Vijay Singh Meena · Pankaj Kumar Mishra
Jaideep Kumar Bisht · Arunava Pattanayak
*Editors*

# Agriculturally Important Microbes for Sustainable Agriculture

## Volume 2: Applications in Crop Production and Protection

Springer

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# Part I

# Microbes for Sustainable Food Production

# Importance of Soil Microbes in Nutrient Use Efficiency and Sustainable Food Production

# 1

Sunita Kumari Meena and Vijay Singh Meena

**Abstract**

Microorganisms that sustain the fertility of soils, resulting in improved plant nutrition, have continued to magnetize attention because of the increasing cost of agricultural inputs and some of their negative impacts on environmental sustainability. The continuous increase in the world population at an alarming rate requires more food for nutritional security. A doubling in global food demand projected for the next 50 years poses huge challenges for agricultural sustainability. Nowadays, plant growth is enhanced by the increasing input of agrochemicals, which act as plant growth regulators (PGRs) and as nutrients. Excessive/injudicious use of chemicals increases the chances of deteriorating soil and environmental quality. Rhizospheric plant growth–promoting microorganisms (PGPMs) are increasingly and promisingly being distributed in world agriculture. Meanwhile, current use of these efficient PGPMs may offer agronomic, pathogenic, and environmental benefits for intensive agricultural production systems. PGPMs are exhibiting a gradual increase in demand on the world market as sustainable and eco-friendly tools. Possible mechanisms for the effectiveness of biofertilizers are mobilization of the scarcely available plant nutrients nitrogen (N), fixer phosphorus (P), potassium (K), and zinc (Zn) solubilizers; production of plant growth–promoting substances; enhanced and induced resistance to environmental multistress factors; and direct or indirect suppression of harmful microbes. Research activities are currently limited by the lack of standards for production and quality control of different commercially used biofertilizers.

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**Keywords**
Nutrient use efficiency • Food production • Soil fertility • Environmental sustainability

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## 1.1 Introduction

The agricultural sector plays a key role as a backbone in the process of economic development of a country. A doubling in global food demand projected for the next 50 years poses huge challenges for agricultural sustainability. According to estimates from the Food and Agriculture Organization of the United Nations (FAO) (2014), ~ 11% (1.5 billion ha) of the globe's land surface (~ 13.4 billion ha) is used in crop production. This area represents slightly over one third (~36%) of the land estimated to be to some degree suitable for crop production. To fulfill the demand of food requirements, agriculture farmers have used injudicious application of agrochemicals such as fertilizers and pesticides for crop production. However, in most countries, there is no scope to further increase the availability of agricultural land.

Nowadays, global agriculture is faced with the serious challenge of providing adequate and sustainable food production for over ~7 billion people and the next generation (FAO 2012). Such an increase in population growth will intensify the pressure on the global resource base to achieve sustainable food production by intensifying or increasing the cropping intensity to improve productivity. On the other side, there is an increasing pressure on land to build new homes, public institutions, roadways, railways, and other developments to accommodate the growing population, and that may decrease the availability of agricultural land for the next generation (Hole et al. 2005; Bahadur et al. 2017; Verma et al. 2017b).

Sustainable management of soil health, crop residue management, water dynamics, soil loss, and carbon (C) sequestration are all dependent on sustainable crop production systems. Sustaining food production is an urgent need for global food security for future generations (Meena et al. 2013a, 2017; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015; Ahmad et al. 2016a). However, there is an urgent need to search for possible ways of sustaining food production without deteriorating soil and environmental quality. Modern large-scale traditional agriculture with intensification of crops has resulted in unacceptable soil erosion, runoff, and associated losses of soil nutrients. Balanced utilization of agricultural inputs and improved technologies introduced over the past few decades are now almost reaching a point of diminishing returns (Cassman et al. 1995; Dawe et al. 2000). The possibilities of converting marginal lands into productive arable land (Crosson and Anderson 1992; Das et al. 2000; Karforma et al. 2012) as an option for productivity improvement are now becoming more and more limited. Genetically engineered plants also may not be major factors in increasing food grain production in the near future (Peng et al. 1994; Hazell 1995). A sustainable system that can maintain agricultural productivity at a higher level without causing deterioration of the ecosystem is the need of the hour (Dobermann and White 1999; Kumar et al. 2017; Nath et al. 2017).

## 1.2 A Way Toward Healthy Soil: Integrated Soil Fertility Management

Integrated soil fertility management (ISFM) is a set of agricultural practices adapted to general conditions to enhance nutrient use efficiency (NUE) and improve agricultural productivity in a sustainable manner. Combinations of mineral fertilizers and various locally available soil amendments (such as lime and phosphate rock) and organic matter (crop residues, compost, and green manure) are used to replenish lost soil–plant system nutrients. By sustaining soil health with efficiency of agricultural inputs, in addition, ISFM enhances the germplasm, agroforestry, and use of crop rotation and/or intercropping with legumes. In addition, efficient soil health management plays a key role in sustainability (Power and Prasad 1997). The potential activity of biofertilizers depends on numerous factors, such as rhizosphere competence in competition with other microorganisms, soil type, management practices, and climatic factors (Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Priyadharsini and Muthukumar 2016; Kumar et al. 2017).

Traditional as well as organic farming systems aim to produce food with minimal harm to the overall ecosystems. They have also been found to improve soil–plant system biodiversity (Mader et al. 2002). Different organic amendment treatments have been suggested as one of the practices leading to sustainable agriculture (Burton and Turner 2003). However, critics argue that the lower yields in comparison with inorganic and traditional farming would necessitate greater expansion of the areas under agriculture to produce the same amount of food as conventional farms produce. Meanwhile, this would undermine the environmental benefits of organic management practices (Trewavas 2001). However, organic production systems can nearly match conventional/traditional yields under certain conditions such as best management practices (BMPs) with specific crop types and growing conditions. Results from long-term fixed plot studies have shown that the productivity on the basis of area and working hours was comparable for organic and conventional/traditional crops (Pimentel 2006). Moreover, hybrid management systems also promote soil health in a much better way in comparison with their individual effects

(Meena et al. 2015a, b, 2016c; Raghavendra et al. 2016; Zahedi 2016; Rawat et al. 2016; Yasin et al. 2016; Kumar et al. 2016b). Most of the long-term results have shown that organic systems are less productive than conventional systems but will be more sustainable (Birkhofer et al. 2008). Soil health has been identified as an indicator of agricultural as well as environmental sustainability (Papendick and Parr 1992; Doran et al. 1994), which shows better quality with high nutrition in organic farming systems as compared with conventional/traditional systems (Reganold et al. 2010). This farming system provides soil sustainability as it reduces soil erosion, in contrast to conventional systems, thus increasing soil productivity (Reganold et al. 1987).

To establish an organic production system as an important eco-friendly tool in a sustainable food production system, factors that limit organic yields need to be fully understood, in conjunction with assessment of the socioeconomic and environmental benefits of traditional/organic farming systems. On the other hand, scientists are now moving toward conservation technologies/agricultural practices that involve minimum soil disturbance, providing soil cover through crop residue management or other cover crops, with crop rotations for achieving higher productivity and minimizing adverse environmental impacts (Saha et al. 2016a; Yadav and Sidhu 2016; Meena et al. 2016d; Jaiswal et al. 2016; Jha and Subramanian 2016; Kumar et al. 2016a).

In conventional/traditional systems involving intensive tillage, there is a gradual decline in soil organic matter (SOM) through accelerated oxidation and burning of crop residues, causing pollution, greenhouse gas (GHG) emissions, and loss of valuable soil–plant system biodiversity. Retention of crop residues on the soil surface, in combination with no tillage (NT), initiates processes that lead to improved soil health, which is related to soil sustainability and overall natural resource enhancement, while conservation agriculture (CA) may lead to sustainable improvements in NUE (Sharma et al. 2005).

Sustainability of SOM encompasses all organic constituents and fractions in mineral soil, including plant and animal tissue at variable stages of decomposition, soil biomass of rhizospheric microorganisms, plant root and microbial exudates, and well-decomposed and highly stable SOM. Although SOM consists of many C compounds, it is often divided conceptually into three pools with different magnitudes and turnover times. The turnover time represents the time during which C resides in a certain pool and hence is a measure of the stability of carbon pools (Saha et al. 2016b; Verma et al. 2014, 2015b; Meena et al. 2014a, 2015f; Sharma et al. 2016; Dotaniya et al. 2016). The lability of carbon in the active pool is due to microbial biomass C (MBC) and easily decomposable compounds from leaf litter and root-derived material with short turnover times (from weeks to years); the slow (also called intermediate C) pool has turnover times from 10 to >100 years, and the passive pool of C has a turnover time on the order of >103 years (Parton et al. 1987; Trumbore 1997). The C input, the magnitude of soil organic carbon (SOC) pools, and finally C mineralization depend on many factors. However, the changing patterns of land use types and land use management (LUM) practices can have significant direct and indirect effects on SOC pools, while the impact of land use type changes on SOC pools in mineral soil depends also on long-term, site-specific factors and is often overridden by the high spatial heterogeneity of SOC in the soil–plant system.

## 1.3 Soil Carbon: A Key to Sustainability

SOM formed from dead plants—containing carbon (C), oxygen (O), and hydrogen (H)—is the basic food and energy source for soil/rhizospheric microorganisms. The essential minerals required for the growth of rhizospheric microorganisms are similar to the needs of plants. Most of the macro- and micronutrients are needed in small amounts and are mainly found in the proteins and nucleic acids of microbes. Therefore, an adequate supply of N is needed for rapid decomposition of SOM. The optimum C to N ratio is 30:1. However, straw and similar mulches have a high C to N ratio (100:1), and when the soil is covered with dense straw mulch, plant growth is retarded because of an N deficiency. These efficient microbes growing on the straw consume most of the available form of N, and small plants suffer from N deficiency. The optimum P content in a soil system for decomposition is 100:1. Root exudates provide many nutrients stimulating microbial growth in the rhizosphere surrounding the root tip. These microbes break down SOM, converting nutrients into soluble forms, which are able to be absorbed by plant roots.

## 1.4 The Soil–Microbe System

The soil system is one of the complex systems that comprise a diversity of microhabitats with different physicochemical gradients and discontinuous environmental conditions in an ecosystem. These microorganisms adapt to their microhabitats and live mutually in consortia with more or less sharp boundaries, interacting with each other and with other parts of the soil biota. A number of long-term investigations have emphasized the impact of soil structure and spatial isolation on microbial diversity and community structure (Verma et al. 2015a; Meena et al. 2013b, 2015c; Shrivastava et al. 2016; Velazquez et al. 2016; Das and Pradhan 2016; Dominguez-Nunez et al. 2016). Results from different analyses of the spatial distribution of bacteria/rhizobacteria at microhabitat levels showed that in soils subjected to different fertilization treatments, ~80% of the rhizobacteria were located in micropores of stable soil microaggregates of 2–20 μm/water-stable aggregates (WSAs). Such microbes offer the most favorable conditions for microbial growth with respect to water and substrate availability, gas diffusion, and protection against predation. Soil aggregation and the size distribution of different aggregates in the soil system had a greater impact on microbial diversity and community structure than did factors such as soil reaction (pH) and the type and amount of organic compound input. The results showed that a biodiversity infraction with small soil particles was higher than that in fractions of water stable aggregates (WSAs). A great diversity of rhizobacteria belonging to the *Holophaga/Acidobacterium* division and *Prosthecobacter* were present in small particles (silt + clay). Large-sized particles (sand) harbored few members of the *Holophaga/Acidobacterium* division and were dominated by rhizobacteria belonging to the Alphaproteobacteria (Sarkar et al. 2017; Verma et al. 2017a).

The interaction of numerous physical, chemical, and biological properties in soils controls plant nutrient availability in the soil–plant system. Understanding these processes and how they are influenced by environmental conditions enables us to optimize NUE, water use efficiency (WUE), and plant productivity. This knowledge is essential for decisions regarding management of nutrients to optimize plant growth and health, and to minimize nutrient application impacts on the environment. The nutrient supply to plant roots is a very dynamic process (Sindhu et al. 2016; Meena et al. 2014b, 2015d, e; Singh et al. 2015, 2016; Teotia et al. 2016; Bahadur et al. 2017). The plant nutrients are absorbed from the soil solution by the plant roots, and as the plant roots absorb the nutrients, the nutrient concentration in the soil solution decreases. As a result, several chemical and biological reactions occur to buffer or resupply these nutrients to the soil solution. The microbial availability, as well as other properties, are related to soil productivity and sustainability. The soil quality of the soil–plant system depends not only on its chemical composition but also on the quantitative nature of the microorganisms inhabiting it.

## 1.5 Seed Inoculants/Biopriming for Use in Agriculture

The application of seed biopriming as a seed treatment delivers efficient microorganisms (Fig. 1.1) directly to the plant rhizosphere (Philippot et al. 2013). Most of the beneficial rhizospheric microorganisms of agricultural importance are rhizosphere-colonizing species, with the ability to increase plant growth promotion (PGP) via a range of mechanisms (Babalola 2010). However, utilization of these efficient and beneficial soil microorganisms as agricultural inputs for improved crop productivity requires selection of rhizosphere-competent microorganisms with PGP traits (Hynes et al. 2008). Beneficial PGP rhizobacteria are disease-suppressive rhizospheric microorganisms that enhance soil–plant system sustainability. The results from previous long-term experiments and documentation abound, and all point toward the need to commercially exploit PGP rhizobacteria as biofertilizers for their agricultural benefits. From a general perspective, however, the problems of variability in colonization efficiency, NUE, field performance, and rhizosphere competence are controversial issues in this system (Meena et al. 2013c, 2016e; Bahadur et al. 2016; Masood and Bano 2016).

Efficient utilization of various bioinoculants under field culture as well as pot culture is seen as being very attractive, since it would substantially reduce the use of mineral fertilizations, and there are now an increasing number of inoculants being commercialized for various agricultural crops (Berg 2009). These efficient microorganisms play a key role in agricultural systems, particularly plant growth–promoting microorganisms (PGPMs) with various mechanisms: (a) PGPMs acting as biofertilizers (such as N-fixing bacteria/rhizobacteria and P, K, and Zn-solubilizing rhizobacteria) assist plant nutrient uptake by providing fixed N and other elements

**Fig. 1.1** Procedure for seed biopriming of a wheat crop with *T. harzianum* (as an efficient bioinoculant) BHU-51: (**a**) *T. harzianum* BHU-51 powder form; (**b**) *T. harzianum* BHU-51 (~$10^8$ spores $mL^{-1}$); (**c**) wheat seed treated with *T. harzianum* BHU-51; (**d**) effect of seedling growth at 30 effect on seedling growth at 30 days after sowing (DAS) of wheat in black soil influenced by *Trichoderma* formulation under pot culture (Meena et al. 2016c)

(Kennedy and Islam 2001); (b) phytostimulators (microbes expressing phytohormones such as *Azospirillum*) can directly promote the growth of plants (Spaepen et al. 2007; Glick et al. 2007); and (c) biological control agents (such as *Trichoderma*, *Pseudomonas*, and *Bacillus*) protect plants against phytopathogenic organisms (Mohiddin et al. 2010; Dawar et al. 2010). Several reviews have discussed various aspects of PGP by PGPMs (Zhuang et al. 2007; Saharan and Nehra 2011).

These efficient PGP rhizobacteria enhance the availability and uptake of plant nutrients, enhancing NUE (Table 1.1). Unlike the adverse effects of continuous use of mineral fertilization, combinations of PGP rhizobacteria, when applied to the soil, improve the soil structure, leaving no toxic effects (Table 1.1). PGP rhizobacteria are known to fix atmospheric molecular N through symbiotic and asymbiotic or associative N-fixing processes (Anjum et al. 2007). With more and more emphasis being placed on organic farming, PGP rhizobacteria are finding increasing applications today as biofertilizers. These efficient PGP rhizobacterial bioactive factors are substances that impact the growth and development of agricultural/horticultural crops. The potential environmental impacts related to inoculation have always been neglected. Since efficient inoculation consists of supplying high densities of viable

**Table 1.1** Effects of various PGP rhizobacteria with single and co-inoculation with other efficient microbes on crop growth, yield, quality, and rhizospheric nutrient availability under field conditions as well as pot conditions

| Crop | Microorganisms | Effect compared with control | Reference |
|---|---|---|---|
| Peanut | *Bradyrhizobium* sp. | Shoot dry weight (28.9%); shoot N (~40%); shelling (6.8%); pod yield (29%); seed protein (~15%) | Badawi et al. (2011) |
| | *Bradyrhizobium* sp. + *Trichoderma harzianum* | Shoot dry weight (33.9%); shoot N (37.4%); seed protein (13.11%); shelling (7.7%); pod yield (55.14%); seed protein (13.4%) | |
| | *Bradyrhizobium* sp. + *S. marcescens* | Shoot dry weight (38.73%); shoot N (39.9%); seed protein (15.54%); shelling (9.74%); pod yield (36.96%); seed protein (15.54%) | |
| Maize | *Anabaena* sp. + *Trichoderma* sp. biofilm | Plant height (3.6%); ear height (3.8%); yield (3.9%); normalized difference vegetation index (1.11%); available N (17.37%); available P (25.93%) | Prasanna et al. (2016) |
| | *Anabaena* sp. + *Azotobacter* sp. biofilm | Plant height (4.92%); ear height (3.76%); yield (4.73%); normalized difference vegetation index (1.11%); available N (20.03%); available P (9.19%) | |
| Cowpea | *B. liaoningense* | Grain yield (35.4%); grains per plant (12.27%); K (10.31%) | Gómez Padilla et al. (2016) |
| | *B. yuanmingense* | Grain yield (27.72%); grains per plant (11.58%); K (19.62%) | |

(continued)

**Table 1.1** (continued)

| Crop | Microorganisms | Effect compared with control | Reference |
|---|---|---|---|
| Chickpea | *Trichoderma* + *M. ciceri* biofilm | Available P (9.87%); plant N (35.73%); MBC (38.36%); yield (23.69%) | Bidyarani et al. (2016) |
| | *Trichoderma* + *Bacillus* biofilm | Available P (18.68%); plant N (26.75%); MBC (49.82%); dry weight (24.13%); no. of pods/plant (12.45%); yield (42.37%) | |
| | *Anabaena* + *M. ciceri* biofilm | Available P (21.49%); plant N (23.49%); MBC (66.36%); dry weight (33.57%); no. of pods/plant (26.69%); yield (39.22%) | |
| Maize hybrid | *Azospirillum* | Yield (3.92%); total N in shoot (24.40%); shoot dry weight (24.94%) | Marks et al. (2015) |
| | *Azospirillum* + enriched metabolites | Yield (14.41%); total N in shoot (21.79%); shoot dry weight (18.87%) | |
| Green gram | *Azotobacter* + *Bradyrhizobium* | Seed yield (51.43%); seed protein (5.93%); shoot N content (37.14%); shoot P content (44.23%) | Ahmad et al. (2016a, b) |
| Cotton | *Azotobacter chroococcum*, *Azospirillum lipoferum* | Seed yield (~21%), plant height (5%), soil microbial population (~41%) increased | Anjum et al. (2007) |
| Chickpea | *Mesorhizobium* sp. *RC3* | Increased dry matter accumulation (71%), number of nodules (86%), seed yield (36%), grain protein (16%), nitrogen in roots (46%) and shoots (40%) | Wani et al. (2008) |

and efficient microbes for rapid colonization of the host rhizosphere, it induces at least a transient perturbation of the equilibrium of soil microbial communities/biodiversity. The changes in soil microbial composition may be undesirable if important native species/unculturable species are lost, thus affecting subsequent crops. The loss of certain rhizobacterial species may, however, not change the functioning of the system, because of rhizobacterial redundancy, since different rhizobacterial species may carry out the same functions (Nannipieri et al. 2003).

## 1.6 The Rhizosphere: An Unknown World

The rhizosphere is the zone of soil surrounding a rhizospheric biology and chemistry. This zone is ~1 mm wide but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the roots and by microorganisms feeding on the compounds. As plant roots grow through soil, they release water-soluble compounds—such as amino acids, sugars, and organic acids—that supply food for the rhizospheric microorganisms (Fig. 1.2).

Toward the sustainable food production system to supply food for microbes for rhizospheric activity in the rhizosphere is much greater than in soil–plant system. In

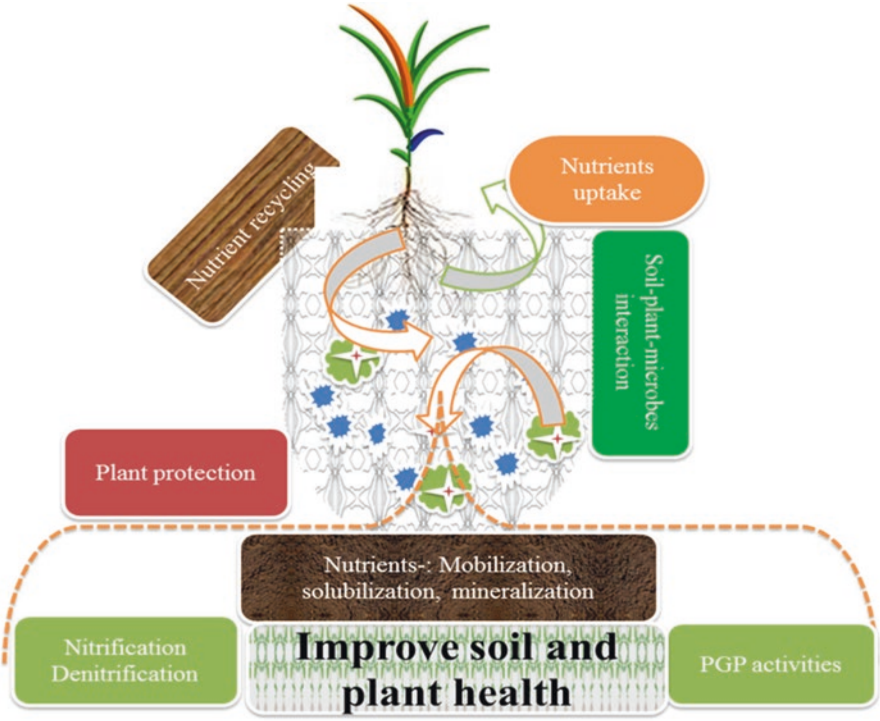

**Fig. 1.2** An overview of rhizospheric phenomena for sustainable food production

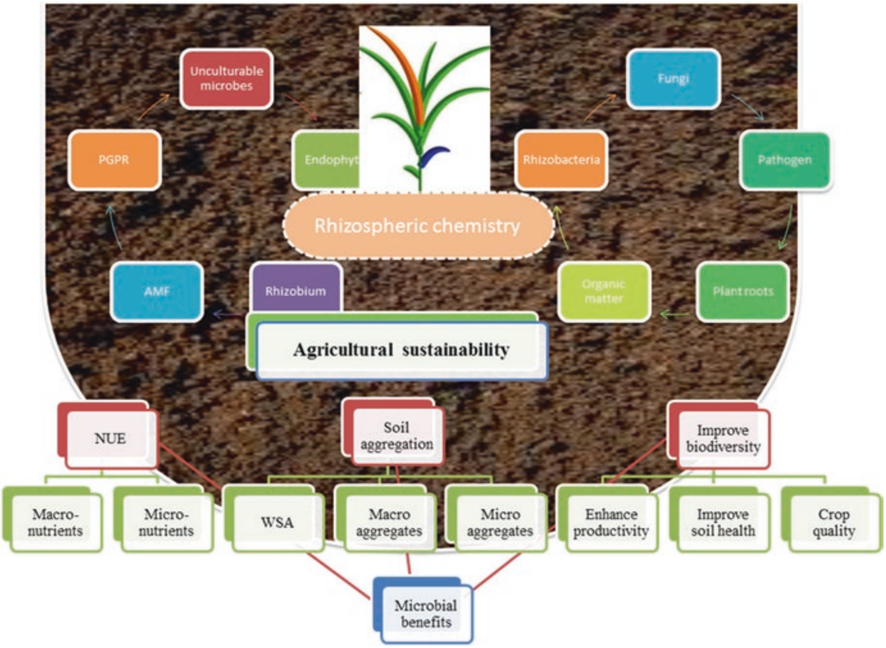

**Fig. 1.3** Role of rhizospheric chemistry in agricultural sustainability/sustainable food production systems

return, these microorganisms provide nutrients for the plants. All of this activity makes the rhizosphere the most dynamic environment in the soil system. Because roots are underground, rhizosphere activity has been largely overlooked, and it is only now that we are starting to unravel the complex interactions that occur (Fig. 1.3).

Rhizospheric biodiversity is complex and dynamic, and it varies in composition between different compartments and levels, which represents a real challenge in soil ecology. The rhizosphere represents one of the most diverse habitats on the planet and is central to ecosystem functioning for sustaining the ecosystem (Fig. 1.3). However, consequently, there is an increasing need to understand its functioning to effectively manage the ecosystem/ecology and harness its potential benefits. In particular, manipulation of the rhizosphere is now considered a key mechanism for solving critical issues facing the planet, including agricultural and forest sustainability, improvement of water quality, mitigation of climate change, and preservation of biodiversity. To face the range of biotic and abiotic stresses, plants interact with different members of the soil biodiversity/microbial community in many ways and in a complex range of trophic cascades. These relations involve positive and negative feedbacks between soil microorganisms, plants, and their chemical environment.

Agriculturally important microorganisms (AIMs) can influence both the efficiency of nutrient availability to crop plants and soil biodiversity, and they also regulate interactions between plants and harmful microflora (Singh 2015; Zeilinger et al. 2016). Interactions among plants and microbes have been reported to influence the physicochemical, biochemical, and microbiological properties of the soil (Dubey et al. 2016). Moreover, root exudates (chemicals secreted into the soil by roots, which work as connecting links between the roots and the soil microbial community), as signaling molecules, can enhance the communication between crops and beneficial microbial agents, and consequently can enhance growth, yield, and immunity in crop plants (Kanchiswamy et al. 2015). Beneficial microbes have advanced abilities to suppress or alter the protective responses of the host plant, permitting them to epiphytically or endophytically colonize their hosts (Bhardwaj et al. 2014).

Efficient microbial communities that can offer services including PGP, NUE/WUE, bioremediation, and control of phytopathogens at the farming level are known as microbial inoculants. A profound understanding of the environmental factors influencing the viability and performance of these microbial inoculants is essential for their large-scale use in sustainable agricultural production systems. *Trichoderma*, commonly used as a biological control agent, employs several mechanisms in influencing seed germination and seedling vigor (Celar and Valic 2005). The seed germination rate, rapidity of root elongation and development during seed germination, plant height, root fresh and dry weight, and shoot fresh and dry weight of the seedling are the most important indicators of seedling vigor. This clearly indicates that the increased growth response of plants caused by *Trichoderma* depend mainly on the ability of *Trichoderma* to survive and develop in the rhizosphere (Harman 2006). Root colonization by *Trichoderma* could be a result not only of root exudates, such as carbohydrates and amino acids, but also of many factors that affect the *Trichoderma*–plant interaction.

## 1.7 Use of Arbuscular Mycorrhizal Fungi

Judicious utilization of commercial arbuscular mycorrhizal (AM) fungus (AMF) inoculants is increasing in horticultural and land reclamation industries (Gianinazzi and Vosátka 2004), and is an emerging technology in field crop production worldwide. AM fungi are able to colonize and establish symbiotic, mutually beneficial associations with the roots of most agricultural crops (Munyanziza et al. 1997) and increase the effective absorptive area of the roots by formation of an extensive extraradical hyphal network, which enhances the efficiency of the absorption of nutrients (George 2000). The importance of AM fungi in improving plant growth and plant resistance to soilborne diseases, and in restoring problematic soils, is well known (Rillig 2004). Many researchers have noted that certain plant species are generally more responsive to mycorrhizal colonization than others (Engqvist et al. 2006; Bonfante and Genre 2008), and the responses may be both host plant and AMF specific. Thus, there is a need to investigate the response of target field crops to AMF inoculants (Gonzalez-Chavez et al. 2004).

