolated from different plant species. As far as the latter are concerned, the rhizosphere is the major source of new *Paenibacillus* species; however, several species have also been isolated from the phyllosphere, plant tissues, and root nodules.

11.3.1 Paenibacillus *in the Rhizosphere*

Multiple *Paenibacillus* sp. can be readily cultured from both bulk and rhizosphere soils. Culturable counts of these bacteria range from log 3 to log 6 cells per gram fresh weight, with soil counts typically exceeding those obtained from the rhizosphere (McSpadden Gardener 2004). Single *Paenibacillus* species can colonize different plant species: *P. polymyxa*, for example, has been recovered from a large variety of plants (Lal and Tabacchioni 2009). While multiple species of *Paenibacillus* can be detected in the soils and rhizosphere, few studies have addressed the question of which are the most commonly isolated species or the relative dominance of single *Paenibacillus* species. Native rhizosphere-colonizing populations of *Paenibacillus* have been shown to vary with time, soil type, crop cultivar, and cropping pattern. da Mota et al. (2005), while studying the *Paenibacillus* community in two different

types of soil in Brazil, found that in one soil, *P. amylolyticus* and *P. graminis* were more abundant, whereas in the other, *Paenibacillus flavisporus* were most prevalent. Also, at the intraspecific level, natural factors can significantly influence the population structure. In fact, when four different cultivars of maize were planted in the same type of soil, the same authors found that the *P. polymyxa* strains isolated from the rhizospheres of the various maize cultivars were genotypically significantly different. Cheong et al. (2005) investigated the diversity of root-associated *Paenibacillus* spp. in winter crops. They found that 56 % of *Paenibacillus* isolates were classified as *P. polymyxa*, the type species for the genus, implying that *P. polymyxa* is the dominant species among root-associated *Paenibacillus* spp. in winter crops. Interestingly, as far as nitrogen fixation ability is concerned, 49 % of the *Paenibacillus* isolates did not contain the *nifH* gene. Moreover, the majority of the *Paenibacillus* isolates exhibited antagonistic activities toward various plant pathogens, suggesting that *Paenibacillus* spp. may be effective as biological control agents; however, no significant relationship between root colonization ability, the presence of the *nifH* gene, and plant growth-promoting effect could be observed. Furthermore, Cheong et al. (2005) tested the *Paenibacillus* strains for their ability to secrete extracellular enzymes, such as amylase, cellulose, and protease and found that the vast majority of isolates exhibited strong hydrolytic activity, indicating that the synthesis of extracellular hydrolytic enzymes would seem to be very common among *Paenibacillus* bacteria isolated from plant roots and that these enzymes are apparently necessary for adaptation to the rhizosphere soil environment.

11.3.2 **Paenibacillus** *in Root Nodules and Plant Tissues*

Prosopis farcta, a woody legume, is a widespread species in North Africa and the Middle East, whose root noodles harbor a variety of nodulating and non-nodulating bacteria. Among the latter, several nitrogen-fixing *Paenibacillus* sp. have been recovered, and a new species, *P. prosopidis*, has been described (Valverde et al. 2010). This is not the only endophytic *Paenibacillus* species found in root nodules. Recently, a new species, *Paenibacillus endophyticus*, has been found in nodules of *Cicer arietinum* and a strain of *P. polymyxa* has been observed to invade roots and root nodules of soybean upon inoculation (Annapurna et al. 2013; Carro et al. 2013). It has been assumed that the common trait of these endophytic bacteria is their inability to nodulate; therefore, the endophytic *Paenibacillus* sp. so far isolated can be considered as nonsymbiotic endophytic bacteria associated to root nodules. In fact, it has been observed that root nodules of legume species can be invaded and massively colonized by different bacterial taxa and that these replace the putative nitrogen-fixing rhizobia symbionts in the nodules. However, in contrast with the general assumption of *Paenibacillus* endophytic strains as nonsymbiotic bacteria, Latif et al. (2013) isolated a strain of *Paenibacillus* capable of nodulating roots of red clover (*Trifolium pratense*). The activity the various endophytic *Paenibacillus*

species exert in root nodules is not always clear. Annapurna et al. (2013) have reported a stimulatory effect on soybean growth by a strain of *P. polymyxa*. Upon colonization of root nodules by this strain alone or in combination with *Bradyrhizobium japonicum*, an increase in both shoot and root dry weight was observed.

Endophytic *Paenibacillus* bacteria are not limited only to root nodules legumes. *Paenibacillus* sp. has been recovered as an endophyte from different plants like pine, ginseng, coffee, and poplar. Recently a *P. polymyxa* strain has been isolated from the internal stem tissue of a lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) seedling naturally regenerating in central British Columbia, Canada (Bal et al. 2012). This strain is unique as it is the only microorganism that has been reported to fix substantial amounts of N_2 in association with tree species (Anand et al. 2013). Reintroduction of the strain to lodgepole seed under controlled environment results in rhizospheric and endophytic colonization of resultant seedlings as well as provision of increasing amounts of foliar N_2 as seedlings develop. Another strain of *P. polymyxa* has been isolated from the internal tissues of ginseng leaves. Inoculation of the same strain by foliar application enhanced plant growth parameters (Gao et al. 2015). A recent study reveals the importance of the *Paenibacillus* strains as endophytic bacteria in micropropagated tissue cultures of woody plants (Ulrich et al. 2008). In fact, high densities of endophytic *Paenibacillus* bacteria were found in plant material from poplar, larch, and spruce that had been micropropagated for at least 5 years. The majority of the isolates showed a close relationship to *Paenibacillus humicus*. Faria et al. (2013) isolated several endophytic strains belonging to the species *P. lentimorbus* and *Paenibacillus macerans* from the meristem tissues of in vitro grown orchid *Cymbidium eburneum*. They were all able to synthesize indole-3- acetic acid (IAA), and when inoculated in *Cattleya* seedlings, they all enhanced biomass in both shoots and roots.

11.3.3 Paenibacillus *in the Phyllosphere*

Various *Paenibacillus* species have been found on the leaves of different plant species (Rivas et al. 2005a, 2007; Valverde et al. 2008), able to excrete a diverse range of extracellular polysaccharide-hydrolyzing enzymes, including cellulases and xylanases. The abundance and hydrolytic ability of these bacteria indicate that they could have a prominent role in the degradation of leaves after drying and detachment from the plant. In fact, it has been suggested that these and other polymer-degrading bacteria can be involved in the first stages of the degradation processes in leaves. For example, in the phyllosphere of palm tree bracts (*Phoenix dactilyfera*), several of these bacteria have been found, mostly belonging to the genus *Paenibacillus* (*Paenibacillus phyllospherae* and *Paenibacillus cellulolyticus*) (Rivas et al. 2007). *P. phyllosphaerae* is able to hydrolyze cellulose and xylan, the most abundant polysaccharides and the major components of the plant cell wall (Rivas

et al. 2005a). In the case of the sweet chestnut (*Castanea sativa*), degradation of leave tissues starts when the leaves are still attached to the tree after drying. Among polymer-degrading bacteria found on the leaves of this plant a new species, *P. castaneae* has been isolated (Valverde et al. 2008).

11.4 *Paenibacillus* spp. as Plant Growth-Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) can promote plant growth either through direct effects or by indirect effects – the former including the production of phytohormones, the solubilization of soil phosphorous and iron and providing the host plant with fixed nitrogen (N_2) and the latter involving the suppression of plant diseases caused by deleterious microorganisms and stimulation of plant host defense mechanisms (Kloepper and Metting 1992; Kuklinsky-Sobral et al. 2004). As shown in Table 11.3, several species of the *Paenibacillus* genus have been described as plant growth promoters (PGPs) exerting their activity by multiple mechanisms such as phytohormone production, N_2-fixation, phosphorous solubilization, and antagonistic activity against soilborne pathogens (Govindasamy et al. 2011).

11.5 Production of Plant Growth Regulators, Nutrient Supply, and N_2-Fixation

Bacteria belonging to different *Paenibacillus* species isolated from soils and the rhizosphere of crop plants and used as rhizosphere inoculum, effectively improve plant growth (von der Weid et al. 2003; Lapidot et al. 2015; Naing et al. 2015). Plant growth promotion by strains isolated from sources other than soil and rhizosphere of crop plants has been also described, providing the evidence for utilizing bacterial strains from unconventional sources for plant growth promotion (Das et al. 2010; Chaudhry et al. 2013).

Although most PGP strains belonging to the *Paenibacillus* genus have been described as N_2-fixing bacteria, their involvement in the soil phosphate solubilization and production of phytohormones has also been reported (Das et al. 2010; Turan et al. 2012; Khan et al. 2012a, b; Kadyan et al. 2013; Faria et al. 2013; Pandya et al. 2015). *P. elgii* isolate SMA-1-SDCH02, recovered from chitin-rich soil, displayed mineral phosphate solubilization apart from its chitinolytic and antifungal activities to promote the growth of groundnut and tobacco plants in greenhouse and under in vitro studies, respectively. This isolate significantly enhanced the growth of the groundnut and tobacco in terms of shoot height, root length, and fresh and dry weight besides increasing the total chlorophyll content in the leaves (Das et al. 2010).

The effect of inoculation of wheat with *P. polymyxa* RC05 on yield and phosphorus solubilization was investigated in field trials. Although this strain resulted to be

Table 11.3 Plant growth promoting activity of *Paenibacillus* sp. bacteria

Strain	Source	Activity	Tests	References
P. alvei K165	Tomato root tips	Biocontrol against *Fusarium oxysporum* f.sp. *melonis* and *Verticillium Dahliae*	Field	Antonopoulos et al. (2008), Charalambous et al. (2013)
P. elgii SMA-1-SDCH02	Soil (premises of a chitin/chitosan producing company)	PGP (groundnut, tobacco)	Gnotobiotic system	Das et al. (2010)
P. elgii HOA73	Tomato field	Biocontrol against root knot nematode *Meloydogine incognita* in tomato	Greenhouse	Nguyen et al. (2013)
P. ehimensis RS820	Vegetable garden	Biocontrol against root knot nematode *M. incognita* in tomato	Greenhouse	Hong et al. (2013)
P. kribbensis PS04	Soil from insecticidal botanical garden	Biocontrol of *Rhizoctonia solani* in rice	Greenhouse	Gao and Liao (2014)
P. lentimorbus B30488^{r}	Cow's milk	Biocontrol against *Alternaria solani* in tomato, PGP (chickpea) in the presence of Cr	Greenhouse	Khan et al. (2012a, b)
P. macerans	Meristems tissues of *Cymbidium eburneum* orchids	PGP (*Cattleya loddigesii*)	Greenhouse	Faria et al. (2013)
P. polymyxa P2b-2R	Internal stem tissues of naturally regenerating pine seedlings	PGP (pine)	Gnotobiotic system	Anand et al. (2013)
P. polymyxa GBR-1	Rotten ginseng roots	Biocontrol against root knot nematode *M. incognita* in tomato	Greenhouse	Khan et al. (2008)
P. polymyxa SQR-21	Watermelon rhizosphere	Biocontrol against *F. oxysporum* f.sp. *niveum*	Greenhouse	Raza et al. (2009)

(continued)

Table 11.3 (continued)

Strain	Source	Activity	Tests	References
P. polymyxa 12.4.1	Marine clay soil	Biocontrol of *F. oxysporum* f.sp. *radicis lycopersici* and *Phytium aphanidermatum* in tomato	Greenhouse	Postma et al. (2013)
P. polymyxa RC05	Wheat rhizosphere	PGP (wheat)	Field	Turan et al. (2012)
P. polymyxa E681	Winter barley rhizosphere	Biocontrol against soilborne pathogens complex of sesame *Pseudomonas syringae*, *Phytophthora capsici*	Field, greenhouse	Ryu et al. (2006), Lee et al. (2012b), (2013b)

effective in enhancing wheat yield and phosphorus (P)-solubilization processes in the soil in comparison to the control, it resulted to be less effective than *Bacillus subtilis* OSU-142 and *Azospirillum brasilense* Sp245 under the same experimental conditions demonstrating that plant growth promotion may vary depending not only on growth conditions and crop management but also on plant and bacterial species (Turan et al. 2012).

P. lentimorbus strain B-30488r (B-30488r) was reported both as plant growth-promoting and bioremediation agent useful in Cr-contaminated rhizosphere soil of chickpea. This strain, isolated from Sahiwal cow's milk, shows antagonism against phytopathogens, *Fusarium oxysporum* f. sp. *ciceri* and *Alternaria solani* (DasGupta et al. 2006; Chaudhry et al. 2013). In vitro studies showed that it can tolerate 200 µg ml-1 of Cr and produce the plant growth-promoting substance indole acetic acid (IAA) in the presence of inorganic Cr. Both in absence and presence of supplemented Cr(VI) the enhancement of biofilm formation by sodium alginate (SA) and calcium chloride ($CaCl_2$) as compared to unsupplemented control was also observed. The plant growth-promoting effects caused by *P. lentimorbus* B-30488^r were evaluated in greenhouse experiments on chickpea plants cultivated in the presence of Cr(VI). Based on the measure of shoot and root length and plant dry matter, the authors suggested a phytoprotective role of the biofilm produced acting as a shield in preventing the direct access of toxic Cr to plant tissues, thus reducing its uptake in plants (Khan et al. 2012a).

P. macerans NBRFT5, a strain isolated from the rhizosphere of *Typha latifolia* grown on fly ash dumps, was used in combination with *Bacillus endophyticus* NBRFT4 and *Bacillus pumilus* NBRFT9 isolated from the same source, in phytoremediation of metals from the fly ash of the wild-type plant *T. latifolia*. Results revealed the potential of this consortium to induce phytoremediation of metal from fly ash as it was able to enhance concentration of both essential and nonessential

metals like Pb, Fe, Cu, Zn, Cr, Ni, and Cd in different plant parts and also to promote the growth of *T. latifolia* plants (Tiwari et al. 2013).

IAA-producing bacteria belonging to the species *P. macerans*, previously isolated from the meristems of *Cymbidium eburneum* orchids, have been reported to promote plant growth during seedling acclimatization in the orchid species *Cattleya loddigesii* (Faria et al. 2013). Experiments were carried out in a greenhouse using bacteria-free micropropagated *C. loddigesii* seedlings. It is noteworthy that these bacteria were not able to solubilize phosphate, thus, the authors suggested IAA production as responsible of plant growth promotion.

Production of IAA was also observed in *Paenibacillus* sp. strain JP44SK7 isolated from the rhizospheric soil of *Phyllanthus amarus*, an important medicinal herb in Indian traditional system cultivated at a commercial level. However, its ability to promote the growth of *Phyllantus amarus* needs further investigation (Kadyan et al. 2013).

Several species of *Paenibacillus* genus have been described to be able to fix N_2 with the *P. polymyxa* species accounting for the majority of N_2-fixing strains isolated so far (Lal and Tabacchioni 2009; Xie et al. 2012a, b). Although the members of N_2-fixing *Paenibacillus* species have great potential uses as biofertilizers in agriculture, the role of N_2-fixation in promoting plant growth needs to be assessed for most isolates. *P. polymyxa* strain P2b-2R is a N_2-fixing bacterium that was isolated from the internal stem tissue of a lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) seedling naturally regenerating in central British Columbia, Canada (Bal et al. 2012). This strain is unique as it is the only microorganism that has been reported to fix substantial amounts of N_2 in association with tree species. Reintroduction of this strain to lodgepole pine seeds under a controlled environment resulted in rhizospheric and endophytic colonization of seedlings as well as provision of increasing amounts of foliar N as seedlings develop (Bal and Chanway 2012a; Anand and Chanway 2013a, b). The foliar N content and biomass of pine seedlings had increased more than twofold during 13 months after seed inoculation (Anand et al. 2013). Similar effects but of lesser magnitude were observed when seedlings generated from western red cedar (*Thuja plicata* Donn.) seeds inoculated with P2b-2R were grown under controlled environmental conditions (Bal and Chanway 2012b; Anand and Chanway 2013b).

Bacteria belonging to *Paenibacillus* genus have also been proved to have a synergistic effect when inoculated with other N_2-fixing bacteria. Cerqueira Rodriguez et al. (2013) performed experiments of co-inoculation of cowpea with *Bradyrhizobium* sp. (BR 3267) and plant growth-promoting bacteria belonging to the genera *Bacillus*, *Brevibacillus*, and *Paenibacillus* in Leonard Jar. They observed synergism in plant growth promotion of cowpeas using the pairs *Bradyrhizobium* BR 3267 + *P. graminis* (MC 04.21) and *Bradyrhizobium* BR 3267 + *P. durus* (C 04.50).

A mixed inoculum containing a N_2-fixing *P. polymyxa* strain, and several other PGPR capable of solubilizing phosphate or producing auxins such as *Rahnella* sp., *Serratia* sp., and *Pseudomonas* sp. have been used to verify its ability to promote the growth of switchgrass (*Panicum virgatum* L.) plants under field conditions with low N_2 inputs (Ker et al. 2012). All these bacteria were isolated from switchgrass

rhizomes that had not received N_2 fertilizers for over 10 years and which increased plant growth in the absence of N_2 fertilizers under growth chamber conditions. Overall, inoculation with the mixed inoculum resulted in a seeding year yield increase of 43 %, compared with the N_2 fertilizer treatment alone with a yield increase of 83 %. A combination of N_2 fertilizer (100 kg N $ha^{-}1$) and the mixed inoculum increased yield by 123 %. More tillers per unit area within a stand, as well as a greater population of medium- and tall-sized tillers were produced within PGPR treated stands. As the inoculum used in this study was a mixture of PGPR, which have been shown to be capable of solubilizing P, producing IAA-like substances, and, in the case of *P. polymyxa*, to fix N_2 the authors hypothesized a combination of these mechanisms responsible for plant growth promotion.

11.6 Biocontrol

Bacteria of the genus *Paenibacillus* are among the most efficient microbial biocontrol agents for crop protection against diseases. Their mode of action is supposed to be related to their production of antibiotics, such as cyclic lipopeptides, lantibiotics, macrolides, and polyketides as well as the production of hydrolytic enzymes, the competition with pathogens for ecological niche and substrate in the rhizosphere, and the reinforcement of the host plant-defensive potential via stimulation of its immune machinery (McSpadden Gardener 2004; Debois et al. 2013).

To determine which compound and/or mechanisms are involved in the antagonism of *Paenibacillus* bacteria toward other microorganisms represents a significant challenge in view of developing more efficient biocontrol agents to be used for crop protection. In many reports, the role in antagonism for a given antibiotic is suggested simply by the fact that the strain (or its crude culture extract), known to produce these compounds, displays some pathogen inhibition potential. To relate the antagonistic activity with specific antibiotics, polymerase chain reaction (PCR) with specific primers for the detection of the corresponding genes in the genome of the specific strain or genome mining using published sequence data of close relatives have also been performed. However, none of these approaches allows to draw any conclusion about the link between antibiotic potential and biocontrol activity, nor does it clearly prove the involvement of a specific compound in the antagonism developed by the strain against phytopathogens. Testing single purified compounds from culture broth necessitates extensive fractionation of crude culture extracts involving the combination of various chromatographic steps. The use of impaired (or overproducing) mutants and correlation with the respective loss (or increase) in the antagonistic activity compared with the wild-type strain may also represent a valuable approach to identify compounds playing a crucial role.

Among the genus *Paenibacillus*, the species *P. polymyxa* accounts for the majority of *Paenibacillus* strains showing the ability to suppress several plant diseases (Ryu and Park 1997; Lal and Tabacchioni 2009). *P. polymyxa* strains, isolated from the rhizosphere of several crops, are capable of producing several hydrolytic

enzymes, including proteases, β-1,3-glucanases, cellulases, xylanase, lipase, amylase, and chitinases which play a significant role in the biocontrol of plant pathogens (Beatty and Jensen 2002; Raza et al. 2008; Choi et al. 2008a; Petersen et al. 1996). *P. polymyxa* is also known to produce mainly two types of peptide antibiotics: polymyxins and fusaricidins. Polymyxins are active against bacteria whereas fusaricidins are active against fungi and actinomycetes (Beatty and Jensen 2002). Bacteria belonging to the species *P. polymyxa* have been proven to be effective in the control of gray mold in strawberries caused by *Botrytis cinerea* (Helbig 2001), as well as of *F. oxysporum* and *Pythium* spp. – causal agents of seedling blight and wilt, root rot of cucumber and watermelon (Dijksterhuis et al. 1999; Yang et al. 2004), and sesame damping-off (Ryu et al. 2006). Furthermore, several strains of *Paenibacillus* sp. are able to control diseases caused by *Phytophthora palmivora*, *Pythium aphanidermatum*, *Erwinia amylovora*, and *Pseudomonas syringae* pv. maculicola ES4326 (Timmusk and Wagner 1999; Lee et al. 2012a; Niu et al. 2013). Recently, the matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometry (MALDIFTICR MS) method was applied to identify the compounds present in the fungus growth inhibition area during simultaneous growth of *P. polymyxa* strain Pp56 and *F. oxysporum* (Debois et al. 2013). Fusaricidins A, B, and C and numerous members of the LI-F antibiotics family were identified. MALDIFTICR mass spectrometry imaging was then used to follow the diffusion of lipopeptides involved in the inhibitory activity over time. The authors concluded that some lipopeptides such as fusaricidin B and a mixture of antibiotics of the LI-F family were mainly involved in the defense mechanism of *P. polymyxa* Pp56. Suppression of a fungal pathogen mediated by the production of fusaricidins was demonstrated for the *P. polymyxa* strain SQR-21 isolated from the rhizosphere of healthy watermelon plant roots in a heavily wilt-diseased field (Ling et al. 2010). MALDI-TOF MS analysis revealed that *P. polymyxa* SQR-21 produces four kinds of fusaricidins: A, B, C, and D. The whole gene cluster for the production of fusaricidin (*fus*A) was isolated and identified from this strain, and its role in fusaricidin biosynthesis was confirmed by gene disruption. Indeed, the lack of fusaricidin production and abolishment of the antifungal activity by the *fus* disruption mutant of SQR21 confirmed the crucial role of the *fus* gene cluster in fusaricidin synthesis, as well as the importance of fusaricidin in antifungal activity (Choi et al. 2008b; Li and Jensen 2008). These results provided useful knowledge about transcription and regulation of the *fus* gene cluster in *P. polymyxa*, as well as the direction of gene engineering for improving the production of fusaricidins.

A study on the influence of ferric ion (Fe^{3+}) on the growth and fusaricidins production by *P. polymyxa* strain SQR- 21 revealed that the production of fusaricidin type increases only up to 50 μM Fe^{3+} and higher levels of Fe^{3+} are inhibitory. As Fe^{3+} concentration increases in the culture medium, the content of intracellular protein, intracellular carbohydrate, extracellular protein, and polysaccharide rises while the intracellular lipid content increases only up to 50 μM Fe^{3+}. Moreover, the authors found that the regulatory effects of Fe^{3+} were reflected by the increase in total RNA content and relative expression of the fusaricidin synthetase gene (*fusA*) up to 50 μM Fe^{3+}, after which a continuous decrease was observed (Raza et al. 2010).

P. polymyxa M-1 is a good candidate for the biocontrol of fire blight, a serious disease of apple and pear caused by the pathogen *E. amylovora*, due to its wide variety of secondary metabolites with antimicrobial activity produced. In particular, this strain synthesizes two components of polymyxin P, polymyxin P1, and P2 that have been characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) in combination with bioautography. Using agar diffusion tests, it was observed that culture supernatants of *P. polymyxa* M-1 suppressed the growth of the phytopathogenic strains *E. amylovora* Ea 273 and *E. carotovora*. The corresponding polymyxin synthetase gene cluster was also identified and further characterized by domain analysis as being different from the *pmx* gene clusters encoding polymyxin A and B, respectively (Niu et al. 2013).

P. polymyxa 12.4.1, known for its ability to mobilize phosphate and for its potential to control plant pathogens (Postma et al. 2010), was tested in greenhouse as biocontrol agent of *P. aphanidermatum* and *F. oxysporum* f. sp. *radicis-lycopersici* causing, respectively, damping-off and crown and root rot of tomato plants (Postma et al. 2013). Animal bone charcoal, a phosphorous-rich waste product, was used as a carrier to deliver the biocontrol agent into the soil, thus combining recycling of phosphorous from animal bones and biocontrol of plant pathogens. Results presented in this study reveal that *P. polymyxa* 12.4.1 controlled *P. aphanidermatum* significantly, whereas it was not effective against *F. oxysporum* f. sp. *radicis-lycopersici*.

The low-rate-sporulating *P. polymyxa* 18SRTS, isolated from the rhizosphere of the medicinal plant *Calendula officinalis*, showed in vitro antagonistic activity against the plant pathogenic fungi *F. oxysporum* and *B. cinerea*. However, the mode of action of this strain needs to be elucidated; indeed, it does not produce siderophores and produces high concentrations of IAA up to 53 μM ml^{-1} (Ait Kaki et al. 2013).

Some PGPR are known to suppress diseases by inducing systemic resistance (ISR) in the plant against both root and foliar pathogens (Wei et al. 1996; Choudhary et al. 2007). This mechanism has also been observed in some PGPR belonging to the genus *Paenibacillus*.

P. polymyxa E681 was isolated from roots of winter barley (*Hordeum vulgare L.*) in southern Korea and is a promising biological control agent that could promote growth and elicit biological control activity in diverse crop systems (Ryu et al. 2006). It is active against the foliar pathogen *P. syringae* pv. maculicola ES4326 (Lee et al. 2012a) and Phytophthora blight of red pepper (Lee et al. 2013b). Suppression of early seedling damping-off in cucumber (*Cucumis sativus L.*) and sesame (*Sesamum indicum L.*) was observed after seed treatments with this strain under greenhouse and field conditions, respectively (Ryu et al. 2005a, b, 2006). Moreover, another possible mechanism for plant growth promotion by *P. polymyxa* E681 is the synthesis of plant growth regulators auxin and cytokinin (Lebuhn et al. 1997; Timmusk et al. 1999). Whole genome sequencing of strain E681 revealed that it encodes an entire set of genes related to the production of indole acetic acid (IAA) (Kim et al. 2013). Recently, Lee et al. (2012b) found that it promotes the growth of *Arabidopsis* seedlings producing volatile organic compounds (VOCs) that prime

transcriptional expression of the salicylic acid, jasmonic acid, and ethylene signaling marker genes *PR1*, *ChiB*, and *VSP2*. They also found that *P. polymyxa* E681 produces a novel class of long-chain bacterial volatile organic compounds (VOCs), i.e., the C13 hydrocarbon tridecane that can also elicit ISR as can C4 alcohols such as 2,3-butanediol. The potential of the antibiotic fusaricidin, produced by this strain, as one of the ISR elicitors for protecting red pepper plants against *Phytophthora* blight under greenhouse conditions, was also reported (Lee et al. 2013b). Indeed, these authors observed that application of fusaricidin at the lowest concentration (0.1 ppm of fusaricidin) by foliar spray significantly reduced *Phytophthora* leaf blight infection when compared with water-treated control plants. Moreover, they proved that fusaricidin is responsible for the activation of the defense gene PR-1a involved in the ISR by means of mRNA accumulation in the leaves of *Arabidopsis thaliana* plants treated with fusaricidin.

P. kribbensis PS04, isolated from soil samples of an insecticidal botanical garden in China showed antagonistic activity against *Rhizoctonia solani* causing rice sheath blight in both in vitro and in vivo experiments (Gao and Liao 2014). Crude metabolites were tested against mycelia in plate assays whereas spray treatments using crude metabolites and fermentation broth were used 24 h before and after an infestation of rice seeds with *R. solani*. The inhibitory effect on *R. solani* observed in both in vitro and in vivo experiments was correlated to the defense-related enzyme activity, i.e., peroxidase and polyphenol oxidase of plants, suggesting that *P. kribbensis* could trigger induced resistance of rice plants to suppress the pathogen (Gao and Liao 2014).

The antagonistic activity of *P. alvei* K165 against the soilborne fungus *Verticillium dahliae* has been reported under greenhouse and field trials (Tjamos et al. 2004, 2005; Antonopoulos et al. 2008). Root dipping and soil drenching of eggplants with this strain resulted in reduced disease severity compared to the control treatment under high *V. dahliae* inoculum.

In heavily *Verticillium*-infested potato fields, experiments with potato seeds dusted with the bacterial talc formulation showed a significant reduction in symptom development and a 25 % increase in yield over the untreated controls (Tjamos et al. 2004). Furthermore, it was observed that *P. alvei* K165 triggers induced systemic resistance (ISR) in a salicylic acid-dependent pathway in *Arabidopsis thaliana* plants against *V. dahliae* (Tjamos et al. (2005). Moreover, this strain was able to reduce the microsclerotia germination of *V. dahliae* in the root tips and the zone of elongation of eggplants as well as in the soil without plants (Antonopoulos et al. 2008). Recently, an in vitro activity of *P. alvei* K165 against Fusarium wilt of melon caused by *F. oxysporum* f. sp. *melonis* was reported by Charalambous et al. (2013). They observed that incorporating *P. alvei* K165 into the transplant soil plug at a ratio of 10 % allowed an adequate biocontrol agent population size in the rhizosphere that reduced Fusarium wilt symptom development and triggered the expression of the plant defense-associated genes *Chi1* and Pal1. Fusarium wilt of melon is a major disease affecting melon production worldwide. Management of this disease is mainly through chemical soil fumigation and the use of resistant cultivars. However, the broad spectrum biocides used to fumigate soil before planting are environmen-

tally damaging, and, on the other hand, resistance appears to be genetically complex and thus a difficult trait to confer by breeding (Berrocal-Lobo and Molina 2008). Therefore, the development and use of biocontrol agents against Fusarium wilt of melon based on *P. alvei* K165 seems an appealing management strategy for both the conventional and organic farming industry.

A multifactorial mode of action of *P. lentimorbus* B-30488^{r} against the tomato plant pathogen *A. solani* including the activation of the plant defense mechanisms and the competition for substrate utilization has been proposed by Khan et al. (2012b). They observed that spray foliar treatments of infested tomato plants grown under greenhouse conditions resulted in the reduction of the incidence of the early blight disease caused by *A. solani* by 45.3 % as compared to the control. The evidence of the upregulating expression of genes encoding defense-related plant proteins and the similarity of the utilization profiles of *P. lentimorbus*B-30488^{r} and *A. solani* of carbon sources presumed to be available in the phyllosphere suggest the involvement of activation of the plant defense mechanisms and the competition for substrate utilization in the biocontrol activity of this strain.

A relationship between the antagonistic activity against the plant pathogenic bacterium *Ralstonia solanacearum*, responsible of tomato wilt, and the in vitro biofilm formation was observed for both *P. polymyxa*and *P. macerans* strains by Li et al. (2011). The ability of seven strains of *P. polymyxa* and nine strains of *P. macerans*, previously isolated from either mycorrhizal or non-mycorrhizal systems, to form biofilm in vitro and to protect tomato plants from *R. solanacearum* under growth chamber conditions was investigated. Results revealed that most *Paenibacillus* strains tested had the ability both to form biofilm in vitro and to protect tomato seedlings from bacterial wilt suggesting a possible role of biofilm in biocontrol activity. Moreover, the *Paenibacillus* strains were able to protect tomato plants independently from the incubation time needed to form biofilm under in vitro conditions.

A bioorganic fertilizer containing *P. polymyxa*SQR21 was used to control Fusarium wilt of watermelon by Ling et al. (2010). They prepared the biofertilizer fermenting aerobically a mixture of amino acid fertilizer and pig manure compost with *P. polymyxa* SQR21 for 6 days at 45 °C. Application of the bioorganic fertilizer both in the seedling nursery soil and in transplanted soil resulted in the lowest Fusarium wilt disease incidence of watermelon in both experiments. Moreover, it not only suppressed Fusarium wilt but also significantly promoted watermelon growth. Nursery applications resulted in the effective colonization by *P. polymyxa*SQR21 in both the rhizosphere and on the root surface, thus protecting plant roots from the invasion of *Fusarium* pathogens before seedlings are transplanted.

Another bioorganic fertilizer based on *P. polymyxa* SQR-21 and two other biocontrol agents (*Bacillus subtilis* SQR-9 and *Trichoderma harzianum* SQR-T037) was used to test its efficacy to control Fusarium wilt on cucumber plants in field experiments (Qiu et al. 2012). It was developed by fermenting mature composts with the three biocontrol agents. Application of the bioorganic fertilizer in combination with a commercial chemical fertilizer suppressed the disease incidence by 83 % and reduced yield losses threefold compared with the use of the commercial chemical fertilizer alone. Analysis of microbial communities in rhizosphere soils by

high-throughput pyrosequencing showed that more complex microbial community was present in the soil treated with the bioorganic fertilizer than in the soil treated with the organic fertilizer (amino acid fertilizer and pig manure composts, 1:1). The dominant taxonomic phyla found in both samples were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* among bacteria and *Ascomycota* among fungi. The abundance of beneficial bacteria or fungi, such as *Trichoderma*, *Hypoxylon*, *Tritirachium*, *Paenibacillus*, *Bacillus*, *Haliangium*, and *Streptomyces*, increased compared to the treatment with the organic fertilizer, whereas the soilborne pathogen, *Fusarium*, was markedly decreased.