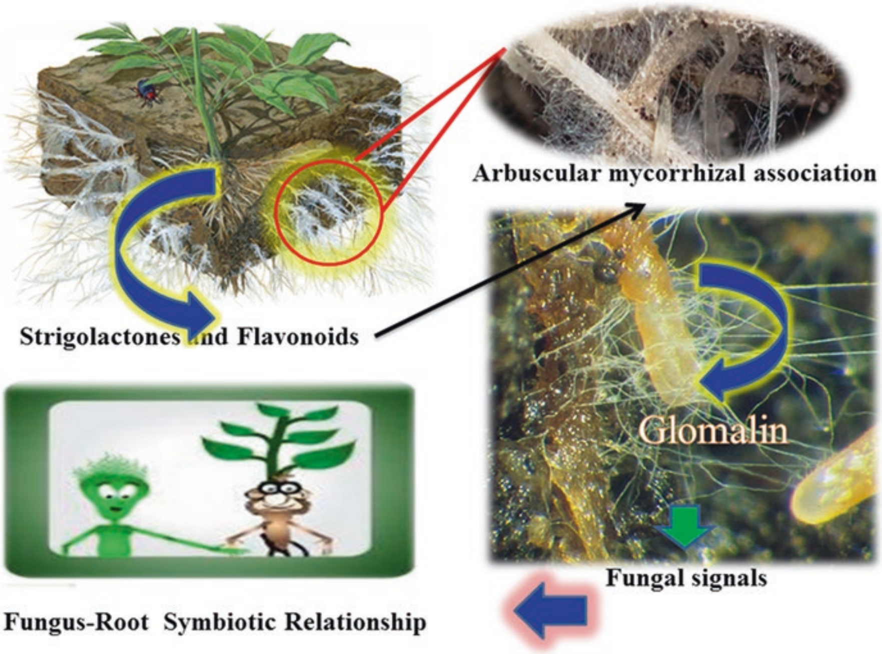

**Fig. 1.4** An overview of AM fungi and the plant root system under the rhizosphere

These efficient AM fungi play a significant ecological role in the phytostabilization of potentially toxic trace element–polluted soils by sequestration and, in turn, help mycorrhizal plants survive in polluted soils. One of these components is glomalin, a glycoprotein produced by the hyphae of AM fungi (Driver et al. 2005). The extraradical mycelium of AM fungi, in addition to its crucial role in enhancing the NUE/WUE of host plants, also plays a role in soil particle aggregation and soil stability, mainly of WSAs (Dodd et al. 2000). All of these efficient mechanisms have implications in reducing plant exposure to potentially toxic elements with use of mycorrhiza remediation technology in sustainable soil–plant systems (Fig. 1.4).

Soil sustainability and productivity are considered important factors for the success of agricultural production rather than soil quality/health. The status of nutrients present in soil–plant systems and their ability to supply the nutrients determines the fertility of the soil, whereas the ability of the soil to produce a higher yield is the soil productivity or the productivity of the system. Production of agricultural crops depends upon many properties of the soil, such as its textural class, structure, acidity, alkalinity, water-holding capacity (WHC), and cation/anion and cation exchange capacity (AEC/CEC), as discussed by Brady and Weil (2002).

The soil system is a vibrant habitat for a huge variety of life-forms; it gives mechanical support to plants, and from it they extract various nutrients. It also provides habitats for rhizospheric microorganisms. All of these forms of life interrelate with each other and with the soil to create continually changing conditions. The

**Table 1.2** Details of crops/plants and associated microorganisms with their nitrogen-fixing capacity

| Crop species | Associated microorganism | $N_2$ fixation (kg N/ha/year) |
|---|---|---|
| *Leucaenaleucocephala* | *Rhizobium* | 100–500 |
| *Medicago sativa* | *Rhizobium* | 150–250 |
| *Trifolium pretense* | *Rhizobium* | 100–150 |
| *Vignaunguiculata* | *Bradyrhizobium* | 50–100 |
| *Cajunus* sp. | *Bradyrhizobium* | 150–280 |
| *Alnus* sp. | *Frankia* | 50–150 |
| *Azolla* | *Anebaena* | 150–300 |
| *Digeteriadecumbens* | *Azospirillum* | 5–30 |

agriculturally beneficial microbial populations are PGP N-fixing cyanobacteria, rhizobacteria, mycorrhiza, plant disease–suppressive beneficial rhizobacteria, stress tolerance endophytes, and biodegrading microbes. The counts of rhizospheric microbes such as *Azotobacter*, *Azospirillum*, *Rhizobium*, cyanobacteria, P-solubilizing microorganisms (PSMs), and K-solubilizing microorganisms (KSMs) and mycorrhizae are high under no tillage (NT) or minimum tillage (MT) soil systems. These are some of the PGP rhizobacteria (Bhardwaj et al. 2014). The bacteria/rhizobacteria are the important soil microorganisms responsible for many enzymatic transformations such as nitrification, ammonification, etc. *Azospirillum* is a microaerobic bacterium, which fixes nitrogen in association with the roots of grasses. Inoculation of grass crops with *Azospirillum* has positive hormonal effects on the roots and plant growth (Table 1.2).

Nonsymbiotic associations of *Azotobacter* and *Clostridium* fix ~5–20 kg N/ha/year, and various species of blue green algae fix ~10–50 kg N/ha/year (Dastager et al. 2010). Nitrifying bacteria/rhizobacteria of the genus *Nitrosomonas* produce nitrite ions from oxidation of ammonia. The efficient rhizobacterium genus *Nitrobacter* and a few other genera can oxidize nitrites to nitrates. N fixers such as *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* are obligate anaerobes (Dastager et al. 2010). The acid products of rhizobacterial fermentation convert insoluble P into soluble phosphates, which are then utilized by plants for growth and development. Some rhizobacteria such as *Thiobacillus ferrooxidans* and iron rhizobacteria of the genus *Gallionella* are capable of oxidizing ferrous ($Fe^{2+}$) iron into ferric ($Fe^{3+}$) iron (Heritage et al. 1999). Thus we can conclude that the presence of microorganisms in soil is beneficial for soil productivity and greater crop yield.

So our effective rhizospheric microorganisms will remain effective only if we manage our pastures with them in mind too. That means not overstocking or baring paddocks. It means allowing pastures to develop enough leaf to do their photosynthesis effectively and fix some carbon for all of the other little "greeblies" further along the food chain. Generally this has been shown to have a reviving action in growing systems. It can enhance soil quality, soil health, and the growth, yield, and quality of crops. It helps in the decomposition of SOM and, during fermentation, produces several normally unavailable organic acids—lactic acid, acetic acid, amino acid, and malic acid—and various other bioactive substances and vitamins. It has an antioxidant effect, which improves the immune systems of both plants and animals.

## 1.8 Conclusions

Rhizospheric microorganisms contribute significantly to fixation of atmospheric nitrogen and solubilization of phosphorous, potassium, iron, and zinc from insoluble forms to plant-available forms. Inoculation of crops with rhizospheric microorganisms has been shown to improve the fertility status of agricultural soils. Apart from their nutrient-solubilizing abilities, rhizospheric microorganisms have the ability to produce plant growth hormones, ammonia, and siderophores. Although rhizospheric microorganisms are abundant in many soils, they have not yet been successfully commercialized, and thus their application under large-scale field conditions is still limited. This communication highlights the contributions of rhizospheric microbes in enhancing soil–plant system productivity. This type of noble microbial consortium is cost effective and eco-friendly in nature for enhancing evergreen agricultural food production systems.

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# Nutrient Solubilizing Microbes (NSMs): Its Role in Sustainable Crop Production

# 2

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## Abstract

Nowadays the land degradation, deteorating the soil health, is the major constraint, which led to the nutrient depletion and limited potential yield of crops toward the food security worldwide. To enhance the crop production, soil sustainability is one of the ideal and sustainable approaches to overcome depleting the soil fertility status and land degradation. The rhizospheric beneficial microorganisms (RBMs) may offers rate of plant growth, agronomically, pathogenically, and environmentally advantage for intensive agricultural production system. The nitrogen-fixing rhizobacteria (NFR) fix atmospheric nitrogen in the soil, while phosphate-solubilizing microorganisms solubilize the insoluble phosphorus (P) in the soil, potassium-solubilizing rhizobacteria (KSR) mobilizes the stable potassium in field, and similarly other microorganisms mobilize nutrients in soil and make it easily available to the crop plants. These NFR include symbiotic $N_2$-fixing forms, in leguminous plants, viz., *Rhizobium*, and obligate symbionts in nonleguminous plant comprising species *Alcaligenes*, *Azomonas*, *Beijerinckia*, *Achromobacter*, *Acetobacter*, *Arthrobacter*, and *Bacillus* spp.; however, P is the primary essential macronutrient for vegetative and reproductive development of the plants. The majority of phosphorus in most soil is insoluble

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form and cannot be used by crops. Some important species are *Aspergillus*, *Bacillus*, *Pseudomonas*, *Penicillium*, etc., which secrete organic acids (products) that lower pH in their vicinity and help to bring the dissolution of fixed phosphates in soil. Additionally seed bio-priming can provide ~30 kg $P_2O_5$/ha. In soil system potassium (K) is associative to movement of water, nutrients, carbohydrates, and cellular and osmotic pressure in plant tissues. Supposing potassium is lacking or not provided in suitable amount, growth of the plants stunts and production reduces. Several bacterial species particularly rhizosphere-colonizing bacteria have been found, which solubilize insoluble inorganic phosphate like that tri-calcium phosphate (TCP), di-calcium phosphate (DCP), hydroxylapatite, and rock phosphate. It is already proved that application of Zn in the form of chemical fertilizer is inappropriate due to its unavailability to crop plants. In the recent past, rhizobacteria have exhibited terrific ability to improve zinc availability in root zone and enhance zinc in plants. In rice, silicate-solubilizing rhizobacteria (SSR) have gained importance in recent times because of their role in solubilization of silicate minerals, rendering potassium silicate ($K_2SiO_3$), and makes readily available potassium (K) and silicon (Si) for crop plants. Recent advancement in biotechnology and genetic engineering have provided new opportunity to find out the presence and abundance of particular microbes or to quantify the expression of target genes directly in soil or in rhizosphere with high levels of sensitivity. Genetically modified strains could be capable of solubilizing more available nutrient from the soil or rhizosphere. Development of genetically modified strains with enhanced mobilization by genetic engineering techniques and DNA technology is needed to maintain an eco-friendly and sustainable agriculture. This chapter focuses on diversifying of nutrient solubilizer/mobilizer microbes, mechanism of solubilization/mobilization, role of various enzymes/auxins/acids effect of various factors on nutrient solubilization, the present and future scenario of their utilization, and potential for application of this knowledge in managing a sustainable environmental ecosystem.

**Keywords**
Microorganism • Nutrient • Nitrogen • Phosphorous • Potassium • Rhizosphere • Silicate

## Contents

## 2.1 Introduction

The major problem in limiting crop yield under the developing countries worldwide and especially among the resource-poor farmers is infertility of the soil. Therefore, improving the soil health can reduce the problems of land degradation, soil fertility, hurriedly declining yield level that occur in wide areas of the world requiring the main principle of better cultivation practices. The minerals, organic components, and microbes are three primary solid components of soil (Mohammadi and Sohrabi 2012). In 2025, food grain requirement for India ~1.4 billion people will be ~300 Mt. This production level will require ~30 Mt of nitrogen (N), phosphorus (P), and potassium (K), including ~9 Mt of $P_2O_5$. In addition, ~14–15 Mt of N-P-K would be needed for vegetable, plantation, sugarcane, cotton, oilseed, potato, and other crops. Thus, ~40–45 Mt of N-P-K, containing 11–13 Mt of $P_2O_5$, will be required just to maintain broad average N-$P_2O_5$-$K_2O$ ratio of 4–2-1. The soil is having 17 essential minerals, but the most important minerals are N, P, and K; these minerals are essential for healthy growth and development of crop plants (Meena et al. 2017). There are 13 nutrients present in the soil, and they are categorized into two groups, macro- and micronutrient; it depends on the quantity required by plants (Lack and Evans 2005). Nowadays, utilization of efficient rhizospheric microbes may offer plant growth promotion (PGP), agronomically, pathogenically, and environmentally friendly for intensive agricultural ecosystem. However, these efficient plant growth-promoting microbes (PGPMs) have shown gradual enhancement in demand to the world market (Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2016b).

Possible mechanisms for the effectiveness of biofertilizers are sparingly available plant nutrient N-fixation; P, K, Zn, and Si solubilizers; manufacture of growth-promoting substances; increased and induced resistance to environmental stresses; and direct or indirect inhibition of plant pathogens (Bahadur et al. 2014; Kumar et al. 2014b, 2015b, c). The role of essential macronutrients, viz., N, P, and K, and other micronutrients is well known for increasing the crop productivity. The modern agriculture gave more emphasis on using hybrid seeds and high-yielding cultivars that gave more response to high doses of chemical/synthetic fertilizers and irrigation resulting soil being deprived of essential element and nourishing organic matter that had always been available to plants, when the natural farming was being practiced historically. However, synthetic fertilizers are those now used widely since green revolution resulting in reduced soil health by making soil ecology non-inhabitable for soil microflora and microfauna, which are responsible for maintaining the soil health and providing many necessary and indispensable elements to the plants (Ahmad et al. 2016; Kumar et al. 2015a, 2016a; Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Jaiswal et al. 2016; Jha and Subramanian 2016).

Some latest studies showed that the use of NPK + *Azotobacter chroococcum* + *Bacillus megaterium* + *Pseudomonas fluorescence* + enriched compost produced the higher growth characters and yield of maize (Umesha et al. 2014). Patra et al. (2013) observed that combined inoculation of PSB + VAM + *Azotobacter* recorded higher growth and yield attributes of hybrid sunflower. It is imperative to restore the soil health with the use of biofertilizers containing live cells of specific isolated strains of bacteria and fungi, which are formulated in suitable carriers. These microorganisms applied in the soil under suitable conditions secrete metabolites, acids, and enzymes, which make deficient nutrients available to plants in assimable form. N-fixation bacteria fix atmospheric N in the soil, while phosphobacteria solubilize insoluble stable phosphorus into soil; KSB solubilize the immobile potassium in soils. Similarly, microorganisms mobilize nutrients into the soil and make them readily available to the plant (Kumar et al. 2013a, b, 2017, Priyadharsini and Muthukumar 2016; Meena et al. 2015a, f; Raghavendra et al. 2016; Zahedi 2016).

This beneficial microorganism works by maintaining the environmental balance by active involvement in C, N, P, and S cycles in the nature. Recent studies showed that application of biofertilizers in combination (*Azospirillum* + *Rhizobium* + *Azoto bacter*) significantly increases the growth, roots, and biochemical parameters of black gram (Maheswari and Elakkiya 2014). In other studies, inoculation of seeds with *Rhizobium* and phosphorus-solubilizing bacteria (PSB) enhances the yields of green gram (Kundu et al. 2013; Gajera et al. 2014).

Plant elements are lost from the soil in many ways. The largest amount of plant nutrient from the soil was removed by cultivation of crops and weeds and the process of leaching and erosion. N is mostly lost by volatilization and denitrification. To increase the production, improve the soil fertility, decrease nutrient losses, and reduce energy utilization, it is most urgent need to use biofertilizers with chemical fertilizers (Ghumare et al. 2014). Similarly, *Azotobacter* makes availability of many plant nutrients such as C, N, P, and S by accelerating the mineralization of organic residues in soil and avoiding uptake of heavy metals (Jnawali et al. 2015). Maheswari and Elakkiya (2014) reported that inoculation of *Azospirillum* + *Rhizobium* + *Azotobacter* improved organic carbon, phosphorus, potassium, and pH in soil. Judicious use of biofertilizers along with chemical and other organic sources of plant nutrients and proper management practices have shown promising results not only in sustaining productivity and soil health but also in meeting a part of fertilizer requirement of different crops. Therefore, the use of nutrient solubilizers/mobilizers in agriculture alone or along with chemical fertilizers improves soil health, reduces the demand of fertilizers, reduces cost of cultivation, increases the production, and is environment friendly (Meena et al. 2015b, 2016c; Das and Pradhan 2016; Dominguez-Nunez et al. 2016; Dotaniya et al. 2016; Rawat et al. 2016; Saha et al. 2016a; Yasin et al. 2016).

## 2.2 Nitrogen

Nitrogen is an essential nutrient for all microorganisms, which composes proteins, nucleic acids, and other major organic compounds. Nitrogen was discovered in 1772 by Daniel Ratherford and independently by Scheele and Cavendish. The origin of name “nitrogen” comes from the Greek words “nitron genes,” meaning “nitre” and “forming.” Nitre is a common name for potassium nitrate (Ohyama 2010). Nitrogen is a major essential element for all organisms and constituent of proteins, nucleic acids, and other indispensable organic compounds. Although highly abundant (~78% by volume) in air, its concentrations in soil, crust rocks, and seawater are relatively low, and availability of N is often limiting factor for plant growth and development in natural habitats as well as agricultural crop production. Major part of elemental N that finds its way into soil is entirely due to its fixation by certain specialized group of microorganisms. Biological nitrogen fixation is mainly known to process for sustainable agriculture and reduced soil fertility decline. Microorganisms and plant able to fix atmospheric N contribute broadly to the production of fertilizers (Bahadur et al. 2016b; Meena et al. 2015e, 2016d; Saha et al. 2016b; Verma et al. 2014, 2015b; Yadav and Sidhu 2016).

The BNF, discovered by Beijerinck in 1901, is carried out by a specialized group of prokaryotes. These organisms utilize enzyme nitrogenase to catalyze conversion of atmospheric nitrogen ($N_2$) to ammonia ($NH_3$). Plants can readily assimilate $NH_3$ to produce aforementioned nitrogenous biomolecules. These prokaryotes include aquatic organisms, such as cyanobacteria; free-living soil bacteria, such as *Azotobacter*; bacteria that form associative relationships with plants, such as *Azospirillum*; and, most importantly, bacteria, i.e., *Rhizobium* and *Bradyrhizobium*, that form symbioses with legumes and other plants (Wagner 2011). These organisms are summarized in Fig. 2.1.

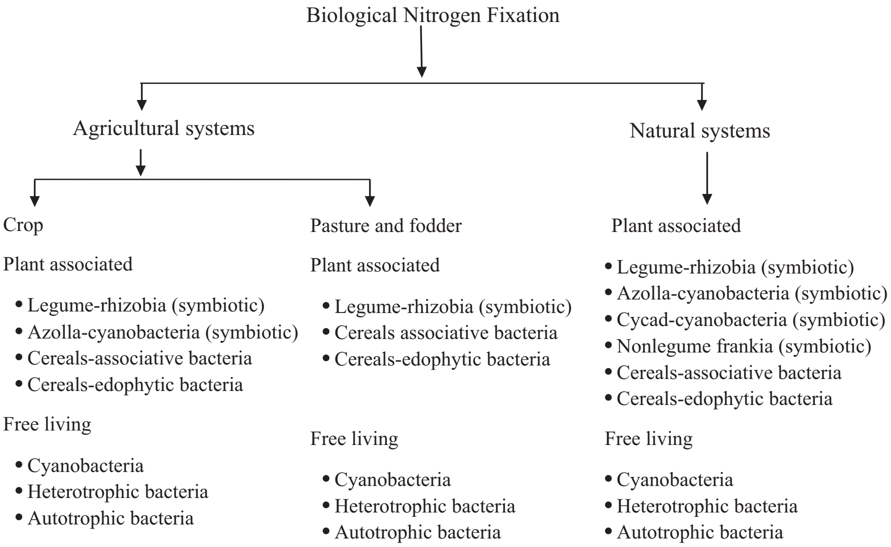

**Fig. 2.1** List of major N-fixer microorganisms isolated from agricultural land and natural systems (Wagner 2011)

The biological nitrogen fixation (BNF) is the process of transforming atmospheric elemental nitrogen into readily available form to the plants (Gothwal et al. 2007). Nitrogen-fixing bacteria/rhizobacteria (NFB/NFR) transform elemental $N_2$ to organic compound (Bakulin et al. 2007). These microorganisms are mainly used for preparation of biofertilizer as a living fertilizer composed of microbial inoculants that are capable of fixating the atmospheric elemental N. They are categoriezed into two groups: (1) free-living bacteria (*Azospirillium* and *Azotobacter*) and (2) symbionts such as *Rhizobium* (Gupta 2004). The nitrogen management is necessary because the total food production in the world has already gone down which must double by 2050 and in the next 40 years or so to level off hunger; the globally will essential to produce annually as much food as in the last 8000 years (Prasad et al. 2014). This would be possible only by the adequate use of fertilizers, especially nitrogen. Further for full exploitation of higher yield potential of traditional, high-yielding varieties as well as genetically modified crops, increased fertilizer nitrogen application is a must (Robert 2009). Nitrogen management of crop is therefore of paramount importance in agriculture. It is estimated that by 2050 about 250 Mt of fertilizer nitrogen may be needed (Tillman et al. 2011) to meet the global increase of food demand as against an estimated demand of about 109 Mt in 2013 (FAO 2011).

### 2.2.1 Nitrogen Status in Soil

The nitrogen in soils occurs in organic and inorganic form.

### 2.2.2 Organic Nitrogen

Most of the N in soil is in organic form (~95–99%), which is protected from various losses but largely unavailable to plants. It mainly depends on environmental and soil conditions; the amount of total soil nitrogen to higher rooting depth (1.0 m) has been determined ranging from 1.0 to 10 t/ha, much of which has occurred in upper ~15 cm. Soils in India, except those in hills, are mostly low in organic matter and total soil N due to high temperature; values of total N in Indian soils vary from 0.02 to 0.1% (Prasad 2007). Many studies have shown ~1.5–3.5% of organic N of soil mineralizes annually (Prasad et al. 2014).

### 2.2.3 Inorganic Nitrogen

In well-aerated soils, nitrate-N is the dominated form and its amount (mg/kg) can vary from 11 to 36 in alluvial soils (Inceptisols), 3–28 in lack cotton soils (Vertisols), 1–29 in red soils (Alfisols), and 12–14 in hill soils (Hapludalf).

### 2.2.4 Role of Nitrogen in Plants

Among all the necessary elements, N is required by plants in the larger amount and is most frequently a limiting component in crop production. Nitrogen concentration in plant ranges from 1.0 to 6.0%. Nitrogen is essential for protein production in plants, which is the direct or indirect source of protein for animals and human nutrition. It is a constituent of amino acids, building blocks of proteins. It is also a component of nucleic acids, which make DNA of all living things and hold genetic code. It is a component of chlorophyll and is required for several enzyme reaction. Chlorophyll is also a substance responsible for green color in leaves. If there is adequate nitrogen, plant photosynthesis occurs at high rates. Plant absorbing sufficient nitrogen will typically show vigorous growth and development, and leaves show dark green color (Ohyama 2010).

### 2.2.5 Deficiency and Excess of Nitrogen

Nitrogen deficiency often occurs especially in crops with a low supply of N fertilizer grown in low-fertility soils. The supply of N is a primary limiting component for crop growth and yields. Under N-deficient conditions, plants exhibit chlorosis, which includes yellowing of leaves and growth retardation. Symptoms can be observed in older leaves because N moves preferentially to young leaves and reproductive organ. With a severe N deficiency, plants can not grow and wither. With milder N deficiency, plant growth is retarded and yields become lower than those under an optimum N supply. Furthermore, senescence and maturation are accelerated by N deficiency. In contrast, excess N promotes shoot growth. In addition, excess N promotes over-luxuriant growth of shoots with lower numbers of seeds and fruits resulting in lower yield. Under excess N condition, plant sometimes becomes soft and weak and susceptible to fungal pests and insects. The metabolic adjustment underlying mechanism is very complex, but recent advances in multi-level approaches using microarrays combined with large number of metabolites and enzyme activities may lead to understanding of plant strategy to N nutrition (Amtmann and Armengaaud 2009).

### 2.2.6 Nitrogen-Fixing Rhizobacteria

Nitrogen-fixing rhizobacteria included symbiotic nitrogen fixer, such as *Rhizobium*, obligate symbionts in leguminous crop plants. The list of $N_2$-fixing rhizobacteria associated with non-legumes includes species of *Achromobacter*, *Arthrobacter*, *Acetobacter*, *Alcaligenes*, *Azomonas*, *Beijerinckia*, *Bacillus*, *Clostridium*, *Campylobacter*, *Herbaspirillum*, *Klebsiella*, *Lignobacter*, *Mycobacterium*, *Enterobacter*, *Erwinia*, *Derxia*, *Desulfovibrio*, *Corynebacterium*, *Rhodospirillum*, *Rhodopseudomonas*, *Xanthobacter*, and *Methylosinus* (Wani 1990). Similarly many genera and species of nitrogen-fixing rhizobacteria are isolated from rhizosphere of many cereals; majority of *Azotobacter* and *Azospirillum* genera have been largely tested to enhance the yield of cereal (Meena et al. 2014a, 2016e; Sharma et al. 2016; Masood and Bano 2016; Teotia et al. 2016).

### 2.2.7 Symbiotic Nitrogen-Fixing Rhizobacteria

There are two major groups of nitrogen-fixing bacteria, viz., *Rhizobia* and *Frankia*, that have been tested on broad level. *Frankia* makes root nodules more than 280 spp. of woody group plants from eight different families, whereas its symbiotic relationship is not well understood. *Alnus* and *Casuarina* are globally known to form effective symbiosis with *Frankia* (Tilak et al. 2005).

### 2.2.8 *Rhizobium* (Family: *Rhizobiaceae*)

*Rhizobium* could be a bridge between removals and additions of nutrients where farmers can scarcely afford costly inputs and that also in a risky environment. Average nitrogen fixation on earth amounts to ~135 Mt annum$^{-1}$. In recent year's application of *Rhizobium* culture as biofertilizer has been recommended a critical input in legume crops. In India ~30 M ha of land is under pulses cultivation. *Rhizobium* inoculatn was first prepared in the USA and commercialized by private sector in 1930, and strange situation at that time has been chronicled by Fred et al. (1932). *Rhizobium* belongs to the family *Rhizobiaceae*, symbiotic in nature, fixing 50–100 kg N/ha associated with legumes only (Mishra et al. 2013). This is mainly useful for pulse crops like chickpea, pigeon pea, pea, lentil, and black gram; oilseed crops such as soybean and groundnut; and forage crops such as berseem and lucerne. These colonize roots of specific legume crops to form tumor-like growth structures called root nodules, which act in production of ammonia.

*Rhizobium* has a capacity to fix the atmospheric N with symbiotic association in legume plants and certain non-legume plants such as *Parasponia* (Saikia and Jain 2007). BNF occurs in free-living microbes, in association or in symbiosis with plants. In ecological point of view, most important N-fixation system is symbiotic. This is a symbiotic biofertilizer used in legume plants and trees (lucerne) and is a crop-specific inoculant, e.g., *Rhizobium trifolii* for berseem, *Rhizobium phaseoli* for green gram, *Rhizobium meliloti* for lucerne and black gram, *Rhizobium japonicum* for soybean, *Rhizobium lupini* for chickpea, and *Rhizobium leguminosarum* for pea and lentil. Of all these are known for their ability to fix atmospheric N with symbiotic association with plants forming nodules in roots (stem nodules in *Sesbania rostrata*). *Rhizobium* is known in agronomic practice to ensure adequate nitrogen for legume plants instead of chemical fertilizers (Gupta 2004).

*Rhizobium* strains are the most efficient biofertilizers as per the amount of N fixed is concerned. In some research findings on biofertilizers, Kumar et al. (2010) observed that seed inoculation with *Rhizobium* and application of molybdenum have significantly higher growth and yield attributes and yields of black gram. Kumari et al. (2010) revealed application of 100 IN + 50% on fertility level with biofertilizer (*Rhizobium* + PSB + PGPR) gave significantly higher grain yield. Further, Kumari et al. (2012) reported that seed inoculation with biofertilizers (*Rhizobium* + PSB + PGPR) markedly enhanced crop growth and yield attributing characters and seed and straw yield of field pea as well as soil fertility status. In oilseed crop like linseed, Bhanwariya et al. (2013) indicated that higher growth, yield attributes and yields, N and P uptake, and oil yield were recorded with dual seed inoculation (*Azotobacter* + PSB). Similarly, Choudhary et al. (2013) inferred that conjunctive application of vermicompost at 0.7 t/ha + 50% RDF or poultry manure at 0.85 t/ha + 50% RDF and seed inoculation with *Rhizobium* or PSB were found to be the most promising as compared to sole application of either manure or chemical fertilizer and no inoculation. Bhanwariya et al. (2013) found application of FYM along with PSM and *Azospirillum* gave higher growth attributes, yields, quality, and profitability of mustard (Bahadur et al. 2016a; Meena et al. 2013b, c, 2015d; Kumar et al. 2013a, b; Kumar et al. 2014a; Verma et al. 2015a; Singh et al. 2015, 2016).

### 2.2.9 Non-symbiotic Nitrogen-Fixing Rhizobacteria

Non-symbiotic nitrogen fixation by rhizobacteria is known to be more important by the point of view of crop production. The major constraint to non-symbiotic nitrogen fixation is the availability of carbon and energy source for energy-intensive N-fixation process. This constraint can be corrected by moving closer to or inside the plants, viz., diazotrophs present in rhizosphere, rhizoplane, or those growing endophytically. Major nonsymbiotic N-fixing rhizobacteria are *Achromobacter*, *Arthrobacter*, *Azospirillum*, *Acetobacter*, *Clostridium*, *Corynebacterium*, *Derxia*, *Alcaligenes*, *Azotobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Klebsiella*, *Rhodospirillum*, *Rhodopseudomonas*, *Pseudomonas*, and *Xanthobacter* (Saxena and Tialk 1998).

#### 2.2.9.1 *Azotobacter* (Family: *Azotobacteriaceae*)

*Azotobacter* species are gram (−), free-living, aerobic soil-dwelling, oval or spherical bacteria, whose resting stage is in a cyst. *Azotobacter* genus was discovered by Dutch microbiologist and botanist Beijerinck in 1901. The family *Azotobacteriaceae* consisted of two genera, i.e., *Azomonas* (non-cyst forming) with three species (*A. agilis*, *A. insignis*, and *A. macrocytogenes*) and *Azotobacter* (cyst forming) having six species, i.e., *A. chroococcum*, *A. beijerinckii*, *A. nigricans*, *A. vinelandii*, *A. paspali*, and *A. armeniacus*. Among the species of *Azotobacter*, *A. chroococcum* was found dominant inhabitant in cultivated land and fixing $N_2$ (2–15 mg $N_2$ fixed/g of carbon source) in culture media (Tilak et al. 2005).