Research on biological control of root-knot nematodes using rhizosphere microorganisms has been well documented (Yoon et al. 2012). Bacteria belonging to the genus *Paenibacillus*are among those bacteria showing nematicidal activity against root-knot nematode, Meloidogyne incognita. It was demonstrated that both under in vitro and in greenhouse conditions *P. polymyxa* strain GBR-1 and *Paenibacillus ehimensis* strain RS820 can suppress root galling and final nematode population of *M. incognita*on tomato plants (Khan et al. 2008; Huang et al. 2013). Culture filtrate of *P. polymyxa* GBR-1 significantly reduced *M. incogni ta* egg hatch and caused substantial mortality of its juveniles. Moreover, it reduced the root galling of tomato and nematode populations in the potting soil in greenhouse experiments and increased tomato plant growth and root mass production compared with untreated control (Khan et al. 2008). A mixed compost containing increased amounts of chitin and inoculated with *P. ehimensis*RS820, a chitinolytic soil bacterium previously isolated from soil in Korea, was used to control the root-knot disease in tomato plants. Results showed reduction of the disease and plant growth promotion in relation to the amount of inoculation with the biocontrol agents (Huang et al. 2013).

Although several strains of the *Paenibacillus* genus are reported to act as PGPR, at this time no *Paenibacillus*-based products have been commercialized in Europe or the United States yet. To our knowledge 12 patents based on *P. polymyxa*, *P. alvei*, and *P. macerans* species related to formulations and methods for controlling and suppressing plant pathogens have been deposited (http://patentscope.wipo.int/). One of the factors limiting the commercialization of products based on *Paenibacillus* bacteria is the poor information available on field tests under production conditions. A more thorough understanding of their ecology can help to figure out which problems to work on, how to approach them, when and where to apply the PGPR and predict situations in which control would not be expected to work.

11.7 Conclusions

The *Paenibacillus* genus includes species that have been isolated from a wide range of sources, and new species colonizing amazingly different habitats are continuously discovered.

An increasing number of studies have recently been published to test the capability of bacteria belonging to the different species of the *Paenibacillus* genus to pro-

mote plant growth and control plant diseases. Most of these studies have been carried out under in vitro and greenhouse conditions, and only a few of them rely on field tests. Patents based on *P. polymyxa*, *P. alvei*, and *P. macerans* species are related to formulations, and methods for controlling and suppressing plant pathogens have been deposited, but no commercial products are available yet. The complete genome sequence of several *Paenibacillus* sp. bacteria isolated from the soil and rhizosphere was recently made available. These data together with those resulting from experiments performed under field conditions will help to clarify the potential of *Paenibacillus* bacteria for developing effective biofertilizers and biocontrol agents to be used in sustainable agriculture.

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Chapter 12
Expanding the Horizons for the Use of *Paenibacillus* Species as PGPR for Sustainable Agriculture

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Abstract Plant growth-promoting rhizobacteria (PGPR) are the group of bacteria residing in the rhizosphere of the plant and impart beneficial effect on the overall health and growth of the plant. Efficient PGPR strains are known to possess plant growth-promoting (PGP) traits which include nitrogen fixation, solubilization of phosphates, production of auxins, cytokines and other phytohormones, production of siderophores, antibiotics, HCN, etc. The well-known PGPR include members belonging to genera *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, *Pseudomonas*, and *Serratia*. Strains belonging to *Paenibacillus* are poorly represented as PGPR despite of them possessing several efficient PGP traits. Species belonging to *Paenibacillus* genus possess essential trait of nitrogen fixation in addition to other mentioned PGP traits, which is not found in the majority of PGPR strains belonging to other genera. Before 20 years, *Paenibacillus* genus was separated from the genus *Bacillus*. Characteristics that distinguish the genus *Paenibacillus* from other members of the family Bacillaceae include rod shaped with a flagellum, producing ellipsoidal spores with swollen sporangia, positive for catalase, negative for H_2S production, variable for oxidase, and DNA G+C content ranging between 45 % and 54 mol%, with anteiso-C15: 0 as the major cellular fatty acid, meso-diaminopimelic acid as the diagnostic diamino acid, and more than 89.6 % intra-genus similarity in 16S rRNA gene sequences. To date 11 species of *Paenibacillus* possessing nitrogen fixation are recognized; they are *P. polymyxa*, *P. macerans*, *P. durus*, *P. peoriae*, *P. borealis*, *P. brasiliensis*, *P. graminis*, *P. odorifer*, *P. wynnii*, *P.*

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_12

massiliensis, and *P. sabinae*. This chapter focuses to portray all PGP traits possessed by species of *Paenibacillus* genus, which will help them to provide better status as efficient PGPR.

12.1 Introduction

Microbes act as the major resource for agricultural, industrial, and medicinal applications; however, large reservoir of microbes is yet to be discovered and the majority of vivid microbial diversity is unexplored (Handelsman et al. 1998; Daniel 2005; Lorenz and Eck 2005; Patel et al. 2011). Microbial populations are found to be present in diverse ecological niches, including extreme environments present in both lithosphere and hydrosphere, where their metabolic abilities play a critical role in geochemical nutrient cycling (Daniel 2005; Aeron et al. 2011; Jha et al. 2011). Rhizosphere is one such well-characterized ecological niche comprising the volume of soil surrounding plant roots with the highest bacterial population influenced by root exudates as defined by Hiltner (1904). Bacterial populations in the rhizosphere harbor 100–1000 times higher bacterial density than in bulk soil, and 15 % of root surface is covered by microbial populations belonging to several bacterial species (Jha et al. 2010; Govindasamy et al. 2011). Plant photosynthetic product (about 5–21 %) is secreted by roots in the form of different sugars which is in turn utilized by microbial populations. Subsequent metabolic activities of these bacteria in the rhizosphere kindle mineral nutrient transport and uptake by plant roots (Glick 1995). The affairs of microorganisms residing in the rhizosphere with plant roots are known since several decades. Microorganisms tend to interact with plant roots and affect plant health in either negative or positive manner. Microorganisms causing negative effect on the health of plant by its interactions are categorized plant pathogens, whereas microorganisms improving health of plants are termed as plant growth-promoting rhizobacteria (PGPR). PGPR include bacteria that reside in the rhizosphere and improve plant health ultimately aiding to augment plant growth. The importance of rhizobacteria for the plant health was showed by Kloepper and Schroth (1978) during Fourth International Congress of Bacterial Plant Pathogens, conducted in France in 1978. However, the term PGPR was coined latterly by the same author in 1980 (Good et al. 1994). Currently, the number of works per year on this topic has increased, creating a new discipline that has changed the basic traditional concepts of plant physiology and microbial ecology. Later, Bashan and Holguin (1998) proposed a revision of the original definition of the term PGPR, since there are a number of bacteria that may have a beneficial effect on the plant even though they are outside the rhizosphere environment. The augmentative effect of PGPR occurs through various mechanisms. This role involves not only the direct effect of a single bacterial strain but also that of the molecular dialogue established among soil microorganisms and plant. A thorough understanding of the plant growth-promoting (PGP) mechanisms is inevitable to manipulate the flora of

microbes in the rhizosphere, in order to maximize the processes that strongly enrich plant productivity. PGP mechanisms have been grouped traditionally into direct and indirect mechanisms. Accordingly, direct mechanisms include those that affect the balance of plant growth regulators, either because the microorganisms themselves release growth regulators that are integrated into the plant or because the microorganisms act as a sink of plant-released hormones, and those that induce the plant's metabolism leading to an improvement in its adaptive capacity, whereas indirect mechanism requires the participation of the plant's defensive metabolic processes, which respond to the signal sent from the bacteria influencing the plant. Two important mechanisms are included in this group: induction of systemic resistance to plant pathogens (biotic stress) and protection against unhealthy environment conditions (abiotic stress) (Solano et al. 2008).

Majority of credible group of PGPR belongs to genera *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Serratia*, *Thiobacillus*, *Pseudomonads*, and *Bacillus* which are widely studied (Glick 1995; Vessey 2003), whereas strains belonging to *Paenibacillus* genus are not vividly described by researchers as efficient PGPR, even when several strains of *Paenibacillus* are reported to possess several direct and indirect PGP traits. To our interest species of *Bacillus* and *Paenibacillus* of aerobic endospore-forming bacterial (AEFB) origin are abundantly found in agricultural systems. The native population of these two genera occurs abundantly in most rhizosphere soils, and plant tissues are differently colonized by distinct subpopulations (Mahaffee and Kloepper 1997; Seldin et al. 1998). The importance of *Bacillus* was well known as PGPR since the past 35 years, and later several species of *Bacillus* were reclassified as *Paenibacillus* in 2004 as reported in the 2nd edition of Bergey's Manual of Systematic Bacteriology. Since then the studies on the strains belonging to *Paenibacillus* genus have gradually increased. Several species of *Paenibacillus* is now reported to augment plant growth in various ways (Goswami et al. 2015). Species belonging to these genera can promote plant growth directly by synthesizing plant hormones or increasing mineral nutrient uptake by fixing atmospheric nitrogen, solubilizing soil phosphorus, and other known mechanics. Some species are known to suppress plant pathogens by producing antibiotic metabolites, whereas other species stimulate plant host defense prior to pathogen infection (Glick et al. 1999; Van loon 2007), indirectly contributing increased crop productivity. Published reports on endophytic colonization and biofilm formation by *Paenibacillus* spp. have suggested that the endophytic colonization and biofilm formation improve the bacterium ability to act as a biocontrol agent against plant pathogens (Hallman et al. 1997; Davey and O'Toole 2000; Timmusk et al. 2005). In recent years *Paenibacillus* spp. attracted considerable attention because of their advantages over other PGPR strains in inoculant formulations, stable maintenance in rhizosphere soil, and greater potentials in sustainable agriculture. The following are the strains belonging to *Paenibacillus* genus that have showed efficient PGP traits and studied thoroughly over the past 10 years: *P. polymyxa*, *P. macerans*, *P. durus*, *P. peoriae*, *P. borealis*, *P. brasiliensis*, *P. graminis*, *P. odorifer*, *P. wynnii*, *P. massiliensis*, and *P. sabinae*.

12.2 Separation of *Paenibacillus* from *Bacillus* and Their Status in Bergey's Manual by 2004

Paenibacillus, a genus separated from the genus *Bacillus*, was first proposed by Ash et al. (1993) where 11 species from *Bacillus* were reclassified as *Paenibacillus*, and they were *P. polymyxa*, *P. alvei*, *P. gordonae*, *P. larvae*, *P. pulvifaciens*, *P. macerans*, *P. azotojixans*, *P. pabuli*, *P. macquariensis*, *P. amylolyticus*, and *P. validus*. Heyndrickx et al. (1995) reported that *P. gordonae* was a synonym of *P. validus*, that *P. pulvifaciens* was a subspecies of *P. larvae* (Heyndrickx et al. 1996a), and that *Bacillus lautus* and *Bacillus peoriae* should be transferred to the genus *Paenibacillus* (Heyndrickx et al. 1996b). In addition, Nakamura (1996) proposed the new species *Paenibacillus apiarius*. Consequently, the genus *Paenibacillus* consists of 13 species and one subspecies. Later, Shida et al. (1997) embedded *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus*. Many species in this genus possess useful properties, such as nitrogen fixation (Achouak et al. 1999) and macromolecule degradation (Ash et al. 1993) which play an economically important role in agriculture. The *Paenibacillus*-specific primer PAEN515F (5′-GCTCGGAGAGTGACGGTACCTGAGA-3′) and universal primer 1377R (5′-GGCATGCTGATCCGCGATTACTAGC-3′) are now available to identify unknown species to be belonging to *Paenibacillus* genus (Shida et al. 1997; Chung et al. 2000). Most recent developments in bacterial taxonomy are comparative sequence analysis of housekeeping genes such as *rpoB*, *gyrA* or *gyrB*, *recA*, etc., to discriminate between species and even within species. Studies in *Paenibacillus* are very recent and mainly concentrate on typing aspects of these genes (Durak et al. 2006; Vollu et al. 2006). Although very promising taxonomic insights can be obtained with this kind of approach, more sequence data are needed before taxonomic rearrangements of *Paenibacillus* will be possible (Bergys manual). Figure 12.1 shows the phylogenetic neighbor-joining (NJ) tree based on 16S rRNA gene sequences showing the relationship between sequences of 16S rRNA genes among the related members of the genus *Paenibacillus* of the strains mentioned in Bergey's Manual of Systematic Bacteriology 2nd edition, 3rd volume: The Firmicutes (Logan and De Vos 2009).

Logan and De Vos (2009), in Bergey's Manual of Systematic Bacteriology (2nd edn., 2004), portrayed phylogenetic classification schemes which landed Clostridia and Bacilli into two different classes which were earlier considered as a single class in endospore-forming Firmicutes. Clostridia include the order Clostridiales and family Clostridiaceae. This class possesses 11 genera including *Clostridium*,

Fig.12.1 (continued) *Paenibacillus* of the strains mentioned in Bergey's Manual of Systematic Bacteriology 2nd edition, 3rd volume: The Firmicutes are shown. *Bacillus subtilis* H6 is taken as an out-group to show the dissimilarity of its 16S rRNA gene sequence with other species of *Paenibacillus*. Gene sequence obtained was aligned by ClustalW using MEGA 4.0 software (Tamura et al. 2007) and a neighbor-joining (NJ) tree. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. Bar 0.5 % sequence variation. GenBank accession numbers are given in *parentheses*

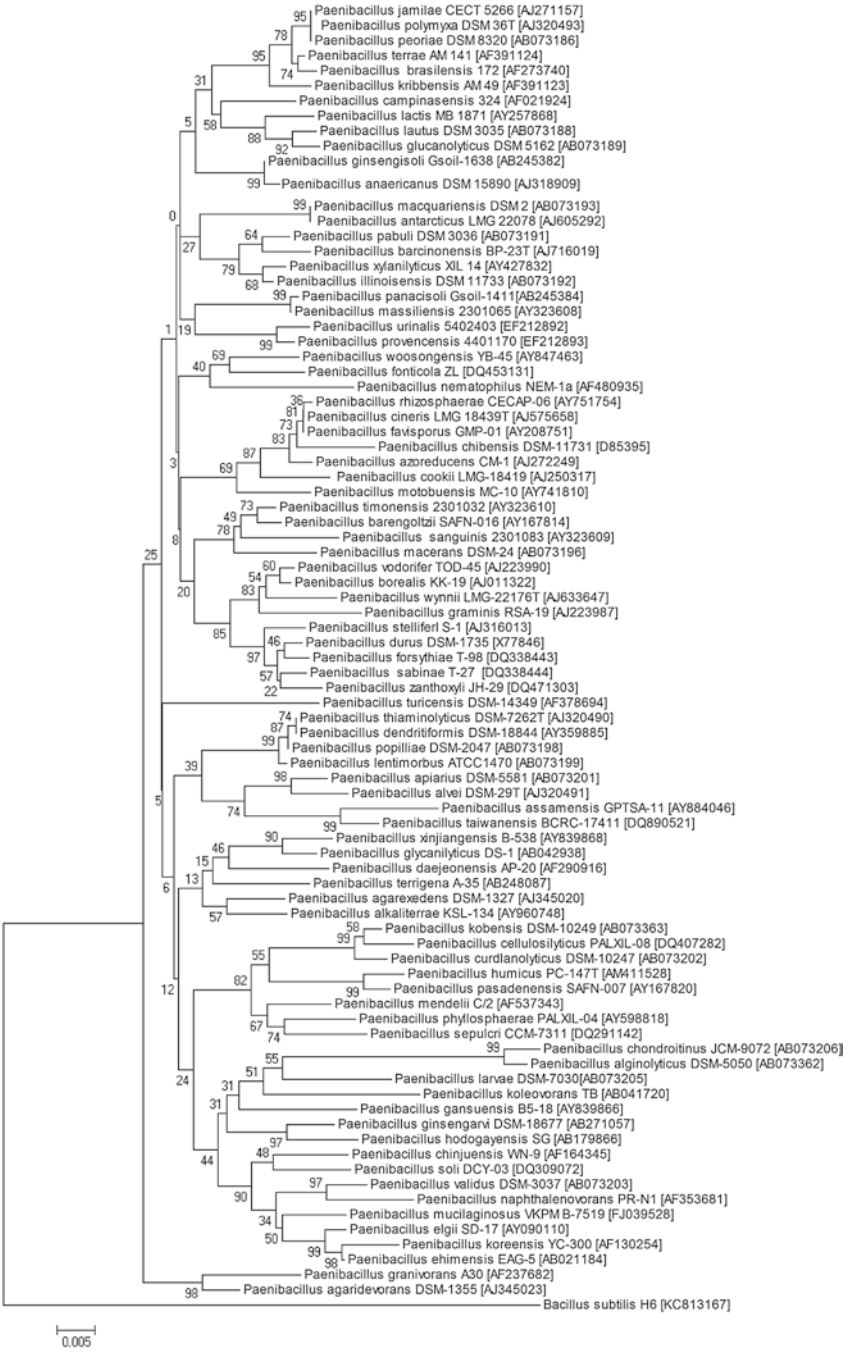

Fig. 12.1 Phylogenetic neighbor-joining tree based on 16s rRNA gene sequences showing the relationship between sequences of 16S rRNA genes among the related members of the genus

whereas Bacilli is included in the order Bacillales and the family Bacillaceae. This class includes 37 new genera on the level with *Bacillus*. This explains the heterogeneity in G+C content observed in the 1986 genus *Bacillus*. Their taxonomic hierarchy is kingdom, bacteria; phylum, Firmicutes; class, Bacilli; order, Bacillales; family, Acyclobacillaceae (genus, *Alicyclobacillus*); family, Bacillaceae (genus, *Bacillus*, *Geobacillus*); family, Paenibacillaceae (genus, *Paenibacillus*, *Brevibacillus*); and family, Planococcaceae (genus, *Sporosarcina*) (Govindasamy et al. 2011).

Bacillus taxonomy is based on phylogenetic approach, which has been attributed largely by the utilizing and analyzing of 16S rRNA molecules by oligonucleotide sequencing. This phylogenetic analysis surprisingly showed *Bacillus* species possessing a large degree of relationship with (1) several nonspore-forming species, including *Enterococcus*, *Lactobacillus*, and *Streptococcus* at the order level, and (2) *Listeria* and *Staphylococcus* at the family level, whereas former members of the genus *Bacillus* gathered into new families, including Acyclobacillaceae, Paenibacillaceae, and Planococcaceae, now on the level with Bacillaceae. Over 200 species of AEFB are now allocated to about 25 genera, and it has been validly published in Bergey's Manual of Systematic Bacteriology (2nd edn., 2004) (Logan and De Vos 2009).

12.3 Cell Structure and Morphology

Paenibacilli are rod-shaped organisms ranging from 2 to 5 μm in length and 0.5 to 0.8 μm in width. They possess Gram-positive cell wall, and they tend to appear Gram-negative when the culture gets old, under microscopic observation. Species of *Paenibacillus* produce endospores which are in greater diameter as compared to the mother cell or sporangium and thus produce swelling of the sporangium. *Paenibacillus macerans* and *Paenibacillus polymyxa* possess spores which show profoundly ridged surface, and those of *Paenibacillus borealis* have a similar striped morphology (Elo et al. 2000). Species belonging to these genus show motility as they possess peritrichous flagella, although in *Paenibacillus popilliae* motility may be restricted to a minority of cells, whereas *Paenibacillus lentimorbus* does not possess any motility. The cell wall peptidoglycan of species studied so far is composed of the *meso*-diaminopimelic acid (DAP) type. Capsules are produced by some species under suitable growth conditions; to illustrate such example, *Paenibacillus polymyxa* produces a levan capsule when sucrose is provided as a carbon source in the growth medium. Some species produce extracellular polysaccharide (Aguilera et al. 2001; Yoon et al. 2002). Recently, *Paenibacillus mucilaginosus* 3016 producing extracellular polysaccharide was portrayed as a PGPR along with its whole genome sequence which was reported recently (Ma et al. 2012). S-layers are present in most of the species of *Paenibacillus*, but they have been not been reported in *Paenibacillus alvei* and *Paenibacillus polymyxa*. The detailed S-layer glycoprotein

structure of and its linkage to the peptidoglycan layer of the cell wall is reported for *Paenibacillus alvei* (Schafferet al. 2000).

The typical biochemical characteristics of species in the genus *Paenibacillus* are as follows: positive for catalase; negative for H_2S production; variable for oxidase, nitrate reduction, and Voges–Proskauer reaction; and DNA G+C content ranging between 45 % and 54 mol%, with anteiso-C15: 0 as the major cellular fatty acid and MK-7 as the major isoprenoid quinone (Shida et al. 1997). In Bergey's Manual of Systematic Bacteriology 2nd edition, 3rd volume, Firmicutes describe 86 species belonging to *Paenibacillus* genus.

12.4 Plant Growth-Promoting Attributes

Paenibacillus has shown the potential to aid in the development of sustainable agriculture. On the whole, *Paenibacillus* functions in three diverse ways to augment plant growth: synthesizing particular compounds for the plants (i.e., nitrogen, indole-3-acetic acid (IAA), etc.), facilitating the uptake of certain nutrients from the soil including phosphate and potassium, and protecting the plants from diseases. The mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood. However, the possible mechanisms include nitrogen fixation; ability to produce hormones such as auxin, i.e., IAA; antagonism against phytopathogenic bacteria by producing siderophores, antibiotics, etc.; and solubilization and mineralization of nutrients, particularly mineral phosphates (Kloepper and Schroth1978; Ahmad et al. 2008; Aeron et al. 2011).

12.4.1 Biological Nitrogen Fixation and Involvement of nif*H Gene*

Some PGPR possess ability of biological nitrogen fixation which is considered an efficient trait by which plant benefits from the association of microbial partners. One of the benefits that diazotrophic bacteria provide to plants is to fix nitrogen in exchange of fixed carbon released as root exudates. Isolates of nitrogen-fixing Paenibacilli from plant rhizospheres were determined by an acetylene reduction assay (ARA) for nitrogenase activity and by amplifying and sequencing part of *nif*H gene. Initially *Bacillus azotofixans*, *Bacillus macerans*, and *Bacillus polymyxa* were identified as nitrogen fixers, based on nitrogenase activity (Seldin et al. 1984); however, after reclassification these organisms are now classified in *Paenibacillus* genus. *Paenibacillus odorifer*, *Paenibacillus graminis*, *Paenibacillus peoriae*, and *Paenibacillus brasiliensis* have been described as nitrogen fixers (Berge et al. 2002; von der Weid et al. 2002). However, *Paenibacillus* species *P. azotofixans*, *P. macerans*, *P. polymyxa*, *P. graminis*, *and P. odorifer* were the only organisms containing

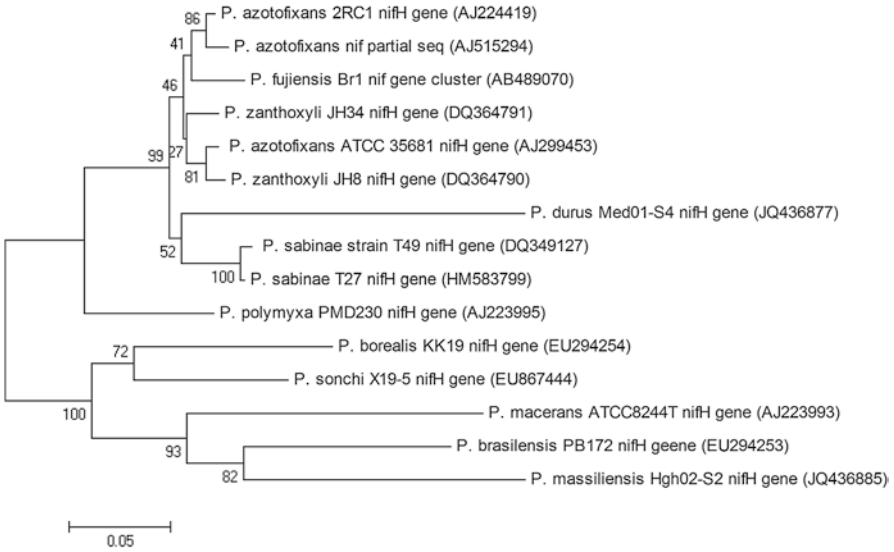

Fig. 12.2 Phylogenetic neighbor-joining tree based on *nif*H gene sequences showing the relationship between sequences of *nif*H genes among the related members of the genus *Paenibacillus*. Gene sequence obtained was aligned by ClustalW using MEGA 4.0 software (Tamura et al. 2007) and a neighbor-joining (NJ) tree. Bootstrap values (expressed as percentages of 500 replications) greater than 50 % are given at nodes. Bar 0.5 % sequence variation. GenBank accession numbers are given in *parentheses*

*nif*H gene (Berge et al. 2002). The presence of *nif*H gene in strains of *Bacillus* and *Paenibacillus* is reported. Ding et al. (2005) isolated and identified nitrogen-fixing strains from plant rhizospheres in Beijing region, reporting the presence of *nif*H gene in both genera *Bacillus* and *Paenibacillus*. Several reports suggesting the presence of nitrogen-fixing ability by *P. polymyxa* are available (Govindasamy et al. 2008). Guemouri-Athmani et al. (2000) measured nitrogenase activity of several strains of *P. polymyxa* recovered from Algerian soil by ARA which showed only 14 of the 23 strains tested were able to reduce acetylene. Some of them were strains SGH1 reduced C2H2 at a similar rate to *P. azotofixans* ATCC 35681T, which is a very efficient nitrogen-fixing bacterium (Seldin and Penido 1986; Govindasamy et al. 2008). Figure 12.2 shows the phylogenetic analysis of *nif*H gene sequences of various strains of *Paenibacillus* in the portrayed form of neighbor-joining tree. *nif*H PAENf: 5′-CTCACGGATTGGCATTGCG-3′and *nif*H PAENr: 5′-TGATCCTGAACACGAAAG-3′ are primers used to amplify *nif*H gene.

12.4.2 Phosphate Solubilization

After nitrogen, phosphorous is the most limiting nutrient for plants. Despite the profound abundant reserves of phosphorous, it is not available in the form suitable for plant uptake. Plants are only able to absorb mono- and dibasic phosphate which are the soluble forms of phosphate (Jha et al. 2012). Microorganisms mineralize organic phosphorous in soil by solubilizing complex-structured phosphates, viz., tricalcium phosphate, rock phosphate, aluminum phosphate, etc. ultimately aiding the phosphate availability to plants. These phosphate-solubilizing bacteria use different mechanism(s) to solubilize the insoluble forms of the phosphate. The primary mechanism phosphate solubilization is based on organic acid secretion by microbes by sugar metabolism. Organisms residing in the rhizosphere utilize sugars from root exudates and metabolize it to produce organic acids (Goswami et al. 2014a, b). These acids released by the microorganisms act as good chelators of divalent Ca^{2+}cations accompanying the release of phosphates from insoluble phosphatic compounds. Many of the phosphate-solubilizing microbes lower the pH of the medium by secretion of organic acids such as acetic, lactic, malic, succinic, tartaric, gluconic, 2-ketogluconic, oxalic, and citric acids (Rodríguez and Fraga 1999), and their detection using high-performance liquid chromatography (HPLC) is also reported (Butch et al. 2010). The involvement of microorganisms in solubilization of inorganic phosphates was known as early as 1903 (Kucey et al. 1989). It is estimated that phosphate-solubilizing microorganisms may constitute 20–40 % of the cultivable population of soil microorganisms and that a significant proportion of these can be isolated from rhizosphere soil (Chabot et al. 1993). Most phosphate solubilizers are isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from sources other than rhizosphere. Phosphate tends to react with calcium (Ca), iron (Fe), or aluminum (Al) leading to its precipitation making itself unavailable for plant uptake. Inorganic phosphate in acidic soils is associated with Al and Fe compounds, whereas calcium phosphates are found in calcareous soils in the form of inorganic phosphates, whereas organic phosphate make up a large fraction of soluble phosphate which is about 50 % in soils with high organic matter (Barber 1984). Hexaphosphate salt of inositol, so-called phytate, is the major form of phosphate in organic form which constitutes up to 80 % of the total organic phosphate (Alexander 1977). Although microorganisms are known to produce phytases that can hydrolyze phytate, however, phytate tends to form in soluble complexes with Fe, Al, and Ca and accumulates in soils (Alexander 1977).

Among the soil bacterial communities, ecto-rhizospheric strains from *Pseudomonas* and *Bacilli* and endosymbiotic rhizobia have been described as effective phosphate solubilizers. *Bacillus megaterium*, *B. circulans*, *B. coagulans*, *B. subtilis*, *P. polymyxa*, *B. sircalmous*, and *Pseudomonas striata* could be referred as the most important strains (Kucey et al. 1989; Govindasamy et al. 2011; Goswami et al. 2013). However, there are several recent reports suggesting species of *Paenibacillus* as potent phosphate solubilizers. Research by Wang et al. (2012)

showed phosphate solubilization by strains of *Paenibacillus*; viz., *Paenibacillus polymyxa* and *Paenibacillus macerans*, which they previously isolated from rhizosphere, hyphosphere, or bulk soil, respectively, from mycorrhizal and non-mycorrhizal cucumber plants, were examined. Result from this study showed that several strains could solubilize both $Ca_3(PO_4)_2$ and $CaHPO_4$, while inositol hexaphosphate could be solubilized by few strains and none of the *Paenibacillus* strains produced clear solubilization halos where growth medium containing $AlPO_4$ or $FePO_4$ as phosphate source. However, quantitative estimation of phosphate solubilization from growth medium (broth) amended with $AlPO_4$ or $FePO_4$, showed that six and ten strains solubilized $AlPO_4$, and $FePO_4$ respectively. Lee et al. (2011) reported the phosphate solubilization by novel *Paenibacillus* strain designated as *Paenibacillus telluris* PS38. Arthurson et al. (2011) reported the strains of *Paenibacillus polymyxa* B1-4 and *Paenibacillus brasiliensis*PB177 for their role in phosphate solubilization. *Paenibacillus kribensis* CX-7 has been reported as a phosphate solubilizer by Ai-min et al. (2013). Thus, from all the reports across the world, it can be deduced that strains of *Paenibacillus* can solubilize various complexes of phosphate making it available to plant like other well-known PGPR.

12.4.3 Phytohormones and Growth Stimulant Production

Plant responds to any phytohormone in the rhizosphere that are supplemented externally or been produced by microbial flora residing in the rhizosphere. The classes of well-known phytohormones include auxins, gibberellins, cytokinins, ethylene, and abscisic acid, and soil microorganisms, particularly the rhizosphere bacteria, do possess the potentials of these hormones (Patten and Glick 1996; Arshad and Frankenberger 1998). These phytohormones can mediate processes including plant cell enlargement, division, and extension in symbiotic as well as nonsymbiotic roots.

12.4.3.1 Indole-3-Acetic Acid (IAA): A Major Auxin Produced by Strains of *Paenibacillus*

Among several known plant growth regulators, most attention has focused on auxins in which the most common and well characterized is IAA, which is known to stimulate both rapid (e.g., increase in cell elongation) and long-term (e.g., cell division and differentiation) responses in crop plants. The production of plant growth-promoting compounds by *P. polymyxa* was reported where indole-3-acetic acid produced by the strain stimulated growth in crested wheatgrass (Holl et al. 1988). It also releases isopentenyladenine and one unknown cytokinin-like compound during its stationary phase of growth which promotes seed germination, de novo bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development, and retardation of senescence in wheat (Mok 1994).

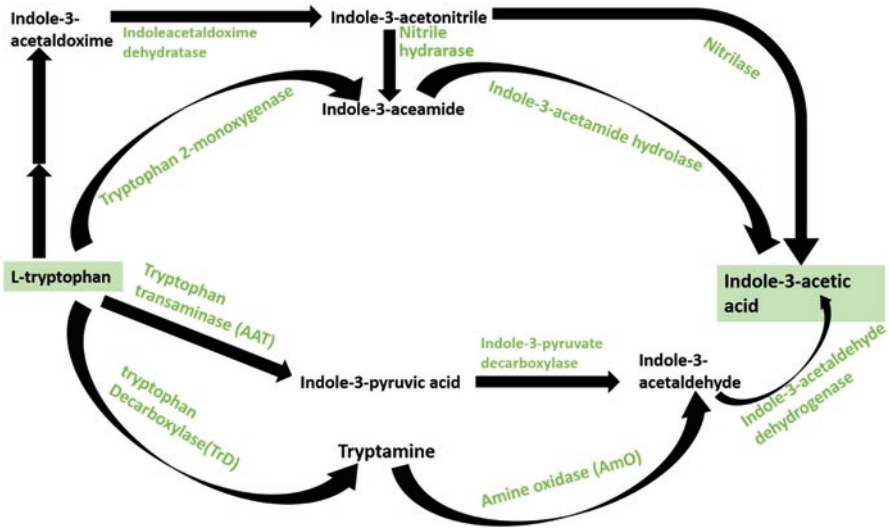

Fig. 12.3 Tryptophan-dependent pathways of bacterial indole-3-acetic acid (IAA) synthesis compiled from Patten and Glick (1996), Steenhoudt and Vanderleyden (2000), and Idris et al. (2007)

Recently, Aswathy et al. (2013) reported isolation of IAA producing *Paenibacillus* strains ClB1 and ClB2 from a medicinal plant *Curcuma longa*. 16S rDNA sequence of ClB1 and ClB2 showed its maximum identity of 99 % to *Paenibacillus favisporus*, and their sequence data were submitted to NCBI under the accession numbers JN835217 and JN835218, respectively; further they also confirmed IAA production using HPLC analysis.