*Azotobacter* is generally regarded as aerobic, free-living, and heterotrophic in nature. The seed inoculations with bacterial culture gave balanced nutrition to the crops, and improving the uptake of nitrogen and phosphorus was the major mechanism of interaction between plants and bacteria. *Azotobacter* generally fixed ~20–30 kg N/ha/year. The culture required for soil application 3–5 kg/ha. *Azotobacter* is commonly used for rice, cotton, and sugarcane. *Azosprillum is* mainly used for cereal crops and mainly for sorghum (Ghumare et al. 2014). *Azotobacter* has favorable effect on growth and yield of crops through biosynthesis of biologically active substances, producing phyopathogenic inhibitors and stimulation of rhizospheric microbes (Meena et al. 2014b, 2015c; Shrivastava et al. 2016; Sindhu et al. 2016; Velazquez et al. 2016) (Fig. 2.2).

The presence of *Azotobacter* in cultivated soil has beneficial effects on crops, but the profusion of bacteria is related to many factors like organic matter, pH, temperature, soil moisture, and biological properties (Jnawali et al. 2015). Seeds inoculated with *Azotobacter* yield of 20% enhancement were recorded by various researchers (Ojaghloo et al. 2007; Naseri et al. 2013; Estiyar et al. 2014; Rinku et al. 2014). Similarly, combined used of *Azotobacter* + PSB significantly increases the growth, yield characters, and yields of linseed (Bhanwariya et al. 2013) (Table 2.1).

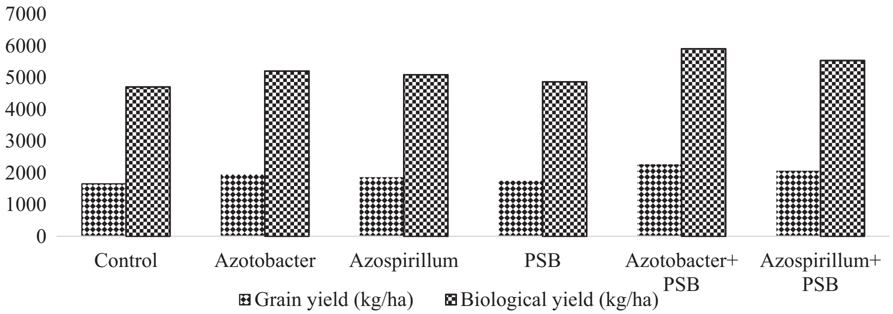

**Fig. 2.2** Effect of biofertilizers on yields of pearl millet (Rinku et al. 2014)

**Table 2.1** Some species involved in biological nitrogen-fixing bacteria

| Genus | Species | Host plant |
|---|---|---|
| | *R. leguminosarum* | |
| | *R. leguminosarum biovar viciae* | *Pisum*, *Vicia*, *Lathyrus*, *Lens*, |
| | *R. leguminosarum biovar trifolii* | *Trifolium* |
| | *R. leguminosarum biovar phaseoli* | *Phaseolus vulgaris* |
| *Rhizobium* (*Croissance rapide*) | *R. galegae* | *Galega orintalis* |
| | *R. tropici* | *P. vulgaris L.*, *Leucaena* |
| | *R. etli* | *P. vulgaris* |
| | *R. gallicum* | *P. vulgaris* |
| | *R. giardinii* | *P. vulgaris* |
| | *R. mongolense* | *Medicago ruthenica* |
| | *R. huautlense* | *Sesbania herbaceae* |
| *Mesorhizobium* (*Crosissance intermediaire*) | *M. loti* | Lotus |
| | *M. huakuii* | *Astragalus sinicus* |
| | *M. ciceri* | *Cicer arietinum* |
| | *M. mediterraneum* | *Cicer arietinum* |
| | *M. plurifarium* | *Acacia*, *Prosopis*, *Leucaena*, *Chamaescrista* |
| *Sinorhizobium* (*Croissance rapide*) | *S. meliloti* | *Medicago*, *Melilotus* |
| | *S. meliloti* | *Trigonella* |
| | *S. medicae* | *Medicago* |
| | *S. fredii* | *Gycine max*, *G. soja* |
| | *S. saheli* | *Sesbania sp.* |
| | *S. kostiense* | *Acacia*, *Prosopis* |
| | *S. arboris* | *Acacia*, *Prosopis* |
| *Allorhizobium* (*Croissance rapide*) | *A.undicola* | *Neptunia natans* |
| *Azorhizobium* (*Croissance rapide*) | *A. caulinodans* | *Sesbania rostrata* |
| | *A.* sp. | *S. rostrata* |
| *Bradyrhizobium* (*Croissance lente*) | *B. japonicum* | *Gycine max*, *G. soja* |
| | *B.* sp. | *Vigna*, *Lupinus*, *Mimosa*, *Acacia* |
| | *B. elkanii* | *Gycine max* |
| | *B. liaoningensis* | *Gycine max, G. soja* |

#### 2.2.9.2 *Azospirillum* (Family: *Spirillaceae*)

There are many species of this genus such as *Azospirillum brasilense*, *A. halopraeferens*, *A. lipoferum, A. amazonense*, and *A. irakense*. Among the species, *Azospirillum lipoferum* and *A. brasilense* are in majority and present in the soil, rhizosphere, and intercellular spaces of root cortex of cereal plants. They are execution associate as symbiotic relation with cereal crop plants. *Azospirillum* fix atmospheric nitrogen in microaerophilic conditions and are frequently associated with root and rhizosphere of many numbers of agricultural crops which are known as associative diazotrophs. The *Azotobacter* colonized roots not only present on root surface, but also a sizable proportion of them penetrates into the root tissues and lives in harmony with the plants (Bahadur et al. 2017; Verma et al. 2017b; Kumar et al. 2017; Nath et al. 2017).

Yields show a discernable improvement with the use of biofertilizers. Studies showed that efficient strain of *Azospirillum* can supply adequate quantity of N to sunflower and can improve the growth and yield attributes (Dhanasekar and Dhandapani 2012). Similarly, combined used of *Azospirillum* + PSB produced higher growth, yield attributes, and yield of pearl millet (Rinku et al. 2014). Various studies have been showing the increase of nodules and vegetative growth of many legumes because of the positive relation between rhizobia and microbes (Cassan et al. 2009). Biari et al. (2008) observed that nutrient uptake (N, P, K, Fe, Zn, Mn, and Cu) by crops was also significantly affected due to application of *Azospirillum.* It also improves the yields, which have been extensively discovered in cereal crops (Kumar et al. 2015d; Sarkar et al. 2017; Verma et al. 2017a).

#### 2.2.9.3 *Acetobacter* (Family: *Acetobacteriaceae*)

The family *Acetobacteriaceae* included genus like *Acetobacter*, *Gluconacetobacter*, *Acidomonas*, and *Gluconobacter* (Yamada et al. 1997). *Acetobacter diazotrophicus* was separated from sugarcane (roots and stem) from Brazil, first reported as nitrogen-fixer bacteria (Cavalcante and Dobereiner 1988). Isolation of these bacteria from tissues of sugarcane and this is absence in soils of sugarcane fields. This organism has been also found in sugar-rich crops like *Pennisetum purpureum* and sweet potato (Paula et al. 1991).

These bacteria successfully colonize sugarcane cultivars, where inorganic nitrogen fertilizers are completely avoided for atleast 2 consecutive years and replaced by organic manures. *Acetobacter* makes significance as biofertilizer for sugarcane crop (Ashbolt and Inkerman 1990). This is mostly adopted endophytically in sugarcane ecosystem and also tolerant of high-sucrose content. These bacteria can fix nitrogen up to ~15 kg/ha/year and produce plant growth-promoting hormones (IAA), which improves the germination and root development and finally help in uptake of plant nutrients.

#### 2.2.9.4 *Azoarcus*

*Azoarcus* is an aerobic microbe N-fixing bacteria which was separated from surface-sterilized tissues of kallar grass [*Leptochloa fusca* (L.) Kunth] (Reinhold et al. 1986), and it can inoculate the roots of rice plants. Kallar grass is a salt-tolerant grass used as pioneer plant on salt-affected low-fertility soils in Pakistan. Again isolation of one genus of diazotrophic rods (Gaur 1990) from kallar grass roots reported that polyphasic taxonomy led to the identification of genus *Azoarcus*, with two categories, i.e., *A. indigens* and *A. communis*, and three additional unidentifed categories, which were distinct at species level. $N_2$ fixation by *Azoarcus* is highly effective such as hyper-induced cells which contain tubular arrays of internal membrane stacks that cover large proportion of the intercellular volume (Tilak et al. 2005).

## 2.3 Phosphorus

Phosphours was first discovered by Brandt in 1669. The word is derived from Greek words “phos” meaning light and “phorus” meaning bringing. It is one of the fifth elements essential for plant growth belonging to the fifth group of the periodic table. It is a major nutrient next to N and plays an important role in plant physiology and biochemistry. It plays a vital role in crop development and is known by many as “Kingpin” in Indian agriculture (Hasan 1996).

### 2.3.1 Phosphorus Status in Soils

Phosphorus is the tenth most important element and constitutes around 0.12% of the earth’s crust (Van Wazer 1958). Soil usually contains ~0.013–0.155% P, and insoluble phosphate compound constitutes ~95–99% of total P (Hayman 1975). It is unevenly distributed along soil profile and usual concentration of total P in top soils is ~500–800 mg/kg (Stevenson and Cole 1999), which is ~100–1800 kg P/ha in plough layer. In India soils, total P content ranges from 120 mg/kg in arid eco-region of Rajasthan to ~2166 mg/kg in sub-humid temperate high lands of Himachal Pradesh. Based on ~10 million soil tests, ~49% of districts and union territories are low in available P, ~49% in medium, and ~2% in high (Hasan 1996). When compared to earlier studies by Ghosh and Hasan (1979), it shows that lower phosphrous fertility class has increased by 3%, while medium and high categories have decreased by 2.7 and 0.3%, respectively.

### 2.3.2 Role of Phosphorus in Plants

Among the 17 essential plant nutrients, phosphorus is the most important nutrient for plant growth and development. Its role cannot be done by any other plant nutrients, and sufficient application of P is required for better vegetative and reproductive growth. It is classified as micronutrient, meaning that it is frequently not sufficient to crop production and is required to crops in relatively higher quanity. Total phosphorus content in arable crops generally ranges from 0.1 to 0.5%. It plays an important role in many biochemical processes of plants, mainly in photosynthesis, carbon metabolism, and membrane formation (Wu 2005). Also, it plays the important role in root elongation and proliferation, and its deficiency affect root architecture (Williamson et al. 2001), seed development, and normal crop maturity. P is easily translocated within the plants and moving from older to newer tissues as the plant forms cells and roots, stems, and leaves. A major portion of P absorbed by the plant is accumulated grains as phytase (Richardson 1994) and its deficiency negatively affects grain yield.

### 2.3.3 Phosphorus Fixation in Soils

Phosphorus is an essential macronutrient for vegetative growth and development of plants; ~95–99% of it, present in soil, is insoluble and can not be absorbed by plants (Vassileva et al. 2000). It is also estimated that in soil up to 75% of applied phosphorus through chemical fertilizers may become unavailable to the plants because of fixation in the soils (Sundara et al. 2002; Ahmed and Shahab 2009). The content of soluble phosphorus in the soil is generally low, normally at levels of 1.0 ppm or less than 1.0 ppm (Goldstein 1994).

The unavailability is due to P-fixation, either it is adsorbed on soil minerals or get precipitated by free $Al^{3+}$ and $Fe^{3+}$ in the soil (Sharma et al. 2013). A higher quantity of P applied as chemical fertilizers goes into the immobile pools through precipitation reaction with highly reactive $Al^{3+}$ and $Fe^{3+}$ in acidic and $Ca^{2+}$ in calcareous soils (Hao et al. 2002). To increase availability of phosphorus to plants, a larger quantity of P is quickly applied to soil, large portion of which is quickly transferred into insoluble form (Omar 1998), and very low percentage of applied P is available to plants making continuous application of fertilizer necessary (Pradhan and Sukla 2005).

### 2.3.4 P-Solubilizing Rhizobacteria (PSR)

Phosphorus-solubilizing rhizobacteria (PSR) are able to convert the insoluble phosphorus into soluble forms, therefore used to increase the solubilility of reprecipitated soil phosphorus for crop production (Shekhar et al. 2000; Kumawat et al. 2009a). Many PSR have shown capacity of various bacteria to solubilize the insoluble phosphate compounds like tri-calcium phosphate, di-calcium phosphate, hydroxylapatite, and rock phosphate. The isolated *Bacilli* are *Bacillus brevis*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus circulans*, *Bacillus polymyxa*, *Bacillus pumilus*, *Bacillus mesentericus*, *Bacillus mycoides*, *Bacillus pulvifaciens*, and *Bacillus subtilis* from rhizosphere of legumes, cereals (rice and maize), areca nut palm, jute, oat, and chilli. *Pseudomonas striata*, *P. cissicola*, *P. pinophillum*, *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. putrefaciens*, *P. syringae*, and *P. stutzeri* have been separated from rhizosphere of chickpea, maize, brassica, soybean, and other cultivated crops. In addition, *Escherichia freundii*, *E. intermedia*, *Serratia phosphaticum*, and species of *Achromobacter*, *Brevibacterium*, *Corynebacterium*, *Erwinia*, *Micrococcus*, *Sarcina*, and *Xanthomonas* are active in solubilizing insoluble phosphates. *Cyanobacteria*, viz., *Anabaena* sp., *Calothrix braunii*, *Nostoc* sp., *Scytonema* sp., and *Tolypothrix ceylonica*, can solubilize phosphate (Fig. 2.3). Phosphorus-solubilizing bacteria are used as biofertilizer in the crops since the 1950s (Kudashev 1956).

In comparison bacteria better perform than fungi in solubilization of phosphorus (Alam et al. 2002). Among all microbes population in soil, PSB having 1–50%, while PSF are ~0.1–0.5% in phosphorus solubilization (Chen et al. 2006). There are adequate colonies of PSB in rhizospheres. There are two types of bacterium aerobic

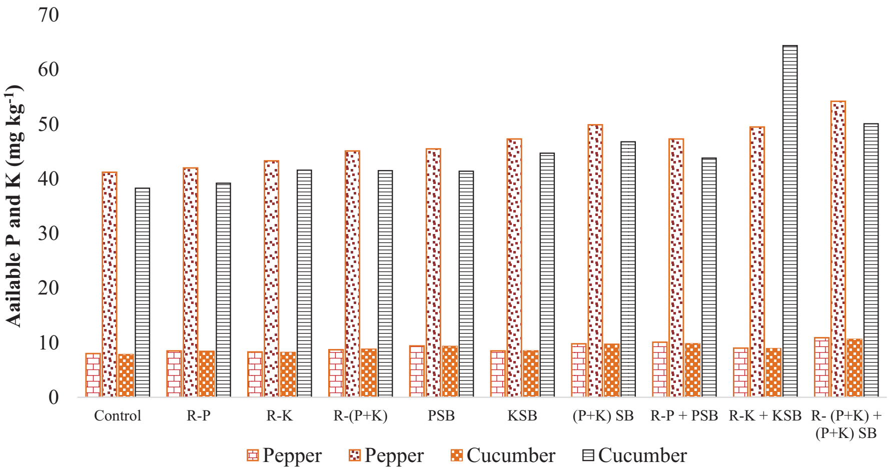

**Fig. 2.3** Effects of PSB and KSB strains on available P and K in soil in pepper and cucumber crops (adopted from Han and Supanjani 2006). *R-P* rock phosphate, *R-P* rock (illite), *PSB* phosphorus-solubilizing bacteria, *KSB* potassium-solubilizing bacteria

and anaerobic, with prevalence of aerobic bacteria which are mostly found in submerged lands. A considerable higher population of PSB is mainly present in plant rhizosphere as compare to non-rhizosphere soil. Among soil bacteria, *Pseudomonas* and *Bacillus* are more common.

### 2.3.5 Mechanism of P-Solubilizing Rhizobacteria

The common species like *Pseudomonas*, *Bacillus*, *Penicillium*, and *Aspergillus* produced organic acids, which have the capacity to lower pH in their vicinity to bring solubilization of bound phosphates in soil. Inoculation of peat-based cultures of *Bacillus polymyxa* and *Pseudomonas striata* increased yields of wheat and potato. The available insoluble phosphorus in soil can be solubilized by PSB; they have the ability to transform inorganic unavailable phosphorus to soluble forms $HPO_4^{2-}$ and $H_2PO_4^-$ by the process of producing organic acid, chelation, and ion-exchange reactions and make them available to plants (Kumar et al. 2014a, Kumar 2015a, b, Shivran et al. 2013). Hence, using PSB in crop production would not only decrease the high cost of manufacturing phosphatic fertilizers but also solubilize insoluble chemical fertilizers that are applied in soil (Chang and Yang 2009; Banerjee et al. 2010, Kumar and Kumawat 2014).

PSB belonging to the genera *Pseudomonas* and *Bacillus* have the capacity to mobilize the unavailable phosphorus in the soil and enhance the availability to plants (Kumari et al. 2010, 2012, Kumawat et al. 2012, Kumawat et al. 2013a, b, 2015, 2017). Inoculation of seed or seedling with microphos biofertilizers can provide 30 kg $P_2O_5$/ha (Ghumare et al. 2014). Kumar and Kumawat (2014) reported that summer mung bean cv. T-1 sown with combined use of chemical fertilizer (10 kg N and 20 kg $P_2O_5$/ha), 50% N as vermicompost (10 kg/ha), and biofertilizers (20 g PSB/kg seed) enhances the crop prouction, economics, and soil fertility. Similarly, Kumar et al. (2015a) noticed that the use of 50% RDF + 50% RDN + PSB produced significantly higher yield attributes, yield, and N, P, and K uptake by mung bean. Kumar et al. (2014b) revealed that application of inorganic fertilizer + 50% N through vermicompost along with biofertilizers (PSB) and Zn application is beneficial in terms of production and economics on green pea in acidic soil. Similarly, Kumar et al. (2015b) observed that application of chemical fertilizer +50% N as organic manures + biofertilizers along with Zn is remunerative in terms of productivity and soil fertility in garden pea under the Eastern Himalayas.

As per the findings of Kumawat et al. (2009b, c), they reported that seed inoculation with PSB significantly increases growth attributes (plant height, number of branches/plant, dry matter accumulation, chlorophyll content, effective nodules, dry weight of nodules/plant, total nodules) and finally yields of mung bean. Kumawat et al. (2010) showed that inoculation of seeds with PSB gave the maximum N, P, and K concentration in seed, straw, their total uptake, and protein content mung bean (Figs. 2.4 and 2.5). Shivran et al. (2012) reported that soybean production is enhanced by application of sulfur at 40 kg/ha and 40 kg $P_2O_5$/ha along with phosphorus solubilizing bacterial inoculation. Kumari et al. (2014) found that use of vermicompost at 1.33 t/ha should be superimposed to recommended dose of fertilizer (40–17–16–20 kg NPKS/ha) along with 5 kg Zn/ha applied to seeds inoculated with biofertilizer consortia comprising of *Rhizobium* + PSB + PGPR for realizing economically optimum yield of field pea.

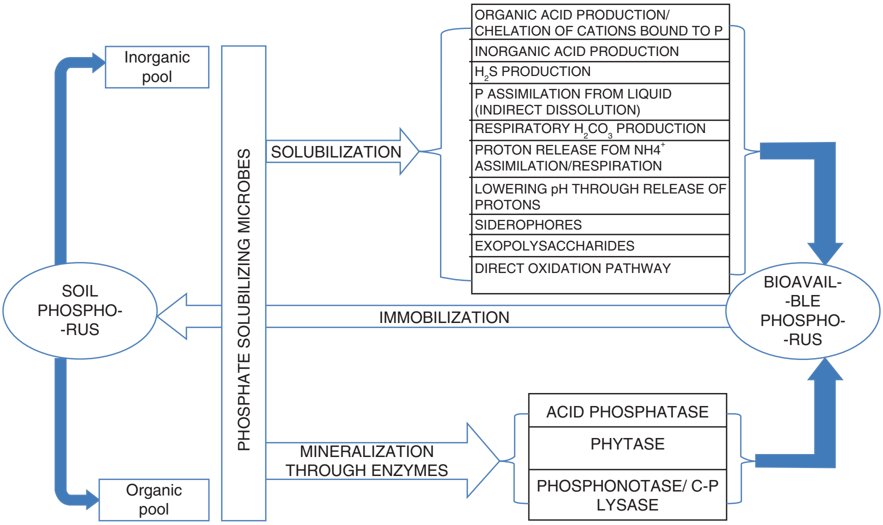

**Fig. 2.4** Systematic representation of processes of soil P mineralization/solubilization and immobilization through PSM

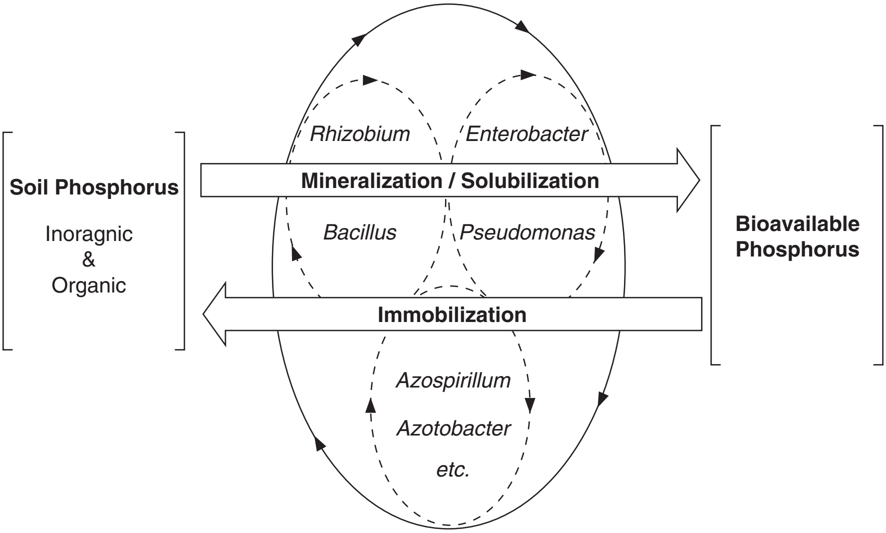

**Fig. 2.5** Systematic sketch of soil P solubilization and insolubilization through bacteria

## 2.4 Potassium

### 2.4.1 Potassium (K) Status in Soil

Out of the major plant nutrients, K is the most adequate nutrient in the soil. It is 7/8 common in ground, whose surface layer (lithosphere) on an average contains ~2.6 K (~3% $K_2O$). It is present in soil in four major pools according to the availability of potassium to plants (Zakaria 2009). Mineral soils are having ~0.04–3% K; total K concentration of upper 0.2 m of most cultivated lands (Sparks and Huang 1987) generally ranges between 10 and 20 g/kg. However, most of potassium in soil (90–98%) is incorporated in crystal lattice structure of minerals and therefore not readily available to the plants.

The availability of potassium depends on soil type and is influenced by physic and chemical properties of soil. Potassium in soil is generally categorized in four groups depending on the availability to plants, i.e., water soluble, exchangeable, non-exchangeable, and structural forms. Water soluble potassium is directly absorbed by plants and microorganism and subjected to leaching exchangeable K which is electrostatically bound on outer sphere to clay surfaces of mineral (Barre et al. 2008). Both fractions are generally considered to be readily available to the crop plants. However, size of both pools is about 0.1–0.2% and 1–2% of total K in soil, respectively (Sparks and Huang 1987).

Inoculation of PSB and KSB combined with rock phosphate or potassium materials increased the availability of phosphorus and potassium in soil; improve N, P, and K uptake; and enhance growth of brinjal (Han and Lee 2005). Similarly, combined application of PSB and KSB resulted in significantly increased availability of

P and K than control (without inoculums) (Han and Supanjani 2006). A research experiment indicated that co-inoculation of phosphorus-diluting bacteria (PDB) and potassium-diluting bacteria (KDB) in combination with direct application of rock phosphate and potassium materials (RP+ K) improves plant growth (shoot and root growth), phosphorus and potassium availability, and uptake by maize grown in P- and K-deficient soils (Seoud and Abdel-Megeed 2012). In response to inoculation with KSB, significant increases in seed germination, root and shoot length, and grain yield were observed over the uninoculated control (Prajapati 2016).

### 2.4.2 Role of Potassium in Plants

Potassium is most important for growth and development of plants. It is participated in adjustment of plant cellular osmotic pressure and the transportation of compounds in plants. It encourages activation of enzymes, the utilization of nitrogen, and the syntheses of sugar and protein. It also helps photosynthesis in plants (Zhang and Kong 2014). Potassium is required for the cell metabolic mechanisms. Potassium is required in huge amount by the crop to obtain its highest yields. It is associated with movement of water, nutrients, and carbohydrates in plant tissues. It also helps in making of protein, photosynthesis, fruit quality, and reduced infection of diseases.

### 2.4.3 K-Solubilizing Rhizobacteria

Potassium-solubilizing bacterium is heterotrophic, which is getting all their energy and cellular carbon from available organic matter in the soil. Therefore, they play a vital role in building of humus, cycling of other minerals tied up in organic matter, and stopping the buildup of dead organic materials (Zakaria 2009). Besides, potassium-solubilizing bacterium is an aerobic bacterium that plays a vital role in improving soil structure by the formation and stabilization of water-stable soil aggregates. In addition, the gram + bacterium can produce substance that promotes plant growth or inhibits root pathogens (Zakaria 2009). A large range of rhizosphere microbes are isolated as potassium mobilizers like *Bacillus mucilaginosus*, *Bacillus edaphicus*, *Bacillus circulans*, and *Arthrobacter* sp. Potassium mobilization is mainly due to the produced organic acids. The use of microbial inoculants as biofertilizers can be an alternative tool of chemical fertilizer (Fig. 2.6). The use of KSB by farmers can solubilize potassium available in their own fields and can save some requirement of potassic fertilizer. Among bacterium genera, these are *Pseudomonas*, *Bacillus*, *Achromobacter*, *Agrobacterium*, *Microccocus*, *Aereobacter*, *Flavobacterium*, and *Erwinia* (Zhao 2008). Earlier research findings have shown that potassium-solubilizing bacterium can stimulate the plant growth (Lin et al. 2002; Basak and Biswas 2009). The studies have shown that inoculum can increase plant growth and increase nutrient concentration in plants (Basak and Biswas 2010).

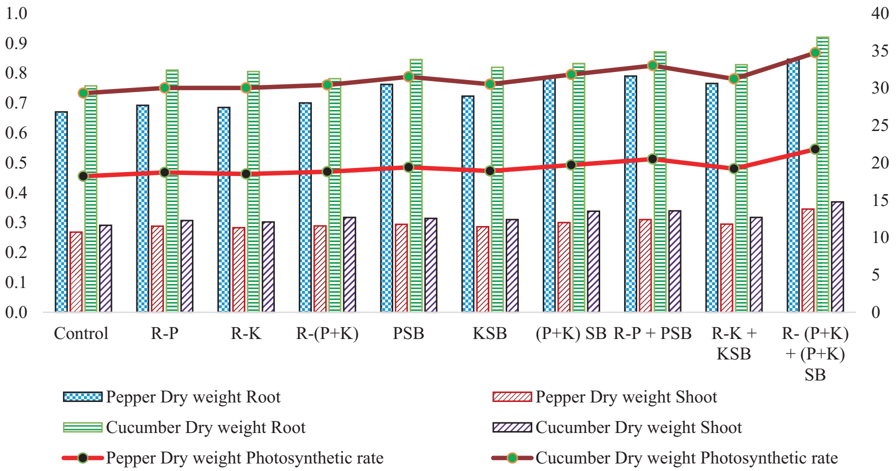

**Fig. 2.6** Reponse of PSB and KSB to dry weight (g/plant) and photosynthetic rate (μmole/cm$^2$/s) of pepper and cucumber (Han and Supanjani 2006)

### 2.4.4 Mechanism of K-Solubilizing Rhizobacteria

Various researchers have found the ability of potassium-solubilizing rhizobacteria to mobilize to unmobilized potassium in liquid Aleksandrov broth medium (Archana et al. 2013; Maurya et al. 2014). The process of potassium mobilization means insoluble potassium and structural unavailable forms of potassium compounds are mobilized and solubilized due to production of different organic acids. These organic acids are accompanied by acidolysis and complexolysis exchange reactions, and these are the main processes attributed to their conversion into soluble form.The organic and inorganic acids transform to insoluble potassium (mica, muscovite, biotite feldspar) into soluble form of potassium with the net result increasing the availability of nutrients to crop plants. It is demonstrated that *Bacillus mucilaginosus* and *Bacillus edaphicus* can produce polysaccharide and carboxylic acids, such as tartaric acid and citric acid, which help to solubilize K compounds in soils (Lin et al. 2002).

## 2.5 Zinc

Zinc (Zn) deficiency has received great attention in India because half of the Indian soils have poor availablility of Zn (Cakmak 2009). This is mainly due to crops grown in Zn deficient soils. In our country, ~50% of soils are low in Zn, and it remains most important nutritional disorder influencing crop production. The major causes of zinc deficiency are the use of chemical fertilizers, intensive agriculture, and poor irrigation facilities that lead to lowering of zinc content in soils (Das and Green 2013). Zn deficiency is estimated to increase from 42 to 63% by 2025 due to continuous decreasing of soil health (Singh 2009).

### 2.5.1 Zinc Status in Soils

Zn is an important micronutrient for all soil microbes and plants. It is found in earth's crust in tune of 0.008%. Zinc has a major role in nutrition of both eukaryotic and prokaryotic microbes as cofactor or metal activator in many enzyme activities (Hughes and Poole 1991). Zinc deficiency is widespread in the whole world, mostly in rice ecosystem of Asia (Tisdale et al. 2009) and in the soil orders like Aridisols, Alfisols, Mollisols, and Vertisols (Srivastava and Gupta 1996). Total Zn (mg/kg) was 47 in Entisols, 60 in Inceptisols, 61 in Aridisols, 36 in Vertisols, 44 in Alfisols, 43 in Ultisols, 30 in Mollisols, and 72 in Oxisols (Katyal and Sharma 1991). Zn > 90% in soils presents as insoluble form and not absorbed by crop plants, while exchangeable Zn ranges from 0.1 to 2 mg/kg soil (Singh 2011). The total area under Zn deficiency is ~10 M ha in India. However, ~85% of rice-wheat cropping system is present in IGP region, and Zn is a limiting factor in crop production due to alkaline and calcareous soil. In India, soybean-wheat cropping system extracts zinc from soil, e.g., harvest of ~7 tonnes grain/ha/year uptake ~416 g Zn/ha/year in Indian soils shows ~50% Zn deficiency which is below critical limit, i.e., 1.5 ppm of available Zn (Prasad 2010).

### 2.5.2 Role of Zinc in Plants

Zinc has pivotal role in plant metabolism by affecting the activities of hydrogenase and carbonic anhydrase, stabilization of ribosomal fractions, and synthesis of cytochrome (Tisdale et al. 1984). It helps in detosification of superoxide radical. The enzyme involved is Cu-Zn-SOD (copper zinc superoxides dismutase). Zn plays a role in carbohydrate metabolism. The enzyme involved is carbonic anhydrase (CA). Zinc has a role in anaerobic root respiration in rice. The enzyme involved is alcohol dehydrogenase (ADH). Zinc also is necessary for the assimilation of tryptophan which is a precursor of indole acetic acid (IAA); it also plays an active role in the secretion of growth-promoting hormones and auxins (Alloway 2004). Zinc also helps in plant diseases resistance, photosynthesis, protein metabolism, pollen development, and cell membrane integrity (Gurmani et al. 2012) and enhances level of antioxidant enzymes and chlorophyll within plant tissues (Sbartai et al. 2011).

### 2.5.3 Deficiency of Zinc in Plants

The essentiality of Zn was first discorved by Maze (1915) in maize, where it is known as "white bud." As already reported, its deficiency in rice was first reported by Nene (1966) at the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar. Since Zn is asssociated with several enzymes, its deficiency leads to several disorders in plants. Also, Zn is relatively immobile in the plants; its deficiency symptoms generally appear on the growing young tissues. Due to deficiency of Zn, development of abnormalities leads to visible deficiency symptoms on plants like stunted growth, chlorosis, smaller leaves, spikelet sterility, etc. Zn deficiency can also negatively influence the quality of grains; plants' susceptibility to injury by high light or temperature intensity and to infection by fungal diseases may also increase (Cakmak 2000). Zn can affect water uptake and transport into plants and also reduce the adverse effects of short periods of heat and salt stress (Peck and McDonald 2010; Tavallali et al. 2010).

### 2.5.4 Zn-Solubilizing Rhizobacteria (ZSR)

Application of chemical fertilizers partially caters the plant need as 96–99% of applied Zn is transformed into different insoluble forms, depending on the soil types and physicochemical reactions within 7 days of application (Saravanan et al. 2004). This crisis can be prevented by zinc solubilizers, which are potential to convert various unavailable forms of metal to available form. These bacteria have capacity to solubilize the insolubilized Zn like zinc phosphate, zinc oxide, and zinc carbonate in appreciable quantity which is not a common feature among bacteria on the topsoil surface. Numerous bacteria, mostly those associated with rhizosphere, have capacity to convert unavailable form of Zn into readily available form through mobilization processes (Cunninghan and Kuiack 1992).

The ZSB are potential alternative that could cater plant Zn essentiality by mobilizing complex Zn in the soil. Many genera of rhizobacteria related to *Thiobacillus thiooxidans*, *Acinetobacter*, *Bacillus*, *Pseudomonas*, and *Thiobacillus ferrooxidans* have been reported as zinc solubilizers (Saravanan et al. 2007). These microorganisms mobilized metal forms by protons, chelated ligands, and oxidoreductive systems present on cell surface and membranes (Table 2.2 and Fig. 2.7). This bacterium also demonstrated many traits beneficial to plants like production of phytohormones, antibiotics, siderophores, vitamins, antifungal substances, and hydrogen cyanide (Goteti et al. 2013).

Rosas et al. (2009) observed wheat grain yield increase by 36% due to seed inoculation with *Pseudomonas aurantiaca* on a sandy loam soil in Argentina. A positive correlation between grain Zn and protein concentration was observed by Cakmak et al. (2010). Inoculations significantly improve the methionine content in grains of both wheat varieties over the control; it is likely that these inoculations would help to produce grains with better Zn bioavailability. Results indicated that seed inoculation with *Pseudomonas* and *Bacillus* significantly improved the nutrient concentration of N and P in leaves of maize (Goteti et al. 2013).

### 2.5.5 Mechanism of Zn-Solubilizing Rhizobacteria

Solubilization of zinc can be done by a range of processes, which are secretion of metabolites like organic acids and proton extrusion or production of chelating agents (Sayer and Gadd 1997). In addition, producing inorganic acids like sulfuric acid, nitric acid, and carbonic acid could also facilitate solubilization (Seshadre

**Table 2.2** Reponse of zinc-solubilizing bacteria to growth of maize seedlings (Hussain et al. 2015)

| | Shoot | Root | Fresh shoot | Fresh root | Dry shoot | Dry root |
|---|---|---|---|---|---|---|
| Treatment | Length (cm) | | Biomass (g plant$^{-1}$) | | | |
| Control | 20.33i | 7.70i | 1.707i | 0.43 h | 0.29f | 0.12a |
| AZ2 | 29.00$^{cd}$ | 10.20de | 1.940e | 0.76f | 0.30f | 0.23 h |
| AZ6 | 32.33a | 12.13a | 2.393a | 1.81a | 0.55a | 0.57f |
| AZ7 | 29.33bc | 10.43 cd | 1.880 g | 0.50 g | 0.41d | 0.15ab |
| AZ8 | 26.53f | 8.70 h | 1.777 h | 0.80e | 0.26 g | 0.25 g |
| AZ9 | 28.50d | 9.90e | 2.060d | 1.57b | 0.41d | 0.53e |
| AZ10 | 27.16e | 9.50f | 2.143c | 1.03d | 0.45c | 0.36b |
| AZ11 | 28.70d | 10.53c | 2.220b | 0.80e | 0.51e | 0.24c |
| AZ12 | 25.50 g | 8.43 h | 2.013e | 1.54b | 0.36d | 0.55ef |
| AZ13 | 29.76b | 11.33b | 2.037de | 1.09c | 0.40c | 0.36c |
| AZ14 | 24.66 h | 9.13 g | 1.967f | 1.01d | 0.34e | 0.32d |

Means sharing the same letter do not differ significantly ($P < 0.05$)

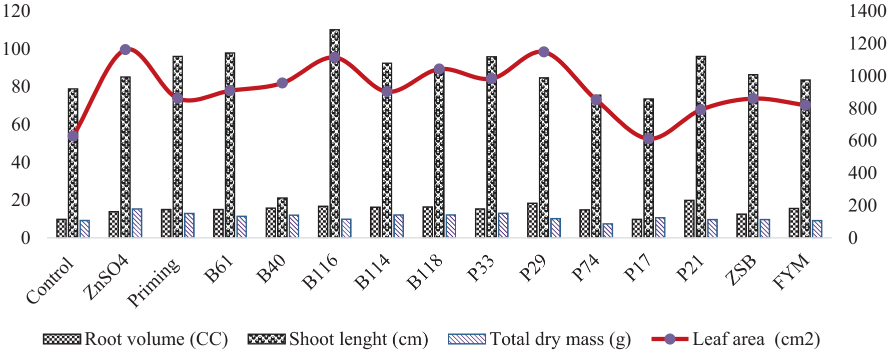

**Fig. 2.7** Biometric growth parameters of maize seed treated with ZSB and inorganic source of Zn (Goteti et al. 2013)

et al. 2002). It is apparent from the zinc solubilization data that the solubilization potential varied with each isolate.

Production of organic acid like gluconic acids (especially 2-ketogluconic acids) by microorganism isolate has been observed to be a major mechanism of solubilization (Fasim et al. 2002). This solubilization property is important in nutrient cycling. Fall in pH and acidification of medium were noted in all cases. Higher solubilization of insoluble zinc sources was achieved in 72 h. The zinc-solubilizing potential also correlated with the zinc levels that are accumulated by plant leaves. The solubilization of zinc phosphate by a strain of *Pseudomonas fluorescens* was reported by Simine et al. (1998). They found that gluconic acids are secreted in culture medium, which helps in solubilization of zinc salts. In their study also reported that acidic pH can solubilize bacterium due to production of organic acids and higher production of available zinc in the culture broth.

Bacteria have demonstrated terrific capacity to solubilize Zn availability in rhizosphere and enhance zinc in plants (Subramanian et al. 2009). *Pseudomonas aeruginosa* has a potential to solubilize ZnO in liquid medium (Fasim et al. 2002). Bacterial inoculation has also the ability to increase bioavailable Zn in rhizosphere soil (Whiting et al. 2001) and Zn content in plants (Biari et al. 2008). PGPR produced siderophores (Saravanan et al. 2011); derivatives of gluconic acids, e.g., 2-ketogluconic acid and 5-ketogluconic acid; and different other organic acids for the mobilization of Zn and iron (Tariq et al. 2007). These bacteria can be used to solubilize insoluble sources of Zn such as ZnO and $ZnCO_3$ because most of soils are rich in Zn contents but less in soluble Zn. *Bacillus* and *Pseudomonas* spp. have much potential to solubilize these sources in soil system for taking economically efficient Zn (Saravanan et al. 2003). Rhizosphere microorganism may benefit plants through different mechanisms including mobilization of nutrients and also acts as a biocontrol agent (Khalid et al. 2009).

## 2.6 Silicon

Rice is a well-known siliceous plant which (Si) accumulates and benefits from silicon nutrition. Si is an element which is useful to healthy growth and development of plants. There is plenty of total Si in soils, but most of them were unavailable to the plant. Silicate mineral-solubilizing bacteria dissolve silicate minerals (such as feldspars and micas) and release elements of potassium and silicon.The SSB are of great interest in recent times because of their role in solubilization of silicate minerals rendering silica and potassium available for crop uptake thus reducing the potash fertilizer requirement (Sheng 2005) and in desilication of ores like bauxite (Zhou et al. 2006).

Studies have shown that these bacteria solubilized silica besides releasing phosphate, potassium, iron, and calcium from soil silicate mineral. Therefore, these microorganisms attracted attention of scientists to advocate these organisms as potassium-mobilizing biofertilizers. Silicon plays an important role in plants like accelerating growth and conferring rigidity to leaves, thus increasing leaf surface area for maximizing photosynthesis and mitigating effects of abiotic stresses such as drought, salt, and metal toxicity in many plants (Ma and Yamaji 2006). Despite of its abundance in earth's crust, its major part occurs in insoluble forms that cannot be taken easily by roots (Vasanthi et al. 2012; Rodrignes and Datnoff 2005). Insoluble form occurs in soil which can be solubilized by weathering of rocks or biological activities of plant roots and soil microbes.

### 2.6.1 Silicon Status in Soils

Silica is the second most important constituent in the earth's crust (Ehrlich 1981). Its content is 27.7% of the total weight of soil after the oxygen ~47% (Datnoff and Snyder 2001). It ranges from 200 to 300 g Si/kg in clay soil and 450 g Si/kg in sandy soils (Kovda 1973; Matichenkov and Calvert 2002). Si content in soil ranged from 1.0 to 45% by dry weight (Sommer et al. 2006). Si is an important fertilizer for cultivation of crops that increases plant tolerance to abiotic stresses (Liang et al. 2005).

### 2.6.2 Role of Silicon in Plants

Silicon concentration in plants may range from 0.1 to 10% (Epstein 1994). Si alleviates physical stress such as drought, radiation, lodging, high temperature, freezing, and ultraviolet and chemical stress which are salt, metal toxicity, nutrient imbalance, etc. (Epstein 1994). It helps in drought resistance by maintaining the plant water balance, photosynthetic rate, errectness of the leaves, and structure of xylem vessels under high transpiration rates due to high temperature and moisture deficient (Hattori et al. 2005). Si makes plant cell walls thick and stronge while enlarging the size of the vascular system of plant. The silicon cellulose membrane in epidermal tissue protects plants against excessive loss of water by transpiration (Meena et al. 2014a, b, c).

### 2.6.3 Si-Solubilizing Rhizobacteria (SSR)

Soil has a lot of various microorganisms, but fewer are able of solubilizing silicates such as *Bacillus caldolytyicus*, *Proteus mirabilis*, *Bacillus mucilaginosus* var. *siliceous*, and *Pseudomonas* was found most effective to release silica from natural silicates (Meena et al. 2014a, b, c). These SSB have the ability to degrade silicate, especially aluminum silicates ($AI_2SiO_5$). These bacteria produced several organic acids during their growth, which can play a role in silicate weathering. These organisms help in releasing potassium from K-containing minerals.

The bacteria identified include different species of *Bacillus*. The improvement of plants and crop yield by releasing the nutrients through seed inoculation and soil application as biofertilizers are practiced by farmers throughout the world. The solubilization of silica by microorganisms is considered as source silicon that supplies to plants. These bacteria increase the growth, chlorophyll content, test weight, filled grains, biomass, and yield of rice crop (Avakyan et al. 1986). Soil application of SSB produced higher yield of maize, potato, wheat, and tomatoes (Aleksandrov 1958) (Table 2.3).

### 2.6.4 Mechanism of Si-Solubilizing Rhizobacteria

Silicon microoganisms released organic acids as part of its metabolism that plays dual role in silicate weathering. These are supplied $H^+$ ions to medium and stimulate hydrolysis and organic acids such as citric acid, keto acids, oxalic acid, and hydroxy carbolic acids that make complexes with cations and made available to plants in

**Table 2.3** Effect of silicon-solubilizing bacteria on yields of rice (Avakyan et al. 1986)

| Treatments | Grains/ panicle (no.) | Test weight (g) | Filled grains (%) | Biomass (t/ha) | Grain yield (t/ha) |
|---|---|---|---|---|---|
| Control | 54 | 22.14 | 76.7 | 10.24 | 3.40 |
| SSB | 62 | 22.84 | 78.1 | 11.27 | 3.80 |

readily form. Joseph et al. (2015) reported that some bacterial isolates can solubilize insoluble minerals like silicates, phosphates, and potash into soluble form by secretion of organic acids (2 ketogluconic acid, alkalis and polysaccharides). Bacteria make available to silicates by producing proton, organic ligands, hydroxyl anion; extra cellular polysaccharides and enzymes (Barker et al. 1998).

## 2.7 Role of Biotechnology to Develop Efficient Strain of Microorganism for Solubilization

In the present era, molecular-based methods have provided latest opportunities to find out the availability and adequacy of specific microorganisms or to quantify the expression of target genes directly in the rhizosphere (Sharma et al. 2013). The knowledge on mechanism of biological nitrogen fixation has been increasing rapidly due to better understanding of mechanism of genetic methodology, function, and regulation of gene responsible for nitrogen fixation. The *nif* genes encoding enzymes that are participated in biological nitrogen fixation converted $N_2$ into available form to all living microorganisms.

The ability to fix atmospheric $N_2$ is found in wider range of prokaryotic microorganisms. Genetic studies showed that structural genes are highly conserved in all known N2-fixing organism (Ruvkun and Ausubel 1980), which suggest that *nif* genes must have evolved and spread throughout virtually to all groups of prokaryotic organisms. Understanding on molecular mechanism of biological nitrogen fixation (BNF) outside legume rhizobium symbiosis could have important agronomic implications and enable the use of N-fertilizers to be reduced or even avoided. There is a need to focus on sustainable exploitation of biodiversity of nitrogen-fixing organisms and, in longer term, transfer of endosymbiotic nitrogen-fixation capacities to major nonlegume crops through genetic engineering.

Overexpression of gene involvement in phosphorus mobilization in soil (both organic and inorganic) under rhizosphere bacteria is a very effective approach for improving the capacity of microorganisms for efficient absorption of phosphorous in soil. Development of genetically modified phosphate-solubilizing microorganism has additional advantage over transgenic plants as modification of bacteria through genetic engineering is much easier than that of complex higher organism. In addition, many plant growth-promoting bacteria (PGPR) may be combined in a single organism. Specific primers based on conserved regions of genomic region have been designed for various microorganisms associated with phosphate mobilization, including mycorrhizal fungi, *Penicillium* sp. and *Pseudomonas* sp. (Oliveira et al.

2009). Recent high-throughput techniques such as microarray-based profiling of bacterial community and pyrosequencing are other promising tools that will certainly add the understanding of microbial communities and their interactions with plants and rhizosphere (Trivedi et al. 2012).

## 2.8 Concluding Remarks

Microbes play a vital role in fixing/solubilizing/mobilizing/recycling of macro- and micronutrients in agricultural ecosystem. Although they occur in soils naturally, their populations are often insufficient to bring about the desire level of nutrient mobilization. There has been a significant enhancement in use of synthetic fertilizer particularly nitrogen after the green revolution, which led to decrease of soil organic matter pool, changing of soil microbe populations and their balance, increase of soil hardness, and overall deteriorated soil health. With increasing cost of chemical fertilizers on account of oil price hikes and degradation of soil and given the reducing yield response to increased use of fertilizer, soil fertility choices available to farmers are limited. The hazards associated with increased use of chemical fertilizers/pesticides in modern agriculture have led to awareness among the people and emphasis on the need for environment friendly agronomic practices.

Nutrient-solubilizing rhizobacteria (NSR) have emerged as one of the alternatives to application of chemical inputs for needs of fertilizers. Their use in agriculture in preference to chemical fertilizers offers economic and ecological benefits by way of soil health and fertility to farmers. Biofertilizers are commonly known as microbial inoculants; they are artificially multiplied cultures of certain soil microbes that can improve soil fertility and crop productivity. This was encouraged through integrated plant nutrient, means together use of chemical fertilizers, organic/green manures and biofertilizers to sustainable crop production, improving soil health and productivity. The new technologies developed using the powerful tool of molecular biotechnology can increase the biological pathways of production of phytohormones. The key challenge in this field is research lies in the fact that along with the identification of different strains of plant growth-promoting rhizobacteria and its characters. Therefore, there is a need to develop collaborative research work including microbiologist, soil scientist, and biotechnologist to assess, refine, and develop potential specific inoculants for several vital nutrients under different environmental conditions.

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# Implementation of Biofortification Technology by Using PGPR for Sustainable Agricultural Production

## 3

Umesh Dhuldhaj and Urja Pandya

**Abstract**

A major challenge in the twenty-first century is to fulfill the hunger need of day-by-day increasing populations. Hence, there is the need to increase the productivity of cultivated land and food fortification. The use of biofortification nutrient and mineral content can be increased in staple food. Economically important crops have limited production yield due to the scarcity and hyper-accumulation of the nutrients and attack of plant pathogen. There are several microbes such as fungi, actinomycetes, cyanobacteria, and plant growth-promoting rhizobacteria (PGPR) possessing plant growth-promoting mechanisms. These microbes help to increase nutrient uptake (e.g., Mn, P, Fe, S, Zn, etc.) by making it phyto-available in rhizosphere. Plant-microbial interaction (mycorrhization and actinomycetes interaction) improves the health of plant by protecting them from attack of phytopathogen and toxicity of hyper-accumulation of nutrient.

**Keywords**

Biofortification • Nutrients • PGPR • Rhizosphere

## Contents

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## 3.1 Introduction

The exploitation of beneficial microbes as a biofertilizer has become paramount importance in agriculture sector for their potential role in food safety and sustainable crop production. The eco-friendly approaches inspire a wide range of application of plant growth-promoting rhizobacteria (PGPRs), and endo- and ectomycorrhizal fungi, cyanobacteria, and many other useful microscopic organisms led to improved nutrient uptake, plant growth, and plant tolerance to abiotic and biotic stress (Bhardwaj et al. 2014). Microorganisms in the rhizosphere mediate the cycling of nutrients and their enhanced mobilization and facilitate their uptake, leading to increased root growth, biomass, and yield of plants (Manjunath et al. 2016) and conferred resistance from pathogens causing diseases to the host plant by the specialized and customized secretions such as antibiotics (Gourion et al. 2015; Bonfante and Genre 2015). The most prevalent phenomena in the biological world are living together. One of the interesting interactions, i.e., plant and microbial interaction in the underground environment, is mycorrhization (Varma et al. 2002). Improving the bioavailability of macro- and micronutrients in soil using microbial inoculants, thereby improving uptake and biofortification of crops or their produce, can be cost-effective options to improve the nutritional quality and reduce malnutrition, particularly in the developing countries (Zhu et al. 2012). Agronomic biofortification represents complementary to the sustainable agricultural approaches (García-Bañuelos et al. 2014). Most of the biocontrol agents comprise bacterial genera such as *Bacillus* and *Pseudomonas*; however, cyanobacteria are also globally gaining attention as inoculants for various crops (Zafar et al. 2012).

All plants need numerous minerals, nutrients, and adequate supply of water, light, and heat from the environment for their proper growth and development. Impaired metabolism (i.e., nutrient uptake and utilization, translocation, and assimilation) and abnormal plant growth result in the reduction in the harvest yield of crop plants due to the deficiency in the minerals and nutrients. Even moderate nutrient deficiency affects the plant growth and development (Grusak 2001; Meena et al. 2017). Marschner (1995) reported that deficiency in nutrient's concentration was not only factor responsible for plant diseases. Plant pathogens are also responsible for the limited plant growth and crop yield (Uchida 2000) by the immobilization or hyper-accumulation of the nutrients in the rhizosphere (Huber and Graham 1999). Microbes and nutrients are playing major role in the disease control of the crop plants (Agrios 2005). In the rhizosphere, a competition for the nutrients among microbes and plant roots makes nutrient less available to the plant (Timonin 1965).

## 3.2 Types of Nutrients and Their Importance for Plant Growth Enhancement

All plants must achieve various types of inorganic minerals from their surrounding environment for victorious vegetative and reproductive growth development. These minerals are responsible for many functions such as (1) structural components in macromolecules, (2) cofactors in enzymatic reactions, and (3) osmotic solutes needed to maintain proper water potential or ionized species to provide charge balance in cellular compartments. Nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), and sulfur (S) are generally considered as macronutrients and found at less than 0.01% concentration of dry tissue weight. Iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), boron (B), chlorine (Cl), molybdenum (Mo), and nickel (Ni) are considered as micronutrients and found at concentrations less than 0.01% of dry tissue weight (Grusak 2001). The important nutrients and their functions are mentioned in Table 3.1.

**Table 3.1** List of important nutrients and their functions (Grusak 2001)

| Name of nutrients | Functions |
|---|---|
| Nitrogen | Cellular metabolism, transcription, translation, and replication of genetic information |
| Potassium | Cell extension and growth, establishment of electrochemical gradient across membrane |
| Calcium | Establishment of stable but reversible intra- and intermolecular linkages, especially in the cell wall and at the surface of membranes |
| Magnesium | Interactions with various ligands through ionic bonding. Various enzymes and enzyme reactions require magnesium |
| Phosphorous | Energy transfer via the pyrophosphate bond in ATP, and the attachment of phosphate groups to many different sugars provides metabolic energy in photosynthesis and respiration |
| Sulfur | Sulfur compounds such as tripeptide glutathione involved in detoxification of oxygen radicals; phytochelatins and metallothioneins, heavy metal detoxification, etc. |
| Iron | When incorporated into proteins, these attributes allow for controlled reversible redox reactions |
| Zinc | Plant growth production and internode elongation |
| Manganese | Redox reactions |
| Copper | Lignin synthesis, photosynthesis, plant respiration |
| Boron | Cell division, reproduction |
| Chlorine | Osmoregulation |
| Molybdenum | Synthesis and activation of nitrate synthetase |
| Nickel | Metabolized urea content |
| Cobalt | Nodulating bacteria for plant growth |

## 3.3 Various Combinations of Interactions for Nutrient Uptake

Different microorganisms have different mechanisms for uptake of nutrients under conditions. They are mentioned as below:

### 3.3.1 Rhizobacterial Interactions

The PGPRs are the group of soil bacteria/rhizobacteria which stimulate growth of host plant and increase nutrient uptake and its mobility (Cakmakci et al. 2006). Flora of the rhizosphere is well known to soil fertility which turns into increase in crop yield. The term plant growth-promoting rhizobacteria was given by Kloepper and Schroth (1978). The important mechanism of PGPR is to increase nutrient availability in the rhizosphere (Glick 1995). There are direct and indirect mechanism of PGPR to improve plant growth and health (Fig. 3.1). Direct mechanism for growth stimulation includes (1) biological nitrogen fixation (Zahran 2001); (2) production of siderophores and enzymes; (3) induction of systemic resistance (Van Loon 2007); (4) varying concentration of phytohormones such as auxins, cytokinins, gibberellins (GA) (Vacheron et al. 2013), or ethylene (Glick et al. 1995); and (5) solubilizing minerals like phosphorus and iron (Delvasto et al. 2006). The indirect mechanism includes (1) production of antibiotics, (2) chelation of iron in the rhizosphere, and (3) synthesis of extracellular enzymes (Van Loon 2007). The rhizosphere supports the growth and multiplication of beneficial microbial community and influence the physiology of whole plant (i.e., improves root functioning and nutrition) (Fig. 3.2) (Vacheron et al. 2013).

There are certain characteristics that can be fulfilled by the PGPR such as (1) they should able to colonize the root; (2) they can survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activity; and (3) they must promote plant growth (Bishnoi 2015). Biofortification of iron in edible plant tissue can be brought about by PGPR (Khalid et al. 2015). The nutrients in the environment are always present in combined form such as minerals and ores (arsenopyrite, iron pyrite). Some of the nutrients are in the gaseous form like nitrogen and carbon dioxide. Some minerals are in combined form with the carbon that are present in the humus-like materials and compost (Kumar et al. 2015a, b, 2016b; Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Ahmad et al. 2016).

Nutrient cycling, organic material decomposing, production of secondary metabolites (such as hormones, antibiotics, enzymes, etc.) can increase bioavailabilty of different nutrients in soil which stimulates the growth of beneficial bacteria and these bacteria plays a major role in enhancement of vegetative growth of plants. The bacteria that are responsible for the increase in plant growth are endophytes, bacteria of rhizosphere, and bacteria of phyllosphere which mediate soil geochemical cycles (Raaijmakers et al. 2009). The most common families of endophytic bacteria

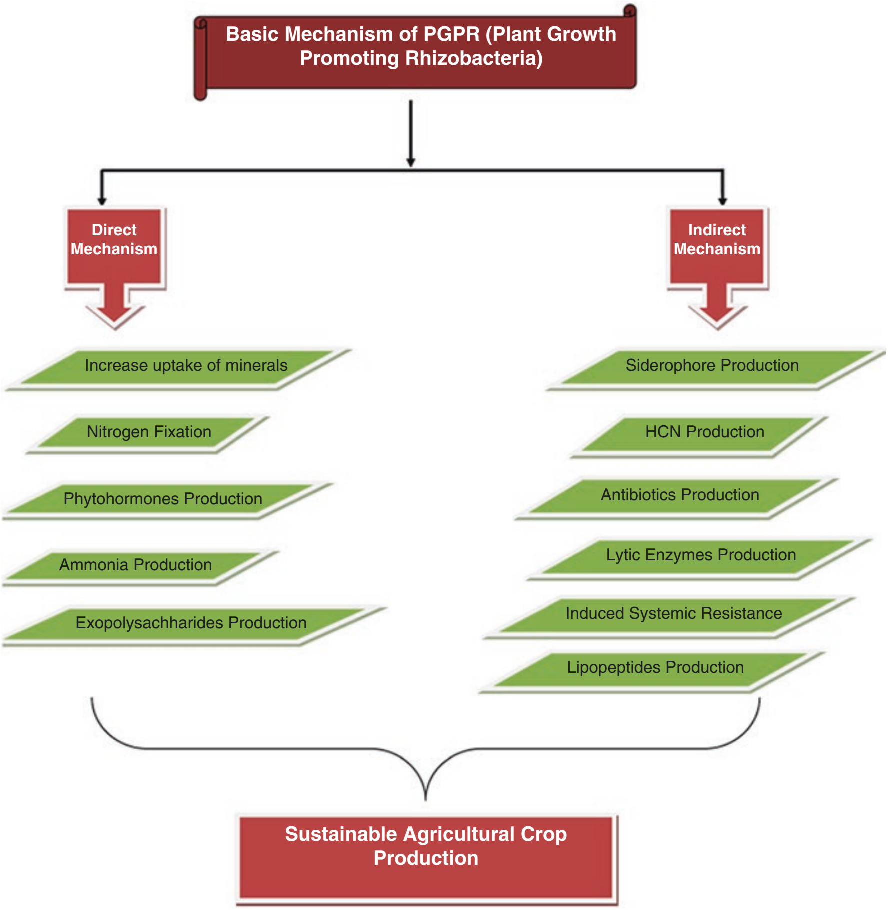

**Fig. 3.1** Direct and indirect mechanism of PGPR for plant growth promotions

are *Pseudomonadaceae*, *Burkholderiaceae*, and *Enterobacteriaceae*. Only the few taxa of bacteria related to the phyllosphere, having relatively large number of individuals. PGPR include bacteria mainly belonging to the genera *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Xanthomonas*, and *Serratia*. Some of these species are directly used as a biofertilizer such as rhizobial and *Azospirillum* strains due to their ability to fix nitrogen in the crop rhizosphere (Shridhar 2012; Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Priyadharsini and Muthukumar 2016; Jha and Subramanian 2016; Kumar et al. 2016a).