IAA production by PGPR and availability of L-tryptophan are important as these microbes produce IAA using tryptophan-dependent route; tryptophan is used as the precursor (Goswami et al. 2014b). There are different pathways which can lead to tryptophan-dependent microbial production of IAA. The various pathways for IAA biosynthesis include tryptophol, tryptamine, indole-3-pyruvic acid, and indole-3-acetamide pathways (Gravel et al. 2007). Figure 12.3 shows the tryptophan-dependent pathway for IAA production found in strains of *Paenibacillus* and other PGPR. The presence of *Paenibacillus* sp. as endophyte and its ability to produce IAA have already been reported from diverse plants (Lebuhn et al. 1997; Timmusk and Wagner 1999). Acuña et al. (2011) reported the increase in the production of IAA by fourfold when grown in tenfold-diluted Luria–Bertani medium than in nutrient-rich medium by *Paenibacillus* sp. However, these microbes may produce other end products other than IAA which may be wrongly interpreted as IAA. Recently Szkop and Bielawski (2013) reported the most efficient HPLC-dependent technique to detect IAA and other related microbial metabolites derived from tryptophan where they suggested bacteria can produce compounds such as indole-3-pyruvate, indole-3-acetamide, tryptamine, indole-3-acetonitrile, indole-3-ethanol, indole-3-lactic acid, etc., which can be identified using their technique.

Lebuhn et al. (1997) showed that IAA production in *Paenibacillus polymyxa* is directly dependent on supplementation of L-tryptophan. They claimed that in L-tryptophan-dependent IAA production, other metabolites from L-tryptophan were also produced that included indole-3-ethanol, indole-3-lactic acid, and indole-3-carboxylic acid.

12.4.3.2 Production of Cytokinins by Strains of *Paenibacillus*

Cytokinins have key regulatory roles in plant growth and development. They promote seed germination, de novo bud formation, release of buds from apical dominance, stimulation of leaf expansion and of reproductive development, and retardation of senescence (Mok 1994). Some of these effects have been observed in wheat when inoculated with *P. polymyxa* strains B1 and B2 (Lindberg et al. 1985; Lindberg and Granhall 1986). One of these strains (B2) was, therefore, chosen for further investigation by Timmusk et al. (1999). As even a limited enhancement of cytokinin production in transgenic plants may increase plant biomass and longevity (Gan and Amasino 1995), there is a renewed interest in the possibility that bacteria supply cytokinins to soil. Because cytokinins are a diverse group of labile compounds and present in small amounts in biological samples, they have often been difficult to identify and quantify. To overcome this problem, Timmusk et al. (1999) have used immunoaffinity chromatography (IAC) to isolate, high-performance liquid chromatography (HPLC) with online ultraviolet (UV) spectrum detection to separate and characterize, and gas chromatography–mass spectrometry to identify cytokinins in culture media of *P. polymyxa*. Various organisms are reported to produce cytokinins, although it is only in higher plants that cytokinins are unequivocally proven to have a hormonal role. Using the defined media $MgSO_4.7H_2O$ (0.2 g/l), NaCl (0.1 g/l), $CaCl_2$ (0.02 g/l), $Na_2MoO_4.2H_2O$ (0.02 g/l), $MnSO_4.H_2O$ (0.01 g/l), FeEDTA (0.125 Fe g/l), glucose (10 g/l), casamino acid (1 g/l), or $(NH_4)_2SO_4$(60 mg/l), the cytokinin-isopentenyladenine (iP) was released by *P. polymyxa* during its stationary phase of growth. However, it was found that when the nitrogen source casamino acid (1 g/l) or $(NH_4)_2SO_4$ (60 mg/l) in the abovementioned medium was replaced with yeast extract, the bacteria could produce cytokinin-isopentenyladenine riboside (iPR). *P. polymyxa* can produce up to 1.5 nM of iP/iPR. However, as little as 1 nM of cytokinins has been shown to influence plant growth in in vitro experiments (Bennici and Cionini 1979; Palni et al. 1984). The amount of iP in our in vitro culture does not, of course, accurately reflect the situation in the rhizosphere. The microenvironments into which bacterial cytokinins are excreted could have very small volumes, resulting in high local concentrations. Another factor that could affect microbial cytokinin production in the rhizosphere is the complex chemical environment; it has been shown that cytokinin production in *Azotobacter* sp. is stimulated by various naturally occurring compounds (Nieto and Frankenberger 1989). It is well known also that other plant hormones, such as auxins, or other growth-regulating substances occur in the rhizosphere. In the vicinity of a plant root, such

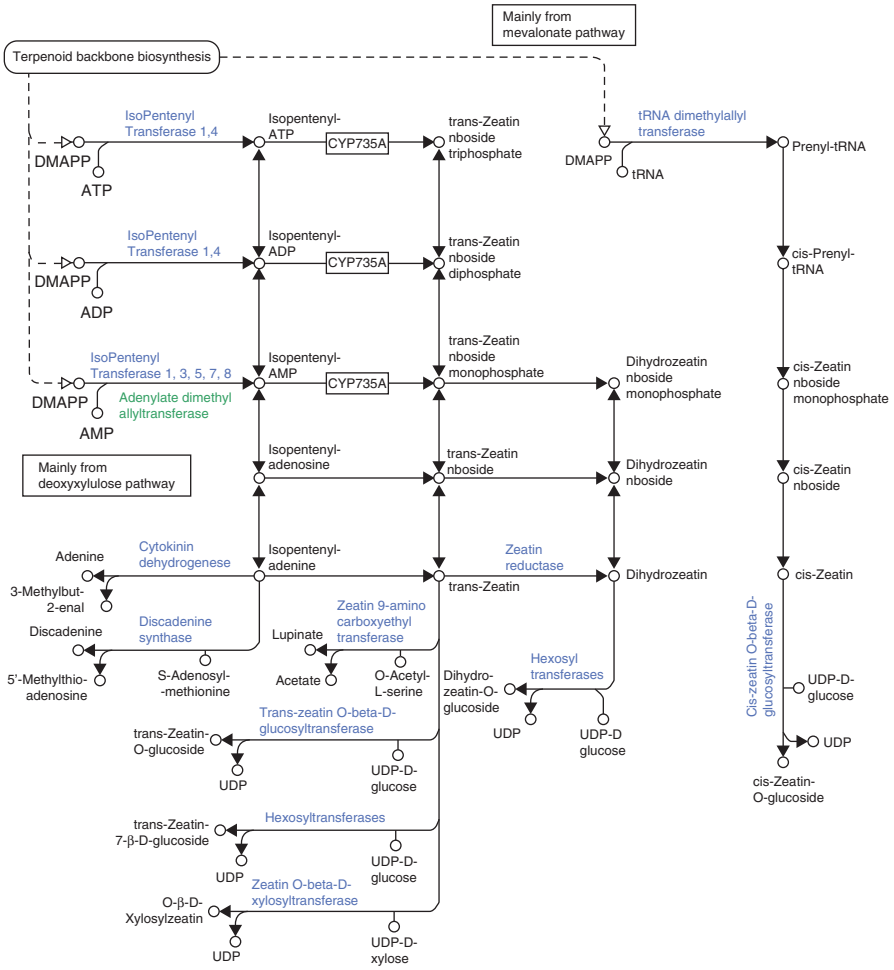

Fig. 12.4 Zeatin biosynthesis pathway studied in *Paenibacillus polymyxa* OSY-DF. The whole genome sequence of the mentioned strain was deposited by Huang and Yousef (2012); taxonomic id. is 1156938 and GenBank accession number is NZ_AIPP01000139.1, and pathway KEGG map as described by PATRIC (Pathosystems Resource Integration Center) is shown (http://patricbrc.org/portal/portal/patric/CompPathwayMap?cType=genome&cId=233737&dm=feature&feature_info_id=85387831&map=00908&algorithm=PATRIC&ec_number=) with modifications

substances could modify the cytokinin effect to the plant in a synergistic way (Stenlid 1982).

Most abundant cytokinins are adenine type, where the N6 position of adenine is substituted with an isoprenoid, such as in zeatin, or an aromatic side chain, such as in kinetin. Zeatin can be synthesized in two different pathways: the tRNA pathway and the AMP pathway (Fig. 12.4). In the tRNA pathway, zeatin is a recycled product of isopentenylated tRNAs. In the AMP pathway, zeatin is synthesized from an isopentenyl donor, dimethylallyl diphosphate (DMAPP), and AMP, ADP, or ATP by isopentenyl transferases. After synthesis cytokinins can be glycosylated.

12.4.4 Biocontrol Traits Possessed by Strains of Paenibacillus

Several PGPR strains show the ability to suppress invasiveness of plant pathogens; such strains are characterized as biocontrol agent. Majority of plant pathogens are fungal strains, and these rhizobacteria show the ability to suppress their growth. These biocontrol agents possess the ability to produce antibiotics and/or possess ability to produce enzymes which degrade fungal cell wall causing its inability to survive in the presence of biocontrol agents. Majority of fungal cell wall-degrading enzymes are chitinases, glucanases (Thakker et al. 2009), and proteases. These are several reports available which show that strain of *Paenibacillus* possesses important traits which make them a biocontrol agent along with PGPR ability. Strains of *Paenibacillus macerans* and *Paenibacillus polymyxa* have been suggested to be involved in plant growth promotion (Jeon et al. 2003; Khan et al. 2008; Zhou et al. 2008) and suppression of pathogens as they possessed trait to produce several antibiotics belonging to the class of polymyxin–colistin–circulin; also these strains possessed ability to produce glucanase and chitinases (Akhtar and Siddiqui 2007; Li et al. 2008; Siddiqui and Akhtar 2009). Timmusk et al. (2005, 2009) found that biofilm aided the colonization of *P. polymyxa*; as a result strains with high biofilm formation ability seem to have potential to colonize and inhibit plant pathogens. Kim et al. (2009) suggested that biofilm-forming strain of *P. polymyxa* showed inhibition of *Phytophthora capsici*. Algam et al. (2010) reported that *Paenibacillus* strain KPB3 suppresses the growth of *R. solanacearum*. There are several reports for strains of *Paenibacillus* producing fungal cell wall-degrading enzymes acting as biocontrol agent. Naing et al. (2013) reported *Paenibacillus ehimensis* strain KWN38 as a potential biocontrol agent against *Phytophthora capsici* which is a pathogen causing late blight disease in pepper, where the authors claim strain to produce glucanase, chitinase, and protease. Similarly, *Paenibacillus macerans* has also been reported to suppress the growth of *Alternaria solani* and *Xanthomonas vesicatoria* which causes bacterial spot and early blight in tomato (Lanna-Filho et al. 2010). Jung et al. (2003) reported *Paenibacillus illinoisensis* KJA-424 causing inhibition in the growth of *Rhizoctonia solani* by its chitinolytic activity suppressing damping-off disease in cucumber seedlings. Another mechanism by which PGPR improves plant health is by induced systemic resistance (ISR) which is defined by the effect of various exogenous or endogenous factors that substantially affect host physiology, leading to rapid and coordinated defense-gene activation in plants normally expressing susceptibility to pathogen infection. This phenomenon, by which resistance of plant to pathogens can be enhanced by the application of various living or nonliving biotic agents, is called ISR in plants (Kothari et al. 2008; Kothari and Thakker 2009; Thakker et al. 2011, 2012). The biocontrol bacterium *Paenibacillus alvei* K165 has been reported to protect *Arabidopsis thaliana* against vascular wilt-causing pathogen *Verticillium dahliae* via induction of ISR in *Arabidopsis thaliana*. PGP traits of other well-known strains of *Paenibacillus* are described in Table 12.1. Thus on the whole, there are several reports across the globe suggesting several strains of *Paenibacillus* that possess biocontrol abilities as they possess important

Table 12.1 PGP traits shown by various strains of *Paenibacillus*

Strain	Locus of isolation	PGP trait	References
*P. sabinae*T27^T	Rhizosphere of plant *Sabina squamata*, *Weigela florida*	Possess the activity of nitrogen fixation and possess *nif*H gene	Ma et al. (2007)
P. jilunlii sp. Be17^T	Rhizosphere of *Begonia semperflorens* Beijing Botanical Garden, PR China	Possess the activity of nitrogen fixation and possess *nif*H gene	Jin et al. (2011)
P. polymyxa (SQR-21)	Rhizosphere of healthy watermelon plants in a heavily wilt-diseased field, People's Republic of China	Fusaricidin-type antibiotic production and suppression of *Fusarium oxysporum* f. sp. niveum	Raza et al. (2009) and Ling et al. (2011)
P. taohuashanense gs 65^T	Rhizosphere soil of the root of *Caragana kansuensis Pojark*, Gansu, China	Possess the activity of nitrogen fixation and possess *nif*H gene	Xie et al. (2012)
P. typhae xj 7^T	Roots of *Typha angustifolia* L. growing in Beijing Cuihu Wetland, China	Possess the activity of nitrogen fixation and possess *nif*H gene	Kong et al. (2013)
P. polymyxa P2b-2R	Endophytic from lodgepole pine, Canada	Possess nitrogen fixation ability which can provide up to 79 % of foliar nitrogen in lodgepole pine Strain also possesses *nif* operon	Anand and Chanway (2013)
P. polymyxa (PMD216, PMD230, PMD112, and PMD128)	Isolated from wheat rhizosphere, Germany, UK	Production of IAA and other indole-containing compounds from tryptophan and production of phenolics	Lebuhn et al. (1997)
P. mucilaginosus 3016	Isolated from rhizospheric soil, China	Release nutritional ions from soils and minerals, produce extracellular polysaccharides, and possess nitrogen fixation trait	Ma et al. (2012)
*P. edaphicus*B-7517^T	Isolated from rhizospheric soil in China	Assist potassium solubilization and uptake by wheat	Hu et al. (2010)
P. polymyxa E681	Isolated from winter barley roots, Republic of Korea	An antibiotic – fusaricidin biosynthesis and biocontrol of fungal pathogens on sesame plants	Choi et al. (2007)

(continued)

Table 12.1 (continued)

Strain	Locus of isolation	PGP trait	References
P. lentimorbus B-30488^{r}	Isolated from milk, India (data from GenBank)	Produce IAA, possess PGP traits, enhances growth of chickpea, and tolerates chromium stress	Khan et al. (2012)
P. elgi SMA-1-SDCH02	Soil samples from chitin/chitosan-producing company, Mahtani Chitosan Pvt. Ltd., Gujarat	Possess phosphate solubilization ability, chitinase activity, glucanase activity suppression, root colonization on tobacco	Das et al. (2010)
P. polymyxa ATCC 12321	Starch-contaminated soil, Japan	Production of 2, 3-butanediol (BDL)	Ui et al. (1983)
P. montaniterrae RMV1	Isolated from red mud pond, India	Alkalitolerant bacteria that possess xylanase activity, IAA production, phosphatase activity	Arora et al. (2013)
*P. telluris*PS38^{T}	Isolated from farm soil, Daejeon, Korea	Phosphate-solubilizing bacteria designated as PGPR	Lee et al. (2011)
P. polymyxa SCE2	Isolated from soil, Brazil	Production of proteases and antimicrobial compounds	Alvarez et al. (2006)
P. peoriae NRRL BD-62	Isolated from soil, Brazil	Production of proteases	Alvarez et al. (2006)
P. mucilaginosus (KNP413 and KNP414)	Isolated from the soil of Tianmu Mountain, Zhejiang Province (China)	Phosphate and potassium solubilization along with function as PGPR	Hu et al. (2006)
P. polymyxa B2	Isolated from wheat rhizosphere, Uppsala, Sweden	Production of various cytokinins	Timmusk et al. (1999)
*P. illinoisensis*KJA-424	Soil from the west coast of Korea	Inhibition of *Phytophthora capsici* (P), the causal agent of *Phytophthora* blight in pepper (*Capsicum annuum* L. Chungok)	Jung et al. (2006)
P. polymyxa strains (B5 and B6)	Isolated from wheat rhizosphere, Uppsala, Sweden	Inhibition of the oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*	Timmusk et al. (2009)
P. azotofixans ATCC 35681	Isolated from rhizospheric soil, Pulau Pinang, Malaysia	Possess nitrogen fixation trait and *nifB1H1D1K1* gene cluster	Choo et al. (2003)
P. rhizosphaerae CECAP06^{T} and CECAP16	Isolated from the rhizosphere of the legume *Cicer arietinum* in Argentina	Growth promotion in *Cicer arietinum*	Rivas et al. (2005)

traits of pathogen inhibition by the production of several antibiotics and the production of fungal cell wall-degrading enzymes such as glucanase, chitinase, and protease.

12.5 Description of *Paenibacillus polymyxa* as One of the Most Potent Strain Portrayed as PGPR

Paenibacillus polymyxa was earlier known as *Bacillus polymyxa* (Prazmowski 1880), and it was reclassified to genus *Paenibacillus* by Ash et al. 1994 (Effective publication: Ash et al. 1993). The name *polymyxa* was designated as *poly* which means much or many, and *myxa* represents slime or mucous, so the *polymyxa* is much slime.

In Bergey's Manual of Systematic Bacteriology (2nd edn., 2004), phenotypic, morphologic, and biochemical properties of *Paenibacillus polymyxa* are as follows: Colonies on nutrient agar are thin, often with amoeboid spreading. On glucose agar, colonies are usually heaped and mucoid with a matt surface. Large capsule of levan capsule is synthesized from sucrose. Facultative anaerobe, ferments glucose to 2,3-butanediol, ethanol, CO_2, and H_2 as well as reduces nitrate to nitrite. Sugars and carbohydrates being fermented by strain include pectin, pullulan, starch, and xylan, but action on cellulose is weak. Most strains fix atmospheric nitrogen under anaerobic conditions. Biotin (or a trace of yeast extract) is required for growth in minimal medium. Major fatty acids are C15:0 anteiso and C17:0 anteiso. The strains isolated from decomposing plants and soil as well as found to be associated with the rhizosphere where, they provide protection to the plant and enhance plant growth. DNA G+C(mol %) content ranges from 43 to 46 (Bd). Lists of type strain are NRRL NRS-1105, ATCC 842, DSM 36, NCIMB 8158, KCTC 3858, and LMG 13294, and EMBL/GenBank accession number (16S rDNA) of the type strain DSM 36 is AJ320493.

Out of several species of *Paenibacillus*, *P. polymyxa* is one of the most widely studied species for its plant growth-promoting traits. *P. polymyxa* is adapted to flourish in various niches such as soils, roots, and rhizosphere of various crop plants including wheat, maize, sorghum, sugarcane, and barley (Guemouri-Athmani et al. 2000; von der Weid et al. 2000); they have also been found to acquire their healthy survival in forest trees such as lodgepole pine (Holl and Chanway 1992) and douglas fir (Shishido et al. 1996). Nitrogen fixation is one of the omnipresent observed in strains of *P. polymyxa* residing in the rhizosphere (Lindberg et al. 1985; Heulin et al. 1994). These authors measured nitrogenase activity of some representative isolates of *P. polymyxa* recovered from Algerian soil by acetylene reduction assay (ARA). Nitrogen-fixing ability by *P. polymyxa* demonstrated by Guemouri-Athmani et al. (2000) showed that only 14 of the 23 strains tested were able to reduce acetylene indicating that 60 % of total strains possessed trait of nitrogen fixation. Also, traits such as soil phosphorus solubilization (Singh and Singh 1993) and production

of antibiotics (Rosado and Seldin 1993; Kajimura and Kaneda 1996; Kajimura and Kaneda 1997; Choi et al. 2007), exopolysaccharides (Haggag et al. 2007), chitinase (Mavingui and Heulin 1994), hydrolytic enzymes (Nielsen P and Sorensen 1997), β-1,3-glucanases (Dunn et al. 1997; Budi et al. 2000), and xylanase (Pham et al. 1998) have been observed among the strains of *P. polymyxa*. All these traits constitute to enhance plant growth, both by direct and indirect mechanisms (Gouzou et al. 1993). Before two decades from now, evidences of *P. polymyxa* showing efficient antagonistic activity against soilborne fungal and oomycetic pathogens have been shown by Heulin et al. (1994); such evidences were later reported by Timmusk et al. (2003, 2005), Haggag (2007), etc. The production of plant growth-promoting compounds by *P. polymyxa* similar in activity to indole-3-acetic acid has been suggested to stimulate growth in crested wheatgrass (Holl et al. 1988). It has also been reported to release isopentenyladenine along with unknown cytokinin-like compound during its stationary phase of growth which promotes seed germination, de novo bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development, and retardation of senescence in wheat (Lindberg et al. 1985; Lindberg and Granhall1986; Mok 1994). Most studies on the biocontrol activity of *P. polymyxa* have been concentrated on the production of different antibiotic substances. Fusaricidin, a peptide antibiotic consisting of six amino acids, has been identified as a potential antifungal agent from *P. polymyxa* E681 (Choi et al. 2007). Various analogs of fusaricidins were isolated and characterized from *P. polymyxa*; these analogs were designated as LI-F03, LIF04, LI-F05, LI-F06, LI-F07, and LI-F08 (Kuroda et al. 2001) as well as fusaricidins A–D (Kajimura and Kaneda 1996; Kajimura and Kaneda 1997). Fusaricidins show exceptional antifungal activity against plant pathogenic fungi including *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium thomii*, whereas fusaricidin B shows antagonistic activity against *Candida albicans* and *Saccharomyces cerevisiae*. Fusaricidins also have an excellent germicidal activity to Gram-positive bacteria specifically for *Staphylococcus aureus* (Kajimura and Kaneda 1996; Kajimura and Kaneda 1997). In addition, they have antifungal activity against black root rot-causing *Leptosphaeria maculans* in canola (Beatty and Jensen 2002). Antagonistic activity of *P. polymyxa* was also demonstrated against the nematode *Meloidogyne javanica*, whereas *P. polymyxa* strain P13 isolated from Argentinean regional fermented sausages was found to produce antibiotic compound named polymyxin which inhibited the growth of *Lactobacillus* strains (Lal and Tabacchioni 2009). Thus, from all the literature studied so far, the strain of *P. polymyxa* is one of the most widely studied strains as PGPR showing several potent traits for plant growth promotion and plant protection.

12.6 Conclusion

Briefly 20 years ago, *Paenibacillus* genus was separated from the genus *Bacillus* as they had different DNA G+C content; DNA G+C content is ranging between 45 % and 54 mol% in *Paenibacillus*. Studies on strains of *Paenibacillus* which possess trait of PGP have increased after its separation from *Bacillus* genus; consequently the awareness to introduce new and more potent strains in sustainable agriculture has increased. Over the period of the last 10 years, new strains belonging to *Paenibacillus* have been identified and characterized as PGPR. The most unique trait of *Paenibacillus* includes nitrogen fixation. Strains gaining importance in PGPR research as they are known to possess *nif*H gene for nitrogen fixation include *P. polymyxa*, *P. macerans*, *P. durus*, *P. peoriae*, *P. borealis*, *P. brasiliensis*, *P. graminis*, *P. odorifer*, *P. wynnii*, *P. massiliensis*, and *P. sabinae*. However, P. polymyxa is the only strain which is recognized as the potent PGPR along with the traits of phytopathogen suppression and antibiotic production. Other strains with nitrogen fixation trait have been reported as PGPR, but due to the lack of research on such strains, they are not able to gain considerable recognition in the field of biofertilizers as gained by strains of P. polymyxa. This lack of research on newly identified strains of Paenibacillus has opened a new horizon to portray efficient strains as PGPR for the development of sustainable agriculture. Some key points that would provide future direction for studying Paenibacillus strains would include:

- Comparing strains of P. polymyxa with biocontrol potentials with B. subtilis, plant growth promotion with Pseudomonas sp., and nitrogen fixation with free-living nitrogen fixers like Azotobacter and Azospirillum.
- Nitrogen fixation ability possessed by strains of *Paenibacillus* should be further studied.
- Commercialization of P. polymyxa and other strains of *Paenibacillus* should be initiated.
- Strains of Paenibacillus should be reputed as efficient PGPR and efforts should be made to isolate more efficient strains belonging to this genus.

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Chapter 13
Bacillus spp.: A Prolific Siderophore Producer

Arif Khan, Hiren V. Doshi, and Mukund Chandra Thakur

Abstract *Bacillus* species comprises of several hundred species and is characterized as non-spore- or endospore-forming, straight or slightly curved Gram-positive rods, which may turn Gram-negative with age, and single or multi-flagellate and grows in aerobic or facultative anaerobic conditions. *Bacillus* spp. include xenobiotic biodegraders, plant growth promoters, siderophore producers and human & plant pathogens.

Iron is a micronutrient and the fourth most abundant element in the earth's crust. Bacteria need iron for a range of metabolic and signaling functions including electron transport, peroxide reduction, amino acid & nucleoside synthesis, DNA synthesis, photosynthesis and most importantly – some virulence traits. *Bacillus* spp. have developed a mechanism for acquiring iron by the use of siderophores. Siderophores are small iron-chelating molecules that have high affinity for iron. Siderophores show a wide range of variety in their structure. Some siderophores are comprised of a peptide backbone with various coordinating iron-ligating groups. *Bacillus* spp. produce a wide variety of siderophores such as bacillibactin, pyoverdine, pyochelin, schizokinen, petrobactin, etc. which play a crucial role in its existence.

Keywords *Bacillus subtilis* • Siderophore • Iron chelator • Bacillibactin • Iron-chelating receptor

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_13

13.1 Introduction

Bacillus is a genus of Gram-positive, rod-shaped (bacillus) bacteria and a member of the phylum *Firmicutes*. *Bacillus* species can be obligate aerobes (oxygen reliant), or facultative anaerobes (having the ability to be aerobic or anaerobic), and are ubiquitous in nature. They give positive test for the enzyme catalase when oxygen is used or present. *Bacillus* include both free-living (nonparasitic) and parasitic pathogenic species. Under stressful environmental conditions, bacteria can produce oval endospores that are not true *spores* but which the bacteria can reduce themselves and remain in a dormant state for very long periods. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera of *Firmicutes*.

Many species of *Bacillus* can produce copious amounts of enzymes which are made use of in different industries. Some *Bacillus* species can form intracellular inclusions of polyhydroxyalkanoates under certain adverse environmental conditions, as in a lack of elements such as phosphorus, nitrogen or oxygen combined with an excessive supply of carbon sources.

B. subtilis has proved to be an invaluable model for research. Other species of *Bacillus* are important pathogens causing anthrax and food poisoning. Many *Bacillus* species are able to secrete large quantities of enzymes. *B. amyloliquefaciens* is the source of a natural antibiotic protein barnase (a ribonuclease), alpha amylase used in starch hydrolysis, the protease subtilisin used with detergents, and the *Bam*HI restriction enzyme used in DNA research. A portion of the *Bacillus thuringiensis* genome was incorporated into corn (brinjal and cotton) crops. The resulting GMOs were then found to be resistant to some insect pests.

B. subtilis is one of the best understood prokaryotes, in terms of molecular biology and cell biology. Its superb genetic amenability and relatively large size have proved to be powerful tools required to investigate a bacterium from all possible aspects. Recent improvements in fluorescence microscopy techniques have provided novel and amazing insight into the dynamic structure of a single-cell organism. Research on *B. subtilis* has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene/protein regulation, and cell cycle events in bacteria.

Two *Bacillus* species are considered medically significant: *B. anthracis*, which causes anthrax and *B. cereus*, which causes food poisoning similar to that caused by *Staphylococcus*. A third species, *B. thuringiensis*, is an important insect pathogen and is sometimes used to control insect pests. The type species *B. subtilis* is an important model organism. It is also a notable food spoiler, causing ropiness in bread and related food. Some environmental and commercial strains such as *B. coagulans* may play a role in food spoilage of highly acidic tomato-based products.

An easy way to isolate *Bacillus* is by placing non-sterile soil in a test tube with water, then shaking it, ultimately plating it in melted mannitol salt agar and incubating at room temperature for at least a day. Colonies are usually large, spreading and

irregularly shaped. Under the microscope, *Bacillus* cells appear as rods and a substantial portion usually contain an oval endospore at one end, making it bulge.

The cell wall of *Bacillus* is a structure on the outside of the cell that forms the second barrier between the bacterium and the environment and at the same time maintains the rod shape and withstands the pressure generated by the cell's turgor. The cell wall is composed of teichoic and teichuronic acids. *B. subtilis* was the first bacterium for which the role of an actin-like cytoskeleton in cell shape determination and peptidoglycan synthesis was identified. It was also the first bacterium for which the entire set of peptidoglycan synthesizing enzymes was localized, thus, paving the way for understanding the role of cytoskeleton in shape generation and maintenance.

The genus *Bacillus* was named in 1835 by Christian Gottfried Ehrenberg to contain rod-shaped (bacillus) bacteria. He had 7 years earlier named the genus *Bacterium. Bacillus* was later amended by Ferdinand Cohn to further describe them as spore-forming, Gram-positive, aerobic or facultatively anaerobic bacteria (Xu and Côté 2003). Like other genera associated with the early history of microbiology, such as *Pseudomonas* and *Vibrio*, the 266 species of *Bacillus* are ubiquitous. The genus has very large ribosomal 16S diversity and is environmentally diverse.

One of the studies reconciles the exception that *Lactobacillus plantarum* does not entail iron. It might apparently manage to stimulate all its enzymatic functions with metals other than iron. After complexing iron, the ferric-siderophore complexes are taken up into the cell. The specific siderophore and its chirality are recognized by highly specific receptors in the outer membrane of bacteria. In an active and energy-dependent way, they transport the ferric complexes into the periplasm. Once the complexes are collected there, they are then handed over to the intracellular transport and storage components and finally integrated into proteins to accomplish their enzymatic functions (Archibald 1983).

Several studies have tried to reconstruct the phylogeny of the genus as mentioned in Fig. 13.1. The *Bacillus*-specific study with the most diversity covered is by Xu and Côté (2003) using 16S and the ITS region, where they divide the genus into ten groups, which includes the nested genera *Paenibacillus*, *Brevibacillus*, *Geobacillus*, *Marinibacillus* and *Virgibacillus*. However, the tree constructed by the living tree project, a collaboration between ARB-Silva and LPSN where a 16S (and 23S if available) tree of all validated species was constructed, the genus *Bacillus* contains a very large number of nested taxa and majorly in both 16S and 23S is paraphyletic to *Lactobacillales* (*Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Listeria*, etc.), due to *Bacillus coahuilensis* and others. A gene concatenation study found similar results to Xu and Côté (2003), but with a much more limited number of species in terms of groups, but used *Listeria* as an outgroup. So in light of the ARB tree, it may be "inside-out."

One clade, formed by *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis* under current classification standards, should be a single species (within 97 % 16S identity), but due to medical reasons,

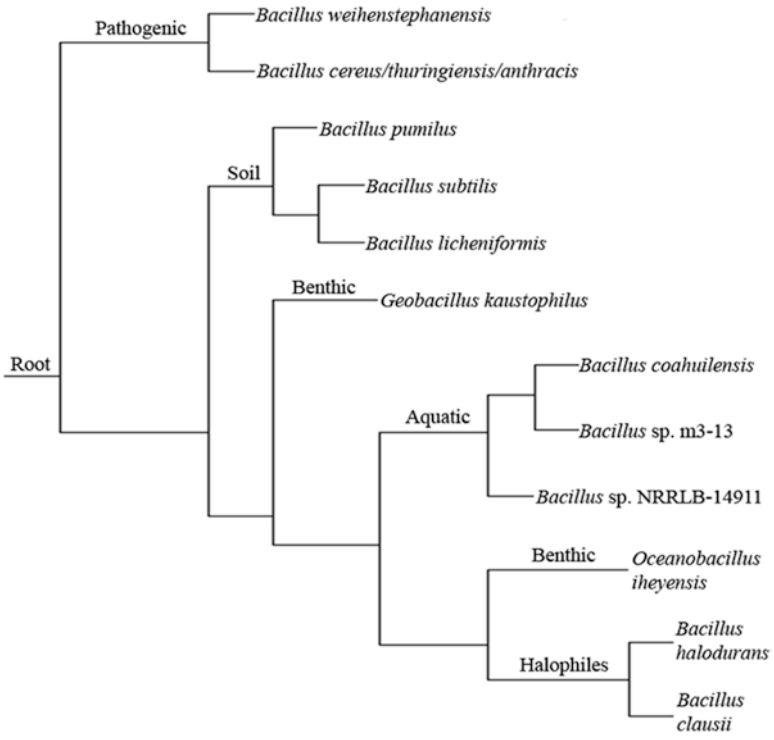

Fig. 13.1 Phylogenetic tree of genus *Bacillus*

they are considered separate species, an issue also present for four species of *Shigella* and *Escherichia coli*.

Much corroboration confirms that corynebactin was isolated from Gram-positive *Corynebacterium glutamicum* and *B. subtilis* incorporates a threonine trilactone and glycine spacers, which elongate the three chelating arms as compared with enterobactin. In *B. subtilis* (DNA with low G + C content), three Fur-like proteins have been characterized (Bsat et al. 1998). One, called Fur, regulates mainly iron uptake and siderophore biosynthesis. A second one, called PerR, regulates peroxide stress response genes and acts with manganese as corepressor. A third one, Zur, regulates genes for zinc uptake. The Zur protein found in *E. coli* shows only 25 % identity to the *B. subtilis* Zur, while the two Fur proteins have 32 % identical amino acids.

Apart from producing siderophores, some *Bacillus* spp. even help in the release of iron from siderophores by using ferri-reductase. Ferri-siderophore reductase of *B. megaterium* has been partially purified and it shows wide range of substrate specificity. The putative Mn oxidase CumA (Okazaki et al. 1997; Brouwers et al. 1999; Francis and Tebo 2001) of *P. putida*GB-1 and MnB1 is a multicopper-type oxidase enzyme that utilizes oxygen as an electron acceptor. Same is also true in other well-studied systems (Brouwers et al. 2000; Tebo et al. 2004) including *Bacillus* sp. spores (Van Waasbergen et al. 1996; Dick et al. 2006).

Webb et al. (2005) have elegantly demonstrated that the oxidation of Mn(II) to Mn(IV) by *Bacillus* spores is a two-step process involving a transient Mn(III) intermediate. Further, an enzymatically produced Mn(III) intermediate of Mn(II) oxidation by *Bacillus* sp. strain SG-1 spores has been trapped by pyrophosphate under conditions that minimized abiotic processes (Webb et al. 2005). The above observation suggests that ligands can trap Mn(III) when interacting with living systems. The differing origin of the Mn(III) in the *Bacillus* and *Pseudomonas* cases could involve the greater stability constant of PVD Mn(III) (Parker et al. 2004) than of pyrophosphate-Mn(III) (Webb et al. 2005). Beside chelation of ferric ions (iron chelator), degradation of textile dyes (Thakur et al. 2012; Joshi et al. 2013) and several other heavy metal ions such as nickel and chromium chelation have also been reported for *B. megaterium* in various studies.

Among pathogenic bacteria, characteristic features of the tubercle bacillus include its slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. Generally bacteria are denoted as pathogenic because they have found out an easy way in animal system to survive where suitable temperature/environment (warmth) is always available for them. Plentiful of nutrition is also available for their survival and growth. Here the pathogenic organisms can acquire all the required minerals from the host body tissues apart from one, i.e., iron which is generally present in oxidized form Fe (III) at pH 7 and is difficult to utilize directly. The uptake mechanism of iron in *Bacillus* spp. is mentioned in Fig. 13.2.

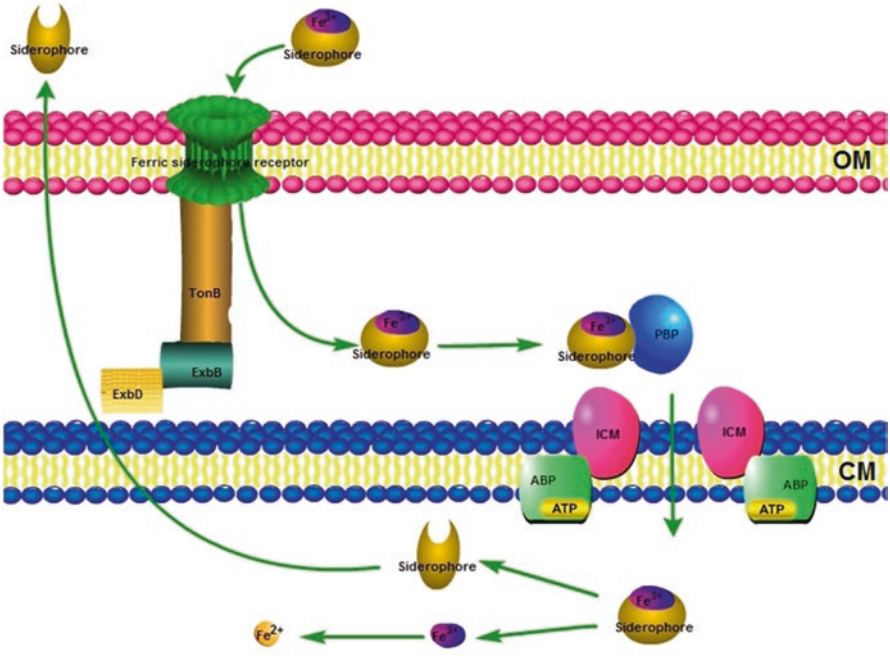

Fig. 13.2 Uptake mechanism of iron in *Bacillus* spp.

13.2 Transport of Substrates into *Bacillus* spp.

The uptake of substrates into the best-studied organism, *Bacillus subtilis*, has been outlined in general terms. The uptake of substrates take place in three steps: (1) uptake across outer membrane, (2) transport across the cytoplasmic membrane and (3) periplasmic binding protein dependent transport.

13.3 Uptake of Ferri-siderophore Complex Across Outer Membrane

Gram-negative *Bacillus* sp. is surrounded by two membranes, the outer membrane and the cytoplasmic membrane (Braun and Hantke 1981; Braun et al. 1985; Lugtenberg and Van Alphen 1983). Hydrophilic substrate not larger than 600–700 Da diffuses through water-filled pores of the outer membrane found by the most abundant proteins in this membrane (Nikaido and Vaara 1987). However, for some substrates, stereochemical recognition takes place between the substrate and an outer membrane protein (Ferenci 1989). This has been demonstrated for maltodextrins, which are recognized by the LamB protein, and for nucleosides which interact with Tsx protein (Hantke 1976; Krieger-Brauer and Braun 1980; Maier et al. 1988; Benz et al. 1988). The PhoE protein forms more efficient pore than the porin (Lugtenberg and Van Alphen 1983) both for inorganic and organic phosphate but does not seem to specifically recognize phosphate, but rather it displays a broad specificity for anions (Benz and Bauer 1988). Before uptake studies had been performed, genetic evidence pointed to the role of these proteins in transport of certain substrates. Synthesis of these proteins was regulated at the transcriptional level by maltodextrins, nucleosides, and phosphates. Maltodextrins (the intracellular regulatory compound is maltotriose) convert a protein (MalT) to an activator, nucleosides inactivates two repressor proteins (DeoR, CytR), and phosphate starvation induces a complex regulatory network resulting in PhoE synthesis. These proteins facilitate the diffusion of the substrate across the outer membrane. The smaller homologues of maltodextrins, phosphate, and nucleosides can also pass through the porins so that the specific porins are not absolutely required. They increase the rate of diffusion and are essential for the uptake of larger homologues across the outer membrane into the periplasmic space.

13.4 Transport Across the Cytoplasmic Membrane

Stereochemical recognition between the substrate and the transport proteins and actual transport against a concentration gradient occurs in the cytoplasmic membrane. Energy required for the active transport is provided in the form of an electrochemical potential across the cytoplasmic membrane by electron transport chain

located therein or by the ATP hydrolysis through the membrane-bound ATPase. Certain substrates such as lactose are transported across the cytoplasmic membrane of *B. subtilis* by FhuBC protein (Ollinger et al. 2006), by a process driven by the electrochemical potential (Hengge and Boss 1983).

13.5 Periplasmic Binding Protein-Dependent Transport (PBT)

Transport of many amino acids, peptides, certain sugars and anions follows a so-called binding protein-dependent mechanism in which protein in the periplasmic space (located between the outer and the cytoplasmic membrane) is involved (Hengge and Boss 1983; Ames 1986). The binding proteins recognize the certain substrates and delivered them to the integral membrane proteins of the cytoplasmic membrane. They are essential constituents of the transport system. The periplasmic binding proteins can be released by an osmotic shock treatment involving plasmolysis of cells in 15 % sucrose (which counterbalances the internal osmotic pressure) in the presence of EDTA (releasing Mg(II) ions supposed to stabilize the outer membrane). Upon rapid dilution of the plasmolyzed cells in to a low-salt Tris/Mg(II) buffer, periplasmic proteins are released from the cells. Alternatively, cells are converted to spheroplasts by a similar procedure but with the inclusion of lysozyme to degrade the murein (peptidoglycan) layer. Such treated cells show greatly reduced transport rates which can be restored by adding back the binding protein in the presence of Ca(II) (increases the permeability of the outer membrane) (Hengge and Boss 1983). Substrates bound to the binding protein rather than the free substrate are accepted by the cytoplasmic transmembrane protein. Usually the periplasmic proteins are synthesized in a large excess with respect to the membrane proteins and are the best characterized transport proteins. The structure of several such proteins (for arabinose, ribose, galactose, sulfate) has been resolved to the atomic scale by X-ray analysis. They exhibit similar conformations composed of two globular domains forming a cleft at the substrate binding sites, linked by a flexible hinge (Quiochio 1988). The very hydrophobic integral cytoplasmic membrane proteins accept substrates from the binding proteins. Usually two such proteins are found in a single system which exhibit the sequence similarity, for example, HisQ and HisM of histidine, MalF and MalG of maltose, PstA and PstC of phosphate, and OppB and OppC of the peptide transport system. But there are also exceptions to this rule. The high-affinity arabinose system contains only one hydrophobic protein of the usual size (34 kD) (Scripture et al. 1987), and variations also occur in the iron transport system.

Characteristic of the PBT system is the involvement of a polar but nevertheless membrane-bound protein which displays sequences also found in nucleotide-binding proteins. In fact, it has long been known that ATP either directly or indirectly

serves as an energy source for PBT. ATP binding, but not ATP hydrolysis, has been demonstrated for the MalK, HisP, and OppD proteins (Higgins et al. 1988). There is evidence that these proteins are bound to the inside of the cytoplasmic membrane (Gallagher et al. 1989).

The PBT system is a high-affinity transport system (K_m 1 μM and below) which concentrates substrates inside the cell against a very large gradient (in the order of 10^5).

13.6 Iron(III) Transport in *B. subtilis*

Iron in the ferric form in aerobic conditions and physiological pH (around 7.00) is extremely insoluble and only scarcely available for bacteria under all natural conditions. As a response to iron starvation, bacteria synthesize elaborate iron supply systems which are composed of low-molecular-weight ferric complexing compounds, termed siderophores and ferric-siderophore transport system. A number of iron(III) transport systems have been characterized in *B. subtilis* (Braun and Winkelmann 1987). Iron uptake in *B. subtilis* using various different siderophores such as bacillibactin, enantio-enterobactin, itoic acid, etc. has been reported by several researchers (May et al. 2001). Further this organism can recognize a variety of catecholate siderophores, but does so through the expression of several and sometimes overlapping membrane transport proteins.

Bacillus subtilis is prototypical for studying iron uptake in Gram-positive organisms. Recent studies of its iron metabolism have elucidated multiple aspects of siderophore synthesis, transport and regulation (Dertz et al. 2006; Miethke et al. 2006; Ollinger et al. 2006; Gaballa and Helmann 2007). *Bacillus subtilis* is a model system which produces a number of hydroxamate- and catecholate-type siderophores such as shizokinen, itoic acid, petrobactin, corynebactin, bacillibactin, etc. Corynebactin siderophore was produced by *Corynebacterium glutamicum* (Abergel et al. 2008), and later genomic data and biochemical analysis revealed that this organism is producing bacillibactin type of compound (Barbeau et al. 2002). Thus, the bacillibactin was the preferred siderophore name for the *B. subtilis* siderophore (Heinrichs et al. 2004; Koppisch et al. 2005).

Bacillibactin is a catecholate siderophore produced by many *Bacilli* (May et al. 2001). It is a cyclic trimeric ester made of three units of 2,3-dihydroxybenzoate-glycine-threonine (Fig. 13.3), joined by lactone linkages in a cyclic manner, similar to the enterobacterial siderophore enterobactin (May et al. 2001). The linear component 2,3-dihydroxybenzoylglycine, also known as itoic acid, also has iron-chelating capabilities (Ito 1993).

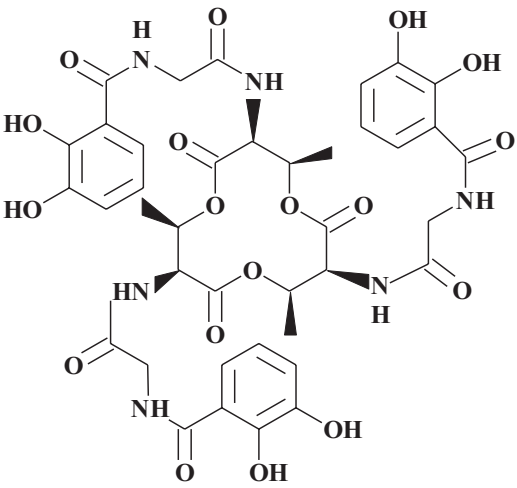

Fig. 13.3 Chemical structure of bacillibactin

13.7 Biosynthetic Pathway of Bacillibactin

Bacillibactin is synthesized by *B. subtilis* under iron-deficient conditions and secreted into the external environment where it binds with Fe(III) with high affinity and specificity. This Fe(III)-bound siderophore is called ferri-siderophore complex which is taken up into the cell by specific transport components.

Bacillibactin synthesis (Fig. 13.4) (http://www.metacyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5903&detail-level=3) can be divided into two parts:

1. Conversion of chorismate to 2,3-dihydroxybenzoate: 2,3-dihydroxybenzoate is synthesized from chorismate through isochorismate and 2,3-dihydroxy-2,3-dihydrobenzoate. Chorismate plays a major role as a key intermediate and branch point in the biosynthesis of many aromatic compounds.
2. Synthesis of bacillibactin from 2,3-dihydroxybenzoate, glycine and L-threonine: Synthesis of bacillibactin is a complex process catalyzed by the bacillibactin synthetase multienzyme complex and is presented by a single pathway reaction. The synthesis starts with the activation of 2,3-dihydroxybenzoate, catalyzed by 2,3-dihydroxybenzoate-AMP ligase, encoded by dhbE (May et al. 2001) in the following reaction:

$$2,3\ \text{dihydroxybenzoate} + \text{ATP} + \text{H}^{+} \rightarrow\ 2,3\ \text{dihydroxybenzoyladenylate} + \text{PPi}$$

The product (2,3-dihydroxybenzoyl adenylate) is transferred onto the aryl carrier protein (ArCP) domain of a bifunctional protein dhbB, whose other function is isochorismatase (May et al. 2001), where it is attached to the free thiol group of its cofactor, 4′-phosphopantetheine. The 4′-phosphopantetheinyl transferase protein (sfp gene product) catalyzes the cofactor attachment (Grossman et al. 1993).

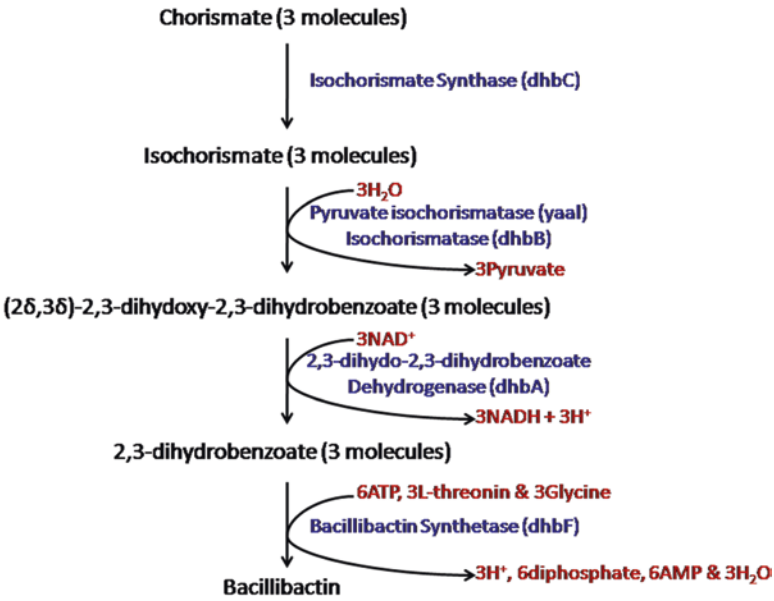

Fig. 13.4 Pathway for bacillibactin biosynthesis

The glycine and L-threonine amino acids are initially bound to 4′-phosphopantetheine cofactors via thiol groups. These 4′-phosphopantetheine cofactors carrying glycine and L-threonine then bind to the seven-domain holo-DhbF protein, thereby activating the holo-DhbF protein. The 4′-phosphopantetheinyl transferase then catalyzes the transfer of glycine and L-threonine amino acids to two peptidyl-carrier-protein domains of the seven-domain holo-DhbF protein and subsequently to the activated 2,3-dihydroxybenzoate. The reaction for binding of glycine and L-threonine amino acids to the thiol groups of the 4′-phosphopantetheine cofactors are as follows:

$$\text{glycine} + \text{ATP} = \text{glycyl-AMP} + \text{PPi}$$

$$\text{L-threonine} + \text{ATP} = \text{L-threonyl-AMP} + \text{PPi}$$

Two additional domains of 4′-phosphopantetheinyl transferase enzyme catalyze the condensation of the activated amino acids to the activated 2,3-dihydroxybenzoate which ultimately form Dhb-glycine-threonine product (May et al. 2001). The product is transferred to the last domain of holo-DhbF protein, named as Te domain. This protein then trims off the three such moieties and releases the trilactone bacillibactin.

13.8 Mechanism of Ferri-bacillibactin Uptake

The bacillibactin synthesizing operon consists of five gene sets (*dhbACEBF*) whose function is given in Table 13.1 and Fig. 13.5.

The detailed molecular analysis of iron uptake pathways in *B. subtilis* was first described by Schneider and Hantke (1993). They suggested that ABC transporter subunits are needed for the uptake of iron siderophore compounds. This hypothesis was generated independently by an analysis of the protein similarities for all of the ABC transport systems (Fig. 13.6), and corresponding surface-binding proteins, in *B. subtilis* (Quentin et al. 1999). The uptake of iron(III) by *B. subtilis* using bacillibactin siderophore requires involvement of a number of membrane-bound proteins such as substrate-binding proteins, ATPase, permeases and transporters. FeuABC is one such membrane-bound bacillibactin transporter of *B. subtilis*. FepDG is another membrane-bound heterodimeric inner membrane permease involved in transport of

Table 13.1 Functions of genes involved in biosynthesis of bacillibactin

Genes	Functions
dhbA	–
dhbC	Isochorismate synthetase
dhbE	DHB-AMP ligase
dhbB	Isochorismate lyases/ArCP
dhbF	Functionally similar to dimodular actinomycin synthetase-II of *Streptomyces chrysomallus*

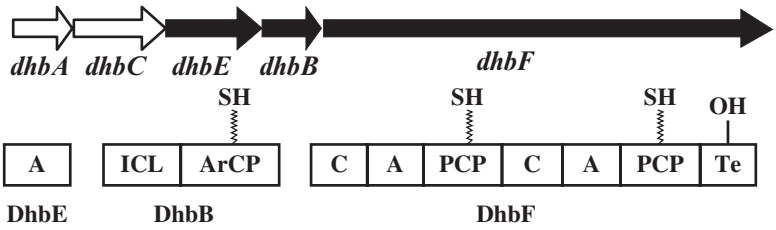

Fig. 13.5 Operon for biosynthesis of bacillibactin

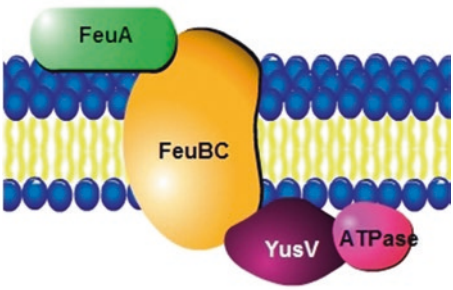

Fig. 13.6 Pathway for iron uptake in *B. subtilis*

bacillibactin. YusV acts as ATP-binding protein and provides energy for transport of ferri-bacillibactin. It is hypothesized that YuiI finally hydrolyzes bacillibactin and ferri-bacillibactin so that iron becomes available for cellular processes and bacillibactin may be recycled for further use. The whole process of iron uptake by bacillibactin in *B. subtilis* is regulated by a Fur homologue which binds directly to a Fur box. Another bacillibactin pathway regulator Mta has been recently discovered which is a MerR-type transcriptional regulator. It activates bacillibactin secretion (Miethke et al. 2008). The exact mechanism of Fur homologue and Mta binding with Fur box and metal ions is poorly understood. Hence, further studies are required in this direction to fully comprehend the regulation of metal uptake in *B. subtilis* or Gram-positive organisms in general.

13.9 Role of Siderophore in Plant Growth Promotion

Siderophores are synthesized by microorganisms and released into the environment which then can bind to iron more specifically and lead the iron unavailable for other microorganisms in the vicinity and thereby limiting their growth. This approach may be used in the biological control of plant diseases (Raymond et al. 2003).

Microorganisms that prosper in the rhizosphere employ a number of different mechanisms to kill or evade pathogens. These microorganisms and their mechanisms can be used as first line of defense and hence become biocontrol agents for plants (Walsh et al. 1971). Plant roots normally exude a variety of different secondary metabolites which chemotactically attract bacteria and fungi toward itself. These bacteria and fungi produce secondary metabolites such as siderophores like bacillibactin which play an important role in competition between microorganisms in soil. These secondary metabolites may act as plant growth promoters (PGP) (Schneider and Hantke 1993). Fusarium wilt is widely reported to cause extensive damage to pepper crop. It was recently reported by Yu et al. (2011) that *B. subtilis* CAS15 significantly suppresses the spore germination of Fusarium wilt in pepper by 8–64 %. *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, and *B. anthracis* have been reported to produce siderophores such as bacillibactin, schizokinen, petrobactin, etc. and have also been used for biocontrol against soilborne pathogens, postharvest fungal pathogens, and even foliar pathogens (Ito and Neilands 1958; Xiong et al. 2000; Barbeau et al. 2002; Moore and Helmann 2005).

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Chapter 14
Bioprospecting of Multiple Hydrolytic Enzymes from Antagonistic *Bacillus* spp. for Biodegradation of Macromolecules

Naser Aliye Feto and Teboho Motloi

Abstract The author of this article and colleagues earlier reported the role played by rhizosphere bacterial antagonists, *Bacillus subtilis* PFMRI and *Paenibacillus macerans* PF9, as bioprotectant and plant growth-promoting rhizobacteria (PGPRB). Since the strains were isolated from the rhizosphere, a diverse and complex environment, it was hypothesized that the strains that are able to survive in such competitive environment could be of potential source of multiple hydrolytic enzymes with ability to biodegrade macromolecules as well. Accordingly, a number of hydrolytic enzymes of the two strains were extracted using carboxymethyl cellulose (CMC), pectin, starch and birchwood xylan as substrates and comparatively analysed for their respective catalytic activities and enzyme kinetics. Consequently, a number of hydrolytic enzymes, namely, cellulase, pectinase, xylanase and amylase, with important physiochemical properties were extracted from *B. subtilis* PFMRI and *P. macerans* PF9. Accordingly, the optimal pH and temperature of enzymes from the former strain were found to vary from 5.0 to 9.0 and 50 °C to 65 °C, while for the ones from the latter, strain varied from 5.5 to 9.0 and 40 °C to 55 °C, respectively, whereas the maximum velocity (Vmax), the amount substrate needed to reach half Vmax (Km) and the time needed to reach half Vmax under optimal condition (Km*t*) for enzymes from the former strain varied from 1128.64 to 13241.86 μmol.min^{-1}.L^{-1}, 1.81 to 205.1 mM and 0.19 to 8.04 min, while for the ones from the latter strain, values varied from 3565.10 to 15366.68 μmol.min^{-1}.L^{-1}, 6.1 to 114.6 mM and 1.54 to 2.86 min, respectively. The present study is the first of its kind in reporting the bioprospecting of multiple hydrolytic enzymes from bacterial antagonists for biodegradation of macromolecules. Accordingly, a number of hydrolytic enzymes stable at elevated temperatures and pH extremes as well as with higher catalytic dynamics and substrate affinity were identified. Besides, it is anticipated that the new parameter, Km*t*, would help us know the time limit of an enzymatic reaction

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_14

and manipulate the reaction as needed. Thus, such enzymes would be of potential role in the white industry. Yet, further study should be conducted to reverse engineer and work on heterologous expression of such enzymes so as to manipulate them for improved physiochemical as well as kinetic traits.

Keywords Bioprospecting • Biochemical • Bacterial antagonists • Enzyme • Macromolecule • Biodegradation • Enzyme kinetics

14.1 Introduction

Lignocellulosic biomass is the most abundant plant by-products rich in energy sources; especially it is the most copiously available raw material on Earth for the production of biofuels, especially bioethanol. Lignocellulose is composed of carbohydrate polymers that contain cellulose, hemicellulose and an aromatic polymer (lignin). Hemicelluloses include xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan, of which xylan is the predominant form of hemicellulose found in hardwood trees with some glucomannan (Wilkie 1979). Hemicelluloses are embedded in the cell walls of plants, sometimes in chains, which are interwoven with pectin to cellulose to form a network of cross-linked fibres (Balan et al. 2009). Besides, starch is the most abundant form of storage polysaccharides in plants and constitutes an inexpensive source for production of syrups containing glucose, fructose or maltose, which are widely used in food industries (Roy and Gupta 2004). Thus, the role of multiple hydrolytic enzymes is crucial in order to convert the macromolecules into simple forms and increase their bioavailability.

Largely, microbial enzymes, among others, play a critical role in biodegradation or bio-fermentation of such macromolecules. Among microbial enzymes reported to have been playing indispensable roles are cellulase, pectinase and xylanase (Balan et al. 2009; Vaseekaran et al. 2010; Ancharida et al. 2014).

The β-1,4-glycosidic linkages in the cellulose can be hydrolysed by cellulolytic enzyme, cellulase. Cellulase is a group of enzymes, which is composed of at least three different enzymes as detailed by Ancharida et al. (2014). Microbes are generally known to be ample sources of a number of industrially important hydrolytic enzymes. Sizable species belonging to *Bacillus* and *Paenibacillus* genera have been reported to be source of cellulase (Ancharida et al. 2014; Eudo et al. 2001; Hakamada et al. 2002; Ogawa et al. 2007). The other enzyme involved in hydrolysis of lignocellulosic biomass, reported in this study, is pectinase. Pectinase is an enzyme that breaks pectic substances by hydrolysis of pectin and breakdown of complex polysaccharides in plant tissues into simpler molecules with extraordinary specificity, catalytic power and substrate specificity (Chaudhri and Suneetha 2012). Pectinase has a number of versatile applications like aiding in fruit juice extraction, textile processing and bioscouring of cotton fibres, degumming of plant bast fibres, retting of plant fibres, treatment of waste water rich in pectin, concentration of coffee and

tea fermentation, paper and pulp industry, animal feed, purification of plant viruses, oil extraction from citrus peels and improvement of chromaticity and stability of red wines (Kashyap et al. 2001; Ahlawat et al. 2011). The other enzyme crucial for biodecomposition of lignocellulosic biomass, also covered by this report, is xylanase. Xylanase (EC 3.2.1.8) is a class of enzymes that hydrolyse the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls (Beg et al. 2001). A number of *Bacillus* spp. were reported to be crucial in the production of xylanase (Bernier et al. 1983; Lee et al. 2008). Besides, because of the size, bacteria cannot transport starch molecules through their cell wall, and thus, bacteria release α-amylase and oligo-1,6-glucosidase into the extracellular space in order to convert starch into a bioavailable form (Vasantha and Hemashenpagam 2012).

Microorganisms have become increasingly important as producer of industrial enzymes. One of the main advantages of using microorganisms for production of any enzyme is the feasible bulk of production and ease to genetically engineer the microbial enzyme of interest for the needed traits (Aliye et al. 2008). A number of studies have been reported regarding the role of microbes as source of catalytically active enzymes (Bernier et al. 1983; Aliye et al. 2008; Chaudhri and Suneetha 2012). However, earlier studies largely dealt with an individual enzyme at a time or a microbe as a source of a given enzyme. Therefore, here we report bioprospecting of multiple hydrolytic enzymes from microbial antagonists, *Bacillus subtilis* PFMRI and *Paenibacillus macerans* BS-DF9 (Aliye et al. 2008), for biodegradation of cellulose, xylan, pectin as well as starch. As far as literature reviewed is concerned, this is the first study of its kind that comprehensively characterized and comparatively analysed multiple hydrolytic enzymes from bacterial antagonists, *Bacillus subtilis* and *Paenibacillus macerans*.

14.2 Materials and Methods

14.2.1 Bacterial Strains

Bacterial strains used in the study were rhizosphere bacterial antagonists, *Bacillus subtilis* PFMRI and *Paenibacillus macerans* PF9, reported by the author of this chapter and colleagues (Aliye et al. 2008).

14.2.2 Biochemical and Physiological Characterization

Biochemical test: different biochemical and physiological characterization were conducted for the strains. The characterization involved KOH solubility test (Gram reaction), spore staining, catalase, oxidase, levan production and tyrosinase activity.

The strains were also evaluated for their ability to liquefy gelatin. Besides, utilization of D-glucose, lactose, cellobiose, mannitol and dulcitol were also studied in broth. The tests were performed according to Goszczynska et al. (2000).

14.2.3 Preliminary Evaluation of Extracellular Hydrolytic Enzyme Production

Preliminary tests for production of extracellular enzymes like cellulase, pectinase, xylanase and amylase were performed on agar plates supplemented with carboxymethyl cellulose (CMC) (Shaikh et al. 2013), pectin (Janani et al. 2011), birchwood xylan (Nagar et al. 2011) and starch (Goszczynska et al. 2000) as sole substrate, respectively.

14.2.4 Enzyme Preparation and Activity Assay

Fresh overnight culture of each strain (*B. subtilis* PFMRI and *P. macerans* PF9) was cultivated overnight in a 5 ml nutrient broth. Then 1 ml of the overnight culture was withdrawn and inoculated into a 100 ml of enzyme production nutrient broth (SIGMA ALDRICH, USA) supplemented with 1 % of either of the substrates, like CMC, pectin, birchwood xylan and soluble starch, for the production of cellulase, pectinase, xylanase and amylase, respectively. The inoculated broth was incubated at 40 °C for 48 h shaking at 100 rpm. Then the 48 h culture was centrifuged at 5000 rpm for 20 min at 4 °C, and the supernatant, which was the enzyme extract, was carefully collected and used immediately to have clear picture of the biological potential of each enzyme and so as not to expose the enzyme for any apparent loss of activities that could result from prolonged storage at 4 °C.

14.2.5 Enzyme Activity Assay

The activity assays for each enzyme were performed following protocols specific to each enzymes.

14.2.5.1 Effect of pH and Temperature on Activity of Enzymes

The activities of the enzymes at different pHs (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and temperatures (37, 45, 50, 55, 60 and 65 °C) were investigated. All tests were done by following the particular assay condition indicated for each enzyme except that either pH or temperature was changed at a time while the other factor kept constant.

Cellulase Assay

The assay was done by the method described by Ghose (1987). Reaction mixture composed of 0.5 ml of 2% (w/v) carboxymethyl cellulose (CMC) in 100 mM sodium phosphate buffer pH 6.5 and 0.5 ml of enzymes from *B. subtilis* PFMRI and *P. macerans* PF9 was incubated at 60 and 55 °C for 30 min, respectively. The formation of glucose was determined spectrophotometrically at absorbance of 540 nm. The amount of reducing sugar released was quantified by a Somogyi method as modified by Nelson (Somogyi-Nelson method) (Nelson 1944) using glucose as standard sugar. One unit of cellulase was defined as the amount of enzyme that yields 1 µmol of glucose within 1 min under the assay condition.

Pectinase [Polymethyl Galacturonase (PMG) and Polygalacturonase (PG)] Assay

Pectin was used as substrate to measure pectinase activity especially those represented by polymethyl galacturonase (PMG) and polygalacturonase (PG) as per methods used by Somogyi (1952), Naidu and Panda (1999) and Weihong and Peilin (2005). The activity of PMG or PG in the broth was determined by measuring the rate of D-galacturonic acid formation from 24.02 mM pectin (MW 208.166 $g.mol^{-1}$) in citrate buffer at pH 6.5 and incubated at 50 and 45 °C for enzymes of former and latter strains, respectively. The formation was determined spectrophotometrically at absorbance of 235 nm. One unit of PMG or PG activity was defined as the amount of enzyme needed for the release of 1 µmol of D-galacturonic acid per minute under assay condition.