The PGPR bacteria are having close interaction with plant roots and exert significant effect on plant growth. They promote nitrogen fixation (Glick 1995) and availability of phosphorous and iron in the rhizosphere (Zahir et al. 2003; Vessey 2003; Kennedy et al. 2004; Welbaum et al. 2004). The diazotrophic bacteria result in the

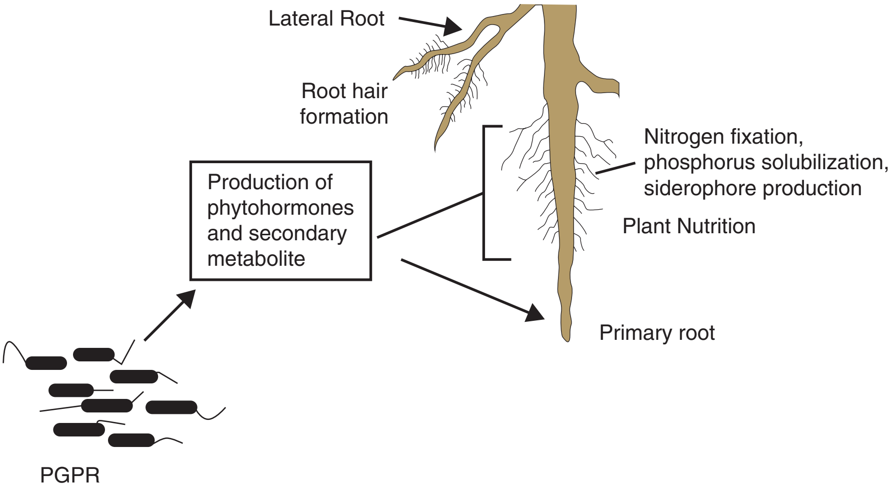

**Fig. 3.2** The possible mode of actions used by PGPR toward plant growth promotions (Modified from Bishnoi 2015)

rice growth promotions (Biswas et al. 2000). The species of *Pseudomonas* and *Bacillus* are chiefly in the solubilization of inorganic phosphate. The uptake of phosphorus can be increased significantly up to 13–23% in response to rhizobial inoculation of rice (Biswas et al. 2000). Toro et al. (1997) reported that tratment with *Enterobacter* sp., *Bacillus subtilis* and *Glomus intraradices* significantly enhanced vegetative biomass of onion as well as accumulation N ans P concnetration in plants. Also these PGPR microbes can create acidic environment to nutrient solubilization (Kumar et al. 2017; Meena et al. 2015a, b, f; Raghavendra et al. 2016; Zahedi 2016; Rawat et al. 2016; Yasin et al. 2016; Jaiswal et al. 2016).

### 3.3.2 Fungal Interactions

The term symbiosis which refers to living together was first introduced by Anton de Bary in 1979 (Begon et al. 1996). In broad sense, the symbiosis is categorized with the situations like parasitic to mutualistic. Two organisms are directly involved, or sometimes they formed interfaces termed as biotrophs. Fungi are involved in parasitic (e.g., rust fungi) as well mutualistic symbiosis (mycorrhizal fungi) with plants. Mutualism between plants and fungus is particularly found in their root. When these mycorrhizae inhabit the absorbing organs of plant like roots, rhizome, and thalli, they behave like dual organ of absorption (Trappe 1996).

Mycorrhizae are distinguished according their interaction with the plants such as endomycorrhizae, ectomycorrhizae, or ectendomycorrhizae. The commonly found mycorrhizae are arbuscular mycorrhizae (AM) and ectomycorrhizae (ECM). The arbuscular mycorrhiza (AM) belongs to the phylum Glomeromycota, and in symbiosis, it plays vital role in the nutrient uptake for the land as well as economically important plant species (Fellbaum et al. 2012). These symbionts are the primary site of the nutrient transfer (Harrison 1999). In the mutualistic symbiosis, both partners get benefitted like fungi obtaining their food (reduced carbon) and shelters (Leigh Jr 2010), while plants will acquire increased rate of photosynthesis and uptake of P and other immovable elements like Zn and Cu and other necessary nutrients. The photosynthetically fixed carbon obtained from host plants acts as a trigger for the N uptake and transport. In case of leguminous plants, their nitrogen-fixing capacity is increased. This symbiosis will also lead to the tolerance of salinity, drought and high soil temperature, maintenance of water balance, and increased rate of growth and development. There is also adoption of defense mechanism against soil- and root-borne pathogens (Azcón-Aguilar and Barea 1996) and also high tolerance to the heavy metal toxicity (Sarmantry et al. 1998; Meena et al. 2016c; Saha et al. 2016a; Yadav and Sidhu 2016; Das and Pradhan 2016; Dominguez-Nunez et al. 2016; Dotaniya et al. 2016).

There are two uptake pathway generally observed in the mycorrhizal plants like direct and mycorrhizal pathway. In the direct pathway, mycorrhizal plant has high affinity for the P and N transporters in the root nodules and root hair. In mycorrhizal pathway, nutrients are obtained by the translocation from extraradical mycelium (ERM) to the intraradical mycelium (IRM) mycorrhizal plants also having inducible P and N transporters in the periarbuscular membrane (Harrison et al. 2002; Guether et al. 2009). There is common depletion in nutrients of plants; therefore, it makes plant to depend on a fungus.

During the active plant growth, the root fungi, i.e., mycorrhizae, actually colonize into the living roots tissue by the integration into the physical structure of the root. The mycorrhization of the plant is one of the methods to make plant susceptible to increase plant defense (including production of fungal antibiotics) and increment in the nutrient uptake such as P, Zn, and Cu from the soil; in return fungi will get sugars and other nutrients. Because of mycorrhization, hyphae (i.e., long thread-like structure of fungi) are spread all over the nutrient depletion zone; indirectly, it will increase surface area of the root. These hyphae have an access to P and nutrient from the soil pores which is generally not available to plants. Direct attack of the plant pathogen is also prevented by the mycorrhiza which acts as physical barrier (Zeilinger et al. 2016).

### 3.3.3 Actinomycetes Interactions

Metal resistance of actinobacteria, including *Streptomycetes* sp., and their potential application for remediation has been recurrently considered in recent years (Haferburg et al. 2007; Benimeli et al. 2011; Schutze and Kothe 2012). Many researchers reported 16S ribosomal DNA analysis which revealed that one of the most abundant groups in heavy metal-contaminated soils is *Actinobacteria* (Gremion et al. 2003; Navarro-Noya et al. 2010). Schutze et al. (2014) isolated *Streptomyces mirabilis* P16B-1 from heavy metal-contaminated soil from uranium mining site in Ronneburg, Germany. This strain produced different types of siderophores such as ferrioxamines E, B, D, and G. Promotion of *Sorghum bicolor* growth (dry biomass) was recorded on metal-contaminated soil by inoculation with *Streptomyces mirabilis* P16B-1. Nickel-resistant *Streptomyces acidiscabies* E13 strain produced siderophores, and this strain is studied for *Vigna unguiculata* growth promotion in the presence and absence of the elicited siderophores. Results showed that hydroxamate types of siderophores produced by this strain promoted *Vigna unguiculata* growth under nickel contamination by binding iron and nickel; thus, iron played dual role, i.e., (1) iron is needed by plant for growth and (2) protection against nickel toxicity (Dimkpa et al. 2008). Three hydroxamate siderophores secreted by *Streptomyces tendae* F4 significantly promoted sunflower (*Helianthus annuus*) and enhanced uptake of Cd and Fe by the plant as compared to control (Dimkpa et al. 2009).

### 3.3.4 Cyanobacterial Interactions

The application of $N_2$-fixing cyanobacteria (as individual organism or in consortia) as a potential $N_2$-biofertilizer source in the field, especially paddy as an alternative to the commonly used organic fertilizers, can be of great value and is becoming popular day-by-day (Thajuddin and Subramanian 2005; Choudhary 2011; Prasanna et al. 2011; Hasan 2012; Sahu et al. 2012). Cyanobacteria were also used as biofertilizer in wheat crops (a major staple crop) (Abd-Alla et al. 1994), lettuce (Sukor 2013), and maize to enhance the nutrient use efficiency by increasing the nitrogenase activity. Cyanobacteria, being the producers of mucilage and enhancers of organic matter content in the soils, also help in the slow release process of the nutrients and minerals present in the soils or being absorbed by themselves and made available to the plants after the decay of their cells. The application of biofertilizers based on cyanobacterial inoculants enhanced microbial population through increased organic matter content and enzymatic activities (dehydrogenase and nitrogenase) (El-Gaml 2006). Phytohormones such as indole-3-acetic acid (IAA) and amino acids are also excreted by cyanobacteria which can also stimulate microbial growth in soil (Song et al. 2005; Karthikeyan et al. 2007). Cyanobacteria have the ability to sequester carbon which leads to a significant role in the carbon enrichment of soils (Jaiswal et al. 2010). Cyanobacteria can mineralize carbon dioxide by calcium carbonate precipitation, and this process offers strategies for point-source carbon capture and sequestration (Tabita and Colletti 1979; Jansson and Northen 2010).

## 3.4 Role of Biofortifications for Enhancement of Nutrients

The process to produce micronutrient-enriched staple food through mineral fertilizer, conventional breeding, and transgenic approaches is termed as biofortification (Murgia et al. 2012; Meena et al. 2013c, 2015d; Singh et al. 2015). Enrichment of nutritional value can be aided by the application of PGPR to breeding varieties which can improve yield and soil fertility (White and Broadley 2005). Biofortification is getting much attention to increase the availability of micronutrients especially Zn and Fe, in the major food crops (Stein 2010). Nutritional value can be increased by adding essential micronutrients and other health-promoting compounds to crops or foods (Meena et al. 2014b, 2015c; Sindhu et al. 2016; Singh et al. 2016). The routine diets or foods or the foods produced in high quantity always lack one of the essential nutrients, which ultimately lead to the hunger starvation of the nutrients. The major stable foods used by the world populations are rice, maize, and wheat, which hardly satisfy the complete nutrient supply (Fischer et al. 2014). There are various approaches to increase the nutritional value of food products like plant breeding and agronomic management practices. Other methods are also available like dietary diversification, mineral supplementation, and food fortification. However, among these biofortification strategies are less explored approach and need to be included in agronomic and breeding approaches. Hence, it is to be achieved by the treatments of microbial inoculants such as biofertilizer. In biofortification, the varieties having high nutrient vigor are to be selected with; these are crossbred with the high yielding plant to make the crops highly biofortified (Meena et al. 2014a, 2015e, 2016d; Saha et al. 2016b; Verma et al. 2014, 2015b; Sharma et al. 2016; Bahadur et al. 2016b).

The essential elements that are necessary for the normal growth of plants are categorized into macronutrients such as C, H, O, P, K, N, S, Ca, Fe, and Mg. There are certain categories into micronutrients such as Cu, B, Mn, and Zn. Some of the elements are needed by few plants such as Na, Cl, and I. Hence, the availability of these nutrients through the soil is very important. The bacteria present in the rhizosphere can easily make available nutrients to the plants by sequestration and mobilization (van Der Heijden et al. 2008). These essential elements are always present in the combined state or in the form of mineral ions or precipitates of soil biota. The acquisition of mineral elements with restricted mobility in the soil, such as P, K, Fe, Zn, and Cu, can be improved by developing a more extensive root system, with the application of biofertilizers (Rana et al. 2012a, b) in wheat crop. Another strategy employed by microorganisms for nutrient acquisition is the secretion of phytosiderophores by microorganisms and plants in drought- and salt-affected areas. This represents an efficient strategy for the uptake of micronutrients by plants from the rhizosphere (Dotaniya and Meena 2015; Verma et al. 2015a; Meena et al. 2013b, 2016e; Shrivastava et al. 2016; Velazquez et al. 2016; Bahadur et al. 2016a; Masood and Bano 2016; Teotia et al. 2016).

## 3.5 Current Perspectives of PGPR Toward Biofortifications

Many microorganisms play an important role in Se cycling in nature, and selenobacteria are also used for the bioremediation of selenium-contaminated soils (Lampis et al. 2009). Yasin et al. (2015) investigated the effect of two selenium-tolerant bacterial strains *Bacillus cereus*-YAP6 and *Bacillus licheniformis*-YAP7 on the growth and Se uptake by wheat plants. Inoculated Se-treated plants also showed increased stem Se, S, Ca, and Fe concentrations, by up to 375%, 40%, 55%, and 104%, respectively, and increased kernel Se, S, Ca, and Fe concentrations by up to 154, 85, 60, and 240%, respectively, compared to un-inoculated Se-treated plants. The consortia of *Anabaena oscillarioides* CR3, *Brevundimonas diminuta* PR7, and *Ochrobactrum anthropi* PR10 (T6) significantly increased nitrogen, phosphorus, and potassium (NPK) content and improved rice yield by 21.2% as well as enhancement of 13–16% in Fe, Zn, Cu, and Mn concentrations, respectively, in rice grains (Rana et al. 2015). Microorganisms such as *Bacillus*, *Providencia*, *Brevundimonas*, *Ochrobactrum*, *Azotobacter*, and *Anabaena* have been reported for enhancing the mineral density of wheat, rice, maize, and chickpea (Rana et al. 2012a, b, 2015; Khalid et al. 2015; Prasanna et al. 2015). Plant growth-promoting actinobacteria significantly increased seed mineral density such as Fe (10–38%), 17 for Zn (13–30%), 16 for Ca (14–26%), 9 for Cu (11–54%), and 10 for Mn (18–35%) and Mg (14–21%) of chickpea under field conditions (Sathya et al. 2016).

Gopalakrishnan et al. (2016) evaluated the effect of seven strains such as *Pseudomonas plecoglossicida SRI-156*, *Brevibacterium antiquum SRI-158*, *Bacillus altitudinis SRI-178*, *Enterobacter ludwigii SRI-211*, *E. ludwigii SRI-229*, *Acinetobacter tandoii SRI-305*, and *Pseudomonas monteilii SRI-360* for their PGP and biofortification traits in chickpea and pigeon pea under field conditions. Results showed that harvested grains contained iron (up to 18 and 12%), zinc (up to 23 and 5%), copper (up to 19 and 8%), manganese (up to 2 and 39%), and calcium (up to 22 and 11%) contents in chickpea and pigeon pea, respectively, which were found enhanced in test bacteria inoculated plots over the un-inoculated control plots.

## 3.6 Conclusions and Future Prospective

Balanced nutrition for the crop protection is the principal component of sustainable agriculture. Application of PGPR as a biofertilizers provides essential nutrients and increase tolerance and defense mechanisms against plant pathogen (Hafeez et al. 2013). Instead of harmful artificial fertilizer and pesticide, the eco-friendly approach with the beneficial microbes such as PGPR, mycorrhiza, actinomycetes, and cyanobacteria inspires plant growth and is cost-effective. Consortia of such microbes over single inoculum will be more effective to reduce the harmful stress of plant (Gupta et al. 2015). There is a need to further focus on the rhizosphere biology to achieve an integrated management of rhizosphere population and effect of environmental condition on PGPR and survival of their inoculum in adverse conditions (Gopalakrishnan et al. 2016). The classical approach (i.e., plant breeding, transgenic and field trials) along with modern technique such as recombinant DNA technology, mathematical modeling, and computer technology will take biocontrol research and technology to higher level (Gupta et al. 2015). To provide health benefits on the global scale needs collective effort in the molecular biology, plant breeding, and nutrition (Weale 2010).

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# Biological Nitrogen Fixation for Sustainable Agriculture

# 4

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## Abstract

Worldwide the increasing use of inorganic nitrogenous fertilizer becomes a threat to the environment and consequently to the mankind. It is high time to think about the alternate nitrogenous source for assuring sustainable agriculture. Biological nitrogen fixation (BNF), involving beneficial microorganism, is a process of converting the free atmospheric nitrogen into plant available form. This biological process harmonizes the ecosystem and offers an economic and environment-friendly approach for reducing the external inputs and improving internal sources. In nature a symbiotic relationship exists between most of the agriculturally important leguminous plants and beneficial microorganism, where bacteria can fix atmospheric free $N_2$ and provide it to the plant in available form in exchange of nutrition and shelter. These rhizobia (bacteria) dwell in the nodule present in the roots of leguminous plants. This type of symbiosis also exists between free-living microorganisms, viz., *Azolla* and *Anabaena*, and with rice plants in anaerobic condition. Therefore, BNF is considered as an important biological process for harnessing soil health as well as for assuring economic,

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environmental, and agronomic benefit. However, the necessity of inclusion of leguminous crop in the cropping system due to BNF concern is not getting popularize among the farming community particularly to the marginal and low-income group farmer due to lack of availability of specific rhizobia strain for specific crop, as well as socioecomic constraints. The present book chapter is focusing on the importance of BNF in agricultural system and the effectiveness of various legume species and their beneficial microsymbiont. The genetics, biochemistry behind the BNF, and the probable strategy for improving the $N_2$ fixation process are also getting concern for understanding these important biological phenomena.

**Keywords**
Biological nitrogen fixation • Nitrogenase • Legume-*Rhizobium* • *nif* gene

## Contents

## 4.1 Introduction

Many agriculturally important plants in the legume family can use nitrogen ($N_2$) from the atmosphere for their growth and development through biological nitrogen fixation (BNF). Legume BNF involves a symbiosis between legume plants and the rhizobia that live in nodules on their roots. There are economic, environmental, and agronomic benefits from using BNF in cropping systems (Anglade et al. 2015). The "Green Revolution" has been spurred by the development of high yielding varieties of cereal crops that responded favorably to high $N_2$ fertilization rates. The striking rise in cereal grain yields by the use of high yield varieties in developing countries between 1950 and 1990 is directly attributable to a tenfold increase in N fertilizer use (Socolow 1999). The demand for total fertilizer nutrients is estimated to grow at ~2% per annum globally between 2011 and 2015, while the demand particularly for N is forecasted to grow annually by 1.7% globally and by 2.6% in South Asia (FAO 2011).

Intensive cropping with the concomitant high application of N fertilizer in developed countries brings with it the substantial economic and environmental nitrogen pollution effects, viz., volatilization of nitrogen oxides (greenhouse gases) into the atmosphere, depletion of nonrenewable resources, an imbalance in the global N cycle, and leaching of $NO_3^-$ into groundwater. Thus, the excessive use of chemical fertilizers in agriculture has polluted the environment and is causing slow deterioration in soil health (Rao 2013; Singh and Ryan 2015). As the population continues to expand almost exponentially, there is an urgent need to consider novel ways of increasing food grain production that are compatible with sustainability and the retention of environmental quality (Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015, 2016b; Ahmad et al. 2016).

Next to plant photosynthesis, BNF is probably the most important biochemical reaction for life on earth. Through this process, certain organisms convert the inert dinitrogen ($N_2$) gas of the atmosphere to nitrogen-containing organic compounds that become available to all forms of life through the nitrogen cycle. Globally enormous amounts of nitrogen are fixed biologically each year. It has been estimated that ~$2.5 \times 10^{11}$ kg $NH_3$ is fixed annually from the atmosphere through BNF (by legumes and cyanobacteria) and ~$8 \times 10^{10}$ kg $NH_3$ are manufactured by ammonia industry (Cheng 2008). Globally, BNF in natural terrestrial ecosystems contributes ~$10^7$ Tg of nitrogen (1 Tg = 1 Mt), while marine N fixation contributes 121 Tg of nitrogen each year (Galloway et al. 2004).

The sustainable agriculture involves the successful management of agricultural resources to satisfy the changing human needs, while maintaining or enhancing the environmental quality and conserving natural resources. Consequently, sustainability considerations demand that alternative sources to the fertilizer nitrogen must be explored (Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Priyadharsini and Muthukumar 2016; Kumar et al. 2016a). The BNF can offer this alternative in farming practices as it uses the capacity of certain nitrogen-fixing bacteria (NFB) to convert atmospheric nitrogen into the plant usable, ammonia (Rao 2014; Meena et al. 2017). The use of nitrogen-fixing species in cropping systems reduces the dependency of agricultural crops on fossil fuel-derived nitrogenous fertilizers and increases soil sustainability. Additionally, biologically fixed nitrogen is bound in soil organic matter and thus is much less susceptible to soil chemical transformations and physical factors that lead to volatilization and leaching. In that way, BNF may have an important role in sustaining productivity of soils

(Kumar et al. 2017; Meena et al. 2015a, b; Raghavendra et al. 2016; Zahedi 2016; Jaiswal et al. 2016; Jha and Subramanian 2016).

Some prokaryotes – a few bacteria and cyanobacteria – have acquired the ability to reduce dinitrogen from the atmosphere to ammonical form using the bacterial enzyme nitrogenase and furnish this essential nutrient into agricultural soils. A variety of NFB have been used in soil inoculations intended to improve the supply of fixed nitrogen as nutrients to crop plants. In symbiotic system, *Rhizobium* species have been successfully used worldwide as a bioinoculant leading to effective establishment of $N_2$-fixing symbiosis with leguminous crop plants (Rawat et al. 2016; Yasin et al. 2016; Meena et al. 2016c, f; Saha et al. 2016a).

The BNF also occurs in the free-living state or in association with plants where the bacteria do not live in a direct symbiotic association with vascular plants. A number of genera have been identified as free-living as well as associative NFB which are capable of fixing atmospheric N into a plant available form (Reed et al. 2011). The most well known include *Azotobacter*, *Beijerinckia*, *Clostridium*, *Azospirillum*, *Bacillus*, and *Klebsiella* sp. which are being inoculated with the aim of enhancing plant productivity, and their fixation rates of up to 60 kg N $ha^{-1}$ $year^{-1}$ have been recorded (Orr et al. 2011).

## 4.2 Diversity Among Nitrogen-Fixing Bacterial (NFB) Groups

The diazotrophic bacteria are widely distributed in nature. Bacteria containing nitrogenase enzyme complex occupy an indispensable ecological niche, supplying fixed nitrogen to the global nitrogen cycle. Due to this inceptive role in the nitrogen cycle, diazotrophs are present in virtually all ecosystems, with representatives in environments as varied as aerobic soils (e.g., *Azotobacter* sp.), the ocean surface layer (*Trichodesmium*), and specialized nodules in legume roots (*Rhizobium*). The confirmed free-living $N_2$-fixing organisms belong to the kingdoms Eubacteria and archaebacteria (Table 4.1). Nitrogen fixation generally occurs only under anaerobic or microaerophilic conditions. Many $N_2$-fixing bacteria are heterotrophic and need a supply of reduced carbon, e.g., *Azotobacter* and *Azospirillum*. A few, such as *Bradyrhizobium japonicum* and *Arthrobacter fluorescens* can grow autotrophically on hydrogen and carbon dioxide. Most diazotrophs reduce nitrogen at 30–35 °C; however, the thermophilic genera of archaebacteria are known to have $N_2$-fixing ability even at 65 °C, e.g., *Methanococcus* and *Methanosarcina* (Yadav and Sidhu 2016; Meena et al. 2016d; Saha et al. 2016b; Verma et al. 2014, 2015b; Das and Pradhan 2016; Dominguez-Nunez et al. 2016; Dotaniya et al. 2016).

Beijerinck discovered the aerobic bacterium *Azotobacter chroococcum in the year 1901*, which was capable of fixing molecular nitrogen. Significant increases in crop yields after inoculation with *Azotobacter* were attributed to $N_2$ fixation. The members of the *Azotobacteraceae* have evolved several mechanisms of protection of nitrogenase from oxygen while growing in an aerobic environment. Facultative anaerobes like *Klebsiella* can grow in both aerobic and anaerobic conditions, but

**Table 4.1** List of genera of microbes, which include free-living, $N_2$-fixing species or strains

| Microbes | Genus or type | Species (examples only) |
|---|---|---|
| Aerobes | *Azotobacter* | *A. chroococcum*, *A. vinelandii* |
| | *Azotococcus* | *A. agilis* |
| | *Azomonas* | *A. macrocytogens* |
| | *Beijerinckia* | *B. indica*, *B. fluminis* |
| | *Derxia* | *D. gummosa* |
| | *Pseudomonas* | *P. stutzeri*, *P. saccharophila* |
| | *Azoarcus* | *A. communis*, *A. indigens* |
| | *Acetobacter* | *A. diazotrophicus* |
| Facultative anaerobes | *Klebsiella* | *K. pneumoniae*, *K. oxytoca* |
| | *Bacillus* | *B. polymyxa*, *B. macerans* |
| | *Enterobacter* | *E. agglomerans* (*Erwinia herbicola*) |
| | *Citrobacter* | *C. freundii* |
| | *Escherichia* | *E. intermedia* |
| | *Propionibacterium* | *P. shermanii*, *P. petersonii* |
| Microaerobes | *Xanthobacter* | *X. flavus*, *X. autotrophicus* |
| | *Thiobacillus* | *T. ferrooxidans* |
| | *Azospirillum* | *A. lipoferum*, *A. brasiliense* |
| | *Aquaspirillum* | *A. peregrinum*, *A. fasciculus* |
| | *Methylosinus* | *M. trichosporium* |
| Obligate anaerobes | *Clostridium* | *C. pasteurianum*, *C. butyricum* |
| | *Desulfovibrio* | *D. vulgaris*, *D. desulfuricans* |
| | *Methanosarcina* | *M. barkeri* |
| Phototrophs | | |
| 1. Cyanobacteria | | |
| Aerobes | *Anabaena* | *A. cylindrica*, *A. inaequalis* |
| | *Nostoc* | *N. muscorum* |
| | *Calothrix* | |
| | Gloeothece | *G. alpicola* |
| Microaerobes | *Plectonema* | *P. boryanum* |
| | *Lyngbya* | *L. aestuarii* |
| | *Oscillatoria* | |
| | *Spirulina* | |
| 2. Bacteria | | |
| Facultative anaerobes | *Rhodospirillum* | *R. rubrum* |
| | *Rhodopseudomonas* | *R. palustris* |
| Obligate anaerobes | *Chromatium* | *C. vinosum* |
| | *Chlorobium* | *C. limicola* |
| | *Thiopedia* | |
| | *Ectothiospira* | *E. shapovnikovii* |

Modified after Orr et al. (2011) and Shridhar (2012)

they need anaerobic environment for nitrogen fixation. However, rhizosphere-based diazotrophic bacteria tend to retain the products of nitrogen fixation for their own use, and any benefit to the crop is only realized after the bacteria die (Bruijn 2015).

### 4.2.1 Blue-Green Algae (Cyanobacteria)

Cyanobacteria can fix nitrogen in both aerobic and anaerobic conditions. The aerobic $N_2$-fixing cyanobacteria have to cope not only with external oxygen but also with that generated intracellularly by the operation of photosystem II (PS II). The remarkable way of protection of the $N_2$ fixation machinery against oxygen is the differentiation of specialized cells called heterocysts which have anaerobic environment (Wolk et al. 1994). In the course of differentiation process, heterocysts acquire supplemental envelope layers, lose activity of PS II and of photosynthetic $CO_2$ fixation, and acquire specific hydrogenases and oxidases that contribute to the generation of ATP by the demanding diazotrophic metabolism.