Amylase Assay

Amylase enzyme activity was estimated by reducing sugar method (Miller 1959) using 3,5-dinitrosalicylic acid (DNS). The assay mixture containing 250 µl of 50 mM Tris/HCl buffer (pH 7.0), 250 µl of 1% soluble starch (substrate) and 500 µl of appropriately diluted enzyme solution and the mixtures were incubated at 60 and 55 °C for 30 min for enzymes from the *B. subtilis* PFMRI and *P. macerans* PF9, respectively. The reaction was stopped by adding 3 ml of DNS reagent and maintained in boiling water for 3 min, and 1 ml of Rochelle salt solution was added. The OD of the reaction mixture was measured at 540 nm. The OD values were converted as per standard graph obtained with different concentration of D-glucose. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of glucose per min under the assay condition.

Xylanase Assay

The substrate solution was prepared by homogenizing 1.0 % birchwood 4-O-methyl glucuronoxylan in 0.05 M Na-citrate buffer at pH 7. One gram of xylan was homogenized in 80 ml of buffer at 60 °C with a high-shear blender and heated to its boiling point on a heated magnetic stirrer. The substrate was then cooled with continued stirring, covered and stirred slowly overnight. The solution was then made up to 100 ml with buffer and stored at 4 °C until use. All enzyme and xylose dilutions were performed in 0.05 N sodium citrate buffer. The enzyme assay was performed by adding 1.80 ml of substrate solution to a 15 ml test tube. The sample was allowed to equilibrate in water bath to 60 and 40 °C for enzyme from *B. subtilis* PFMRI and *P. macerans* PF9, respectively. Then 200 µl of enzyme, diluted appropriately in citrate buffer, was added and mixed, and the mixture was incubated for 30 min at the same equilibration temperatures and then followed by addition of 3.00 ml of DNS. The reaction was then boiled for 15 min to deactivate the enzyme, followed by cooling in cold water. Finally, the absorbance was read against a blank containing only the reagent and another blank containing only the enzyme at 540 nm and corrected accordingly. One unit of xylanase activity was defined as the amount of enzyme needed for the release of 1 µmol of D-xylose acid per minute under the customized assay condition.

14.2.6 Enzyme Kinetics

The concentration-dependent enzyme kinetic parameters like Vmax and Km were determined by varying concentration of the substrates at optimal pH and temperature. Besides, the same model (Michaelis-Menten) was used to compute the rate of enzymatic activity of a given enzyme under optimal pH, temperature and substrate concentration at a given time, and the Km*t* value or the time needed for a given enzyme to reach its half Vmax(*t*) was determined. Accordingly, the data of enzyme activity throughout the experiment were normalized as needed against the respective maximum value. Then the data were fit into Michaelis-Menten model using the following equation:

$$Y = \mathrm{Vmax} * X / \left(\mathrm{Km} + X\right) \tag{14.1}$$

where 'Vmax' is the maximum value of rate of reaction expressed in $\mu M.min^{-1}.L^{-1}$, 'X' is either the substrate concentration in mM or $mg.ml^{-1}$ or time in minute and 'Km' is the Michaelis-Menten constant that represent either the concentration of the substrate or the time needed to reach half Vmax, and the unit of Km is the same as the unit on the 'X' axis.

14.2.7 Statistical Analysis

All experiments were done in triplicates and repeated trice. The mean and standard error of mean were computed using the GraphPad Prism Software (2007). The obtained values were fitted into Michaelis-Menten model using Eq. 14.1. The absorbance values were converted to the actual amount of reducing sugar by fitting the values into the D-glucose standard curve obtained at $A_{540\ nm}$ for cellulase, xylanase and amylase and standard curve of D-galacturonic acid obtained at $A_{235\ nm}$ for values obtained for pectinase.

14.3 Results and Discussion

Initially, bacterial antagonists, *B. subtilis* PFMRI and *P. macerans* PF9, were evaluated for their biochemical and physiological characteristics. Accordingly, liquefaction of gelatin in broth and hydrolysis of starch on solid media as well as utilization of D-glucose, lactose, cellobiose, mannitol and dulcitol in broth under aerobic and anaerobic conditions were reported. Eventually, extraction and detailed and comparative analyses of the extracellular multiple hydrolytic enzymes like cellulase, pectinase, xylanase and amylase from both strains are detailed.

14.3.1 Biochemical and Physiological Characterization

14.3.1.1 Gelatin Liquefaction and Hydrolysis of Starch

Accordingly, gelatin liquefaction was observed in both tubes inoculated with *B. subtilis* PFMRI and *P. macerans* PF9, while the non-inoculated control remained solidified (picture not shown). As for starch hydrolysis, both strains showed clear zones around their growth, which is an indication of starch hydrolysis, and the non-hydrolysed part of the plate stained blue-black (picture not shown). The gelatin liquefaction result implies that the strains have the ability to produce extracellular hydrolytic enzymes to act upon such macromolecules. Brunel et al. (1994) also reported that isolates belonging to genus *Bacillus* like *Bacillus cereus* were able to hydrolyse starch and glycogen. The finding is also in agreement with Touzel et al. (2000) who reported that an aerobic, thermophilic, xylanolytic, spore-forming bacterium, *Thermobacillus xylanilyticus* gen. nov., sp. nov. utilized starch.

14.3.1.2 Oxidative and/or Fermentative Hydrolysis of Some Carbohydrates and Their Derivatives

Accordingly, *B. subtilis* PFMRI showed oxidative utilization of glucose, lactose and cellobiose, but not *P. macerans* PF9, which utilized glucose and lactose under both aerobic and anaerobic conditions and cellobiose aerobically (Table 14.1). This implies that the strains have apparently different pathway to utilize the said carbon source. Similar result was also reported by Reva et al. (2001) that *B. subtilis* and *B. pumilus* are obligate aerobes and unable to grow under anaerobic condition. The same was also reported by Noeth et al. (1988) that species belonging to the genus *Bacillus* like *B. subtilis*, *B. pumilus*, *B. sphaericus*, *B. laterosporus* and *B. cereus* did not show growth under anaerobic condition, implying the need for the biological availability of sufficient oxygen for growth of those members of *Bacillus* spp. However, *P. macerans* PF9 was able to hydrolyse the sugars under anaerobic condition as well. In line with our finding, Reva et al. (2001) also reported that *P. macerans* can grow under anaerobic condition and hence that the bacterium is facultatively anaerobic. On the other hand, both strains failed to utilize sugar alcohols, mannitol and dulcitol, implying the apparent absence of the metabolic pathway to hydrolyse the said sugars in such strains, unless proven otherwise.

Table 14.1 Biochemical and physiological characteristics of *B. subtilis* PFMRI and *P. macerans* PF9

Test	*B. subtilis* PFMRI		*P. macerans* PF9	
Biochemical:				
KOH solubility	+		+	
Spore staining	+		+	
Catalase	–		–	
Oxidase	–		+	
Levan production	–		–	
Tyrosinase activity	–		–	
Physiological:				
Decomposition of macromolecules:				
Gelatin liquefaction	+		+	
Starch hydrolysis	+		+	
Utilization of:				
Glucose	O+	F–	O+	F+
Lactose	O+	F–	O+	F+
Cellobiose	O+	F–	O+	F–
Mannitol	O+	–	–	–
Dulcitol	V	–	–	–

Goszczynska et al. (2000)

[a]Keys: – no/negative reaction, + positive reaction, ± weak reaction/particle colour change, V variable reaction, F+ fermentative reaction/anaerobic growth (where both the 'open' and the oil-covered test tubes turned *yellow*), and O+ oxidative reaction/aerobic growth (where only the 'open' test tubes turned yellow and the oil-covered ones remained *dark green*)

Most importantly, characterization and comparative analyses of extracellular hydrolytic enzymes from the potent bacterial antagonists, *B. subtilis* PFMRI and *P. macerans* PF9, were also made in order to investigate their versatile use in biodegration of macromolecules as detailed in the following sections.

14.3.2 Comparative Analyses of Cellulase, Pectinase, Amylase and Xylanase from B. Subtilis *PFMRI and* P. Macerans *PF9*

14.3.2.1 Stability of the Enzymes at Different pH and Temperature

The four hydrolytic enzymes, cellulase (CMCase), pectinase, amylase and xylanase, from both species, *B. subtilis* PFMRI and *P. macerans* PF9, showed different and sometimes overlapping reaction to changing pH and temperature.

Cellulase Accordingly, cellulases from both *B. subtilis* PFMRI and *P. macerans* PF9 maintained more than 80 % of their activities at pH 6.5 and were thermostable at 60 and 55 °C for 30 min, respectively.

It is worth mentioning that the enzymes maintained more than 55 % of their activity over pH range 5–8.5 for 30 min (Figs. 14.1a and 14.2a).

This shows that cellulases from such strains have thermophilic nature and can function at pH extremes at more than half their biological potential, thus implying potential roles in a number of industrial applications that might involve elevated temperature and pH fluctuation. In line with this finding, Horikoshi et al. (1984) reported that two cellulases (CMCases), E1 and E2, from *B. subtilis* N-4 were active over pH range 5–10 and stable at 60 and 80 °C, respectively. Recent report by Rawat and Tewari (2012) indicated that *B. subtilis* strain LFS3 produced thermophilic cellulase, implying that *B. subtilis* strains are potential source of thermostable cellulase.

On the other hand, Emtiazi et al. (2007) reported that different strains of *Paenibacillus* spp. showed optimal CMCase activity at pH 7.0 and 50 °C, a condition, which is almost comparable to our finding that indicated optimal pH and temperature for CMCase from *P. macerans* PF9 to be 6.5 and 55 °C, respectively. In addition, Asha et al. (2012) recently reported that another *Paenibacillus* sp. called *Paenibacillus barcinonensis* produced thermophilic cellulase. Such kind of reports further confirm that *Bacillus* and *Paenibacillus* spp. are potential sources of thermostable hydrolytic enzymes of potential industrial importance.

Pectinase Pectinase from *B. subtilis* PFMRI showed maximum activity at pH and temperature of 7.0 and 50 °C, respectively, while maintaining greater than 90 % of its activity over the pH range 5.5–7.0. However, pectinase from *P. macerans* PF9 showed the same pH preference as cellulase from the same strain and optimum temperature of 45 °C (Table 14.2, Figs. 14.1b and 14.2b). This apparently indicate

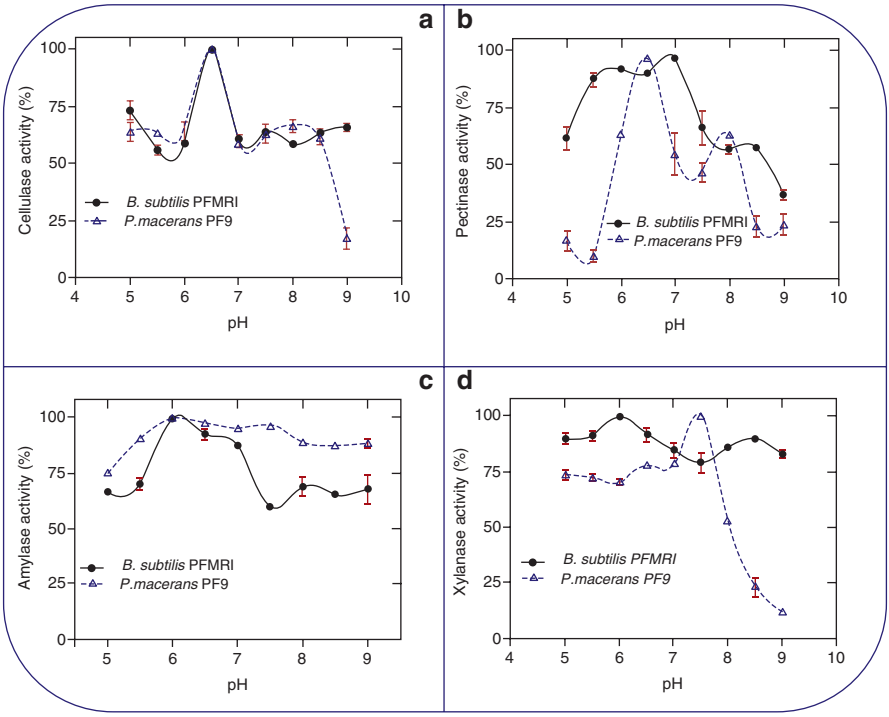

Fig. 14.1 The enzymatic activities of hydrolytic enzymes from *B. subtilis* PFMRI and *P. macerans* PF9 at different pH. The data was computed using GraphPad Prism Software (2007)

that pectinase from the latter strain is less thermostable than the one from the former strain, at least according to our finding. Moreover, Takami and Horokoshi (1999) reported that pectinase from *Bacillus halodurans* C-125 had pH and temperature optima of 9 and 50 °C, respectively. Interestingly, pectinase from *B. halodurans* had the same optimum temperature as that of the one from *B. subtilis* PFMRI though the optimum pH varied, which could be due to the former strain adaptation to a more alkaline environment (Takami 2000), implying that physiochemical properties of the enzyme might have been influenced by the species identity and habitat.

Besides, Zou et al. (2014) reported that pectinase from *B. subtilis* 7-3-3 strain isolated from soil effectively degraded pectin, further confirming the pectin-degrading ability of pectinase isolated from *Bacillus* spp. of soil origin and apparently implying pectinase production being an intrinsic behaviour of such strains.

So long as pectinase or pectate lyase from *Paenibacillus* is concerned, Li et al. (2014) very recently reported that a pectinase enzyme called pectate lyase (Pel, EC 4.2.2.2) from *Paenibacillus* sp. 0602, a strain that was originally isolated from high alkaline soil, had alkalithermophilic nature with pH and temperature optima of 9.8 and 65 °C, respectively. On the other hand, *Paenibacillus amylolyticus* 27C64 showed maximum pectate lyases activity on a highly methylated pectin at pH and

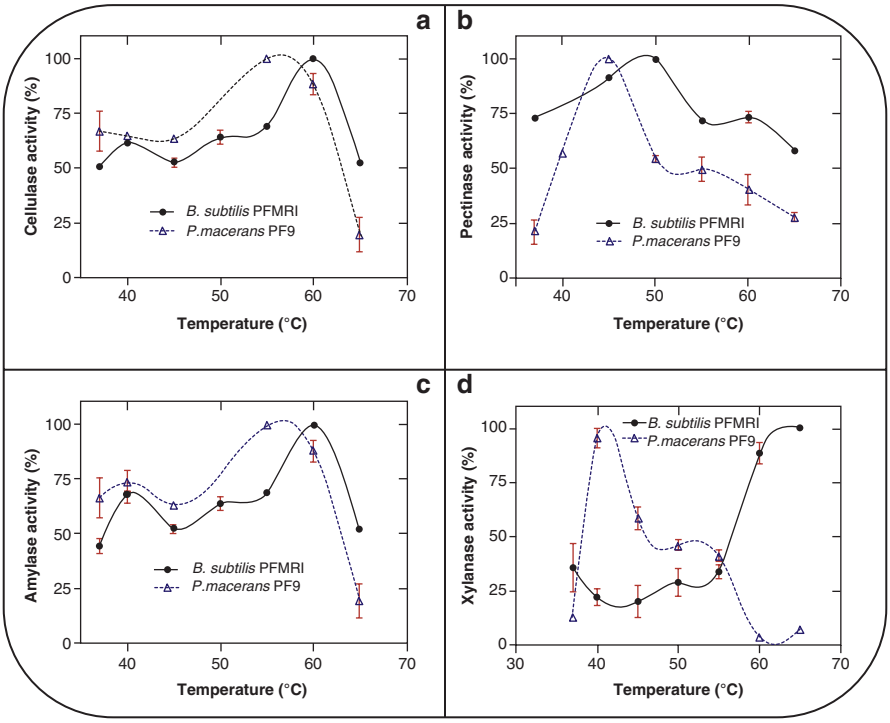

Fig. 14.2 The enzymatic activities of hydrolytic enzymes from *B. subtilis* PFMRI and *P. macerans* PF9 at different temperatures. The data was computed using GraphPad Prism Software (2007)

Table 14.2 The optimal pH and temperature of hydrolytic enzymes from *B. subtilis* PFMRI and *P. macerans* PF9

Enzyme	*B. subtilis* PFMRI		*P. macerans* PF9	
	Optimum[a]		Optimum[a]	
	pH	Temperature (°C)	pH	Temperature (°C)
Cellulase	6.5	60	6.5	55
Pectinase	5.5–7.0	50	6.5	45
Amylase	6.0–7.0	60	5.5–9.0	55
Xylanase	5.0–9.0	65	7.0–7.5	40

[a]The pH and substrate (CMC, pectin, starch and xylan) concentration at which at least 80 % of activity was observed considered as an optimum one, while for temperature, the listed optimum value was the temperature (°C) at which 100 % of activity was recorded

temperature of 10.5 and 45 °C, respectively (Boland et al. 2010). Our result as well as other reports indicates that the physiochemical properties, especially the pH and temperature preference of pectinase from *Paenibacillus* spp., are apparently dependent on the type of the species and the environment it dwells in, as is the case for others.

Amylase Consequently, amylase from *B. subtilis* PFMRI showed narrow pH preference (pH 6.0–7.0), but the same enzyme from *P. macerans* PF9 kept its optimum activity over a wide pH range (pH 5.5–9.0), while the optimal temperatures for both strains were 60 and 50 °C, respectively (Table 14.2, Figs. 14.1c and 14.2c). However, it is worth mentioning that both strains kept their significant level of activities at greater than 66 and 75 % at pH 5, respectively. Moreover, the displayed pH and temperature preference of *B. subtilis* PFMRI amylase shows that the enzyme has acidothermophilic nature. This implies that amylase from the former strain could be of potential use in the white industry that functions at acidic and elevated temperature. In line with our finding, Teodoro and Martins (2000) reported the production of thermostable amylases by a *Bacillus* sp. A number of other reports registered the production of amylase from *Bacillus* spp., including the chimeric one. For instance, Konsoula and Liakopoulou-Kyriakides (2007) reported the co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. In addition, Barros et al. (2013) reported the production of different enzymes including amylase from agroindustrial wastes by biosurfactant-producing strains of *Bacillus subtilis*. On top of potential industrial role, *B. subtilis* PFMRI endowed with amylase of such character could be of potential use as a probiotic supplement, pending experimental verification, to aid digestion of starch for people with gastrointestinal disorder, besides its role in triggering the host immune response and help develop acquired immunity (Hosoi and Kiuchi 2004).

On the other hand, Rajesh et al. (2013) reported identification and functional characterization of α-amylase, Amy1, from *Paenibacillus* sp. with broad temperature (30–90 °C) and pH (4–10) stability. Besides, Haq et al. (2012) reported the production of α-amylase with pH optimum of 8 from *Paenibacillus amylolyticus* using solid-state fermentation.

Our result as well as other reports, thus, indicates that both *B. subtilis* and *P. macerans* are potential sources of thermostable amylase with a number of indispensable downstream industrial applications.

Xylanase Interestingly, xylanase from *B. subtilis* PFMRI showed impressive stability at all pH ranges tested unlike its counterpart from *P. macerans* PF9, which showed optimum activity at nearly neutral condition, pH 7.0–7.5. Besides, xylanase from the former strain showed maximum activity at 65 °C, while the one from the latter strain showed nearly mesophilic preference, showing maximum activity at 40 °C (Table 14.2, Figs. 14.1d and 14.2d). This clearly implies that the ability of *B. subtilis* PFMRI xylanase to function at high temperature as well as pH extremes as compared to the one from the latter strain is more pronounced. In line with our finding, Wang et al. (2010) reported halostable xylanase extracted from *Bacillus* sp. NTU-06. For a xylanase to be used in bleaching process, it should be stable at high temperature and alkaline pH (Viikari et al. 1990; Srinivasan and Rele 1999; Kaur et al. 2010). The xylanase from *B. subtilis* PFMRI was even more thermostable than its equivalent from *Bacillus licheniformis* strain isolated from hot spring (Lee et al. 2008). As per their report, the enzyme which was cloned and transformed into

E. coli showed optimum activity at pH 5–7 and 40–50 °C. This shows that xylanase from *B. subtilis* PFMRI has potential use in the bleaching process that requires the enzyme to have alkalithermophilic nature.

In addition, there are a number of reports regarding xylanases from *Paenibacillus* spp. For instance, Dheeran et al. (2012) reported that *Paenibacillus macerans* IIPSP3 isolated from the gut of the wood-feeding termite produced thermostable xylanase that was active over a broad range of temperatures (40–90 °C). However, according to their report, the strain showed maximum xylanase production at its optimum growth temperature, 50 °C. Moreover, Valenzuela et al. (2010) reported recombinant expression of an alkali stable GH10 xylanase from *Paenibacillus barcinonensis*, a new species isolated from rice farm. According to the report, GH10 xylanase was highly active at 60 °C in alkaline pH values up to 9.5 and remained stable for at least 3 h under alkaline conditions. Apparently, our finding as well as the discussed reports indicates that xylanases from such strains have intrinsic alkalithermophilic nature, if not proven otherwise.

Therefore, the multiple extracellular hydrolytic enzymes from both strains of bacterial antagonists showed interesting physiochemical properties heralding potential downstream applications in the white industry.

14.3.2.2 Enzyme Kinetics

Concentration-Dependent Enzyme Kinetics The enzyme kinetics at optimal pH and temperature but different concentration of substrates were evaluated. Accordingly, xylanases from both *B. subtilis* PFMRI and *P. macerans* PF9 showed the highest Vmax of 13,241.86 and 15,366.68 $\mu mol.min^{-1}.L^{-1}$, respectively (Fig. 14.3 a–d). Accordingly, the Km (concentration of substrate needed to reach half-maximal velocity) values of 1.811, 48.72, 159.88 and 205.1 mM and 6.1, 29.22, 8.28 and 114.6 mM were recorded for cellulase, pectinase, amylase and xylanase from *B. subtilis* PFMRI and *P. macerans* PF9, respectively (Tables 14.3 and 14.4).

The result of Km values indicates that cellulase from both strains has the lowest Km values indicating that the enzyme has the highest substrate affinity as compared to others. On the other hand, xylanase from both species has the highest Km values implying the lowest affinity the enzyme has for its substrate, which means it requires a greater concentration of substrate to achieve Vmax. It is important to mention that despite the lowest substrate affinity, the rate of enzymatic activity of xylanase as explained by Vmax was consistently high, despite being from different isolates. However, it needs further study to ascertain if xylanase has the highest rate of activity regardless of its origin as compared to other hydrolase enzymes or not. A number of reports on enzymatic kinetics of xylanase registered much lower Km values as compared to ours; however, much of such reports were results of solid-state fermentation unlike ours, which was otherwise. For instance, Lee et al. (2008) reported that xylanase from *Bacillus licheniformis*, which was cloned and transformed into *E. coli*, said to have Km and Vmax of 6.7 $mg.ml^{-1}$ and 379 $\mu mol.min^{-1}.mg^{-1}$,

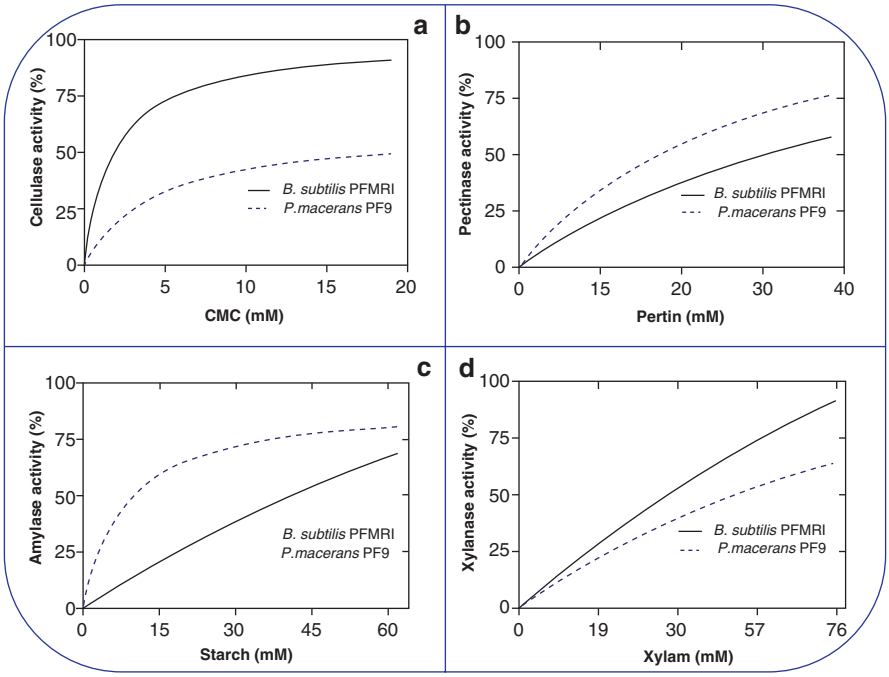

Fig. 14.3 Kinetics of the substrate affinity of the hydrolytic enzymes from *B. subtilis* PFMRI and *P. macerans* PF9. The data were fitted to Michaelis-Menten model using GraphPad Software (2007)

respectively. Moreover, Kamble and Jadhav (2012) reported that the Km and Vmax values of xylanase from a new sp. of *Bacillus* were 5.26 mg.ml^{-1} and 277.7 μmol. min^{-1}.mg^{-1}, respectively.

Nonetheless, it is worth emphasizing on the need to use appropriate unit when explaining the Km values; for instance, in our study, the Km values of amylase and xylanase were 26.84 and 23.14 mg.ml^{-1}, respectively; according to the unit used, it looks that amylase has higher Km values than xylanase even though the reverse is true. However, the use of appropriate unit like millimolar (mM) gives better picture. For instance, the Km values of the aforementioned enzymes are 159.88 and 205.1 mM, respectively, clearly showing that the latter enzyme has higher Km values. Thus, consistent use of appropriate unit is extremely important to avoid misrepresentation and interpretation of results.

Enzyme Velocity in a Given Time or Vmax(*t*) In this case, the enzyme kinetics over time at optimal pH, temperature and concentration were studied. Accordingly, xylanases from *B. subtilis* PFMRI and *P. macerans* PF9 showed the highest Vmax(*t*) of 9840.36 and 11345.72 μmol.min^{-1}.L^{-1}, respectively (Fig. 14.4 a–d), whereas cellulase from both strains showed the least Vmax(*t*) with 2762.96 and 2955.56 μmol min^{-1}.L^{-1}, respectively.

Table 14.3 Kinetic parameters of hydrolytic enzymes from *B. subtilis* PFMRI

	Enzyme kinetics*			
	Kinetics based on concentration		Kinetics based on speed of conversion	
Enzyme	Vmax (μmol.min^{-1}.L^{-1})[a]	Km (mg.ml^{-1}) (mM)[b]	Vmax(*t*) (μmol/min/L)[c]	Km*t* (min)[d]
Cellulase	3018.52 ± 21.12	0.477 ± 0.20 (1.81)	2762.96 ± 13.08	0.43 ± 0.003
Pectinase	4366.49 ± 31.98	9.81 ± 0.95 (48.72)	1834.49 ± 20.78	4.39 ± 0.003
Amylase	1128.64 ± 19.80	26.84 ± 2.6 (159.88)	1017.63 ± 11.84	8.04 ± 0.543
Xylanase	13241.86 ± 20.40	23.14 ± 1.4 (205.1)	9840.36 ± 12.09	0.19 ± 0.009

[a]Vmax is the maximum enzyme velocity in the same units as Y, which in this case is relative amount of reducing sugar molecule released by a given amount of enzyme. It is the velocity of the enzyme extrapolated to very high concentrations of substrate at optimal pH and temperature. **Km** is the Michaelis-Menten constant, in the same units as X, which in this case is in mg.ml^{-1} or millimole (**mM**). It is the substrate concentration needed to achieve a half-maximum enzyme velocity

[b]The value in parenthesis is **Km** value in millimole (**mM**)

[c]**Vmax(*t*)** is the **Vmax** version obtained at optimal pH, temperature and substrate concentration in a given time (**min**)

[d]**Km*t*** is the time elapsed by a given enzyme to reach its half **Vmax(*t*)** at optimal temperature, pH and substrate concentration

*Data represent means ± SE (n = 3). The significance level of α = 0.05 was used throughout the analysis. The analysis was made using GraphPad Prism Software (GraphPad 2007)

As long as the time needed for an enzyme to reach its half-maximum velocity at optimal pH, temperature and substrate concentration or Km*t* value is concerned, the least value was recorded for xylanase from the former strain and amylase of the latter strain with 0.19 and 1.54 min, respectively (Tables 14.3 and 14.4), whereas the maximum Km*t* value was computed for amylase from *B. subtilis* PFMRI and xylanase from *P. macerans* PF9 with 8.04 and 2.86 min, respectively (Tables 14.3 and 14.4). The other details are as listed in Tables 14.3 and 14.4. This implies that xylanase from *B. subtilis* PFMRI has the fastest catalytic activity followed by amylase from *P. macerans* PF9, even though the enzyme from the former strain is at least 3x faster than the one from the latter strain.

Interestingly, cellulase from *B. subtilis* PFMRI and *P. macerans* PF9 has nearly equal Vmax(*t*); however, the one from the former strain was nearly 5x faster to reach its half Vmax than the one from the latter strain. This shows that even if the enzymes have the same Vmax(*t*), the one from the former strain could catalyse 5x more reaction than the one from the latter strain resulting in a higher turnover. Conversely, amylase from *P. macerans* PF9 was computed to have 5x faster catalytic activity than the one from *B. subtilis* PFMRI under optimal conditions. Showing that not all enzymes from *B. subtilis* PFMRI have faster catalytic activity than the ones from *P. macerans* PF9, even though, two out of four enzymes from the former strain have faster catalytic activity than their counterparts from the latter strain. This apparently shows that cellulase and xylanase from *B. subtilis* PFMRI can catalyse reaction within shorter span of time though have lower Vmax(*t*) than the same enzymes from *P. macerans* PF9. Albeit, it should further be proven whether such phenomenon is

Table 14.4 Kinetic parameters of hydrolytic enzymes from *P. macerans* PF9

	Enzyme kinetics*			
	Kinetics based on concentration		Kinetics based on speed of conversion	
Enzyme	Vmax ($\mu mol.min^{-1}.L^{-1}$)[a]	Km ($mg.ml^{-1}$) (mM)[b]	Vmax(*t*) ($\mu mol.min^{-1}.L^{-1}$)[c]	Km*t* (min)[d]
Cellulase	4114.81 ± 20.79	01.12 ± 0.59 (6.1)	2955.56 ± 11.66	2.09 ± 0.69
Pectinase	4610.31 ± 12.54	4.78 ± 0.47 (29.22)	1462.84 ± 12.36	2.82 ± 0.07
Amylase	3565.10 ± 10.51	0.65 ± 0.04 (8.28)	1268.87 ± 22.86	1.54 ± 0.25
Xylanase	15366.68 ± 19.9	18.88 ± 1.1 (114.6)	11345.72 ± 17.14	2.86 ± 0.02

[a]**Vmax** is the maximum enzyme velocity in the same units as Y, which in this case is relative amount of reducing sugar molecule released by a given amount of enzyme. It is the velocity of the enzyme extrapolated to very high concentrations of substrate at optimal pH and temperature. **Km** is the Michaelis-Menten constant, in the same units as X, which in this case is in $mg.ml^{-1}$ or millimole (**mM**). It is the substrate concentration needed to achieve a half-maximum enzyme velocity

[b]The value in parenthesis is **Km** value in millimole (**mM**)

[c]**Vmax**(***t***) is the **Vmax** version obtained at optimal pH, temperature and substrate concentration in a given time (**min**)

[d]**Km*t*** is the time elapsed by a given enzyme to reach its half **Vmax**(***t***) at optimal temperature, pH and substrate concentration

*Data represent means ± SE (n = 3). The significance level of $\alpha = 0.05$ was used throughout the analysis. The analysis was made using GraphPad Prism Software (GraphPad 2007)

specific to enzymes from such strains or not by screening cellulases and pectinases from a number of different strains of *B. subtilis* and *P. macerans* and evaluate for their Vmax as well as speed of catalytic activity.

Unfortunately, it was not possible to find any literature to compare and appreciate the results regarding the new parameter, Km*t*, which is the time needed for a given enzyme to reach its half Vmax at optimal temperature, pH and substrate concentration. Since such approach is new as far as literature reviewed is concerned, it is anticipated that the knowledge about the Km*t* would help us know the time limit of an enzymatic reaction, as it would help us know the time needed to reach half Vmax or Vmax for that matter and manipulate the reaction as needed. Besides, knowledge of both Vmax and time needed to reach the half Vmax would help us have comprehensive understanding of the overall dynamics involved in the enzyme kinetics. For instance, an arbitrary enzyme 'x' might have as twice Vmax as that of 'y' but at the same time as twice slower as the latter enzyme to reach half Vmax, which in effect means both enzymes have the same catalytic activity as the lower Vmax of 'y' is apparently complemented with the relatively shorter time span needed to reach its half Vmax making product of both reaction at a given time comparable. However, further rigorous experimentation would help us better understand the dynamics involved and come up with a model.

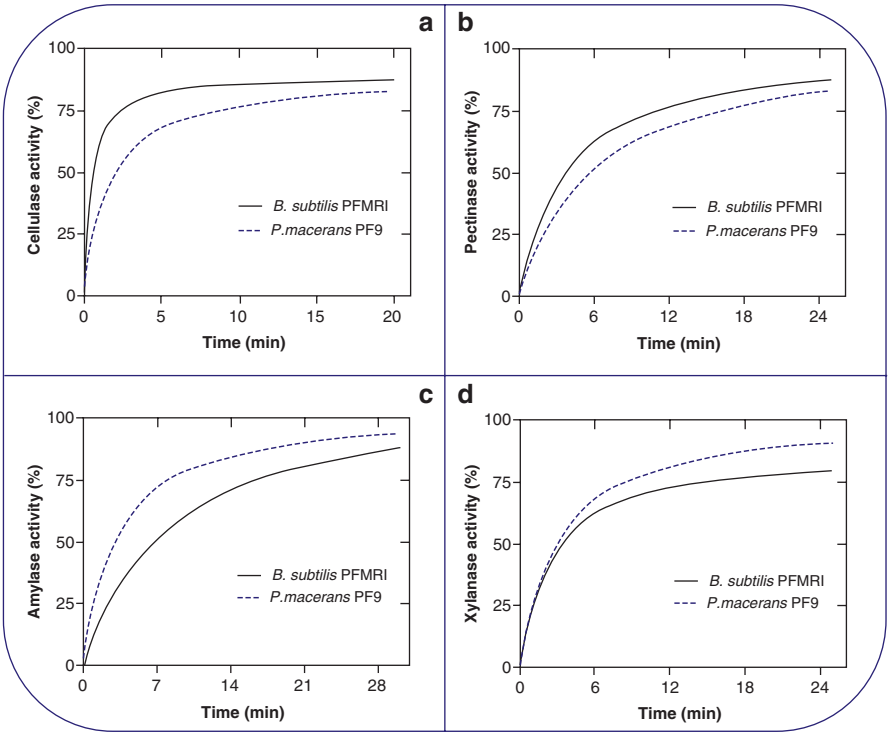

Fig. 14.4 Kinetics of the velocity of hydrolytic enzymes in a given time. The data were fitted to Michaelis-Menten model using GraphPad Software (2007)

14.4 Conclusion

In conclusion, the present study is the first of its kind in reporting the bioprospecting of multiple hydrolytic enzymes from bacterial antagonists for biodegradation of macromolecules. Accordingly, a number of hydrolytic enzymes stable at elevated temperatures and pH extremes as well as with higher catalytic dynamics and substrate affinity were identified. Hence, it is anticipated that such enzymes would be of potential role in the white industry and in green agriculture, whereby the antagonistic bacteria could play dual roles as both bioprotectant and decomposer of macromolecules thereby increasing the bioavailability of the macromolecules to rhizosphere microbial communities and, thus, enhancing the fertility of the soil. Yet, further study should be conducted to purify and reverse engineer and work on heterologous expression of such enzymes so as to manipulate them for improved physiochemical as well as kinetic traits.

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Chapter 15
Effect of Organic Biostimulators with *Bacillus amyloliquefaciens* ssp. *plantarum* (Former *Bacillus subtilis*) *as* the Main Agent in Vegetable Cultivation

Michael H. Böhme, Ina Pinker, and Helmut Junge

Abstract In the last decade, investigations were carried out to analyze the effects of biostimulators (e.g., humate, lactate, *Bacillus amyloliquefaciens* ssp. *plantarum*, formerly *Bacillus subtilis*) in hydroponic systems especially under stress conditions (nutrient deficiency, temperature, salt, and pH stress) on the growth of different vegetables (tomatoes, cucumbers, water spinach, sweet potatoes) in different substrates. Applications of biostimulators are reported to reduce abiotic stress in plants. The physiological effects of these substances, however, are little investigated so far. For biostimulators as K-humate, LACTOFOL, and FZB24®, there were reports about their beneficial effects. Application of different biostimulating substances in diverse combinations, however, could be even more beneficial to plants' growth and development than their separate utilization. Effects of humate, lactate, and *Bacillus amyloliquefaciens ssp. plantarum* FZB24® as single treatment and as mixture on pH and EC values in substrate culture confirmed this assumption. After application of each biostimulator to the roots, the number of marketable cucumbers was enhanced in total and in relation to the nonmarketable fruits. The application in the root zone had a stronger effect than the application on the leaves and should be preferred. This was also visible under stress condition, e.g., under growing conditions with suboptimal pH value and temperature. In previous experiments, we used therefore a bioactive complex containing K-humate, LACTOFOL, and FZB24® and recorded positive effects on plant growth and yield. If biostimulator mixture (humate, lactate, and *Bacillus amyloliquefaciens* ssp. *plantarum*) was applied, no

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_15

plant growth reduction under pH and temperature stress was determined, in comparison to plants without biostimulator treatments. Results show that there is strong correlation between green biomass of treated plants and their root mass. It can be assumed that the effect of stress reduction by use of this biostimulator mixture is based mainly due to enhancing the root growth.

Keywords Biostimulator • *Bacillus amyloliquefaciens ssp. plantarum* • FZB24® • Humic acid • Lactic acid • Soilless culture • Vegetable cultivation • Abiotic stress • Nutrient uptake

15.1 Introduction

Intensively cultivated crops such as vegetables suffer often from inadequate abiotic or biotic growth conditions. Many studies were done to find growth promoters or biostimulators to stabilize the production process or to enhance plant growth.

Most investigations were carried out to find powerful and effective agencies for plant protection from pest and diseases. In this regard also different bacteria species and their strains were investigated and practically used (Krebs et al. 1998; Elsorra et al. 2004; Chen et al. 2009).

Many microorganisms from the rhizosphere can influence plant growth and plant health positively and are therefore often referred to as "plant growth-promoting rhizobacteria" (Schippers 1992 in Kilian et al. (2000). However, their effects must be seen as the complex and also as a cumulative result of various interactions between plant, pathogen, antagonists, and environmental factors (Schippers 1992). There are various effects induced by *Bacillus amyloliquefaciens* ssp. *plantarum*, and the mechanisms of these effects as well as the interactions between them are postulated in Fig. 15.1.

Following the investigations and practical experiences, it can be concluded that *Bacillus amyloliquefaciens ssp. plantarum* has different effects:

- Growth promoting
  - Based on formation of growth hormones and enzymes for nutrient mobilization, there is an effect in enhancing the nutrient availability and nutrient uptake by plant.
  - Improved seed and tuber germination
  - Improved rooting of plants by supporting root formation
  - Improved plant growth, biomass production, and yields
  - Enhance the earliness of vegetative growth and generative development
- Plant health promoting
  - Improving plant strength
  - Reduced disease impact regarding intensity and frequency
  - Induce resistance of the plants

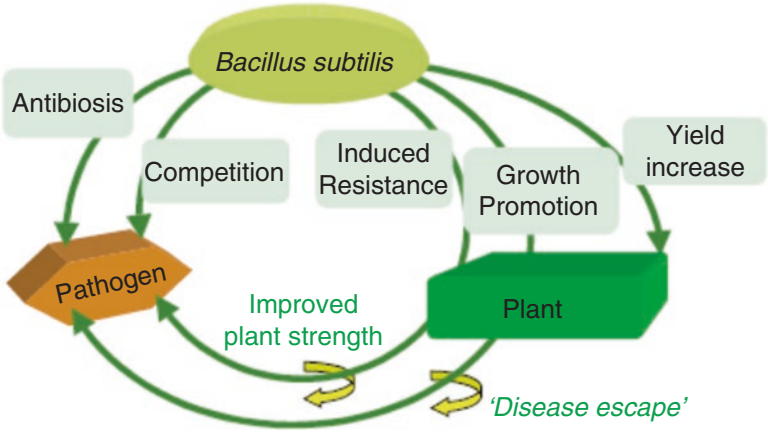

Fig. 15.1 Overview of the modes of action of *Bacillus subtilis* (now *B. amyloliquefaciens ssp. plantarum*) and the interaction between *Bacillus subtilis*, the plant, and the pathogen (Kilian et al. 2000)

Because there are many statements regarding the mentioned effects, several investigations and experiments were carried out in order to use them in different plant cultivation systems. The focus of all these studies was to investigate the specific effect of *Bacillus amyloliquefaciens ssp. plantarum* in different cultivation systems as single treatment and in combination with other agents as biostimulator. In particular, in soilless culture systems, the interaction between plants and rhizosphere is very sensitive. The main aim was to stabilize plant growth in different cultivation systems in a sustainable and environmentally friendly way. Three different biostimulators were used because of their specific effects (Böhme 1999).

In previous investigations, we found beneficial effects in soilless culture systems of the gram-negative rhizobacterium FZB 24® regarding the reduction of salt stress (Böhme 1999). These positive effects under salinity conditions could be confirmed by Bochow et al. (2002) in open-field research with eggplant and bell pepper in Egypt. Plants irrigated with salty water and treated with FZB24® had a four times higher yield for eggplants and five times for bell pepper. It could be shown that treatment with FZB24® stimulated the root growth of the stressed plants. The effects of *Bacillus* strains against fungal and bacterial diseases caused by *Streptomyces scabies*, *Erwinia carotovora*, and *Agrobacterium tumefaciens* are well known for a long time and practically used (Loeffler et al. 1968; Bochow 1995; Krebs et al. 1998; Schmiedeknecht et al. 1998; Grosch et al. 1999). The commercialized rhizobacterium *Bacillus amyloliquefaciens* ssp. *plantarum* FZB24® is capable for evolving different kinds of stress protective mechanisms including stimulation of plants' self-defense mechanisms. Furthermore, FZB24® is efficient regarding different kinds of stress factors such as suboptimal pH, EC, and temperature (Petersohn et al. 2001; Beckering et al. 2002). *Bacillus amyloliquefaciens ssp. plantarum* is well known for its antiphytopathogenic activities (Böhme 1999; Böhme and Vorwerk 2003).

Humates are substances with high cation absorption capacity and therefore balancing salt concentration in growing media (Hoang and Böhme 2001). The effects of these substances applied separately or as complex on growth and yield of vegetables in soilless culture (hydroponic) systems and on plants under extreme growing conditions were studied.

Positive effects of humates on plant growth using NH_4-, K-, or Ca-humates could be proved (Hoang 2003), whereas the source of humate plays an important role for its effects. Application of soluble iron humate to leaves and roots counteracted iron deficiency in the nutrient solution (Böhme et al. 2005a). These findings supported the assumption that humic acids have special importance for transportation and availability of microelements in the plants (David et al. 1994). Also other authors reported prevention of chloroses by humate application, probably because the availability of iron was enhanced (Fortun and Lopez 1982; Kreij and Hoeven 1997). The beneficial effect of humates on nutrient uptake was reported in tomato and other plants for N, P, Fe, Cu, and Zn (Fortun and Lopez 1982; Tattini et al. 1989; Adani et al. 1998).

Lactates, salts of lactic acid, can be used to chelate nutrients, especially micronutrients. Stress-reducing effects of lactates could be found especially in nutrient solutions with too low or too high pH values and also in stress situations because of extreme temperature (Böhme et al. 2000). Several products of a Bulgarian company ECOFOL are offered as foliar fertilizer with the brand name LACTOFOL. Application of these substances as mixture with *Bacillus amyloliquefaciens ssp. plantarum* and humates had positive effects on plant growth and yield in rockwool and other substrates too (Böhme et al. 2005b, 2008). Reduced stress symptoms in photosynthesis after temperature, drought stress, or pH stress and enhanced microbial activity of the substrates could be proved (Schevchenko et al. 2006).

It was essential in all experiments for the treatments with biostimulators to determine the appropriate concentration and frequency of their application. Furthermore, the type and place (plant organ) of application of the biostimulators are very important. The biostimulators can be applied as liquids to the rhizosphere together with the nutrient solution or they can be sprayed on the leaves. Also in this case, there are two possibilities to spray the biostimulator to the upper or to the lower part of the leaves, respectively. Investigations are important which treatment is the most effective one.

15.2 Development Concept for Biological Products

Bacteria are present in the soil in a mean amount of 6×10^8 cells/g of soil, and with a live weight of about 10,000 kg/ha, they are the most abundant microorganisms in soil samples. However, the numbers of bacteria vary by up to a factor of 50, depending on biotic and abiotic environmental factors. *Bacillus* species are among the most common organisms isolated from soil samples. Within this microbiological context, FZB24® must temporarily establish itself in the rhizosphere of the cultivated plant

(Kilian et al. 2000). *Bacillus subtilis*, known as hay bacterium, is a well-known microorganism, and various strains are already used industrially in diverse fields (Junge et al. 2000). *Bacillus subtilis* is available in the nature, but it is important to find the promising active strains and then to develop marketable products.

Biological control of farming and organic farming is nowadays strongly increasing. Microorganisms of the genus *Bacillus* can be used in biological and integrated plant protection. The necessary criteria for the successful development and marketing of biological products are reliable effect, suitability for application by conventional techniques, and competitive treatment costs per ha for both the farmer and the gardener. A low-cost production method, constant product quality, satisfactory storage stability, and formulation capability are indispensable too. FZB24® or other *Bacillus* strains meet these criteria, as explained below. The strain selection process that led to FZB24® took several years. Numerous experiments had shown that FZB24® was the strain that reliably promoted growth and yield and reduced losses due to soilborne pathogens in many species of plants. In addition, this strain also fulfilled all the requirements on the production and formulation sides.

15.2.1 Strain Selection and Systematic Identification of Bacillus amyloliquefaciens ssp. plantarum *FZB24*®

So far lots of fully characterized species have been assigned to the genus *Bacillus*. With new diagnostic methods, it is now possible to determine greater genetic and physiological diversity within individual species. As a consequence, new taxonomic groups could be separated in the future, and some species could be assigned to different groups as it happens to the *Bacillus subtilis* group. The *B. subtilis* group was taxonomically renewed and includes now species like *Bacillus amyloliquefaciens* ssp. *amyloliquefaciens* and *Bacillus amyloliquefaciens* ssp. *plantarum* which were formerly known as *Bacillus subtilis* (Priest et al. 1987; Borriss et al. 2011), and *B. pumilus*, *B. licheniformis*, and *B. firmus*, which are characterized by acid formation from various sugars, including glucose, under aerobic conditions, and which have ellipsoid spores that are not larger than the parent cell (Priest 1993).

Bacillus amyloliquefaciens ssp. *plantarum* FZB24®, which had been isolated from natural habitats in Brandenburg, Germany, was identified as *B. amyloliquefaciens* ssp. *plantarum* (formerly *Bacillus subtilis*) by the methods of Bergey (1986) on the basis of its morphological, biochemical, and serological properties. The identification as *Bacillus amyloliquefaciens ssp. plantarum* has been confirmed by its genome sequence. The *Bacillus amyloliquefaciens ssp. plantarum* strain FZB24® forms the typical wrinkled colonies of *Bacillus subtilis* in surface cultures on solid PD agar (Fig. 15.2). Vegetative cells or spores are found in liquid cultures, depending on the age of the culture (Fig. 15.3).

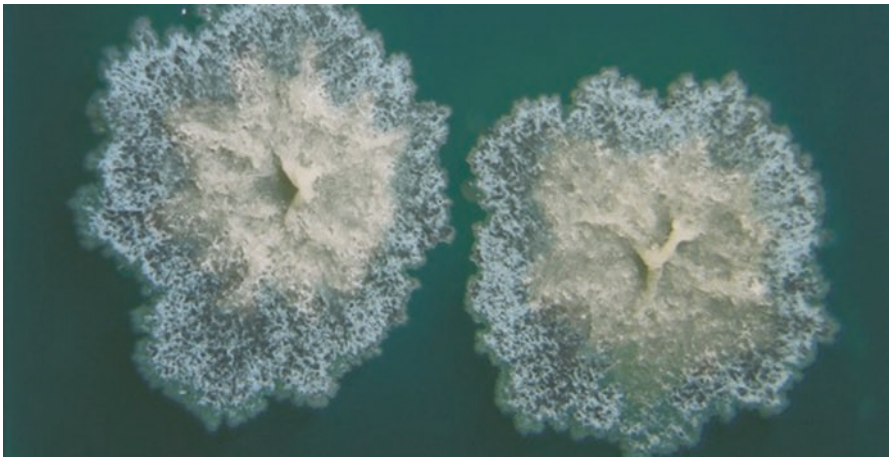

Fig. 15.2 Colony forms of *Bacillus amyloliquefaciens ssp. plantarum* FZB24® on solid PD agar after incubation for 5 days at 25 °C (Photo: Dr. U. Steiner, University of Bonn)

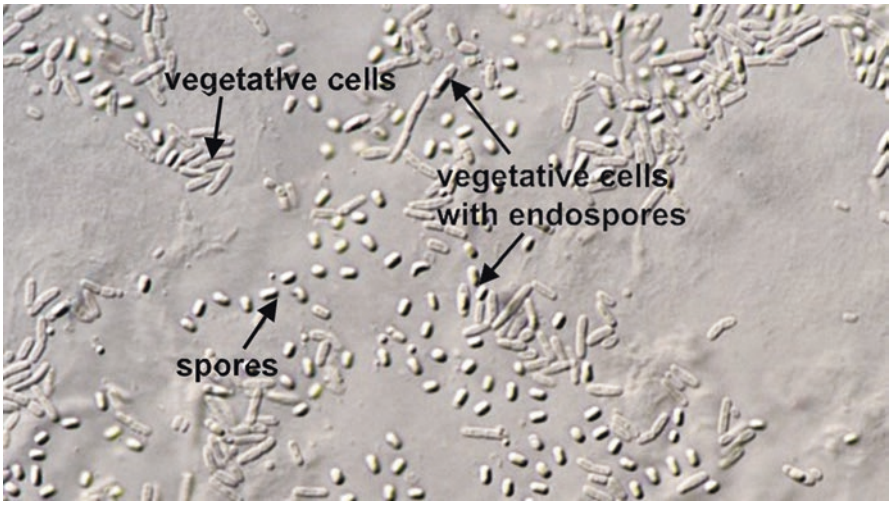

Fig. 15.3 Vegetative cells and spores of *Bacillus amyloliquefaciens ssp. plantarum* FZB24® (Photo: Dr. U. Steiner, University of Bonn)

15.3 Effects of Organic Biostimulators on the Nutrient Supply in Vegetables

Investigations with the organic biostimulators were undertaken regarding the effects:

- Of different humate and lactate types, whereas from *Bacillus* was always used in the strain FZB24®.
- To concentrations and frequencies of applications (humates, lactates, and FZB24®).

– To identify the best ways of applications (in the rhizosphere, to the growing media or in the nutrient solution, or direct to the leaves adaxial or abaxial).
– In stress situations, e.g., salt stress (EC values), suboptimal pH, and suboptimal temperature
– On the nutrient uptake of N, P, K, Ca, Mg, and Fe

15.3.1 Effects on EC Values of the Substrate

Imbalanced EC values can be problematically in hydroponic system and may reduce plant growth and yield as shown previously for tomato (Böhme 1999). The negative effects of very high EC values (EC 8 mS cm^{-1}) could be reduced by application of humate and FZB24® separately or combined. This could be due to the encouraging of root growth. LACTOFOL had no positive effect but also no negative effect.

Even if the nutrient solution has the appropriate EC, in substrate culture, with increasing cultivation duration, there could be an accumulation of salts in the rhizosphere (Böhme et al. 2008). This could lead to salt stress and reduced yield in crops like cucumber. Application of humate and/or FZB24® reduced this salt accumulation (Fig. 15.4).

Lactate had less stabilizing effect on EC value. Most effective for EC stabilization was K-humate even more effective than FZB24®. The mixture of all three compounds, however, was as effective as humate alone and stabilized the EC value at about 2. Interestingly, the stabilizing effect could be maintained over weeks after the last application indicating that there could be a sustainable culture of the bacte-

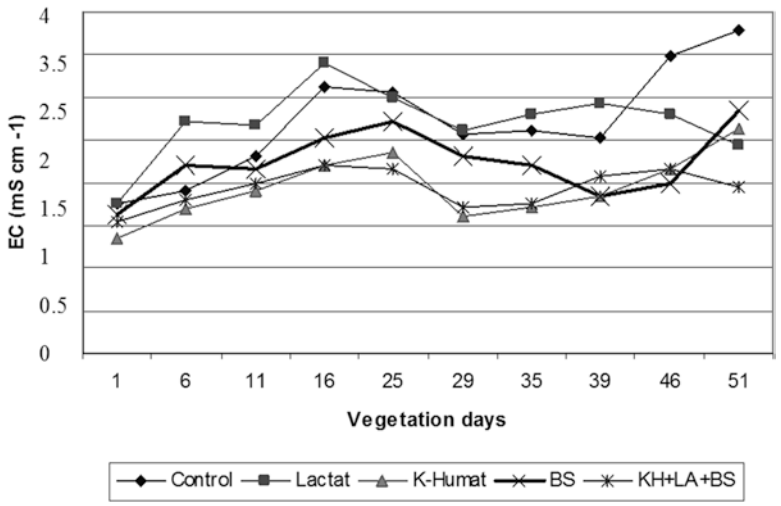

Fig. 15.4 Effect of biostimulators (0.1 % lactate, 0.01 % K-humate, 0.2 % FZB24®) on EC development in the substrate during cultivation of cucumber (Böhme et al. 2008)

Table 15.1 Growth parameters of cucumber plants treated or non-treated with biostimulators (different letters indicate significant differences, LSD, $p \leq 0.05$); *ns* = nonsignificant) (Böhme et al. 2008)

Variants	Shoot weight (g plant^{-1})	Leaf weight (g plant^{-1})	Harvest fruits/plant
Control	676.00 d	172.33 c	7.0 ns
0.1 % LACTOFOL	817.50 bc	203.00 b	10.0 ns
0.01 % K-humate	776.33 c	203.67 b	7.8 ns
0.2 % FZB24®	839.33 b	202.83 b	10.4 ns
Mixture of K-humate, LACTOFOL, FZB24®	911.83 a	235.33 a	7.4 ns

ria and adsorption of the humate on the perlite. The increase of EC from 1.5 to 3.6 was not so strong, but plants treated with biostimulators grew more vigorously and had in tendency a higher yield (Table 15.1).

15.3.2 Effects on pH Values of Substrate

For various plants like tomato and bean (Böhme 1999; Böhme et al. 2000), it was shown that pH values higher than 5.7 in substrate culture can inhibit plant growth. In some growing media as rockwool or perlite, the initial pH is often higher than 6.5. To regulate the pH by changing the nutrient solution is sometimes difficult. Therefore different treatments also with biostimulators were tested. Application of LACTOFOL stimulated root growth and also shoot development even at pH 7.5. Lower pH values had no negative effects in tomato. In general, pH values affect the nutrient availability and uptake. As recorded in experiments with cucumber (Böhme et al. 2008), the pH of substrates changed with the duration of cultivation (Figs. 15.4 and 15.5) and declined in the control to 5.2. The pH of the substrates treated with biostimulators was more stable, especially if FZB24® was added alone or in combination with the other compounds. The nutrient uptake was positive influenced even the pH was higher than recommend.

15.3.3 Effects on Plant Growth

The final evaluation of plants showed that plants treated with biostimulators had significant higher shoot and total leaf weight than non-treated plants especially the plant treated with the mixture (Table 15.1). For the mean number of fruits harvested per plant in this short term experiment, no significant differences could be found. It can be assumed that the pH and EC stabilizing effects of the biostimulators contributed to the development of the plant.

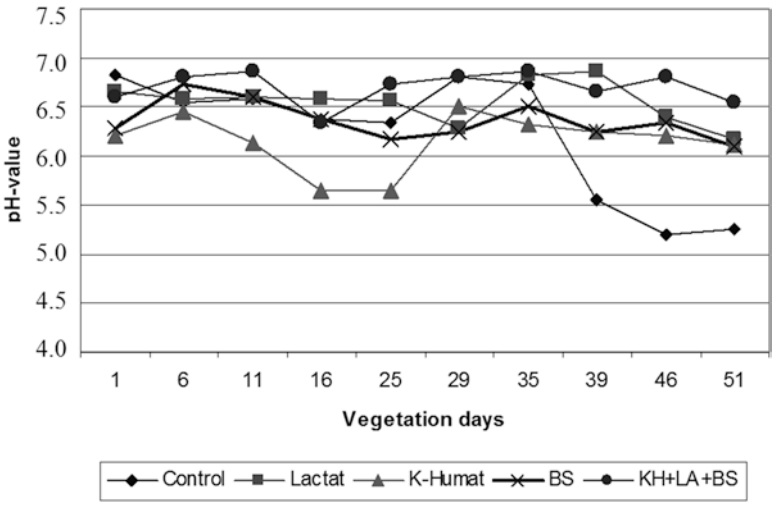

Fig. 15.5 Effect of biostimulators (0.1 % lactate, 0.01 % K-humate, 0.2 % FZB24®) on pH change in the substrate during growth of cucumber (Böhme et al. 2008)

15.3.4 Effect of Different Treatment Methods Using Organic Biostimulators on Growth of Vegetables

All substances described have beneficial effects on plants in stress situations, however, their main effects they have in different stresses. Therefore, the combination of these substances should be investigated more in detail. The experiment presented here was done with cucumber, a very sensitive plant, even under standard conditions. It should be found out if such combination of these substances supports their specific beneficial effects or if they interfere with each other. The biostimulators can be applied in the root zone or on the leaves. It should be investigated which treatment is the most effective one.

15.3.5 Experimental Design

Eight different treatments were compared with the control (Table 15.2).

K-humate (Fa. Humintech), LACTOFOL (O) (Fa. ECOFOL, Table 15.3), and FZB24® (Fa. ABITEP GmbH) was applied on leaves or in the nutrient solution. Quantity and concentration of applied substances were deduced from either previous experience (Böhme 1999) or by using application instructions of manufacturer (FZB 24®).

Timing of application coincided with transplanting of seedlings into big vegetation pots (Mitscherlich Pots with volume of 8 l). Plants were treated three times in weekly intervals in the following development stages: first treatment, five to six leaf

Table 15.2 Concentrations and application patterns of biostimulators used in the experiment (Böhme et al. 2005b)

Treatment	Concentration of substances	Leaf application	Root application
Control	–		
LACTOFOL	0.08 %	X	X
K-humate	0.2 %	X	X
FZB24®	Spore suspension (10/cfu/ml)		
LACTOFOl + K-Humate + FZB24®			

Table 15.3 Composition of LACTOFOL®

Components	Unit	LACTOFOL®	Components	Unit	LACTOFOL®
Lactic acid	%	10	Magnesium	%	0.1
Riboflavin	mg/l	0.5	Iron	%	0.4
Ascorbic acid	mg/l	3	Boron	mg/l	300
Thiamine	mg/l	0.1	Copper	mg/l	200
Nitrogen	%	30	Manganese	mg/l	250
Phosphorus	%	7.5	Zinc	mg/l	125
Potassium	%	15	Molybdenum	mg/l	18
Calcium	%	0.5	Cobalt	mg/l	6

stage; second, seven to eight leaf stage; and third, nine to ten leaf stage. Quantity of substances applied is 20 ml per pot and plant. Leaf application was conducted through spraying of the solution of given substances on surface of the leaves. Watering in substrate was conducted through direct application of solutions into the root area of the plants.

15.3.6 Shoot Development

A three-times application of biostimulators in the growing stage (weeks 4, 5, and 6) affected growth and yield of cucumber plants.

The application of all substances tested stimulated the shoot development represented by a higher fresh matter of shoots and leaves in most variants (Fig. 15.6a). Obviously the location of application was important for the effect of the biostimulators. The application in the root zone led in each case to a higher fresh matter compared to the control. If the substances were applied over the leaves, the effect on shoot fresh matter was not as strong as if they were applied in the root zone. The application of FZB24® even resulted in a lower shoot fresh matter. The effect on leaf fresh matter was also a stimulating one. It should be stressed, however, if the combination of all substances was applied the effect was opposite stimulating if applied over the roots and inhibiting if applied over the leaves.

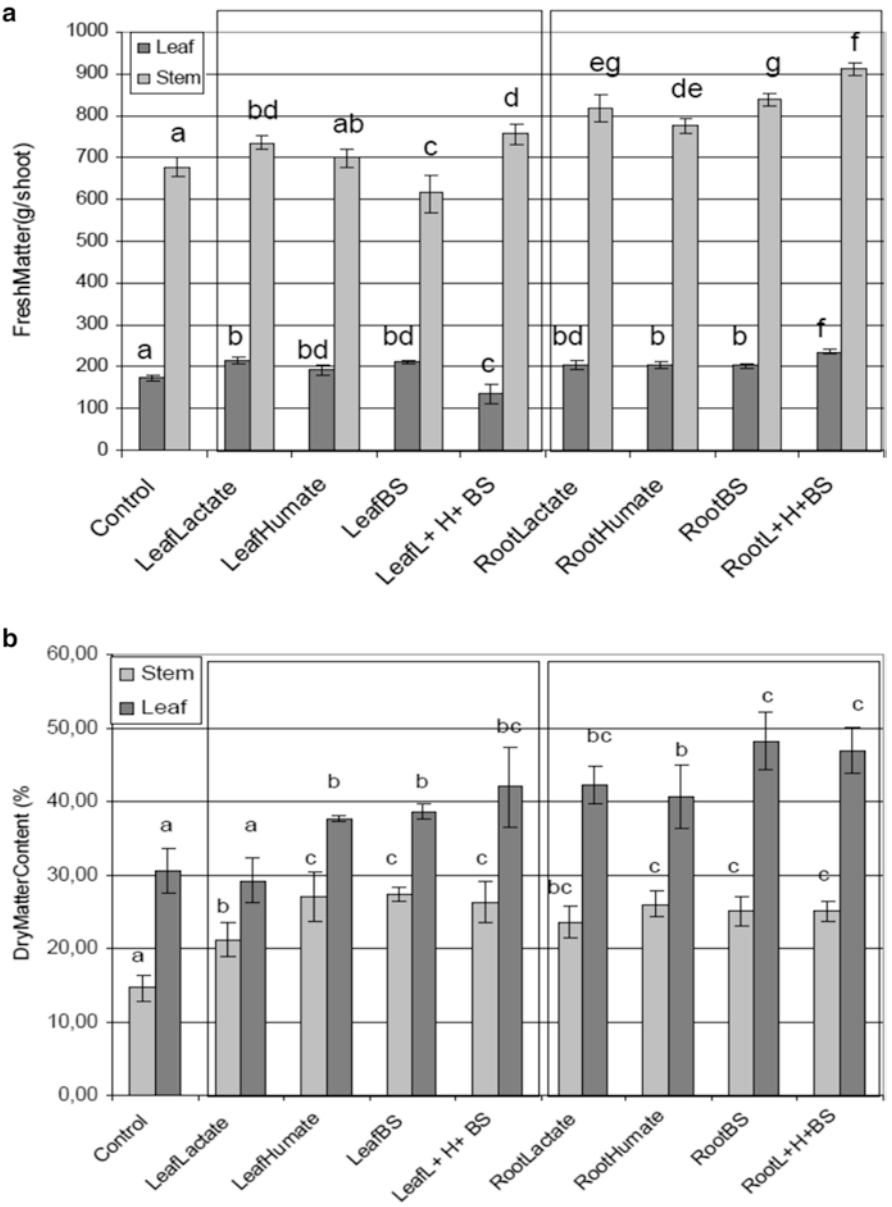

Fig. 15.6 (**a**, **b**) Effect of application biostimulators (lactate, K-humate, FZB24®) on leaves and roots, respectively, on biomass of shoots and leaves after finishing the experiment (Fig. 15.1a = FM; Fig. 15.1b = DM). *Different letters* indicate significant differences (LSD, $P = 0.05$) (Böhme et al. 2005b)

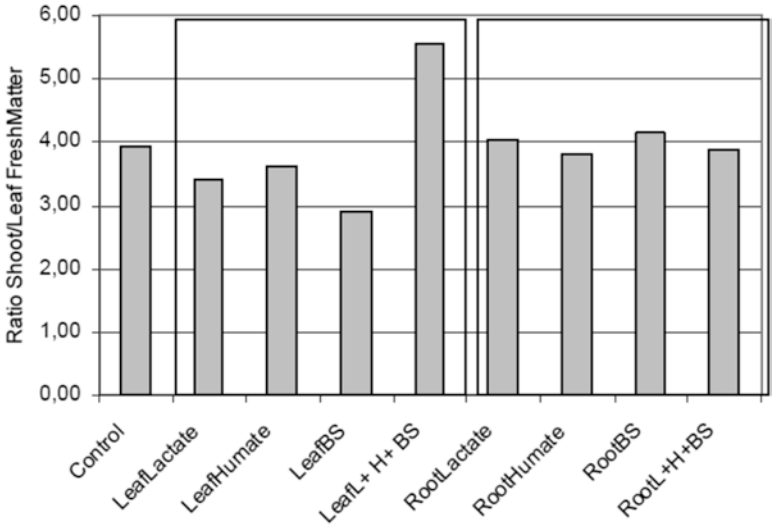

Fig. 15.7 Effect of application of biostimulators (lactate, K-humate, FZB24®) on leaves and roots, respectively, on the ratio of shoots and leaf biomass after finishing the experiment. No significant differences (Böhme et al. 2005b)

The application of biostimulators enhanced in most cases the dry matter content of shoots and leaves (Fig. 15.6b). Therefore, also the quality of shoots and leaves seems to be different, and effects on the weakness against fungi's could be expected. This effect was also found in experiments with water spinach (Hoang 2003); however, in these experiments, the effect on the root growth was much stronger than on the shoot growth. In this respect much more investigations are necessary and the results are only a first advice.