### 4.2.2 Lichens

There are some blue-green algae (BGA) which exist in association with fungi, liverworts, ferns, and flowering plants. Some of them fix atmospheric nitrogen. The alga-fungus association to form lichens occurring on soils, rocks, and tree crops is yet another instance of symbiosis wherein the genus *Nostoc*, *Calothrix*, or other unidentified blue-green algae fix nitrogen and, in turn, obtain protection and space from fungal partner. The ability of lichens to fix nitrogen has been proven by the use of $^{15}N$ in genera of lichens such as *Collema*, *Stereocaulon*, *Leptogium*, *Lichina*, and *Peltigera*. Recently, the list of lichens capable of fixing nitrogen has been expanded to include *Lobaria*, *Massalongia*, *Nephroma*, *Pannaria*, *Parmeliella*, *Placopsis*, *Placynthium*, *Polychidium*, and *Sticta* which reduce acetylene to ethylene and thus demonstrate nitrogenase activity (Seneviratne and Indrasena 2006).

### 4.2.3 Associative $N_2$ Fixers

Endophytic bacteria that manage to enter and colonize the inside of roots may have an advantage over surface-associated diazotrophs in that they do not suffer from heavy competition with other microorganisms for nutritional sources. Moreover, bacteria located within roots and xylem vessels are likely to be growing in low concentrations of oxygen that are necessary for the expression and functioning of nitrogenase (James et al. 1994). A variety of endophytic NFB have been found to colonize the interior roots of rice, maize, and grass (Barraquio et al. 1997) and are capable of contributing directly to the nitrogen requirement in sugarcane, rice, and wheat (Webster et al. 1997). *Azospirillum* was discovered by Beizerinck in the year 1925 as free-living N-fixing bacterium, to which the name *Spirillum lipoferum* was given initially. Subsequently, *Azospirillum* was found to have association with growing root system of a variety of crop plants (Okon and Hzigsohn 1995).

Some other examples of beneficial endophytic diazotrophs are *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azoarcus* sp., some traits of *Burkholderia* sp., and *Enterobacter asburiae.* The diazotrophs *Herbaspirillum seropedicae* have been reported to be endophytic in 13 members of the Gramineae, particularly within roots as well as in stems and leaves of rice, maize, and sugarcane (Kirchhof et al. 1997). A saccharophilic bacterium *Acetobacter diazotrophicus* has been reported as the main contributor of endophytic BNF in sugarcane and sweet sorghum (Dobereiner et al. 1993). *Gluconacetobacter diazotrophicus* (formerly *Acetobacter*) has also been found in endophytic association with *Coffea arabica* (Jimenez-Salgado et al. 1997) and *Ananas comosus* (Tapia-Hernandez et al. 2000), all of which were cultivated in very distant geographical regions.

The first *Azoarcus* species, *Azoarcus indigens* and *A. communis*, were described in association with Kallar grass (*Leptochloa fusca*) cultivated in Pakistan (Reinhold-Hurek and Hurek 1998), and recently Hurek et al. (1994) showed that endophyte *Azoarcus* strain BH72 has the ability to invade and colonize rice roots. Other NFB such as *Rhizobium leguminosarum* bv. *trifolii*, *Azorhizobium caulinodans*, and *Pseudomonas stutzeri* strain A15 have also been found in endophytic association with field-grown rice (Yanni et al. 1997; Rediers et al. 2003).

### 4.2.4 *Rhizobium*-Legume Symbiosis

*Rhizobium*-legume symbiotic relationship is of vital agricultural importance. Specific *Rhizobia* have the ability to infect the plant roots and form nodules in them. It is in these nodules that atmospheric nitrogen is reduced to ammonia. The ammonia is further converted to L-ketoglutamic acid and finally to glutamic acid. However, there is a specific association between the *Rhizobium* species and the legume species. It may be pertinent to mention here that there are 20 species of *Rhizobium*. Most of the nodule-fixed nitrogen is utilized by the plants themselves. Four important $N_2$-fixing associations, i.e., *Rhizobium*-leguminous plants, *Frankia*-actinorhizal plants, *Anabaena-Azolla* symbiosis, and lichen symbiosis involving cyanobacteria, have been studied in great detail. Due to these symbiotic associations, plants are generally able to grow in nitrogen-poor soils. Most of the characterized rhizobial strains had been derived from the limited range of cultivated legume species (Brewin 2002). All these rhizobial isolates belong to three distinct branches within the alpha-2 subgroup of Proteobacteria. In each case, rhizobia are phylogenetically intertwined with nonsymbiotic bacteria (Meena et al. 2013b, 2014a, 2015e, Sharma et al. 2016; Verma et al. 2015a; Teotia et al. 2016; Bahadur et al. 2016b).

The largest branch includes the genus *Rhizobium*, which nodulates peas and clovers, and *Sinorhizobium*, which nodulates alfalfa (Lucerne) and closely related to *Agrobacterium* and to *Brucella*. A second branch includes the genus *Bradyrhizobium*, with species that nodulate soybean, lupin, and many tropical legumes which is closely related to *Rhodopseudomonas* (Stepkowski et al. 2007, 2011). The third group includes *Azorhizobium*, which is closely related to the chemoautotroph *Xanthobacter*. Two completely new rhizobial groupings have been identified recently. *Methylobacterium nodulans*, isolated from *Crotalaria* nodules, represents a fourth class of alpha-2 subgroup of Proteobacteria, while bacteria isolated from *Aspalathus* nodules were found to belong to the genus *Burkholderia*, a member of the distant beta-subclass of Proteobacteria (Brewin 2002).

### 4.2.5 $N_2$-Fixing Nonlegumes

$N_2$-fixing ability is important in nonleguminous crops such as cereals which have long been a major goal of workers in the field of biological nitrogen fixation. Some of those microbes so far identified in nonlegumes include *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) from sugarcane (Riggs et al. 2001; Muthukumarasamy et al. 2002). Strains of *G. diazotrophicus* have also been isolated from roots and stems of coffee (Jimenez-Salgado et al. 1997). *Azospirillum* is another much studied diazotroph, especially the species *lipoferum* and *brasilense*, which have been shown to infect a number of cereal plants including wheat, maize, and sorghum (Malik et al. 1997; Weber et al. 1999; Dobbelaere et al. 2001). Other known diazotrophs include *Herbaspirillum seropedicae* (Weber et al. 1999; Riggs et al. 2001), *Klebsiella pneumoniae* and *Pantoea agglomerans*, *Enterobacter* sp., *Klebsiella oxytoca*, *Azotobacter*, *Arthrobacter*, *Azoarcus*, *Bacillus*, and *Zoogloea* (Santi et al. 2013; Shrivastava et al. 2016; Velazquez et al. 2016; Meena et al. 2014b, 2015c; Sindhu et al. 2016).

In agroforestry systems, both leguminous and nonleguminous trees and shrubs fix considerable amount of nitrogen. *Mimosa* and *Acacia* species are examples of such leguminous plants. In rice-based cropping system, *Gliricidia sepium*, *Leucaena leucocephala*, and *Sesbania bispinosa* are used as green leaf manures. Some nonlegumes, as those belonging to genus *Alnus* (Betulaceae), *Myrica* and *Umptonia* (Myricaceae), *Casuarina* (Casuarinaceae), etc., have root nodules and fix $N_2$ the same way as do legumes. Phyllospheric fixation of nitrogen done by *Frankia,* actinomycetes, is also seen in these plants (Santi et al. 2013).

### 4.2.6 Stem-Nodulating Legumes

There are two genera of legumes which are known to have nodules on stem – *Aeschynomene* and *Sesbania*. Of the 150–250 species of *Aeschynomene*, nodules on stem have been reported in *A. indica*, *A. aspera*, *A. elaphroxylon*, *A. villosa*, *A. evenia*, *A. paniculata*, and *A. afaspera*. These reports have come from India, Mali, Ghana, Venezuela, Brazil, the USA, Japan, Zimbabwe, Java, South Africa, Zambia, South America, Puerto Rico, and Argentina. In India, *A. indica* and *A. aspera* occur widely in waterlogged situations. The genus *Sesbania* has 170 species which are annual as well as perennial and grow in warmer regions of both the hemisphere. *S. rostrata* is an annual plant which bears nodules on stems as well as on roots. There are other green manure and green leaf manure crops which have been used in rice cultivation by several generations of rice farmers in India and other countries. In India, species of *Crotalaria*, *Indigofera*, *Lathyrus*, *Pongamia*, *Tephrosia*, *Trifolium*, and *Sesbania* have been used for a long time. The high N content of these crops and their ability to get easily degraded in wet rice soils of Asia are the main criteria for adopting a given plant species as a suitable green manure candidate of choice. *Astragalus sinicus*, a species widely used in China, has 108 kg N $ha^{-1}$, whereas *S. rostrata* contains 267 kg $ha^{-1}$. The commonly used Indian green manure species, *Sesbania aculeata*, has ~96–122 kg $N_2$ ha$^{-1}$. *Sesbania rostrata* could fix 83–109 kg $N_2$ ha$^{-1}$, whereas *S. sesban* could fix only 7–18 kg $N_2$ ha$^{-1}$ under similar conditions of growth reflecting the superiority of the stem-nodulating legume (Singh et al. 2015, 2016; Meena et al. 2015d, 2016e; Masood and Bano 2016).

## 4.3 Contribution of Fixed Nitrogen by Various Bacteria to Cereal and Legume Crops

The first free-living bacteria (nonsymbiotic bacteria) capable of fixing atmospheric $N_2$ were *Clostridium pasteurianum* discovered in 1890 by the Russian microbiologist Sergei Winogradsky (Dworkin and David, 202) followed by discovery of *Azotobacter chroococcum*, a free-living aerobic $N_2$ fixer in 1901 by Martinus Beijerinck. This discovery had created breakthrough in this area and resulted in discovery of many more microorganisms. The mechanism behind $N_2$ fixation both in the case of free living and symbiont involving the same biochemical pathway and the capability of fixing N by symbionts are much more than the free-living organism. It is not that they are inherently incapable of vigorous fixation, but abundant substrates to support their growth and fixation are commonly lacking in the soil (Bahadur et al. 2017; Verma et al. 2017b; Kumar et al. 2017).

In case of free-living bacteria like *Azotobacter vinelandii* when cultured in in vitro condition supported with carbon sources and other growth regulators can fix more amount of $N_2$ in comparison with free-living state. However, in case of symbionts microorganism, the growth regulators and other beneficial substrates are provided by the host plant therefore facilitating vigorous $N_2$ fixation. This finding was also supported by the study of Burris (2004). The free-living bacteria, including *Azotobacter*, *Beijerinckia*, *Klebsiella*, *Pseudomonas*, and enterobacters are of great significance in the gradual accumulation of fixed nitrogen in undisturbed environments and contribute a significant input of nitrogen to plants (Meena et al. 2013c; Bahadur et al. 2016a).

Potential of the root-associative *Azospirillum* or *Azotobacter paspali* is mainly judged from the gain in crop productivity when these bacteria are colonized in the crop rhizosphere. These microbes have the potential to fix atmospheric nitrogen up to the level of 15–36 kg N ha$^{-1}$ year$^{-1}$ (Table 4.2). N-balance studies indicated that endophytic *Acetobacter diazotrophicus* contribute as high as 150–200 kg N ha$^{-1}$ year$^{-1}$ in sugarcane (Dobereiner et al. 1993).

Legume-*Rhizobium* symbiosis is the most extensively studied nitrogen-fixing system. On the global basis, this association reduces ~70–80% of the total of ~$17.2 \times 10^7$ tons biologically fixed nitrogen per year (Ishizuka 1992). The symbiotic rhizobia have been found to fix nitrogen ranging from 57 to 600 kg N ha$^{-1}$ year$^{-1}$ (Elkan 1992). The effectiveness of different legume species and their microsymbionts have been found variable (Table 4.2). In general, faba bean (*Vicia faba*) and pigeon pea (*Cajanus cajan*) have been found to be very efficient; soybean (*Glycine max*), groundnut (*Arachis hypogaea*), and cowpea (*Vigna unguiculata*) to be

**Table 4.2** Estimated average rate of biological nitrogen fixation for specific organisms and associations

| Organism or system | Dinitrogen fixed (kg ha$^{-1}$ yearr$^{-1}$) |
|---|---|
| Free-living microorganisms | |
| Cyanobacteria (blue-green algae) | 7–80 |
| *Azotobacter* | 0.3–15 |
| *Clostridium pasteurianum* | 0.1–0.5 |
| Grass-bacteria associative symbioses | |
| *Azospirillum*, *Azotobacter paspali* | 15–36 |
| *Acetobacter diazotrophicus* | 150–200 |
| Plant-cyanobacterial associations | |
| *Gunnera* | 12–21 |
| *Azolla-Anabaena* | 45–450 |
| Lichens (fungi + BGA) | 39–84 |
| *Rhizobium*-legume symbiosis | |
| Soybeans (*Glycine max* L. Merr.) | 57–94 |
| Cowpea (*Vigna, Phaseolus*, and others) | 84 |
| Clover (*Trifolium hybridum* L.) | 104–160 |
| Alfalfa (*Medicago sativa* L.) | 128–600 |
| Lupines (*Lupinus sp.*) | 150–169 |
| Nodulated nonlegumes | |
| *Alnus* (alders, e.g., red and black alders) | 40–300 |
| *Hippophae* (sea buckthorn) | 2–179 |
| *Coriaria* ("tutu" in New Zealand) | 60–150 |
| *Casuarina* (Australian pine) | 58 |

average; and common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) poor in fixing atmospheric nitrogen (Hardarson 1993).

The *Azolla-Anabaena* symbiotic system has been reported to contribute 45–450 kg N ha$^{-1}$, and *Frankia*-actinorhizal symbiosis provides 2–362 kg N ha$^{-1}$ (Elkan 1992). Symbiotically fixed nitrogen also becomes available to an intercrop or subsequent crop, and, therefore, ~ 50% of the crops grown in Africa, India, and Latin America are either intercropped or rotated with nitrogen-fixing species. Thus nitrogen supplied to crops by biological nitrogen fixation reduces our dependence on nitrogenous fertilizers and also builds up soil fertility for succeeding crops. Inoculation of legumes with effective strains of rhizobia has also often resulted in significant increases in yields of various legume crops (Thies et al. 1991).

Several experiments conducted in temperate regions of the world show that nitrogen fixation in *Azotobacter* inoculated soils is not more than 10 to 15 kg of N ha$^{-1}$ year$^{-1}$, depending on the availability of carbon sources. Bacterial preparations containing *Azotobacter* cells under the name azotobacterin were being produced and used in the erstwhile USSR and East European countries such as Czechoslovakia, Rumania, Poland, GDR, Bulgaria, and Hungary where bacterization of seeds with azotobacterin has proven beneficial in increasing yields of crops such as wheat, barley, maize, sugar beet, carrot, cabbage, and potato. The increase in yield of field crops was not more than 12% over corresponding uninoculated controls.

The studies done at IRRI further point out that submerged soils have a greater capacity to fix atmospheric nitrogen than non-submerged soil. The amounts of nitrogen fixed in 1 ha area in dry season of the year is approximately 79.8 kg $ha^{-1}$ in planted, submerged fields; 42.5 kg $ha^{-1}$ in unplanted, submerged fields; 5.4 kg $ha^{-1}$ in planted, upland fields; and 2.7 kg $ha^{-1}$ in the unplanted, upland fields. In the field, most nitrogen is fixed in soils during the reproductive and ripening phases of the growth of rice plants (Subba Rao 2009).

A variety of endophytic $N_2$-fixing bacteria have already been found that colonize the interior roots of rice, maize, and grass (Barraquio et al. 1997) and are capable of contributing directly to the $N_2$ requirement in rice and wheat (Webster et al. 1997). Nitrogen balance studies showed that BNF in wetland rice fields could yield up to 50 kg N $ha^{-1}$ $yr.^{-1}$ (Roger and Ladha 1992). The responses to inoculation with $N_2$-fixing bacteria have been found to vary with crops, cultivars, locations, seasons, agronomic practices, bacterial strains, levels of soil fertility, and interactions with native soil microflora.

The algal biofertilizer is an easily manageable input forming a perpetually self-generating system, which adds to the nutrient status as well as health of the soil. In addition to contributing 20–25 kg N $ha^{-1}$ $season^{-1}$, they add organic matter to soil, excrete growth-promoting substances, and increase the fertilizer-utilizing efficiency of the crop plants. Cyanobacterial inoculation in rice fields could improve the nitrogen nutrition of crops, and ~25–30 kg N $ha^{-1}$ $season^{-1}$ has frequently been observed.

Coinoculation of *Azotobacter vinelandii* and *Rhizobium* sp. has been found to increase the number of nodules on the roots of soybean, pea, and clover. The coinoculation of *Pseudomonas* and *Bacillus* spp. with *Rhizobium/Bradyrhizobium* has been found to increase nodule number, nitrogen fixation, and plant biomass of green gram, chickpea, and other legume crops (Sindhu et al. 2002b; Goel et al. 2000). Therefore, coinoculation of diazotrophic bacteria could be exploited to enhance nitrogen fixation in the rhizosphere of cereals as well as legumes.

## 4.4 Environmental Factors Affecting Nitrogen Fixation

Various soil environmental factors have been found to affect nitrogen fixation which includes temperature, moisture, acidity, available nitrogen, phosphorus, calcium, and molybdenum content.

(i) *Combined forms of nitrogen*

When ammonium salts, nitrate, or other combined forms of nitrogen are present in culture or in soil, the bacteria or algae preferentially use the ammonium salts than molecular nitrogen from the atmosphere. Thus, urea or nitrates are most effective in inhibition of nitrogen fixation in agriculture, but amino acids have less marked deterring influence. When the concentration of ammonium or nitrate falls to a low concentration through microbial assimilation of the element, nitrogen fixation and its utilization resumes (Shridhar 2012).

(ii) *Availability of energy sources*

Energy source is a major factor limiting the rate of nitrogen fixation and the extent of nitrogen assimilation by heterotrophic populations. Thus, addition of simple sugars, cellulose straw, or plant residues with wide C/N ratios often markedly enhances the transformation either in aerobic or anaerobic conditions.

(iii) *Inorganic nutrients availability*

Some inorganic nutrients such as molybdenum, iron, calcium, and cobalt are indispensable for $N_2$ fixation and proliferation of microorganisms. Molybdenum is required for $N_2$ fixation; microorganisms will not use nitrate unless molybdate is present. Growth on ammonium salts, however, proceeds rapidly in the absence of added molybdenum. For some microorganisms, vanadium could replace molybdenum, but it is never fully effective.

Similarly, iron salts are implicating the nitrogen metabolism of *Azotobacter*, *Clostridium*, algae, and *Klebsiella*. A requirement of Ca has been demonstrated during nitrogen assimilation by cyanobacteria and some species of *Azotobacter*, but the calcium can sometimes be replaced by strontium. Similarly organisms making use of $N_2$ must have cobalt (Co) available to them, although a lesser concentration of this element may be required for growth on combined nitrogen. A role of Co in the $N_2$ fixation process has been established for *Azotobacter*, *Beijerinckia*, clostridia, and several algal genera (Weisany et al. 2013).

(iv) *Soil pH*

Prevailing pH has a profound influence on the abundance of these $N_2$-fixing organisms. For example, *Azotobacter* is characteristically sensitive to high hydrogen ion concentrations. Ecological investigations showed that many soils contain none or insignificant number of *Azotobacters*. Their abundance is associated directly with pH. In the environments having pH less than 6.0, very few *Azotobacter* cells are found. Similarly, the bacteria generally will neither grow nor fix $N_2$ in the culture media having a pH below 6.0, but an occasional variant will tolerate greater hydrogen ion concentrations. *Beijerinckia* sp. could grow and fix $N_2$ from pH 3.0 to 9.0. BGA develop poorly in media and are sparse in soils having pH less than 6.0. The acid tolerance of *Clostridium* falls between *Azotobacter* and *Beijerinckia* (Narula and Vasudeva 2007).

(v) *Moisture*

The rate of nitrogen fixation is frequently determined by soil moisture. The rate and magnitude of $N_2$ fixation process increases as moisture becomes abundant. Sometimes the activity is especially great at or near field capacity. The changes associated with excessive moisture are intimately linked with shift from aerobiosis to anaerobiosis, and the $N_2$ fixation reaction sequence is affected by the $O_2$ status of the environment. It has also been proposed that waterlogging promotes $N_2$ gains because complex organic nutrients are decomposed to simple products, either in the aerobic top portion of the flooded soil or in the underlying anaerobic zone, and the simple products are then diffuse to the adjacent microhabitat where these are metabolized by the $N_2$ fixers.

(vi) *Temperature*

Temperature also has a profound influence of $N_2$ metabolism. Little activity is observed at low temperature, and warming promotes the microbial $N_2$ fixation and uptake of the fixed gas. In some regions of the northern temperate zone, $N_2$ fixation occurs even during winter. This $N_2$ fixation results from indigenous algae or lichens containing blue-green algal symbionts. These photosynthetic microorganisms or symbioses may still be active during parts of winter when the temperature is somewhat below 0 °C (Belnap 2001).

## 4.5 Biochemistry, Genetics, and Regulation of Nitrogen Fixation

### 4.5.1 Biochemistry of Nitrogen Fixation

Biological nitrogen fixation is an energy-driven complex phenomenon where $N_2$ is reduced into $NH_3$. This process basically acts as an entry of free atmospheric $N_2$ into the biosphere, thereby reducing the loss through denitrification. In the nitrogen fixation, basically the dinitrogen, i.e., inert "dihydrogen," or nitrogen gas are converted into usable ammonia form. This process requires high energy input because the triple bond of atmospheric $N_2$ is stable and therefore give the molecule a stable and inert property. Therefore, the free $N_2$ does not combine with other elements easily. However, breaking the triple bond to generate the usable form, i.e., ammonia, requires a series of reduction steps involving high input of energy. The most common process for ambient nitrogen reduction is the Haber process, invented in 1910. The Haber process involves high pressure, high temperatures, and possibly an iron or ruthenium catalyst to produce $NH_3$. Biological nitrogen fixation is run by a microbial enzyme nitrogenase which is a complex metalloenzymes and causes breakdown of covalent bond of free $N_2$ through reduction process. The nitrogenase complex has multiple redox centers due to presence of two proteins. The first part, a reductase, provides electrons, while the second part, nitrogenase, uses these electrons to turn nitrogen into ammonia. The transferring of electrons, from reductase to nitrogenase, in this process is coupled with the hydrolysis of ATP by the reductase. Therefore, reduction of nitrogen to ammonia is an exothermic reaction, yet it requires energy in the form of ATP due to high activation energy (Brill 1980). In this process the Fe protein delivers one electron at a time to MoFe protein which requires the hydrolysis of two MgATP molecules (Seefeldt and Dean 1997). Two MgATP molecules are bound to the reduced form of Fe protein. The reaction stoichiometry (known for only Mo-nitrogenase) for the conversion of nitrogen to ammonia by the nitrogenase enzyme is as follows:

In biological $N_2$ fixation, the $N_2$-fixing microorganisms, the eight high-potential electrons, come from reduced ferredoxin, generated by photosynthesis or oxidative processes. Two molecules of ATP are hydrolyzed for each molecule of $N_2$ reduced.

$$N_2 + 8e^- + 8H^+ + 16\,ATP \rightleftarrows 2NH_3 + H_2 + 16\,ADP + 16P_i$$

Under ideal condition this process is driven by energy from hydrolysis of 16 molecules of ATP. However, under native ecosystems, the energy source requirement generally varies between 20 and 30 molecules of MgATP because the process is less efficient under natural conditions than when observed under optimum laboratory conditions. The ATP molecules supporting nitrogen fixation are derived primarily either directly from photosynthetic processes in $N_2$-fixing photoautotrophs or from decomposition of organic compounds in nitrogen-fixing heterotrophs, supported indirectly from photosynthetic processes.

Conformation of dinitrogenase reductase (Fe protein) is altered upon ATP binding which lowers its reduction potential and permits it to interact with dinitrogenase (Brill 1980). For this purpose the Fe protein complex binds with the ATP molecule. In this state it forms a transient association with αβ unit of MoFe protein. Two MgATP molecules are hydrolyzed into two MgADP molecules during the association event, and a single electron is transferred from the 4Fe-4S cluster of Fe protein to MoFe protein. The oxidized Fe protein ($[4Fe\text{-}4S]^{2+}$) with MgADP bound to it and then dissociates from the MoFe protein. The release of Fe protein is the rate-limiting step for nitrogenase catalysis (Fig. 4.1).

The reduction process of free atmospheric $N_2$ by nitrogenase enzyme requires two or more electrons; thereby, the reaction is needed to be repeated several times for conversion of $N_2$ into $NH_3$, and during the electron transfer, it reacts with $H^+$ and produces hydrogen. Besides hydrogen and nitrogen, many low molecular weight compounds can also be reduced to ammonium (Swain and Abhijita 2013).

### 4.5.2 Nitrogenase Enzyme Complex

The nitrogenase enzyme system is widely distributed among Bacteria and the Archaea, but no eukaryotic system has been shown to contain a nitrogenase. Till date nearly four different classes of nitrogenase have been identified; among them three are almost similar features only having different heterometal atom (Mo, V, Fe) present in the active site of the metal cluster of protein 1 (Eady et al. 1978). Therefore, nitrogenase-I, nitrogenase-II, and nitrogenase-III are known as molybdenum (Mo)-dependent nitrogenase, vanadium (V)-dependent nitrogenase, and iron (Fe)-dependent nitrogenase, respectively. The Mo-nitrogenase is the principal one and abundantly distributed, and in case of Mo-limiting condition, V-nitrogenase and Fe-nitrogenase are synthesized. The vanadium-nitrogenase is encoded by the vnf regulon, and the iron-only nitrogenase is encoded by the anf regulon. The fourth

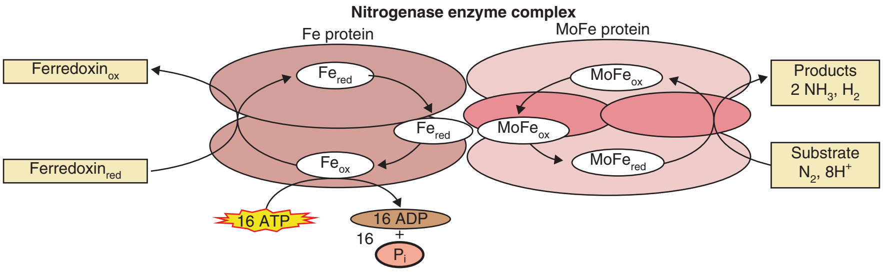

**Fig. 4.1** Schematic representation of the electron flow in nitrogen fixation (Source: Taiz and Zeiger 2006)

class isolated from *Streptomyces thermoautotrophicus* (Hoffmann-Findeklee et al. 2000) is a superoxide-dependent nitrogenase. Mo-dependent nitrogenases consist of two component proteins called as component I and component II; both are soluble in nature. Component I is also known as MoFe protein or dinitrogenase, whereas component II is referred as Fe protein or dinitrogenase reductase (Burris 1991).

The MoFe protein, i.e., component I (dinitrogenase), is responsible for substrate reduction and is a heterotetramer of $\alpha_2$ and $\beta_2$ subunits with total molecular weights varied from 180 kDa to 220 kDa in rhizobia and *Klebsiella* and *Clostridium*, respectively (Table 4.3). Dinitrogenase is organized as an $\alpha_2\beta_2$ heterotetramer (where $\alpha$ = NifD and $\beta$ = NifK protein) (Raymond et al. 2003). The α subunit has a molecular mass of about 56 kDa, and the β subunit has a molecular mass of approximately 60 kDa. The MoFe protein contains two atoms of Mo and 30–34 atoms of each of Fe and acid-labile S (Dean and Jacobson 1992). Dinitrogenase is the site of substrate reduction, and this protein contains two each of two unique metal clusters, the P-cluster and the iron-molybdenum cofactor (FeMo cofactor) (Dos Santos et al. 2004). The actual reduction occurs in the FeMo center (Rees et al. 2005). There are two groups of metal center in the MoFe component which are known as FeMoCo and P-clusters. Two copies of P-clusters are present per MoFe-protein tetramer, and each cluster pair contains two-bridged 4Fe-4S clusters (Peters et al. 1997; Kim and Rees 1992). In some microorganisms particularly in some diazotrophs, the Mo is replaced by vanadium in Mo-limiting conditions which are commonly known as alternative nitrogenase. The specificity and efficiency of FeMo nitrogenase in binding nitrogenase are more than the alternative nitrogenase (Raymond et al. 2003). Component I is less sensitive to oxygen and in free air the half-life is 10 min.

The Fe protein or component II (dinitrogenase reductase) acts as an ATP-dependent electron donor to the dinitrogenase and exists as a homodimer consisting of γ subunits (*nif*H protein) with a molecular weight of 55,000–65,000 Da (Christiansen et al. 2001). Each dimer contains four Fe and four acid-labile S atoms referred as [4Fe-4S] cluster which can bind two molecules of MgATP dinitrogenase reductase and binds two molecules of MgATP at a pair of sites distal from the site of the Fe4S4 cluster. Furthermore, dinitrogenase reductase is required for the proper insertion of the iron-molybdenum cofactor into the dinitrogenase protein. The

**Table 4.3** A comparison of the two components

| Composition | Component I | Component II |
|---|---|---|
| Molecular weight | 200,000–250,000 | 55,000–73,000 |
| Number of units | 4 | 2 |
| Molecular weight of subunits | 595,000 and 52,000 | Both with 27,500 |
| Fe per molecule | 30–34 | 4 |
| $S^{2-}$per molecule | 18–19 | 4 |
| Mo per molecule | 2 | Nl |
| $Mg^{2+}$, $Ca^{2+}$ | Present | Absent |

Adapted from Swain and Abhijita (2013)

Fe-protein complex is extremely oxygen sensitive and has a half-life of 0.5 to 0.75 seconds in air.