Comparing the ratio between shoot and leaf fresh matter (Fig. 15.7), it is obvious that after application of biostimulators via roots, more or less the same ratio was found as in the control indicating the shoot and leaf growth was stimulated in the same manner. After application of biostimulators to the leaves, the leaf growth was more encouraged than shoot growth resulting in a lower ratio apart from leaf treatment with the combination of all substances. In this treatment leaf development was inhibited, and therefore the shoot/leaf ration increased.

15.3.7 Fruit Harvest and Quality

The total yield after 1 month harvesting (Fig. 15.8) was affected after the application of biostimulators. The fresh weight of all cucumbers with market quality was about 500 g; therefore, the number of fruits is representative for yield. The number

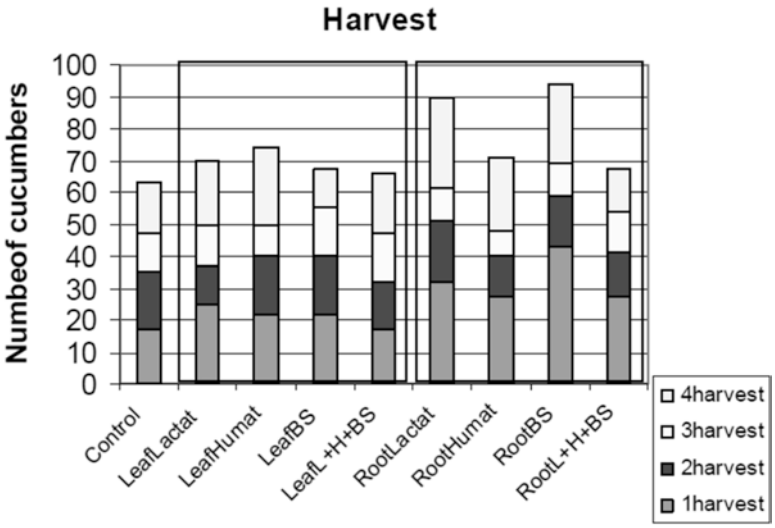

Fig. 15.8 Effect of application biostimulators (lactate, K-humate, FZB24®) on leaves and roots, respectively, on number of marketable fruits in four harvesting periods of 9 days each. No significant differences (Böhme et al. 2005b)

of marketable fruits was higher than in the control in most variants treated with biostimulators. In particular, the treatment with biostimulators on the roots resulted in a higher yield at the first harvest. The number of fruits finally harvested was considerably higher after treatment of roots with LACTOFOL and FZB24®.

For the following experiments, further application also during the fruit set should be taken into consideration because these additional applications could enhance the yield further. This could be especially important in long time cultivation. A very important result of this experiment is that the relative amount of marketable and nonmarketable fruits is affected by treatments with biostimulators (Fig. 15.9).

The ratio of nonmarketable fruits (C class) was more than 25 % in the control and could be reduced by leaf application of each substance investigated till 20 % and even till 10 % if substances were applied over the roots.

The application of humates affected also the quality of cucumbers as indicated by the dry matter content (Fig. 15.10). The dry matter content increased after the treatment with humates in the root zone or on leaves and also in combination with the other substances.

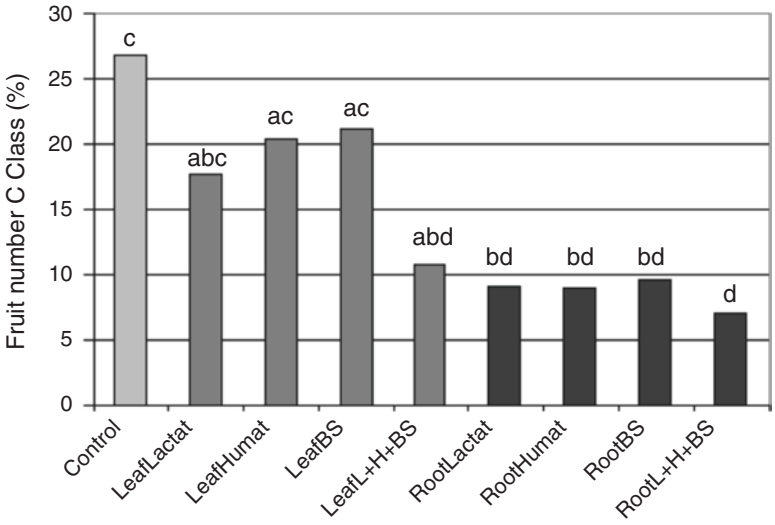

Fig. 15.9 Effect of application biostimulators (lactate, K-humate, FZB24®) on leaves and roots, respectively, on the percentage on nonmarketable fruits. *Different letters* indicate significant differences (Chi-square test, $P = 0.05$) (Böhme et al. 2005b)

15.4 Use of Organic Biostimulators to Reduce Abiotic Stress in Cucumber Plants (*Cucumis sativus* L.)

As already highlighted *Bacillus amyloliquefaciens* ssp. *plantarum* is well known for its effects against soilborne fungal and bacterial diseases, but the strain *Bacillus amyloliquefaciens* ssp. *plantarum* FZB24 is capable of evolving different kinds of stress protective mechanisms including stimulation of plants' self-defense mechanisms, e.g., in case of suboptimal pH, EC, and temperature. Furthermore, *Bacillus amyloliquefaciens* ssp. *plantarum* also accelerate plant growth, stimulate the process of formation of plant organs, and increase the unspecific resistances of plants against stress conditions, such as extreme high temperatures, frost, drought, strong radiation, and deficiency of plant nutrients (David et al. 1994; Hoang and Böhme 2001; Hoang 2003).

Application of lactates in the form of LACTOFOL tends to reduce a plant's stress under suboptimal pH levels of nutrient solution (Böhme 1999; Böhme et al. 2000; Shaban et al. 1995). Introduction of LACTOFOL into the growing system increases availability of micro- and macronutrients for plants. Investigations have shown that lactates have more stable bonds with several metal ions than other chelates do. Therefore, lactates have been used as fertilizers and as bioregulators.

The experiment reported here aimed to investigate the physiological effect of a biostimulating complex consisted with FZB24®, K-humate, and LACTOFOL on the growth of cucumber (*Cucumis sativus* L.) cultivated in perlite with pH and

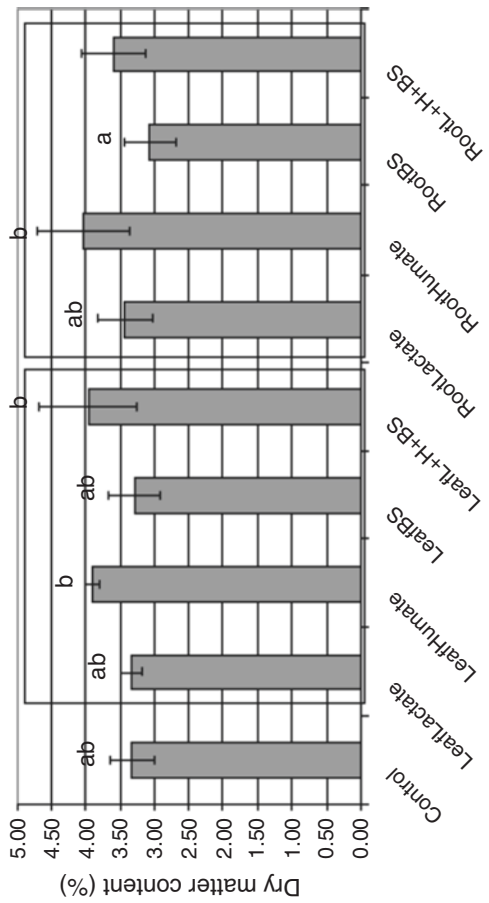

Fig. 15.10 Effect of application biostimulators (lactate, K-humate, FZB24®) on leaves and roots, respectively, on dry matter content of marketable cucumbers. *Different letters* indicate significant differences (LSD, $P = 0.05$) (Böhme et al. 2005b)

temperature stress. The aim was to investigate the photosynthetic reactions to clarify first stress responses in plants. Chlorophyll fluorescence has long been regarded as a very useful method for evaluation of plants' photosynthetic conditions and a tool in noninvasive stress detection and its subsequent evaluation (Böhme et al. 2008).

15.4.1 Experimental Design

Plants were divided into two equal groups. The control group was not treated with biostimulators. The other group was treated with 300 ml biostimulator mixture (FZB24® (0.2 %) + K-humate (0.01 %) + LACTOFOL "O" (0.1 %) per pot once a week by watering to the substrate. Treatments coincided with the following plant developmental stages, i.e., first treatment at five to six leaf stage (week 1), second at seven to eight leaf stage (2 weeks), and the third at nine to ten leaf stage (3 weeks).

After the last treatment with the complex biostimulator (4 weeks), the stress factor was applied. For the pH experiment, pH values were adjusted to a suboptimal level (pH 3.2) by adding H3PO4 to the nutrient solution. This pH stress was maintained for 1 week. For temperature stress, temperature in the growth chamber was lowered from 25 to 6 °C for 3 h.

15.4.2 pH Stress

After transplanting the cucumber plants, the chlorophyll fluorescence Fv/Fm value increased from 0.760 (Fig. 15.11) to 0.790 in plants treated with biostimulating complex and 0.770 in plants without treatment. A drastic decrease in electron efficiency was observed after imposition of a strong lowering the pH value. Between the fourth and fifth measurements, Fv/Fm of treated plants decreased to 0.747 and that of the non-treated ones even to 0.654.

Without biostimulator treatment, the stress effect was much stronger as shown in Fig. 15.11 where the lowest Fv/Fm value was 0.620. Four weeks later the plants in both were able to recover from the stress but to a different extent. The plants treated with biostimulator mixture showed a higher electron efficiency of photosystem II (0.765 Fv/Fm value) at the end of the experiment as compared to the plants without treatment (0.670 Fv/Fm value).

The final evaluation of plants showed that plants treated with biostimulators had significant shorter shoot length and heavier weight than non-treated plants (Table 15.4). The roots were longer than non-treated plants, and treated plants also yielded some marketable fruits (data not shown). Obviously the biostimulator mixture was effective for stress reduction.

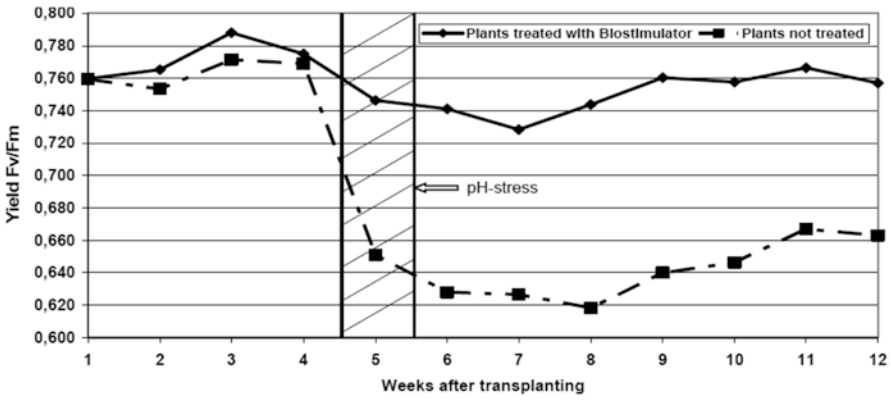

Fig. 15.11 Electron efficiency in photosystem II of cucumber plants treated with biostimulator mixture before and after pH stress (pH 3.2) for 1 week (Böhme et al. 2008)

Table 15.4 Growth parameters of cucumber plants treated with biostimulator mixture prior to pH stress (pH 3.2) for 1 week

Variants	Shoot length (cm)	Shoot weight (g/plant)	Leaf weight (g/plant)	Leaf area (cm^2/plant)	Root weight (g/plant)	Root length (m/plant)
Not treated	325.75 b	230,25 b	460 ns	7002.3 ns	275.75 ns	45.08 ns
Treated with biostimulator	315.25 a	350 a	477.75 ns	7105.2 ns	265.5 ns	52.93 ns

Different letters indicate significant differences (LSD, $p = 0.05$); and *ns* nonsignificant) (Böhme et al. 2008)

15.4.3 Temperature Stress

Temperature stress was applied by lowering the air temperature in the growth chamber right after the third treatment with the biostimulating mixture.

The Fv/Fm parameter had the same pattern as in case of pH stress. However, Fv/Fm development showed its peculiarities (Fig. 15.12). Measurement 1–4 gave equal electron efficiency levels. After temperature stress, Fv/Fm values decreased considerably indicating a reduction in photosystem II efficiency. Only the treated plants were able to reach higher levels of Fv/Fm after stress and could recover better than non-treated plants.

At the end of this experiment, the plant growth parameters were also determined (Table 15.5). The effect of the biostimulator mixture led to a significant difference in all parameters as compared to the non-treated plants, except for the leaf area.

It can be assumed that plants with well-developed root systems have higher resistance against different stress situations. The biostimulator mixture used in these experiments had also shown a positive reaction on the root growth in a previous experiment (Böhme 1999). Therefore, the correlation between the biomass of the

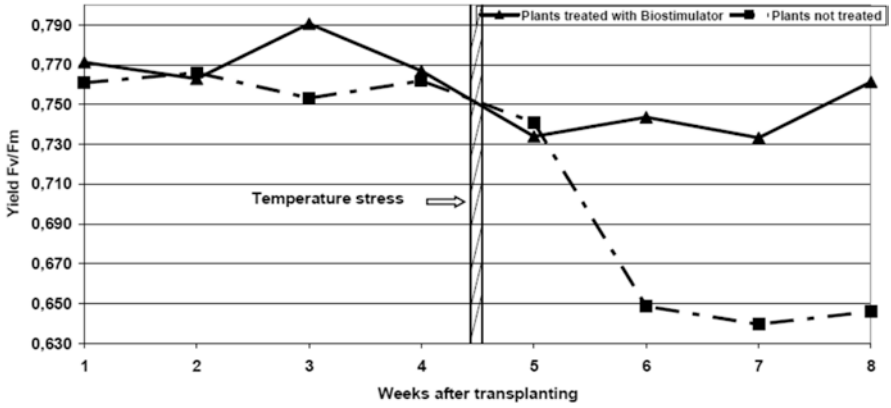

Fig. 15.12 Electron efficiency in photosystem II of cucumber plants treated with biostimulator mixture before and after low temperature treatment with 6 °C for 3 h (Böhme et al. 2008)

Table 15.5 Growth parameters of cucumber plants treated with biostimulator mix prior to temperature – stress at 6 °C for 3 h

Variants	Shoot length (cm)	Shoot weight (g/plant)	Leaf weight (g/plant)	Leaf area (cm^2/plant)	Root weight (g/plant)	Root length (m/plant)
Not treated	341,75 b	244,5 b	477,75 ns	7,333,7 b	206 b	4,86 b
Treated with biostimulator	390 a	358,5 a	548,25 ns	9,637,4 a	321,25 a	6,98 a

Different letters indicate significant differences (LSD, $p = 0.05$); and *ns* nonsignificant) (Böhme et al. 2008)

cucumber plants and the root mass were calculated. In the experiments where biostimulator mixture was not used, no correlation was found (Fig. 15.13).

On the other hand, those treated with biostimulator plants showed a very close correlation (R^2 linear = 0.949) between green biomass and mass of roots (Fig. 15.14). This close relationship confirms the hypothesis that increases in root mass lead to formation of larger shoots and leaf mass even under stress conditions if treated with some biostimulators.

15.5 Conclusions

The *Bacillus amyloliquefaciens* ssp. *plantarum*, in particular, strain FZB24®, is the result of years of study in which various strains of *Bacillus* were selected on the basis of biological activity and suitability for production. FZB24® is produced in a multistage liquid fermentation process from a stock culture that guarantees a uniform strain identity. The spores formed in this process are separated from the culture broth and then dried and formulated together with protective colloids, inert material,

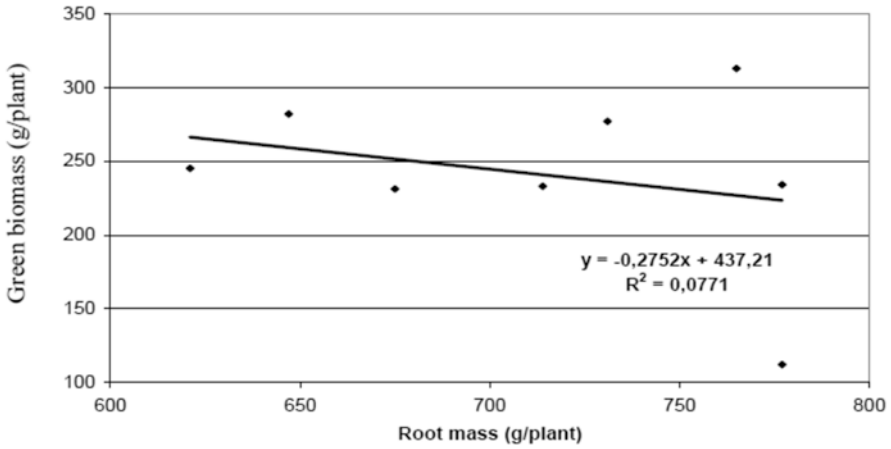

Fig. 15.13 Correlation between green biomass and root mass of cucumbers in plants exposed to pH and temperature stress condition, without biostimulator mixture treatment (Böhme et al. 2008)

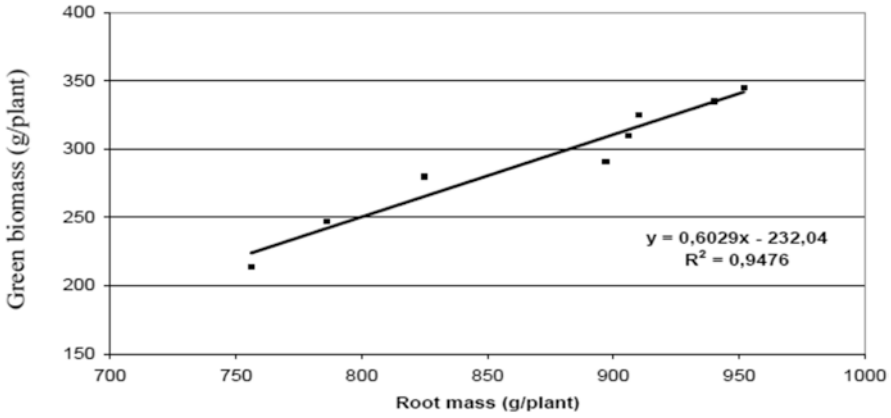

Fig. 15.14 Correlation between green biomass and root mass of cucumbers in the experiment with pH and temperature stress condition, with biostimulator mixture treatments (Böhme et al. 2008)

and other additives. The formulated end product has a storage stability of at least 2 years.

Some effects of biostimulators could be determined or approved; in particular in soilless cultivation systems (hydroponic systems), different effects of humates, lactates, but in particular of *Bacillus* strains like FZB24® were visible. In general can be assumed, an effect of biostimulators is strong apparent if some stress situations occur, as light, pH, or salt (EC) stress. The application of biostimulators can stabilize pH and EC values in the substrate and support thereby the plant growth. Because there were no negative effects found in combination of the biostimulators, this com-

bination could be used to prevent different kinds of stresses. Effects on nutrient availability and uptake have to be evaluated in further experiments. Based on the results, the application of biostimulators in hydroponics seems to be useful and should investigate further. It should be stressed that all different biostimulators in most cases had a stimulating effect on shoot development and number of marketable fruits.

Effects on shoot growth and yield were dependent on the biostimulator used. It is surprising that all substances tested stimulated the vegetative growth, while yield was higher if LACTOFOL and FZB24® were applied. In former experiments, however, a combination of all substances was even more effective than separate use (Böhme 1999). In this experiment a combination only in some cases was better than single substances.

Application of a biostimulator mixture containing FZB24® (0.2 %), K-humate (0.01 %), and LACTOFOL "O" (0.1 %) proved to be useful in reducing stress influence on the growth of cucumber plants. Application of either pH or temperature stress markedly reduced the growth if no biostimulator was applied. The chlorophyll fluorescence Fv/Fm value showed the positive effect of the curative biostimulator treatments for stress counteraction in plants. Cucumber plants with no biostimulator treatment showed very low Fv/Fm values implying that they were not able to recover from pH as well as temperature stress. Results showed that there was strong correlation between green biomass of treated cucumber plants and their root mass. It can be assumed that the effect of stress prevention by the biostimulator was based mainly on enhancing the root growth. This combined use of biostimulators is strongly recommended for the further research.

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Chapter 16
Can *Bacillus* Species Enhance Nutrient Availability in Agricultural Soils?

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Abstract One major challenge for the twenty-first century will be the production of sufficient food for the global human population. The negative impacts on soil–plant–microbes–environmental sustainability due to injudicious use of chemical fertilizer, pesticide, insecticide, etc. by the unaware farmers deteriorate soil and environment quality. One possible way to use efficient soil microorganisms to remediate nutrient deficiency in agricultural soils and other plant growth-promoting (PGP) activities

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3_16

that can be of help for plant growth and development. The *Bacillus* species is one the most dominant rhizospheric bacterial/rhizobacteria species like *Bacillus subtilis*, *B. cereus*, *B. thuringiensis*, *B. pumilus*, *B. megaterium*, etc. that can help enhance the plant growth and development by different mechanisms, which PGPR can inhibit phytopathogens is the production of hydrogen cyanide (HCN) and/or fungal cell wall degrading enzymes, e.g., chitinase and ß-1,3-glucanase. Direct plant growth promotion includes symbiotic and non-symbiotic PGPR which function through production of plant hormones such as auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Mitigate the challenge by adopting eco-friendly crop production practices. Some *Bacillus* species function as a sink for 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene in higher plants, by hydrolyzing it into α-ketobutyrate and ammonia and in this way promote root growth by lowering indigenous ethylene levels in the micro-rhizo environment. *Bacillus* species also help in solubilization of mineral phosphates, potassium, zinc, and other nutrients; rhizobacteria retain more soil organic N and other nutrients in the soil–plant system, thus reducing the need for fertilizers and enhancing release of the nutrients from indigenous or mineral sources, enhancing the economic and environmental sustainability.

Keywords *Bacillus* spp. • Mineral solubilization • Rhizosphere • Fe sequestration • Efficient microorganisms • Nutrient uptake

16.1 Introduction

World food insecurity is a chronic problem and is likely to worsen with climate change and rapid population growth. It is largely due to poor yields of the cereal, pulse, and millet crops caused by factors including soil–plant–environment system. The world's population is assumed to increase from ~7 billion now to 8.3 billion in 2025. The world will need 70–100 % more food by 2050 (Godfray et al. 2010). The increasing human population is placing greater pressure on soil and water resources and threatening our ability to produce sufficient food, feed, and fiber. As a result, there is a growing consensus within our global community that the protection of natural resources and implementation of environmentally and economically sound agriculture practices is of the utmost priority (Ahmad et al. 2016; Bahadur et al. 2016a).

Nowadays world agriculture is facing new challenges in which ecological and molecular approaches are being integrated to achieve higher crop yields while minimizing negative impacts on the environment. In this direction, enhancing nutrient availability, plant growth and yield, and plant multi-stress resistances are key strategies. Root-, soil-, and plant-associated eco-friendly numerous microorganisms produce plant growth-promoting activities with specific action against coexisting microorganisms toward the soil sustainability (Raaijmakers et al. 2009; Combes-Meynet et al. 2011; Genilloud et al. 2011; Pineda et al. 2012; Meena et al. 2013; Maurya et al. 2014; Kumar et al. 2015; Verma et al. 2015b). Global agriculture has to double food production by 2050 in order to feed the world's growing population and at the same time reduce its reliance on mineral/inorganic agricul-

tural inputs. To achieve this goal, there is an urgent need to harness the multiple beneficial interactions that occur between soil microorganisms, plant, and the environment. Beneficiary impacts of soil microorganisms enhance the sustainability of soil–plant–environment ecosystem (Gupta 2012; Bahadur et al. 2016b; Das and Pradhan 2016; Dominguez-Nuñez et al. 2016).

16.2 Soil Microbial Diversity

The beneficial influences of soil microorganisms on plant growth and development include nitrogen fixing (Peix et al. 2001; Riggs et al. 2001; Marino et al. 2007), phosphorus solubilization (Yasmin et al. 2004; Tajini et al. 2012; Verma et al. 2013), potassium solubilization (Phua et al. 2012; Yadegari et al. 2012; Zhang et al. 2013: Meena et al. 2014; Maurya et al. 2014; Saha et al. 2016a), zinc solubilization (Mäder et al. 2010; Saravanan et al. 2007; Bahadur et al. 2016b), and indirect mechanisms such as productio n of phytohormones (Rashedul et al. 2009; Abbasi et al. 2011) such as auxins (Verma et al. 2013), siderophores (Filippi et al. 2011; Yu et al. 2011a, b), and PGPR from the rhizosphere to screen for their growth-promoting activity in plants under axenic conditions (Datta et al. 2011; Meena et al. 2015a, 2016; Singh et al. 2015; Verma et al. 2015a;).

16.2.1 Agricultural Important Soil Microorganisms

It has been reported that biological fertilization is an efficient method to supply plants with their necessary nutrients. It is economically and eco-friendly recommendable, because its results improved the agricultural and environmental sustainability. During the past century, industrialization of agriculture has provoked a significant and essential productivity increase, which has led to a greater amount of food available to the general population. Along with this abundance, the appearance of serious environmental and social problems came with the package: problems that must be faced and solved in the not too distant future. Nowadays, it is urgent to maintain that high productivity, but it is becoming urgent to alter as little as possible the environment. Clearly we must then head for a more environmentally sustainable agriculture while maintaining ecosystems and biodiversity. One potential way to decrease negative environmental impact resulting from continued use of chemical fertilizers, herbicides, and pesticides is the use of plant growth-promoting rhizobacteria (PGPR). This term was first defined by Kloepper and Schroth (1978) to describe soil bacteria that colonize the rhizosphere of plants, growing in, on, or around plant tissues that stimulate plant growth by several mechanisms. Since that time, research activities aimed at understanding how these bacteria perform their positive (or negative) effect have steadily increased, and many reports have been published on these microorganisms. Although interactions between soil microorganisms, plants–rhizosphere, and the environment have important consequences for

ecosystem dynamics and changes in plant communities with time occur in concert with changes in soil properties, the relationships between soil microbial community and plant community dynamics are not fully understood (Van Der Putten 2003; Saha et al. 2016b). Plants are able to modify the structure of microbial communities in their rhizosphere (Berg and Smalla 2009), while soil microbes are important regulators of plant productivity, both through direct effects and through regulation of nutrient availability (Meena et al. 2014). However, the role of such interactions in plant community dynamics with time has received little attention (Bartelt-Ryser et al. 2005; Meena et al. 2015b, c).

16.2.2 The Bacillus *Diversity in Agricultural Soils*

Bacillus is the most abundant genus in the rhizosphere, and the PGPR activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved (Probanza et al. 2002; Mañero et al.2003). Naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth (Dotaniya et al. 2016; Jaiswal et al. 2016; Jha and Subramanian 2016). In a micro-propagated plant system, bacterial inoculation at the beginning of the acclimatization phase can be observed from the perspective of the establishment of the soil microbiota rhizosphere. *B. licheniformis* when inoculated on tomato and pepper shows considerable colonization and can be used as a bio-fertilizer without altering normal management in greenhouses as well as field condition (Bacon et al. 2001; Sessitsch et al. 2002; Wu et al. 2005). *B. megaterium* is very consistent in improving different root parameters in mint. Phosphorus-solubilizing bacteria (PSB) *B. megaterium* var. *phosphaticum* (Lavakusha et al. 2014) and potassium-solubilizing bacteria (KSB) *B. mucilaginosus* (Meena et al. 2014; Maurya et al. 2014) when inoculated in nutrient-limited soil showed that rock materials (P and K rocks) and both bacterial strains consistently increased mineral availability, uptake, and plant growth of pepper and cucumber, suggesting its potential use as bio-fertilizer (Han et al. 2006; Supanjani et al. 2006).

Soil is the main reservoir of the potential bacterial rhizosphere community (Berg and Smalla 2009). Evidence is increasing that plants actively select specific elements of their bacterial rhizosphere micro-flora, establishing a habitat which is favorable for the soil–plant–environment system (Robin et al. 2007; Houlden et al. 2008; Rudrappa et al. 2008). The soil–matrix is a favorable niche for bacteria since both temperature and humidity are relatively sustainable (Ranjard et al. 2000; Sessitsch et al. 2001), mineral composition (Carson et al. 2009), and agricultural practices (Rooney and Clipson 2009; Saha et al. 2016b). The neutral soil reaction is the most favorable condition for higher bacterial diversity, whereas acidic soils were least diverse; it's favorable for fungus growth and development. Bacterial population revealed by culture-dependent techniques represents only 1–10 % of the total bacterial micro-flora present in soil and is now known as the great plate count anomaly (Amann et al. 1995; Meena et al. 2015d, e).

16.2.3 Soil–Plant–Microbe System

Soil–plant–microbe interactions in the rhizosphere soils are responsible for various processes that influence plant growth and development and nutrient mobilization (Awasthi et al. 2011; Singh 2013); a wide range of beneficial microorganisms (e.g., bacteria, fungi, and actinomycetes) associated with plant roots have the ability to promote the growth of the host plant under natural as well as agroecosystem by various mechanisms, namely, fixation of atmospheric nitrogen (Glick et al. 2007), phosphorus (Verma et al. 2012a), potassium (Zhang et al. 2013), and zinc solubilization (Bapiri et al. 2012), and production of plant growth regulators (Meena et al. 2012; Miransari 2011; Rajkumar et al. 2012; Verma et al. 2012b). Besides, the plant-associated microbes residing in the rhizosphere enhance the mobility and availability of plant nutrients to the plants through release of chelating agents, acidification, and redox changes (Glick et al. 2007; Rajkumar et al. 2012). It is also well known that these microbes can utilize the plant-derived substances (e.g., root exudates) comprising different compounds (e.g., organic acids, sugars, vitamins, and amino acids) as major nutrients for their growth and development (Berendsen et al. 2012; Dakora and Phillips 2002; Ryan et al. 2001). On the other side, plants stimulate or inhibit the growth of specific microorganisms through releasing secondary metabolites (e.g., pyrones, sesquiterpenes) into the rhizosphere (Reino et al. 2008; Berendsen et al. 2012; Chakraborty et al. 2012). An example of bacterial stimulation of maize plant root shoot growth is shown in Fig. 16.1.

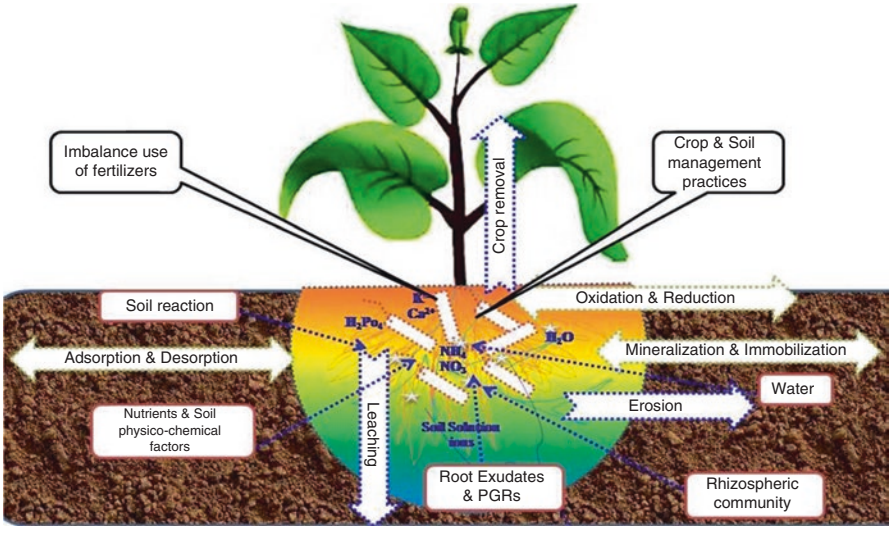

Fig. 16.1 Schematic illustration of how soil and crop management practice factors influence nutrient availability under soil–plant system

16.3 Current Nutrient Status of Agricultural Soils

A recent review of worldwide data on N use efficiency for cereal crops from researcher-managed experimental plots reported that single-year fertilizer N recovery efficiencies are ~65 % for corn, ~57 % for wheat, and ~46 % for rice. Differences in the scale of farming operations and management practices (i.e., tillage, seeding, weed and pest control, irrigation, harvesting) usually result in lower nutrient use efficiency (Kumar et al. 2016; Masood and Bano 2016; Meena et al. 2016). Nitrogen recovery in crops grown by farmers rarely exceeds ~50 % and is often much lower. A review of best available information suggests average N recovery efficiency for fields managed by farmers ranges from about 20 % to 30 % under rainfed conditions and 30 to 40 under irrigated conditions. Looked at N fertilizer recovery under different cropping systems and reported 37 % recovery for corn grown in the north central USA. They found N recovery averaged 31 % for irrigated rice grown by Asian farmers and 40 % for rice under field-specific management. In India, N recovery averaged 18 % for wheat grown under poor weather conditions, but 49 % when grown under good weather conditions (von Braun 2007; Rajkumar and Freitas 2008a, b; Khamna et al. 2010). Phosphorus (P) efficiency is also of interest because it is one of the least available and least mobile mineral nutrients. First year recovery of applied fertilizer P ranges from less than 10 % to as high as 30 % (Fig. 16.2).

However, because fertilizer P is considered immobile in the soil and reaction (fixation and/or precipitation) with other soil minerals is relatively slow, long-term recovery of P by subsequent crops can be much higher. There is little information available about potassium (K) use efficiency. However, it is generally considered to have higher use efficiency than N and P because it is immobile in most soils and is not subject to the gaseous losses that N is or the fixation reactions that affect P. First year recovery of applied K can range from 20 % to 60 % (Fig. 16.3).

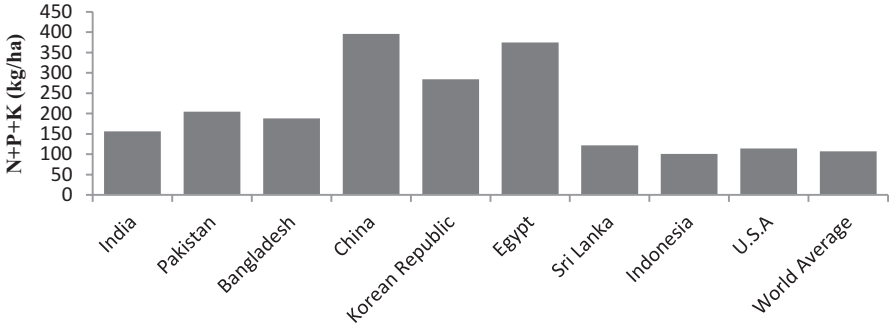

Fig. 16.2 The worldwide nutrients (NPK) consumption in agricultural production system

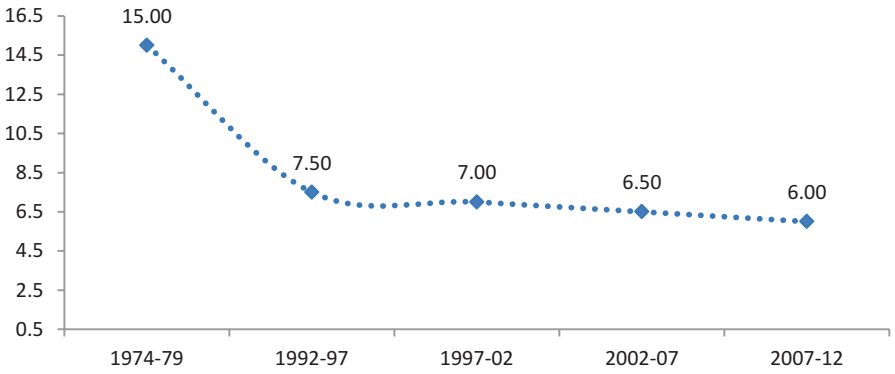

Fig. 16.3 Crop response to fertilizer (kg of food grain/kg NPK)

16.4 PGPR Mechanism of *Bacillus* Species

Bacillus species have the potential to act as a PGPR, nutrient solubilization, and bioremediation, to enhance crop growth, yield, and nutrient uptake by different mechanisms that contributed through direct and indirect mechanisms in the development of sustainable soil–plant–environment systems (Schippers et al. 1995). The generally plant growth-promoting bacteria function in three different ways – synthesizing particular PGR compounds for the growth and development of plants (Zahir et al. 2004), facilitating the mineralization or solubilization of mineral from fixed form to plant available form or soil solution that can help to enhance the nutrients' uptake from the soil (Cakmakci et al. 2006), and helping to reduce the chances of disease infection or preventing the agricultural crops from insect, pest, and diseases (Raj 2004; Saravanakumar et al. 2008; Meena et al. 2015f; Prakash and Verma et al. 2016; Priyadharsini and Muthukumar 2016).

16.4.1 Direct and Indirect Mechanisms

The mechanisms of PGPB-mediated enhancement of plant growth and yield of many crops are not yet fully understood (Dey et al. 2004). However, possible explanations include (a) the ability to produce a vital enzyme, 1–aminocyclopropane–1–carboxylate (ACC) deaminase, to reduce the level of ethylene in the root of developing plants thereby increasing the root length and growth (Li et al. 2006; Meena et al. 2013; Verma et al. 2013); (b) the ability to produce hormones like auxin, i.e., indole acetic acid (IAA) (Patten and Glick 2002), abscisic acid (ABA) (Dangar and Basu 1987; Dobbelaere et al. 2003), gibberellic acid (GA), and cytokinins (Dey et al. 2004); (c) a symbiotic nitrogen fixation (Kennedy et al. 2004); (d) antagonism against phytopathogenic bacteria by producing siderophores, ß-1,3-glucanase, chitinases, antibiotic, fluorescent pigment, and cyanide (Cattelan et al. 1999; Pal et al. 2001; Glick and

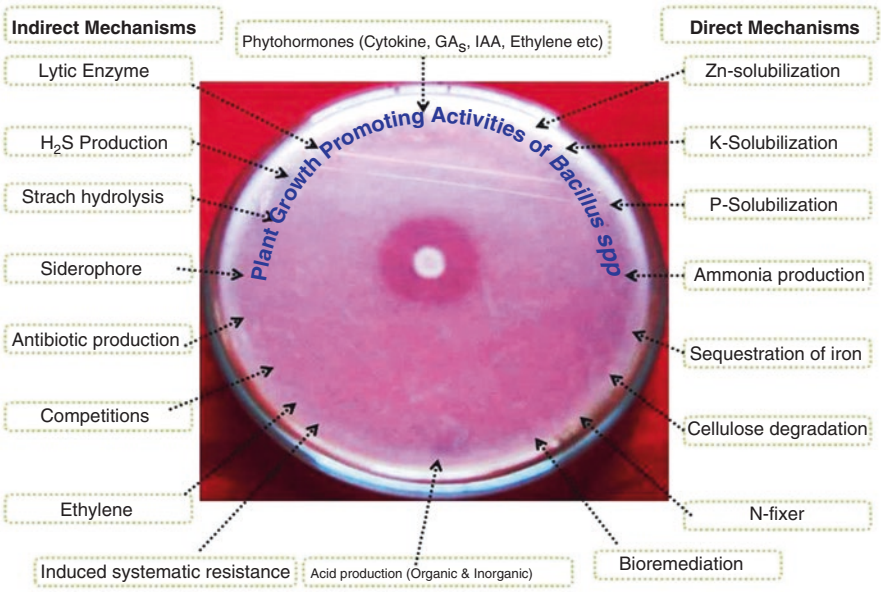

Fig. 16.4 Mechanism of plant growth-promoting *Bacillus* species. (**a**) Direct mechanism (e.g., N_2-fixer, phosphorus, potassium and zinc solubilization, etc.). (**b**) Indirect mechanism (e.g., IAA, GAs, cytokinins and certain VOCs, etc.), both mechanism enhance plant mineral uptake and productivity of crop

Stearns 2011); (e) solubilization and mineralization of nutrients, particularly mineral phosphates and potassium (Maurya et al. 2014; Lavakusha et al. 2014; Meena et al. 2014); (f) enhanced abiotic stress (Saleem et al. 2007; Stajner et al. 1997); and (g) production of water-soluble B group vitamins such as niacin, pantothenic acid, thiamine, riboflavin, and biotin (Revillas et al. 2000; Zhuang et al. 2007; Raghavendra et al. 2016; Rawat et al. 2016; Saha et al. 2016a) (Fig. 16.4).

16.4.2 Nitrogen Fixer

The mineralization of soil organic nitrogen (N) through nitrate to gaseous N_2 by soil microorganisms is a very important process in global N cycling. This cycle includes N mineralization, nitrification, denitrification, and N_2 fixation. A number of bacterial species belonging to the genera *Bacillus*, *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Yu et al. 2012; Braghini Sa et al. 2012; Thepsukhon et al. 2013) are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth and development. Nowadays new techniques have identified a wide range of organisms with the plant rhizosphere with the capacity to carry out biological nitrogen fixation (BNF) – greatly expanding our appreciation of the

diversity and ubiquity of N fixers – but our understanding of the rates and controls of BNF at ecosystem and global scales has not advanced at the same pace. Nevertheless, determining rates and controls of BNF is crucial to placing anthropogenic changes to the N cycle in context and to understanding, predicting, and managing many aspects of global environmental change. Here, we estimate terrestrial BNF for a preindustrial world by combining information on N fluxes with 15 N relative abundance data for terrestrial ecosystems. Our estimate is that preindustrial N fixation was 58 (range of 40–100) TgN fixed yr 21; adding conservative assumptions for geological N reduces our best estimate to 44 TgNyr 21. This approach yields substantially lower estimates than most recent calculations; it suggests that the magnitude of human alternation of the N cycle is substantially larger than has been assumed (Saha et al. 2016b; Sharma et al. 2016; Shrivastava et al. 2016).

16.4.3 Phosphorus Solubilizers

The role of phosphorus mobilizers and solubilizers is more important in soil–plant system because only ~15 % of the phosphorus fertilizer is directly available to the plant growth and development and the rest of the 85 % is lost by different processes like runoff and P fixation due to unfavorable soil conditions. However, eminent soil fertility scientists recognize that soil reactions with applied phosphate limit its direct uptake by plants in the short term; the long-term recovery can approach 90 %, because phosphorus is retained in the soil in slowly available forms (Syers 2003; Panhwar et al. 2012).

Phosphate solubilization by rhizospheric microorganisms in mineral phosphate solubilization was known as early as 1903. Since then, there have been extensive studies on the mineral phosphate solubilization by naturally abundant rhizospheric microorganisms (Fig. 16.5). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw 2000). *B. megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, and *B. sircalmous* could be referred as the most important strains (Verma et al. 2013; Meena et al. 2014; Yu et al. 2012).

16.4.4 Potassium Solubilizers

K-solubilizing bacteria are able to release potassium from insoluble minerals (Sugumaran and Janarthanam 2007; Basak and Biswas 2009, 2012; Kalaiselvi and Anthoniraj 2009; Parmar and Sindhu 2013; Zarjani et al. 2013; Prajapati et al. 2013; Zhang et al. 2013; Gundala et al. 2013; Archana et al. 2012, 2013; Sindhu et al. 2012). In addition, researchers have discovered that K-solubilizing bacteria can provide beneficial effects on plant growth through suppressing pathogens and improving soil nutrients and structure. For example, certain bacteria can weather silicate minerals to release potassium, silicon, and aluminum and secrete bioactive

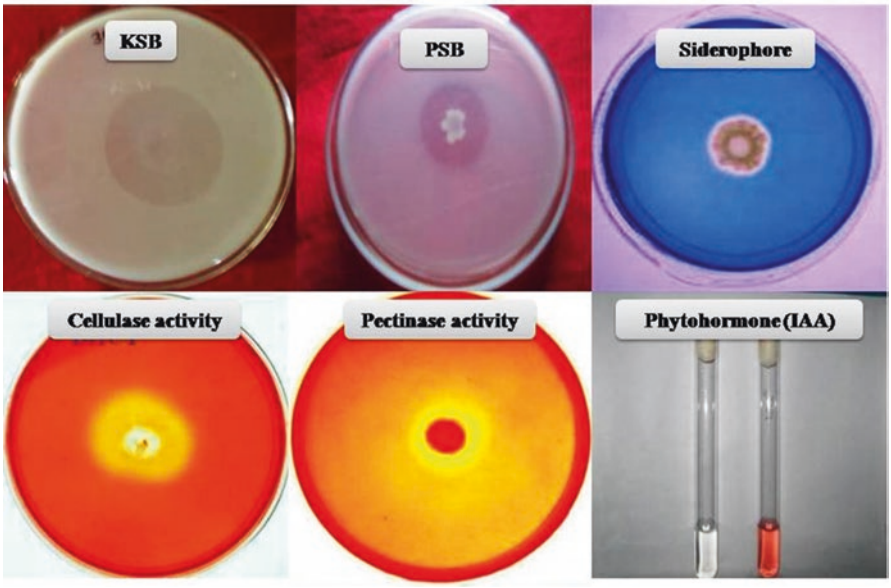

Fig. 16.5 The plant growth-promoting activities of *Bacillus* species, like potassium-solubilizing bacteria (*KSB*), phosphorus-solubilizing bacteria (*PSB*), iron-sequestering bacteria (siderophore-producing bacteria), cellulose-degrading activities, pectinase-producing bacteria, and phytohormone-producing bacteria (IAA, GA_3, ethylene, etc.)

materials to enhance plant growth (Fig. 16.5). These bacteria are widely used in biological K fertilizers and biological leaching (Lian et al. 2002; Bosecker 1997). The considerable populations of potassium-solubilizing microorganisms are present in rhizospheric soil which promotes the plant growth (Sperberg 1958).

It is generally accepted that the major mechanism of mineral K solubilization is the action of organic acids synthesized by rhizospheric microorganism. Productions of organic acids result in acidification of the microbial cell and its surroundings environment which promote the solubilization of mineral K. Silicate bacteria were found to resolve potassium, silicon, and aluminum from insoluble minerals. Silicate bacteria exert beneficial effects upon plant growth and yield. The KSB can promote K solubilization from silicate mineral and is very important to enhance the fertility status of soils. Rhizospheric microorganisms contribute directly and indirectly to the physical, chemical, and biological parameters of soil through their beneficial or detrimental activities (Meena et al. 2015g, h; Sindhu et al. 2016; Teotia et al. 2016).

16.4.5 Zinc Solubilizers

Zinc is predominantly taken up as a divalent cation, Zn^{2+}, but in some cases of calcareous and high pH, it is believed to be taken up as a monovalent cation $ZnOH^{+}$. Zinc interactions in both plants and soils are quite complex and play a major role in

how and when we should apply zinc to a crop. Increasing the Zn and Fe concentration of food crop plants, resulting in better crop production and improved human health is an important global challenge. Among micronutrients, Zn deficiency is occurring in both crops and humans (White and Zasoski 1999; Welch and Graham 2004). Zinc is required in relatively small concentrations in plant tissues (5–100 mg/kg). Zn deficiency is well reported in the soils of much of the world. The deficiency of Zn in cereals especially rice is nutritionally a major problem. Cereals play in satisfying daily calorie intake in the developing world, but the Zn concentration in the grain is inherently very low, particularly when grown on Zn-deficient soils.

The major reason for the widespread occurrence of zinc deficiency problems in crop plant is the low solubility of Zn in soils rather than low total amount of Zn. Zinc-solubilizing bacteria (ZSB) help to solubilize the fixed form of Zn and increase uptake of Zn leading to fortification of grains with Zn (Bapiri et al. 2012). Soil microorganisms require various nutrients for their growth and metabolism. Among the nutrients, zinc is an element present in the enzyme system as cofactor and metal activator of many enzymes (Parisi and Vallee 1969). This causes transformation of about 96–99 % of applied available zinc to various unavailable forms (Fig. 16.5). The zinc thus made unavailable can be reverted back to available form by inoculating a bacterial strain capable of solubilizing it. Since zinc is a limiting factor in crop production, importance of ZSB has an immense in zinc nutrition to plants (Bapiri et al. 2012; Verma et al. 2013).

16.4.6 Fe Sequestration

Iron (Fe) deficiency is a worldwide problem that is directly correlated with poverty and food insecurity. Approximately one third of the world's population suffers from Fe deficiency-induced anemia, 80 % of which are in developing countries (Boccio and Iyengar 2003; Miethke and Marahiel 2007). Total Fe content in soil is relatively high, but its availability to soil microorganisms is low in aerated soils because the prevalent form (Fe^{3+}) is poorly soluble. Plants and microorganisms have developed mechanisms to increase Fe uptake (Marschner 1995; Rajkumar et al. 2010). In plants, there are two different strategies in response to Fe deficiency. Strategy I plants (dicots and non-graminaceous monocots) release organic acid anions which chelate Fe. Iron solubility is also increased by decreasing the rhizosphere pH, and Fe uptake is enhanced by an increased reducing capacity of the roots ($Fe^{3+} \rightarrow Fe^{2+}$). Strategy II plants (*Poaceae*) release phytosiderophores that chelate Fe^{3+} (Von Wiren et al. 1993; Sinha and Mukherjee 2008; Sullivan et al. 2012). Under Fe deficiency stress, soil microorganisms release organic acid anions or siderophores that chelate Fe^{3+}. After movement of the ferrated chelate to the cell surface, Fe^{3+} is reduced either outside or within the cell (Neilands 1984). Microorganisms produce a range of siderophores, e.g., ferrichromes by fungi and enterobactin, pyoverdine, and ferrioxamines by bacteria (Von Wiren et al. 1993; Ma et al. 2011). Rhizobacterial strain significantly influences Fe uptake by agricultural crop (Yu et al. 2011a, b; Sadeghi et al. 2012; Socha and Guerinot 2014).

Fe was supplied either as microbial siderophores (pseudobactin [PSB] or ferrioxamine B [FOB]) or as phytosiderophores obtained as root exudates from barley (epi-3-hydroxy-mugineic acid [HMA]) under varied population densities of rhizosphere microorganisms (axenic, uninoculated, or inoculated with different microorganism cultures). When maize was grown under axenic conditions and supplied with FeHMA Socha and Guerinot 2014), Fe uptake rates were 100–300 times higher compared to those in plants supplied with Fe siderophores (Fig. 16.5). Fe from both sources was taken up without the involvement of an extracellular reduction process. The supply of FeHMA enhanced both uptake rate and translocation rate to the shoot (>60 % of the total uptake). However, increased density of microorganisms resulted in a decrease in Fe uptake rate (up to 65 %), presumably due to microbial degradation of the FeHMA. In contrast, when FeFOB or FePSB was used as the Fe source, increased population density of microorganisms enhanced Fe uptake. The enhancement of Fe uptake resulted from the uptake of FeFOB and FePSB by microorganisms adhering to the rhizoplane or living in the free space of cortical cells. The microbial apoplastic Fe pool was not available for root to shoot transport or, thus, for utilization by the plants (Socha and Guerinot 2014). These results, in addition to the low uptake rate under axenic conditions, are in contrast to earlier hypotheses suggesting the existence of a specific uptake system for Fe siderophores in higher plants. The bacterial siderophores PSB and FOB were inefficient as Fe sources for plants even when supplied by stem injection. It was concluded that microorganisms are involved in degradation processes of microbial siderophores, as well as in competition for Fe with higher plants (Crowley et al. 1992; Socha and Guerinot 2014).

Fe sequestration of *B. megaterium* in iron-deficient medium detected in the exponential phase of growth seems not to be affected by the glucose availability and was not related with the onset of endospore formation (Chincholkar et al. 2007). The carbon source affected the siderophore production by *B. megaterium* (Socha and Guerinot 2014). Among the carbon sources tested, the growth on glycerol promoted the highest siderophore production. The increase of argentine concentration in the culture medium did not enhance the siderophore production. The agitation had a positive effect on the growing of *B. megaterium* and siderophore production. To our knowledge, this is the first work that describes the physiological response of *B. megaterium* in terms of siderophore production (Das et al. 2007; Socha and Guerinot 2014).

16.5 Impact of *Bacillus* Species on Yield and Nutrient Uptake

Nowadays rapidly increasing rate of human population with reducing land holding size due to urbanizations, industrialization, and modernization, by all these increasing presser how to we increasing our food grain production in compared to population with soil–plant–environment sustainability (Ilippi et al. 2011; Sullivan et al. 2012). One possible way to use of beneficially agricultural important microorganisms, with judicious application of mineral as well as chemical fertilizer for sustainable crop production. In

this context many research studies reported that *Bacillus* species and other rhizobacterial strains inoculated to soil significantly enhanced crop growth, yield, and nutrient uptake (Yasmeen et al. 2012b; Velázquez et al. 2016; Yadav et al. 2016).

Bacillus spp. are used as PGPR with plant growth-promoting traits like phosphate, potassium, and zinc solubilization; N_2 fixation and phytohormone production (Liu et al. 2006; Lavakusha et al. 2014; Meena et al. 2014; Maurya et al. 2014) are also being used as bio-inoculants for crop production. The *Bacillus* species are reported to increase the yield in wheat (de Freitas et al. 2007; Cakmakci et al. 2007), maize (Pal et al. 2001), sugar beet (Cakmakci et al. 2006), and spinach (Cakmakci et al. 2007). According to Verma et al. (2012a) observed increase in growth and yield of beans by co-inoculating *Bacillus* strains with other rhizobacteria significantly influenced on nodule formation in pulse crops (Lavakusha et al. 2014; Liu et al. 2006; Yadav et al. 2010) and are widely used as plant health-promoting rhizobacteria by reducing diseases and producing antibiotic (Verma et al. 2013) (Table 16.1).

16.6 Implications of Efficient Soil Microorganisms in Sustainable Agriculture

The various ways in which efficient soil microorganisms have been used over the past fifth decade to modern sustainable technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection, agricultural biotechnology, and in more effective treatment of agricultural. However, microbial technologies have been applied to various agricultural and environmental problems with considerable success in recent years; they have not been widely accepted by the scientific community as it is often hard to consistently reproduce their beneficial effects. We can enhance soil–plant–environment sustainability through the use of efficient soil microorganisms for sustainable agricultural production (Godfray et al. 2010). As discussed above, agriculture should consider maximizing the coadaptation between soil–plant–microbes in an effort to promote soil microbial diversity (Badri et al. 2008 ; Yasin et al. 2016; Zahedi 2016).

Which implications does decoupling the coadapted soil–plant–microbial relationship have on sustainable agriculture? The soil environment is likely the most complex biological community. Efficient soil organisms are extremely diverse and contribute to a wide range of ecosystem services that are essential to the sustainable function of natural and managed ecosystems. The efficient soil organism community can have direct and indirect impacts on land productivity. Direct impacts are those where specific efficient soil microorganisms affect crop yield immediately (Broeckling et al. 2008). Indirect effects include those provided by soil organisms participating in carbon and nutrient cycles, soil structure modification, and food web interactions that generate ecosystem services that ultimately affect productivity. Research opportunities and gaps related to methodological, experimental, and conceptual approaches may be helpful to enhance sustainable agricultural production system.

Table 16.1 Impact of *Bacillus* species on growth, yield, nutrient uptake, and plant growth-promoting activities with different crop species

Crop species	*Bacillus* species	Impact	References
Cicer arietinum	*B. firmus* strain NARS1	Cold stress	Khan et al. (2007)
	B. megaterium	Phytohormones	Verma et al. (2012b)
Lolium multiflorum	*B. pumilus* C2A1	Bioremediation	Ahmad et al. (2006)
Cucumis melo	*B. subtilis* Y-IV	Plant growth, root colonization	Zhao et al. (2011)
Triticum aestivum	*B. pumilus strain* S2	Enhance growth, yield, nutrient uptake	Abbasi et al. (2011)
	B. pumilus S6-05		Upadhyay et al. (2009)
Atriplex lentiformis	*B. pumilus* ES4	Phyto-stabilization	De-Bashan et al. (2008)
Glycine max	*B. subtilis* CICC1016	Siderophore, P solubilization, antagonism with *F. oxysporum*, *S. rolfsii*, *R. solani*	Wahyudi et al. (2011)
	B. sphaericus NUC-5		
	B. cereus strain SS-07		
	B. pumilus		
	B. shandongensis SD		
Oryza sativa	*B. pumilus* strain S68	ACC producing, PGRs	Lavakush et al. 2014
	B. sp SB1-ACC3		
Artemisia annua	*B. subtilis* strain Daz26	Nitrogen fixing	Awasthi et al. (2011)
Fragaria spp.	*B. subtilis* NA-101	IAA equivalents, siderophore, strawberry root, and shoot growth	Pereira et al. (2011)
	B. subtilis NA-120		
Solanum tuberosum	*B.* strain	Phosphorus solubilization, IAA	Calvo et al. (2010)
Zea mays	*B.* sp.	Seed germination and root shoot growth	Ngoma et al. (2014)
Prunus cerasus cv. Kutahya	*B. subtilis* OSU – 142	Fruit set, pomological and chemical characteristics, color values	Karakurt et al. (2011)
	B. megaterium M		
Piper nigrum	*B. subtilis* CAS15	Siderophore producing	Yu et al. (2011a)
Lycopersicon esculentum	*B. amyloliquefaciens* QL5	Controlling bacterial wilt	Wei et al. (1996)
	B. amyloliquefaciens QL18		
Juglans spp.	*B. megaterium*	Nitrogen fixating, PSB	Yu et al. (2012)
Lycopersicon esculentum	*B. subtilis*	Antifungal, nutrient availability	Nihorimbere et al. (2010)
Mammillaria fraileana	*B. megaterium* M1PCa	Mobilization of elements from rocks, mineral degradation	Lopez et al. (2012)
			Puente et al. (2009a, b)

(continued)

Table 16.1 (continued)

Crop species	*Bacillus* species	Impact	References
Zea mays	*B. mojavensis*	Maize seedling growth and nutrient uptake	Bahadur et al. (2016b)
Bouteloua dactyloides	*B.* spp.	Phytoremediation, PGPR	Ma et al. (2011)
Zea mays	*B.* spp.	Drought tolerant	Singh et al. (2013)
Brassica juncea	*B.* spp. Ba32	PGRs, P solubilization	Rajkumar et al. (2006)
			Rajkumar et al. (2008a, b)
Brassica juncea	*B. subtilis* SJ-101	IAA, P solubilization, increased shoot length, fresh and dry weights	Zaidi et al. (2006)
			Rajkumar et al. (2008)
Brassica napus	*B. subtilis* RJ16 (RS)	IAA, Cd-mobilization, increased root elongation (gnotobiotic conditions), shoot and root dry weight (pot experiment)	Sheng and He (2006)
			Rajkumar et al. (2009)
Sorghum bicolor	*B. subtilis*	Increase root shoot biomass	Abou-Shanab et al. (2008)
	B. pumilus		
Lycopersicon esculentum	*B. amyloliquefaciens* S499	PGPR, P solubilization	Nihorimbere et al. (2011)
Sorghum bicolor var. sudanense	*B. mucilaginosus*	Potassium solubilizing	Basak and Biswas (2010)
Glycine max	*B. subtilis*	Nutrient uptake, plant growth	Bais et al. (2002)
Pinus thunbergii	*B. cereus*	Growth, nutrient uptake	Wu et al. (2011)
Actinidia deliciosa	*B. subtilis* OSU142,	Rooting and root growth	Erturk et al. (2010)
	B. megaterium RC01		
Musa paradisiaca	*B. amyloliquefaciens* W19	*Fusarium* wilt and plant growth, increased biomass	Baset Mia et al. (2010)
Brassica napus	*B. licheniformis* BLMB1	Cr, Cu, Pb, and Zn phytoextraction	Brunetti et al. (2011)
			Rajkumar et al. (2010)
Triticum aestivum	*B. subtilis*	PGRs, nutrient uptake	Upadhyay et al. (2011, 2012)
Zea mays	*B. megaterium*	Vegetative growth, yield	Singh et al. (2013)
Arabidopsis thaliana	*B. subtilis*	P solubilization, PGRs	Zhang et al. (2008)
Persea gratissima	*B. megaterium*	Phytohormones, growth, yield	Nadeem et al. (2012)
Raphanus sativus	*B. subtilis*,	Bioremediation, yield, PGRs	Kaymak et al. (2009)
	B. megaterium		
Medicago sativa	*B. pumilus*	Growth, yield, nutrient uptake	Medina et al. (2003)
	B. licheniformis		

(continued)

Table 16.1 (continued)

Crop species	*Bacillus* species	Impact	References
Lycopersicon esculentum	*B. megaterium*	PGRs, growth, yield	Singh et al. (2013)
Manihot esculenta Crantz	*B. megaterium* Cav. cy3	P solubilization	Chen et al. (2014)
Oryza sativa	*B. circulans* P2	Increased rice grain yield	Panhwar et al. (2012)
	B. megaterium P5		
Spinacia oleracea	*B. megaterium* RC07	PGRs, vegetative growth, bioremediation	Çakmakçi et al. (2007)
	B. subtilis RC11		
Sorghum bicolor	*B. polymyxa*	Increased grain and dry matter yields and N and P uptake	Alagawadi and Gaur (1992)
Cicer arietinum	*B. megaterium*	Increased dry matter, grain yield and P uptake, nodulation, N fixation	Verma et al. (2013)
Helianthus annuus	*B. megaterium* M-13	Increased yield, oil, protein content	Ekin (2010)
Solanum tuberosum	*B. polymyxa*	Increased yield, P uptake	Kundu and Gaur (1980)
Rubus idaeus	*B. megaterium*	Increased crop yield	Orhan et al. (2006)
Ammi visnaga	*B. simplex*	Increased root, shoot length, dry weight	Hassen et al. (2010)
	B. cereus		
Fragaria ananassa	*B. megaterium*	Increased fruit yield, nutrient contents	Esitken et al. (2010)
Curcuma longa	*B. megaterium*	Plant growth and yield	Sumathi et al. (2011)
Momordica charantia	*B. subtilis*	Enhanced yield, quality, root length, and dry root weight	Kumar et al. (2012a)
Phyllanthus amarus	*B. coagulans*	Improved growth, yield	Earanna (2001)
Begonia malabarica	*B. coagulans*	Biomass yield, nutrients, and secondary metabolites	Selvaraj et al. (2008)
Mentha piperita	*B. megaterium*	Root length, dry matter	Kaymak et al. (2008)
Solanum viarum	*B. coagulans*	P, Fe, Zn, Cu, and Mn content, secondary metabolites	Hemashenpagam and Selvaraj (2011)
Sphaeranthus amaranthoides	*B. subtilis*	Enhanced growth, biomass, nutrition	Sumithra and Selvaraj (2011)
Withania somnifera	*B. circulanse*	Increased plant height, root length, and alkaloid content	Rajasekar and Elango (2011)
Rosmarinus officinalis	*B. megaterium*	Increased oil content, yield in fresh herb, and total CHO	Abdullah et al. (2012)
	B. circulanse		

16.7 Future Prospect

The *Bacillus* species are a major integral component of soil microbial community and play an important role in the N fixation and phosphorus, potassium, zinc, and iron cycles in soil–plant rendering the plants available forms of nutrients. These bacterial strains have enormous potential for making use of fixed form of minerals and very slowly available nutrients under soil–plant systems with low availability in tropical and subtropical countries. The mechanism of mineral solubilization by *Bacillus* species has been studied in detail, but the K and Zn solubilization and Fe sequestration are a complex phenomenon affected by many factors, such as potential of bacterial strain used, nutritional status of soil, mineral type, amount of mineral, size of mineral particles, and environmental factors. Moreover, the sustainability of the *Bacillus* species after inoculation in soil as well as seed and seedling treatment is also important for mineral availability to benefit sustainable crop growth and development. Therefore, further study is needed to understand the problem of development of efficient and indigenous *Bacillus* species with microbial consortium for growth and yield of crops. Another big problem is the commercial propagation of soil microorganism's consortium and their preservation and transportation at farmer's fields for sustainable agricultural production.

16.8 Concluding Remarks

Climate change problems have raised great interest in eco-friendly sustainable agricultural management practices. The use of growth-promoting rhizobacteria is a promising solution for sustainable soil–plant–microbes, environmentally friendly agricultural production system. The studies on *Bacillus* species as plant growth-promoting activities in sustainable agriculture included isolating and screening antagonists targeting different diseases, evaluating their effectiveness in greenhouse as well as field, dissecting their mechanisms, and enhancing nutrient availability in agricultural soils. Research on improvement of *Bacillus* species through genetic engineering is also conducted in order to increase effectiveness under unfavorable conditions. *Bacillus* species control the damage to plants from phytopathogens and promote the plant growth by a number of different mechanisms and enhance the availability of nutrients for sustainable growth and development of agricultural production system.

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M.T. Islam et al. (eds.), *Bacilli and Agrobiotechnology*,
DOI 10.1007/978-3-319-44409-3

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