### 4.5.3 Mechanism of Nitrogenase Action

For efficient nitrogen fixation, the role of enzyme complex nitrogenase cannot be under minded. There is structural similarity of nitrogenase present in both the symbionts and free-living bacteria, though the efficiency of nitrogenase in case of symbionts is more than the free-living organism. Symbiotic microorganisms receive photosynthetic products from the host plant where they dwell within the nodule present in the root system of the host. These photosynthates provide ATP for the nitrogen-fixing process which is an energy-driven phenomenon through oxidative phosphorylation. Nitrogenase enzyme possesses a high affinity toward oxygen, thus requiring an oxygen-protecting system (Downie 2005). An oxygen-binding protein leghemoglobin (LHb) (red pigment of legume root nodule) acts as an oxygen buffer that cycles between oxidized $Fe^{3+}$ and reduced $Fe^{2+}$ and helps to maintain oxygen level sufficiently low inside the nodule (White et al. 2007). Nitrogen fixation starts only after the formation of symbiosomes. The membrane envelope is the primary site of nitrogen fixation by symbionts. The nitrogenase enzyme is concentrated in the bacteroid, whereas LHb lies within the membrane envelope that surrounds the bacteroids (Harriott et al. 1991).

The mechanism of action of nitrogenase complex is a continued area of interest. The overall nitrogenase reaction consists of redox cycle between the dinitrogenase reductase (Fe protein) and dinitrogenase (MoFe protein) and the substrate reduction cycle (Burris and Roberts 1993). Dinitrogenase reductase supplies electrons, one at a time, to dinitrogenase through hydrolysis of two molecules of MgATP. The primary electron donors are ferredoxins or flavodoxins, while sodium dithionite can serve as electron donor under in vitro conditions. Ferredoxins are iron-sulfur proteins with molecular masses between 14 and 22 kDa, whereas flavodoxins are proteins with molecular masses between 5 and 24 kDa and contain the prosthetic group, flavin mononucleotide (Fig. 4.2). Dinitrogen reductase reacts with MgATP, after electron acceptance from reduced ferredoxin or flavodoxin. Since the transfer of

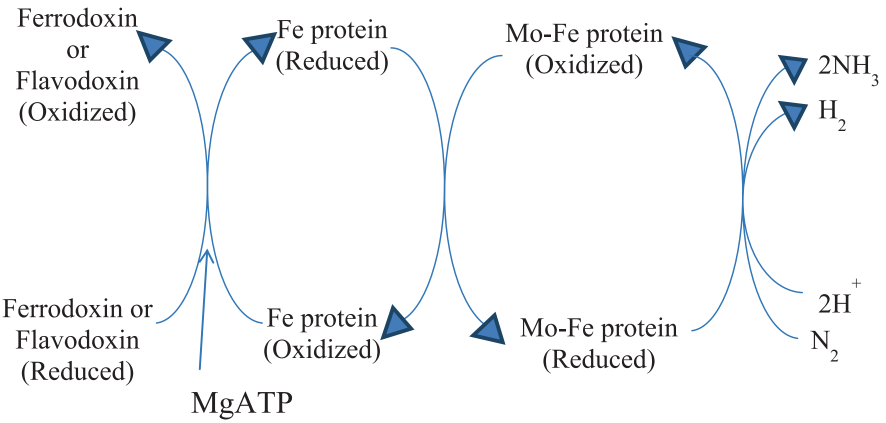

**Fig. 4.2** Mechanism of the nitrogenase enzyme action in biological nitrogen fixation (Source: Taiz and Zeiger 2006)

electrons from Fe protein to MoFe protein is MgATP dependent, therefore it required hydrolysis of two molecules of MgATP for each electron transfer. ATP binding and hydrolysis must be used in inducing conformational changes in the MoFe protein required for electron transfer from P-cluster pair to the FeMo cofactor. When dinitrogenase has collected enough of electron from dinitrogenase reductase, it binds with the $N_2$. As because electrons are transferred to dinitrogenase singularly, and the reduction of $N_2$ plus two protons to yield two molecules of ammonium and one molecule of $H_2$ is an eight-electron process, therefore eight individual transfers must occur before the dinitrogenase enzyme is competent to reduce its substrate. For repetition of the cycle, dinitrogenase has to accept additional electrons from dinitrogenase reductase. The initial reduction of MoFe protein generates a dihydride species at the active site which is released as $H_2$. Therefore, the production of $H_2$ which requires four MgATP is obligatory in the N2 fixation process; thus, ~ 25% of energy from MgATP is lost in the form of $H_2$. Some diazotrophs contain an uptake hydrogenase enzyme, which catalyzes the consumption of the hydrogen produced by nitrogenase and to generate a reduced electron carrier or MgATP, thereby recapturing some of the energy lost in the $N_2$ fixation reaction (Sindhu and Dadarwal 1992; Tamagnini et al. 2002; Bothe et al. 2010).

Several photosynthetic products comprised of glucose, sucrose, and organic compounds translocated from leaves to nodules serve as the substrate supporting nitrogen fixation by bacteria. The enzyme β-hydroxybutyrate dehydrogenase present in bacteria helps to generate the reducing power of ATP for the support of nitrogen fixation (Trainer and Charles 2006). Oxidative phosphorylation is the major source of ATP for nitrogen fixation (Resendis-Antonio et al. 2007). The major organic compounds that are transported across the symbiosome membrane and into the bacteroid are the intermediates of TCA cycle (Lodwig and Poole 2003). Organic acids of $C_4$ pathway such as succinate, malate, and fumarate are also used as electron donors (Fig. 4.3) for production of ATP and finally converted into pyruvate which serves as the ultimate source of electron for nitrogen reduction (Bergersen

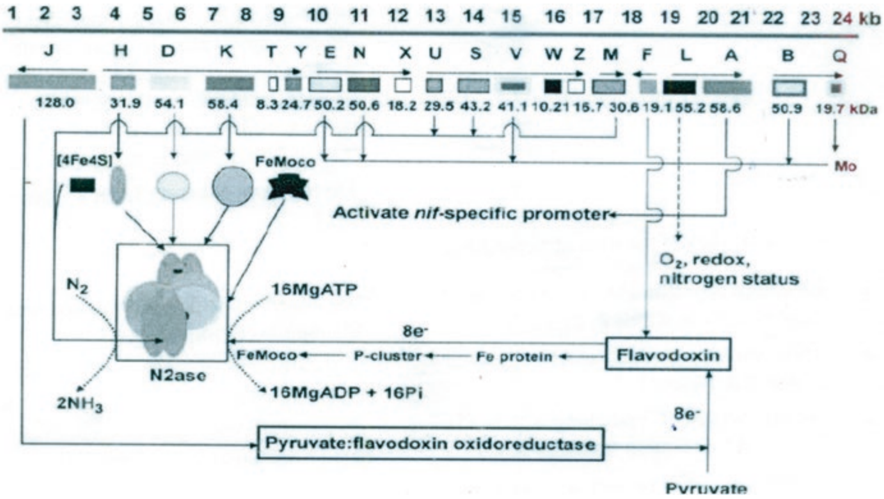

**Fig. 4.3** Organization of $N_2$ fixation genes in *K. pneumoniae* (Adopted from Dixon and Kahn 2004)

1971). The first and primary stable product in nitrogen fixation is $NH_3$ which accounted up to ~90% of the total nitrogen fixed and is rapidly assimilated to organic nitrogen compound after synthesis. Bacteroids have the poor capability to assimilate $NH_3$ into organic compounds as amino acids due to the presence of low amount of ammonia-assimilating enzymes in bacteroid. However, in comparison with bacteroid, the enzymes present in plant's cell cytoplasm are quite high. This $NH_3$ must be transported to plant cell where the assimilation of $NH_3$ is carried out by the assimilating enzymes (Franche et al. 2009). After the transamination reactions, the products of nitrogen fixation are translocated to growing parts of the plant. The incorporation of ammonia into glutamic acid mediated by glutamate synthase (Ott et al. 2005) is the primary assimilation step.

### 4.5.4 Genetics of Nitrogenase

Two major groups of genes are involved in $N_2$ fixation. The ***nif* genes** are genes encoding enzymes involved in the fixation of atmospheric nitrogen into available $NH_3$ and have structural and functional correlates in the free-living diazotroph *Klebsiella pneumoniae*. Another group of *fix* genes essential for symbiotic $N_2$ fixation have been identified in *Rhizobium/Bradyrhizobium*. Mutations in the *fix* genes result in nodules with a $Fix^-$ (non-$N_2$ fixing) phenotype (Fischer 1994). However, these *fix* genes have no correlates in *K. pneumoniae*. The primary enzyme encoded by the *nif* genes is the nitrogenase complex. Besides the nitrogenase enzyme, the *nif* genes also encode a number of regulatory proteins involved in nitrogen fixation. This gene is found in both free-living nitrogen-fixing bacteria and in symbiotic bacteria associated with various plants. The early work established that nitrogen fixation (*nif*) genes mapped close to genes for histidine biosynthesis in *K. pneumoniae* (Streicher et al. 1971; Dixon and Postgate 1971) and that when the histidine (*his*) operon and adjacent DNA were transferred by conjugation into *E. coli*, the recipients gained the ability to fix nitrogen (Dixon and Postgate 1971).

The genetics of $N_2$ fixation was initially studied in *Klebsiella pneumoniae*. The detail analysis of *nif* gene facilitated initial identification of 17 genes required for the synthesis and activity of nitrogenase in *K. pneumoniae* and also helps to disclose the complexity of this gene. However, a combination of genetic mapping, complementation analysis, cloning, and sequencing studies has established that 21 contiguous *nif* genes are clustered and transcribed in eight adjacent operons, which occupy 25 kb of the genome (Dean and Jacobson 1992).

This facilitated the discovery and assay of a Fe- and Mo-containing cofactor, now been considered as the active site of nitrogenase. The genetic control of nitrogen fixation has shown that the *nif* genes are regulated by a cascade mechanism, involving a series of transcriptional control circuits named as *nif* gene regulon. Like the *his* operon, most of the *nif* cistrons are transcribed in the same direction; however, two unlinked genes, *nifF* and *nifJ* are transcribed in the opposite direction. The function of various component of *nif* gene is depicted in Table 4.4. Dinitrogenase is a complex protein consisting of α and β subunits. α subunit is the product of *nif*D gene while β subunit of *nif*K gene (Beringer and Hirsch 1984). There have been indications that some of these proteins might be necessary for full derepression of the regulon (Dixon 1984). The *nif*H gene encodes for the protein dinitrogenase reductase which is a protein dimer made up of two identical subunits (Roberts et al. 1978). Besides these three structural genes, the complete assembly of nitrogenase requires products of *nif* genes that are involved in the synthesis of FeMoCo, information of Fe-S clusters, and maturation of nitrogenase components. The products of three complementation groups (*nif*B, *nif*N, and *nif*E) are necessary for the presence of the FeMoCo center in component I. Some scientists suggested similar roles for the products of *nif*Q, *nif*C, and *nif*J. Genes *nif*S and *nif*U play a part in the assemblage of Fe-S clusters (Hu and Fay 2007). Products of *nif*H, *nif*M, *nif*U, and *nif*S are required for maturation of Fe protein. *nif*E and *nif*N products have been proposed to function as scaffold for FeMoCo biosynthesis (Fig. 4.3). The gene *nifV* encodes homocitrate synthase and is required for the synthesis of FeMoCo. The gene *nif*W is involved in stability of dinitrogenase and protects the protein from oxygen inactivation (Cheng 2008).

Beside these structural genes, *Klebsiella* also contains the genes that mediate electron transport to nitrogenase. *nif*F encodes flavodoxin that transfers electrons to nitrogenase, and *nif*J encodes pyruvate oxidoreductase that transfers electrons to flavodoxin from the pyruvate. The synthesis of the *nif*F protein seems to be under *nifJ* control. *nif*A encodes positive regulatory protein that serves to activate transcription of other genes, while *nif*L acts as repressor of nitrogenase when oxygen is present (Beringer and Hirsch 1984). The genetics of nitrogenase is still an interesting arena of research for manipulation of nitrogenase activity and also helps in guiding for adoption of several strategies for improving the activity of nitrogenase.

**Table 4.4** The component of *nif* gene and their function

| Gene | Product/known function | References |
|---|---|---|
| *nifX* | Not essential for nitrogen fixation; required for FeMo cofactor biosynthesis | Allen et al. (1995) |
| *nifB* | Required for FeMo cofactor biosynthesis | |
| *nifEN* | Required for FeMo cofactor biosynthesis | Allen et al. (1995) and Dean and Jacobson (1992) |
| *nifH* | Fe protein subunit | Dean and Jacobson (1992) |
| *nifDK* | MoFe protein ά and β subunits | Dean and Jacobson (1992) |
| *nifL* | Negative regulatory protein | Dixon (1998) |
| *nifA* | Positive regulator of *nif* transcription | Dixon (1998) |
| *nifM* | Required for Fe protein maturation. Putative peptidyl-prolyl *cis/trans* isomerase | Gavini and Pulukat (2002) |
| *nifY* | Associates with MoFe protein and dissociates upon FeMo cofactor insertion | Homer et al. (1993) |
| *nifV* | Homocitrate is an organic component of FeMo cofactor | Hoover et al. (1987) |
| *nifQ* | Incorporation of Mo into FeMo cofactor | Imperial et al. (1984) |
| *nifW* | Function unknown; interacts with the MoFe protein | Kim and Burgess (1996) |
| *nifZ* | Function unknown; required for full activity of the MoFe protein | Paul and Merrick (1989) |
| *nifJ* | Pyruvate oxidoreductase; couples pyruvate oxidation to reduction of the *nifF* product | Shah et al. (1983) |
| *nifT* | Function unknown; not essential for nitrogen fixation | Simon et al. (1996) |
| *nifF* | Flavodoxin required for electron transfer to the Fe protein | Thorneley et al. (1992) |
| *nifU* | Fe-S cluster biosynthesis | Yuvaniyama et al. (2000) |
| *nifS* | Fe-S cluster biosynthesis | Zheng et al. (1993) |

In case of symbiotic mutants of *Rhizobium*, it is observed that symbiotic genes are not normally expressed in the free-living bacteria. Therefore, to solve this problem, investigators have used transposon Tn5 to generate symbiotic mutants because approximately 0.1% of randomly mutagenized population of cells contains Tn5 insertions in symbiotic genes (Shanmugam et al. 1978). However, most symbiotic genes can be identified only by mutagenizing *Rhizobium* strains and then screening for symbiotic defects on plants, which can be further used to locate and study the genes that code for nitrogenase due to its conserved amino acid sequence. Moreover, a transposon insertion in a symbiotic gene “marks” the symbiotic gene both genetically and physically in such a way that it can be mapped genetically or cloned. Therefore, it is possible to identify and clone the genes encoding this enzyme from other nitrogen-fixing species with cloned *K. pneumoniae nif* genes as hybridization probes.

In this manner, nitrogenase structural genes from *R. meliloti* (Ruvkun and Frederick 1980), *R. japonicum* (Better et al. 1983), and *Anabaena* strain 7120 (Ausubel 1982) have been cloned. In all the abovementioned species, *nif*H (iron-containing protein) and *nif*D (a subunit of molybdenum-iron-containing protein) are closely linked as is the case in *K. pneumoniae*. Interestingly, *R. meliloti*, *nif*HDK are arranged in the same transcriptional order as in *K. pneumoniae* (Ausubel 1982). One of the most interesting features of symbiotic nitrogen fixation is the clustering of symbiotic genes, including *nif* genes, on large (100–500 kb) indigenous plasmids. A plasmid location for symbiotic genes was first discovered by Johnston et al. (1978) who demonstrated that the host-range specificity of *R. leguminosarum* (peas) could be transferred to *R. trifolii* (clover) or to *R. phaseoli* (beans) by conjugal transfer of an *R. leguminosarum* plasmid carrying the kanamycin resistance gene of Tn5.

Direct evidence that *Rhizobium nif* genes are plasmid-borne has been obtained by hybridization of cloned nitrogenase genes to purified plasmid DNA (Nuti et al. 1979). In most of the species, including *R. leguminosarum* and *R. trifolii*, the plasmids carrying *nif* genes are ~200 kb; however, the plasmid containing the *nif* genes in *R. meliloti* is very large (> 300 kb). Till date there is no supporting evidence that the transfer of *Rhizobium* plasmid DNA into plant root cells can differentiate the root cells into symbiotic nodule cells. However, the information regarding success of DNA transfer in the nodule will be difficult, because the nodule, in contrast with a crown gall tumor, is packed with bacterial cells. Nevertheless, the analogy between *Agrobacterium* and *Rhizobium* is a compelling one and deserves careful examination in the future.

### 4.5.5 Regulation of Nitrogenase

In any ecosystem, diazotrophs must response to varied environmental conditions to regulate the tremendously taxing nitrogen fixation process. All characterized diazotrophs regulate nitrogenase at the transcriptional level. A smaller set also possesses a fast-acting posttranslational regulation system. Although there is little apparent variation in the sequences and structures of nitrogenases, there appear to be almost as many nitrogenase-regulating schemes as there are nitrogen-fixing species (Burris and Roberts 1993). The subunits of nitrogenase from different nitrogen-fixing microorganisms can be mixed to produce functional system (Emerich and Burris 1978); structural genes found in nitrogen-fixing species are conservative in nature and the distribution results due to transfer of genes.

Genes can be manipulated to improve the fixation of dinitrogen. The microorganisms are modified in the host so that they are unable to assimilate the fixed nitrogen until the function of the nitrogenase is over and release $NH_4^+$ directly to the plants (Peters et al. 1982). The nitrogen fixation can be regulated by leguminous plants by reducing the number of root nodule formation and by regulating the carbon flow to the microorganisms (Beringer and Hirsch 1984).

Nitrogenase is synthesized when *K. pneumoniae* is grown under anaerobic, N-limiting conditions. The regulation of nitrogenase synthesis is a useful control mechanism in which free-living bacteria do not undergo energy requiring $N_2$-fixation when $NH_3$ is in plentiful supply or oxygen concentration is high.

Transcriptional regulation of the *nif* regulon appears to involve at least three major systems: regulation in both positive and negative fashion by elements of the general nitrogen metabolism system at *nif*R, positive regulation by the *nif*A product at the operator region of each of the other *nif* transcripts, and negative regulation in the presence of oxygen, mediated by some unknown factors. In addition, there is some evidence for regulation of the *nif* region by nitrate, molybdenum (Brill 1980), high temperature (Hennecke and Shanmugam 1979; Zhu and Winston 1981), and amino acids (Shanmugam and Carlo 1976) which are independent of the general nitrogen metabolism.

#### 4.5.5.1 Activation of *nif* Transcription

The detail study regarding regulatory phenomena of $N_2$ fixation by *nif* gene do not appear to be *nif* specific but rather are part of a general nitrogen metabolism regulatory system (Tubb 1974). The majority of loci involved in this system have the designation *gln*, and, until recently, the product of the *gln*A gene (glutamine synthetase) was thought to be the major nitrogen regulatory protein in the cell (Tyler 1978). The *gln*A, which is essential for ammonia assimilation under low nitrogen conditions, determines positive control of nitrogen-regulated operons. The simple model of *nif* regulation had only a short half-life, however, since it became apparent that expression of the *gln*A gene, the structural gene for GS, was itself controlled by a combination of three regulatory genes, viz., *ntr*A, *ntr*B, and *ntr*C. The *nif*LA operon, involved in the regulation of nitrogenase synthesis (Dixon et al. 2002), is functionally regulated by the prokaryotic universal N control system, designated as *ntr*A. The *ntr*B and *ntr*C genes are linked to *gln*A in *K. pneumoniae* and in other enteric bacteria (Pahel et al. 1982).

Under anaerobic, $N_2$-limiting conditions, the gene product of *ntr*A and the *ntr*C which is phosphorylated by *ntr*B product acts as an activator of *nif*LA operon and of *ntr*BC operon at *gln*Ap2 promoter site. The *nif*A protein then activates transcription of all other *nif* operons. Evidence for the involvement of *nif*L and *ntr*B in repression of nitrogenase has been obtained through *nif*L and *ntr*B mutants, which synthesize nitrogenase in the presence of oxygen and/or excess nitrogen condition. Positive control of *nif* transcription is therefore affected by a regulatory cascade mechanism in which *ntr*C activates transcription of the *nif*LA promoter and the *nif*A product in turn activates transcription of the remaining *nif* operon. This is because, nitrogenase is a relatively slow enzyme (Thorneley and Lowe 1983), and large amount are generally needed since nitrogenase can represent as much as ~10% of the cellular protein in nitrogen-fixing cells. Under high $N_2$ conditions, *ntr*C is dephosphorylated by the *ntr*B, and the dephosphorylated *ntr*C acts as a repressor of all the three promoters of *ntr*BC operon. *nif*A is inactivated by altered *nif*L, thus resulting in repression of all the *nif* operons under high N and aerobic conditions (Dean and Jacobson 1992).

When sufficient levels of the *nif*A gene product are expressed, from a constitutive promoter, autogenous activation of the *nif*LA promoter can occur in the absence of the *ntr*C gene (Drummond et al. 1983). Since *ntr*A is required for *ntr*C-mediated activation, therefore, it can be concluded that *ntr*C gene is also required for activation mediated by *nif*A and this is similar at both the *nif*L and *nif*H promoters' site.

### 4.5.5.2 Repression of *nif* Transcription

Since *nif* expression is positively controlled by two transcriptional activators and requires the presence of the *ntr*A gene product, therefore, it can be assumed that one or more components of the positive control system must be overridden in order to obtain repression of nitrogenase synthesis in response to the nitrogen source or to oxygen. There are basically two mechanisms for preventing *nif* transcription. The first involves an anti-activation system in which one or more of the positive control elements are inactivated, and the second assumes a more conventional negative control system in which repressor molecules directly interact with regulatory regions in order to inhibit transcription.

The first evidence that oxygen repression is mediated by a *nif*-specific gene came from an analysis of mutant strains in which transcription of the *nif* structural genes occurred in the presence of oxygen. Such strains carried mutations in the *nif*L gene. Subsequently it was shown that the *nif*L product responds not only to oxygen but it also mediates repression in response to fixed nitrogen (Roberts and Brill 1981).

The *nif*L gene product is apparently more sensitive to nitrogen sources than the *ntr*B product since nitrogenase synthesis is repressed by casamino acids in the growth medium (Shanmugam and Carlo 1976), whereas other nitrogen-controlled operons are not affected. When present on a multicopy plasmid, the *nif*L gene inhibits *nif* transcription in the absence of fixed nitrogen or oxygen. This indicates that the *nif*L gene product is normally maintained in a non-inhibitory state in nitrogen-fixing cells and that factors required for this maintenance can be titrated out by several copies of *nif*L (Roberts and Brill 1981). So, when present in multiple copies, *nif*LA genes autogenously repress transcription from the *nif*LA promoter in the absence of *ntr*BC but do not prevent activation in the presence of *ntr*BC, supporting the hypothesis that *nif*L inactivates *nif*A product rather than directly repressing transcription at this promoter (Dixon 1984).

Transcriptional regulations by oxygen address three factors. First one is that the *nif* proteins are oxygen labile both for activity and stability, and second one is that the oxygen addition must be done under nitrogen-limiting conditions to avoid negative regulation by that system, and the cells are therefore extremely nitrogen starved; and third one is that there seems to be a posttranscriptional oxygen effect that might well affect levels of detectable messages. The *ntr*B product does not apparently respond to oxygen, and many of the nitrogen-regulated operons are expressed in aerobic growth conditions.

The transcription of the *nif*LA operon is relatively insensitive to the presence of oxygen (Dixon 1984), whereas the presence of $NH_4^+$ affects transcription of the all the *nif* operons since it is mediated by the *ntr* gene at the *nif*LA promoters. It is found that the product of the *ntr*B gene has a negative function when ammonia is present in the growth medium. The *ntr*B does not function as a repressor molecule per se but can interact with *ntrC* protein in some way to modulate its activity (Alvarez-Morales et al. 1984), the two proteins forming a regulatory protein complex.

The *ntr*B therefore acts as an anti-activator of *ntr*C in response to the nitrogen source. The lack of nitrogenase synthesis in ammonia-grown cells may therefore be due to lack of activation at the *nif*LA promoter which would consequently result in a lack of activation of the remaining *nif* operons. From this it can be envisaged that *ntr*C product can bind to DNA and repress transcription, independently of its role as a transcriptional activator, and that the latter role can be modulated by the *ntr*B product so that activation does not occur when repressive levels of ammonia are present.

Like activation, repression of *nif* transcription therefore occurs at two levels. Specific *nif* repression results in an inhibition of *nif*A-mediated activation, thus preventing nitrogenase synthesis in the presence of oxygen or low levels of fixed nitrogen, without affecting *ntr*C-mediated activation of other nitrogen-controlled operons. The ammonia repression determined by the *ntr*BC genes ensures that transcription of nitrogen-regulated genes including the *nif*LA operon is inhibited in nitrogen-enriched media. The precision role of *nif*L and *ntr*B in mediating *nif* repression is difficult to determine since they show no apparent phenotype in the absence of the *nif*A and *ntr*C genes, respectively (Dixon 1984).

**Other Regulators** The role of molybdenum in the regulation of nitrogen fixation is also significant. Initially it was assumed that nitrogenase polypeptides are not present in significant amounts in the absence of molybdenum. However, a recent analysis of molybdenum-deficient cells supports the view that *nif*-coded polypeptides are synthesized at low levels in the absence of molybdenum. Very recently, it has been suggested that molybdenum only affects the level of synthesis of the *nif*-HDK transcript and, in fact, full expression of the transcript requires the presence of both molybdenum and "molybdoprotein," presumably component I (Dixon and Kahn 2004). Temperature above 37 °C prevents the synthesis of most *nif* polypeptides (Zhu and Winston 1981). This effect is due to thermolability of the *nif*A product.

Thus, elevated temperature level causes all *nif* operons except *nif*RLA to be turned off. The regulation of nitrogenase in symbiotic root nodules differs from that of the free-living system (Fischer 1994; Merrick and Gibbins 1985). The expression of *nif*A in rhizobial systems is not autoregulatory nor it is under control of the global *ntr* system. Instead, *nif*A is oxygen regulated (Fig. 4.4). Two genes, i.e., *fix*L and *fix*J, which have no homologues in free-living nitrogen-fixing organisms, act as a sensor-transducer of low-oxygen potential in root nodules. The *fix*L product is a transmembrane heme-containing protein, which perceives low oxygen and becomes autophosphorylated. The phosphorylated *fix*L then phosphorylates *fix*J, which activates *nif*A and *fix*K genes. In turn, *nif*A activates transcription of the other *nif* operons, and the *fix*K protein controls the regulation of *fix*NOQP and *fix*GHIS genes (Fischer 1994).

#### 4.5.5.3 Sequencing of *nif* Promoters and Regulatory Genes

The common requirement for the *ntr*A gene product in transcriptional activation and the functional homologies between the *ntr*BC and *nif*LA genes suggests that

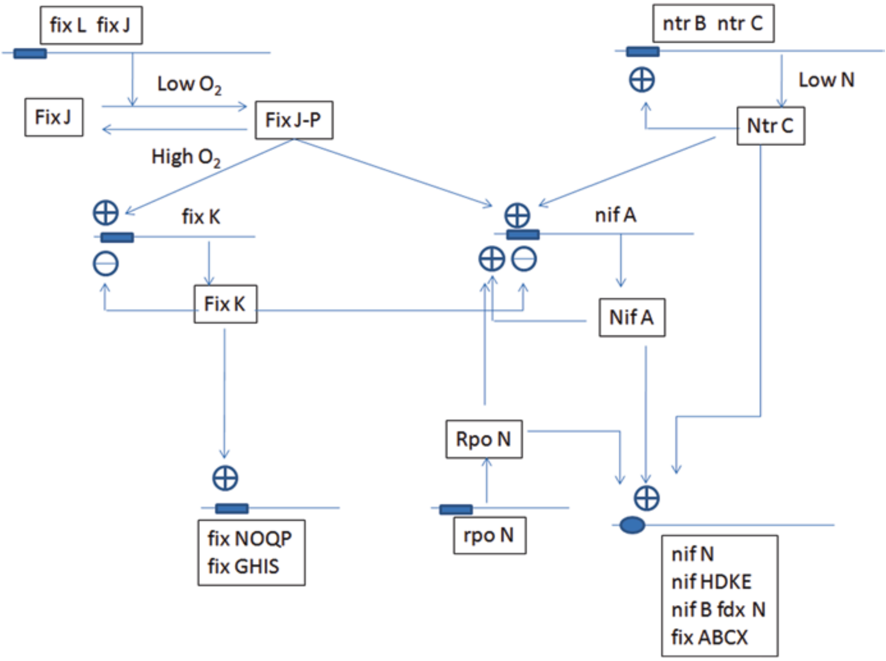

**Fig. 4.4** *nif* and *fix* genes regulation in *R. meliloti* (Adapted from Fischer 1994)

nitrogen-regulated promoters might share some sequence homology (Dixon 1984). Analysis of the *nif*LA promoter demonstrated that it had no typical –35 region and, in agreement with this finding, some positive control by *ntr*C was maintained even in deletions extending to −28 (Drummond et al. 1983). Analysis of promoters activated either by *ntr*C or *nif*A revealed a heptameric consensus sequence, TTTGCA, in the −15 region, which was proposed to be a binding sequence for transcriptional activation (Ow and Ausubelf 1983).

Sequencing of five *nif* promoters revealed a characteristic primary structure with the consensus CTGG at −24 and TTGCA at −12 region. Presence of an upstream AT-rich region in some *nif* promoters was protected from DNase I digestion (Beynon et al. 1983). The spacing between the conserved GG and GC motifs in the −24 and −12 elements was found to be critical for promoter activity, reflecting a stringent spacing requirement (Buck 1986).

The activation of the *gln*A promoter by *ntr*C also occurred at a distance, and that *ntr*C-binding sites could function far upstream of the promoter (Reitzer and Magasanik 1986). *nif*A and *ntr*C were therefore brought into the limelight as eukaryotic-like transcriptional activators that bound to regulatory sequences similar to enhancers (Dixon et al. 2000). These transcriptional activators are classified as enhancer-binding proteins (EBPs). So it can be logically concluded that *nif*A and *ntr*C activate transcription through a DNA looping mechanism. However, activation of transcription was not entirely dependent on the UAS sequences as because some of the C promoter sequences are not strictly dependent on the UAS. Therefore, it can be suggested that a run of T residues between −17 and −14 might be critical in the response of the promoter in the absence of the UAS (Dixon and Kahn 2004).

Conversion of this sequence in the *nif*H promoter from CCCT to TTTT suppressed the requirement for the UAS to be located on the same face of the helix with respect to the −24 –12 sequence, and the promoter was far more responsive to a truncated form of *nif*A lacking the DNA-binding domain (Buck and Cannon 1989). These observations suggested that relatively weak binding of RNA polymerase to the downstream sequence coupled with stereospecific binding of the activator at the UAS ensures the fidelity of activation, thus, ensuring that this *nif* promoter is specifically activated by *nif*A (Morett and Buck 1988). Comparison of the *nif*A and *ntr*C sequences revealed a strongly conserved central domain and a C-terminal domain containing a helix-turn-helix motif proposed to be required for DNA binding (Buikema et al. 1985). The homology between these two proteins suggested a common mechanism of transcriptional activation, corroborate with previous observations that, when overexpressed, *nif*A could substitute for *ntr*C at promoters normally activated by the latter (Drummond et al. 1983). The sequence of *ntr*B initially revealed no homologues, but the subsequent sequencing of *nif*L revealed a common C-terminal domain present in *ntr*B and other regulatory proteins, including *Env*Z, *Pho*R, *Cpx*A, and *Che*A (Drummond and Wootton 1987). These proteins all had a corresponding regulatory partner belonging to the *ntr*C family and originated from same ancestor (Dixon et al. 2000).

## 4.6 Strategies for Improving $N_2$ Fixation

Considerable research efforts have been made recently for improving the efficiency of biological nitrogen fixation because symbiotic and free-living organisms have the potential to reduce our overall dependence on nitrogenous fertilizers. The strategy selected depends primarily on whether the diazotrophic bacteria under study are capable of fixing $N_2$ under free-living or symbiotic conditions and whether nitrogen fixation (*nif*, *fix*) or nodulation (*nod*) genes are targeted. To date, most efforts of improving $N_2$ fixation have been made on symbiotic $N_2$-fixing organisms of the genera *Rhizobium* and *Bradyrhizobium* because they come from symbiotic associations with agronomically important legume crops (Shantharam and Mattoo 1997). Moreover, considerable research progress has also been made in understanding the process of $N_2$ fixation in the cyanobacteria of the genus *Anabaena* (Haselkorn and Buikema 1997), *Frankia* (Clawson et al. 1998), and *Azospirillum* (Okon and Labandera-Gonzalez 1994).

The subunits of nitrogenase from different nitrogen-fixing microorganisms can be mixed to produce functional system (Emerich and Burris 1978). Structural genes in nitrogen-fixing species are conservative in nature and the distribution results due to the transfer of genes. By manipulating genes the fixation of dinitrogen can be improved. The microorganisms could not assimilate the fixed nitrogen until direct the release of $NH^+$ to the plant after the nitrogenase's function is over (Peter et al. 1982; Swain and Abhijita 2013).

Several strategies have been proposed to optimize endophytic nitrogen fixation in nonlegume crops either by (i) altering the receptivity of the host plant to colonization by $N_2$-fixing bacteria, (ii) by exploiting naturally occurring stable plant-diazotrophic endophytic bacterial associations in cereals such as rice and wheat to fix nitrogen endophytically, or (iii) through the genetic alteration of selected endophytic bacteria. Alternative approach used involves the genetic manipulation of nonlegumes to incorporate *nif* genes from bacteria (Dixon and Kahn 2004) or extension of the host range of symbiosis between rhizobia and nonlegume crops (Trinick and Hadobas 1995; Sindhu and Dadarwal 2001).

### 4.6.1 Enhancement of Nodulation and Broadening of Host Range

*Rhizobium*-legume associations are usually host specific, and a given rhizobial strain can infect a limited number of hosts (Brewin 1991). Some specialized rhizobia associated with the tribes Cicereae, Trifolieae, and Vicieae have restricted host ranges (Broughton and Perret 1999). In other symbiotic associations, host specificity varies greatly among the symbionts. *Azorhizobium caulinodans* nodulates only *Sesbania rostrata*; *Rhizobium meliloti* can initiate nodule formation on few host plants (Medicago, Melilotus, and Trigonella), whereas *Rhizobium* sp. NGR234 nodulates more than 137 genera of legumes, as well as the nonlegume *Parasponia andersonii* (Young and Johnston 1989).

Under field conditions, legumes are confronted with a diversity of rhizobial strains, and opportunities for legume-mediated genetic exchange among the rhizobia or genetic exchange between rhizobia and other types of rhizosphere bacteria do exist (Muresu et al. 2008). Kinkle et al. (1993) have reported plasmid transfer between populations of *R. leguminosarum* bv. *Viciae* and *B. japonicum*, respectively, in non-sterile soil. Souza et al. (1994) presented evidence that gene exchange is frequent among local soil populations of *R. etli*. Due to gene transfer and genomic rearrangements between bacteria in soil, sometimes rhizobial populations appear that are different from the original inoculants (Vlassak et al. 1997; Nath et al. 2017; Sarkar et al. 2017; Verma et al. 2017a).

#### 4.6.1.1 Transfer of Symbiotic Plasmid

Many *Rhizobium* strains harbor plasmids, and the genes affecting nodulation (*nod*, *nol*, *noe* genes), nitrogen fixation (*nif* and *fix* genes), polysaccharide production (*exo* and *lps* genes), and other cellular functions are located on the plasmids (Denarie et al. 1992). The symbiotic (*sym*) plasmids of *R. leguminosarum* and *R. meliloti* vary in size from 140 to 1500 kb (Beynon et al. 1980). The number and size of these plasmids vary among different isolates. The well-known nitrogen-fixing bacteria *K. pneumoniae* resembles closely to non-nitrogen-fixing bacterium *E. coli*. The genes of these two species could be transferred and expressed in either of the organisms.

*nif* mutants of *K. pneumoniae* that are deficient in fixing nitrogen are located between genes for histidine biosynthesis (his) and shikimic acid uptake (shi A). The *his* and *nif* regions can be actively transferred from a strain of *K. pneumoniae* to an *E. coli* strain which require histidine. *E. coli* cells that do not require histidine anymore have acquired the ability to fix nitrogen.

The conjugative plasmid pRDI that picked up the nif and his genes was selected and transferred to other bacterial genome (Dixon et al. 1976). These results indicated that infection and nodule initiation genes could be expressed in heterologous rhizobia leading to broadening of host range but bacteroid development leading to formation of effective nitrogen-fixing nodules is difficult to achieve. Rogel et al. (2001) reported that *Ensifer adhaerens* ATCC 33499, Gram-negative soil bacterium, did not form nodules on *Phaseolus vulgaris* (bean) and *Leucaena leucocephala*. When the symbiotic plasmid from *Rhizobium tropici* CFN299 was transferred to *Ensifer adhaerens,* it formed nitrogen-fixing nodules on both the hosts.

*Rhizobium tropici* was selected as the donor because *Rhizobium tropici sym* plasmids conferred on *Agrobacterium tumefaciens* the capacity to form nitrogen-fixing nodules on *Phaseolus vulgaris* and *Leucaena leucocephala* (Martinez-Romero et al. 1991). The plasmids “a” and “b” were cotransferred from *Rhozibium tropici* CFN299 along with plasmid c (which carries *nod-nif genes*) into *A. tumefaciens* (Martinez et al. 1987).

#### 4.6.1.2 Transfer of Cloned *nod* Genes

In rhizobia, the nodulation genes are located either on large symbiotic plasmids (p*sym*) or on chromosome, which are organized into several coordinately regulated operons. So far, over 60 different nodulation genes have been characterized in different rhizobia (Sindhu et al. 2002a; Loh and Stacey 2003). These nodulation genes are basically categorized into three main classes, namely, (a) the regulatory *nod*D and *nod*VW genes which activate the transcription of other common and host-specific nod genes; (b) the common *nod*ABC, *nod*M, and *nod*IJ genes which are functionally and structurally conserved among different rhizobia; and (c) the host-specific *nod* genes which are variable with bacterial species and strains. Mutations in these host-specific nod genes usually do not complement with cloned genes from other *Rhizobia* spp.

The structural *nod* genes are expressed in response to biochemical signals from the plant, usually flavonoid compounds, and a transcriptional activator produced by *nod*D regulatory genes. The proteins encoded by *nod*D genes are activated by particular flavonoid compounds; thus, *nod*D genes are partial determinants of strain/host specificity. Expression of structural nod genes results in the production of specific extracellular lipo-oligosaccharide compounds that elicit root-hair deformation, cortical cell division, and other responses in the susceptible legume root.

Schlaman et al. (1989) constructed chimeric *nod*D gene, consisting of 75% from *nod*D1 gene of *R. meliloti* at the 5′ end and 27% of the *nod*D gene from *R. leguminosarum* bv. *trifolii*. Its expression in *R. leguminosarum* bv. *trifolii* and *R. meliloti* resulted in an extension of the host range for nodulation to the tropical legumes *Macroptilium atropurpureum*, *Lablab purpureus*, and *Leucaena leucocephala*. The expression of chimeric *nod*D gene in *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* also resulted in a significant increase in nitrogen fixation during symbiosis with *Vicia sativa* and *Trifolium repens*. Bender et al. (1988) transferred *nod*D1 gene from Rhizobium strain NGR234 to a restricted host range *R. leguminosarum* bv. *trifolii* strain, and this transfer extended the nodulation capacity of the recipients to new hosts including the nonlegume *Parasponia andersonii*.

The transfer of the *nod*FEGHPQ genes of *R. meliloti* to strains of *R. leguminosarum* bv. *trifolii* or bv. *viciae* conferred to these strains the ability to nodulate alfalfa (Putnoky and Kondorosi 1986) but strongly inhibited nodulation on the normal host plants, white clover, and vetch, respectively (Debelle et al. 1988). Mutations in the *nod*H gene of *R. meliloti* (involved in the transfer of sulfate on lipo-oligosaccharide Nod factor) strongly inhibited nodulation on the normal host *Medicago sativa* and led to delayed nodulation on *Melilotus alba* but conferred the ability to nodulate the nonhost plant, vetch (Faucher et al. 1988; Roche et al. 1991). Mutation in *nod*Q gene also extended the host range of *R. meliloti* to vetch.

The *nod*L gene is required for the addition of an O-acetyl residue at the terminal non-reducing glucosamine residue in *R. meliloti* Nod factors (Ardourel et al. 1994). In strain NGR234, disruption of the flavonoid-inducible *nol*L gene results in the synthesis of NodNGR factors that lack the 3-O- or 4-O-acetate group (Berck et al. 1999). The transconjugants of *R. fredii* strain USDA257 containing *nol*L of NGR234 produce acetylated Nod factors and nodulated the nonhosts *Calopogonium caeruleum*, *L. leucocephala*, and *Lotus halophilus*. Acetylation of the fucose of Nod factors of *R. etli* also conferred efficient nodulation on some *P. vulgaris* cultivars and on the alternate host *Vigna umbellata* (Corvera et al. 1999).

The construction of bacterial strains with an increased copy number to specific genes has been widely used for biotechnological applications. In some cases natural gene amplification, a common feature of the genome of prokaryotic organisms, is associated with adaptive responses in bacteria (Romero and Palacios 1997). Amplification is usually achieved by co-integrating a plasmid carrying the region of interest into the homologous region of the genome. Homologous recombination between the DNA-repeat regions leads to duplication and further amplification of the whole amplicon structure. Castillo et al. (1999) used specific DNA amplification (SDA) strategy to construct *Sinorhizobium meliloti* strains CFNM101 and CFNM103, which contained an average of 2.5 to 3 copies of the symbiotic region (containing *nod*D1, *nod*ABC, and *nif*N of p*sym* plasmid). The inoculation of alfalfa with these strains resulted in an increase in nodulation, nitrogen fixation, and growth of alfalfa plants under environmentally controlled conditions. Similarly, Mavingui et al. (1997) used random DNA amplification (RDA) in the symbiotic plasmid of *R. tropici* to obtain strains with enhanced competitiveness for nodulation.

### 4.6.2 Enhancement of Nitrogen Fixation

The structural or regulatory *nif* genes of the nitrogenase enzyme complex can be manipulated to increase the efficiency of $N_2$ fixation. It has been hypothesized that increasing *nif*A production, which is the transcriptional activator of other *nif* genes, would enhance the expression of the whole $N_2$-fixing system. Preliminary greenhouse studies indicated that some *R. meliloti* strains having enhanced *nif*A gene products showed a 7–15% increase in biomass compared with the wild-type parent (Williams et al. 1990). Since the rate-limiting step in nitrogen fixation appears to be the cycle of binding reduced dinitrogenase reductase (Fe-protein, the *nif*H gene product) to dinitrogenase (MoFe-protein) followed by one electron transfer, it has been proposed that increased copies of the *nif*H gene and its product may result in increasing the turnover rate of nitrogenase. This may be the basis for the presence of more than one copy of the *nif*H gene in some diazotrophs such as *Azotobacter vinelandii* (Jacobson et al. 1986), *Rhizobium phaseoli* (Quinto et al. 1985), and *Azorhizobium sesbaniae* (Norel and Elmerich 1987).

Improved substrate transport through modified expression of the C4-dicarboxylate transport (*dct*) genes has also been used to enhance nitrogen fixation (Ronson et al. 1990). Root nodules are the largest sink of photosynthetic energy and consume approximately 10% of the plant's net photosynthetic output for $N_2$ fixation. Thus, $N_2$ fixation in the Rhizobium-legume symbiosis is presumed to be limited by the amount of plant-derived photosynthate available to bacteroids (Hardy and Havelka 1975). Birkenhead et al. (1988) suggested that increasing the ability of the endosymbiont to utilize photosynthate in the nodule may lead to increased $N_2$ fixation rates.

Nitrogenase activity is correlated with the hydrogenase activity that evolves hydrogen; hence, it requires more energy. The energy can be saved if the evolved hydrogen is further reduced to water releasing electrons. Some diazotrophic bacteria including *Rhizobium*, *Azotobacter*, *Azospirillum*, etc. are able to improve the efficiency of nitrogen fixation by oxidizing hydrogen using uptake hydrogenase enzyme, which is simultaneously produced and evolved during nitrogen fixation (Sindhu and Dadarwal 2000). These bacteria possess "uptake hydrogenases" (Fig. 4.5) which consist of two subunits HupS and HupL, and it is advantageous to introduce it together with the nif genes into hosts that do not possess uptake hydrogenase system.

This oxidation of $H_2$ increases the rate of ATP biosynthesis. Enhanced nitrogen fixation rates have been reported in nodules and bacteroids of soybean, pea, and *Vigna* group of hosts formed by inoculation of $Hup^+$ strains (Emerich et al. 1979; Dadarwal et al. 1985). Mutants were obtained for increased hydrogenase activity in $Hup^+$ *B. japonicum* strains (Merberg and Maier 1983) or *Rhizobium* sp. strains (Sindhu and Dadarwal 1992). The mutants of *Rhizobium* sp. strains showed increase in dry matter yield when inoculated into green gram and black gram. The *hup* genes, encoding the biosynthesis of uptake hydrogenase, have been cloned and used to transform $Hup^-$ strains. These $Hup^+$ recombinants have been shown to exhibit increased nitrogen fixation.

Another strategy of increasing nodule number by manipulations of host genome is also employed for enhancing nitrogen fixation capacity in symbiotic microorganisms. The basis for these approaches lies in several assumptions, including that nodulation in legume is suboptimal and increasing the nodule number will lead to

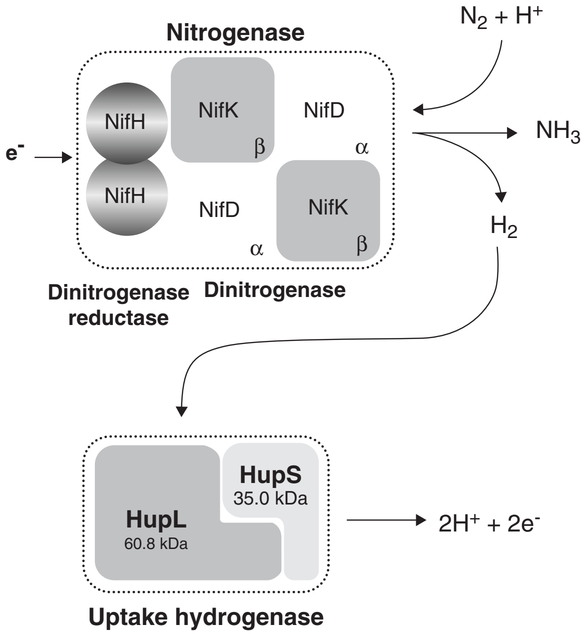

**Fig. 4.5** Schematic representation of the activity of uptake hydrogenase (Source: Sajid et al. 1992)

an increase in total fixed nitrogen. Research efforts to increase the number of root nodules were tested with hypernodulating mutants of soybean. Such mutants produced 100-times more nodules than the parent plant (Carroll et al. 1985; Betts and Herridge 1987). Several soybean mutants with the ability to form large number of nodules even in the presence of nitrate have been isolates (Carroll et al. 1985). These nitrate-tolerant symbiotic (*nts*) mutants usually form 3 to 40 times as many nodules as the parent and showed increased nitrogen fixation ability in the presence of nitrate.

Unfortunately, these mutants turned out to be poor agronomic performers (Pracht et al. 1994). The apparent reason for the failure of this approach was that plant spent considerable amount of energy in bearing root nodules, thus limiting the energy needed for the nitrogen fixation process. Nitrogen fixation is a highly energy intensive process consuming 6.5 g carbon $g^{-1}$ of the nitrogen fixed (Shantharam and Mattoo 1997). Sato et al. (1999) manipulated source-sink relationship in hypernodulating soybeans by decreasing infection dose in such a way that nodulation is reduced to normal level. They concluded that autoregulatory control may play an important role in optimizing the nodule numbers in soybeans to maximize the nodule growth and total nitrogen fixation capacity. The enzyme nitrogenase can be protected from the inhibitory action of oxygen by a protein leghemoglobin. The genes encoding these proteins can be isolated and transferred to other nitrogen-fixing systems so as to protect the nitrogenase from oxygen activity (Beringer and Hirsch 1984).

### 4.6.3 Nodulation and Nitrogen Fixation in Nonlegume Hosts

Some nonlegume plants are capable of establishing a nitrogen-fixing symbiosis. The *Frankia*-induced nodules on predominately woody angiosperms such as *Alnus* or *Casuarina* are of largest significance. These nodules have primitive, branched structures, reminiscent of thickened lateral roots, yet their ability to fix nitrogen is equivalent to that in legumes (Clawson et al. 2004). Similarly, nonlegume nodulation and nitrogen fixation was observed with *Bradyrhizobium* inoculants in the genus *Parasponia* (Trinick and Hadobas 1995; Webster et al. 1997). *Parasponia* nodules induced by *Bradyrhizobium* fixes nitrogen at highly efficient rates and are structurally similar to actinorhizal nodules.

The *nod*D gene of rhizobia has been demonstrated to control the first level of host specificity (Denarie et al. 1992). The mobilization of *nod*D1 of NGR234 into *R. leguminosarum* bv. *trifolii* caused the extension of host range to nodulate nonlegume *Parasponia andersonii* (Bender et al. 1988). Plasmids bearing *nod*DABC genes of *R. leguminosarum* bv. *trifolii* were transferred to *A. tumefaciens*, *Pseudomonas aeruginosa*, *Lignobacter* sp., *Azospirillum brasiliense*, *E. coli*, and different non-nodulating mutant rhizobia (possessing *sym* plasmid deletions). It conferred on these strains the ability to cause root hair curling and distortions on clover and a range of other nonhost legumes (Plazinski et al. 1994), suggesting that the expression of *nod*DABC genes in diverse range of soil bacteria may extend or affect the normal growth pattern of plant root hairs of a wide range of host and nonhost legumes. Although a large amount of research for enhancing nitrogen fixation through alteration of macrosymbiont host plant has been done with leguminous crops such as soybean and alfalfa, improvement of nitrogen fixation is also being examined in nonleguminous systems. The improvement of biological nitrogen fixation in actinorhizal (*Frankia*)-nonleguminous tree associations (Sprent and Sprent 1990) and in cereal plant-*Azospirillum* interactions (Dobereiner 1988) is of particular interest.

The genetics of host and microbes in both of these systems, however, are largely uncharacterized, and more research efforts are needed to enhance biological nitrogen fixation. Recent studies to transfer the nitrogen fixation ability or the capacity to form symbiotic associations to nonleguminous plants have shown that nodule-like structures could be induced on rice and wheat roots with *Rhizobium* strains under certain artificially created conditions using hormones or cell wall-degrading enzymes (Al-Mallah et al. 1989; Cocking 2003). A critical examination of these nodule-like structures revealed that the bacteria accumulated at the site of injury due to emergence of lateral roots. Recently, *Rhizobium* strains isolated from *Aeschynomene indica* (strain ORS310) and from *Sesbania rostrata* (strain ORS571) were reported to form nodule-like structures on emerging lateral roots of rice, wheat, and maize (Cocking 2009).

The nodules showed significant levels of nitrogen fixation activity using acetylene reduction assay. A high level of acetylene reduction activity was reported in lateral roots of wheat plants inoculated with *Azorhizobium caulinodans* when grown in pots under aseptic conditions. Nitrogenase activity, however, was not observed in uninfected plants or plants infected with $Nif^{-}$ strain of *A. caulinodans* (Sabry et al. 1997). *Azorhizobium caulinodans* strain ORS571 carrying a *lac*Z reporter gene was shown to be present within the cracks associated with emerging lateral roots of rice and wheat (Webster et al. 1997). The understanding of the various physiological and genetic processes within the legume plants and the bacteria and the identification of the essential characters that are present in legumes might result in the design of strategies by which nonlegumes like rice can be given the ability to enter symbiosis with nitrogen-fixing bacteria (Kennedy and Tchan 1992).

Therefore, extensive basic studies are needed to understand interactions between *Rhizobium* and cereal plants with special emphasis on signal-exchange mechanisms. Furthermore, these modified lateral roots of cereals having nodule-like structures must contain some sort of a micro-aerobic environment for plant protection of the $O_2$-sensitive nitrogenase. For this requirement, the plant could be engineered so that the intercellular space becomes filled with polysaccharides or other $O_2$-excluding material upon infection. Much efforts and coordination are required in genetics, molecular biology, and developmental biology to achieve a complete understanding of the *Rhizobium*-legume symbiosis and to explore potential avenues for achieving the ultimate goal of expressing active nitrogenase in cereal crops (Shantharam and Mattoo 1997).

### 4.6.4 Incorporation of *nif* Gene in Eukaryotes

Introduction of *nif* genes in eukaryotic cells has been attempted time to time. Eukaryotes have monocistronic mRNAs with a binding site at 5′ end, and for that if *nif* genes from prokaryotes were to be expressed in eukaryotes, it is necessary to fuse the coding sequence of each genes to the promoters of eukaryotes so as to produce a suitable ribosome binding site at 5′ end. The enzymes responsible for chlorophyll biosynthesis in *Chlamydomonas reinhardtii* resemble structural and functional homology with nitrogenase. The genes *chl*L, N, and B of *Chlamydomonas reinhardtii* are similar to the subunits of nitrogenase, but the genes *nif*H and *chl*L have strongest sequence identity, and for that *nif*H gene product can be activated by the genes required for *chl*L protein activity. Also *nif*H gene can substitute *chl*L in its function. The coding region of *chl*L can be replaced with *nif*H gene. It is a great challenge to introduce the *nif* gene in eukaryotes. Although in few cases the expression of the prokaryotic gene can express in eukaryotes such as yeast, the expression of *nif* genes has yet not been reported. Due to unfulfillment of physiological requirements, the enzymes transcribed by the promoter of *nif* genes would not function.

### 4.6.5 Protoplast Fusion Technique

The nodulation and production capacity of *Rhizobium sp.* is enhanced by the action of protoplast. The fusion of protoplasts of two weak strains of *Rhizobium* (Rt11 and Rt12) and one efficient strain (RtAI) resulted in 1.93- to 5.67-fold increase in nodulation number compared to that of parental strains (Sabir and El-Bestawy 2009). The nitrogen-fixing nodule formation is species dependent (Morris and Djordjevic 2006), while the nodule production efficiency is determined by host (Miller et al. 2007). Inappropriate strains or some environmental conditions also fail to produce high nodulation (Mhadhbi et al. 2008).

The nitrate reductase is more active in the plants nodulated by *Azorhizobium* than the non-nodulated ones (Saikia et al. 2006). To enhance the nodulation and nitrogen fixation, strategies have been made for the construction of improved strains of *Rhizobium* and *Bradyrhizobium* (Sabir and El-Bestawy 2009). Protoplast fusion is one of such strategies that can improve the genetic traits and nodulation efficiencies of Gram-positive (Hotchkiss and Gabor 1980) and Gram-negative (Attallah and Abd-El-Aal 2007) bacteria. Fast-growing nitrogen-fixing actinomycetes can be produced by fusing the protoplast of *Frankia* with the fast-growing actinomycete *Streptomyces griseofuscus* (Prakash and Cummings 1988). Introduction of the *nif* genes to protoplasts of nonleguminous plants has been attempted (Shanmugam and Valentine 1975). The cells of nitrogen-fixing bacterium *Azotobacter vinelandii* can be induced into fungus *Rhizopogon* which is mycorrhizal in *Pinus radiata*. Limited fixation can occur by mycelia of the modified fungus that is associated with *P. radiata* (Pandey 1978). *Rhizobium* of cowpea produces nodules on roots of nonleguminous tree *Trema aspera* (Trinick 1973), but these cannot fix nitrogen due to lack of leghemoglobin (Dilworth 1974; Baldani et al. 2000).

### 4.6.6 Performance and Limitations of Genetically Manipulated Inoculant Strains

Release of beneficial microorganisms into soils resulted in the colonization of soil and plant roots significantly. However, application of biofertilizer with commercial inoculant strains often fails to improve crop productivity as the desired result is usually not achieved (van Elsas and Heijnen 1990). The problem associated with the survival of inoculant diazotrophic bacteria under field conditions. Abiotic soil factors such as texture, pH, temperature, moisture content, and substrate availability largely determine the survival and activity of the introduced microorganisms (Hegazi et al. 1979; van Veen et al. 1997). The response of the inoculant diazotrophic bacteria to the prevailing soil conditions depends on its genetic and physiological constitution (Brockwell et al. 1995). The uses of genetic markers like resistance to drugs/antibiotics or introduction of metabolic markers from other bacterial species could help in tracing the introduced strains, whether it is rhizobia, cyanobacteria, *Azotobacters*, or *Azospirilla*.

A key factor involved in the lack of success of rhizobial inoculants is its failure to compete with the indigenous, ineffective, and built-in populations of homologous strains for nodulation (Sindhu and Dadarwal 2000). Production of bacteriocins by rhizobia have been shown to suppress growth as well as nodulation by the indigenous nonproducer strains, thus improving nodulation competitiveness of bacteriocins producing inoculant strains (Goel et al. 1999; Sindhu and Dadarwal 2000).

Transfer and expression of *tfx* genes (involved in trifolitoxin production) in various rhizobia showed stable trifolitoxin production and restricted nodulation by indigenous trifolitoxin-sensitive strains on many leguminous species. However, attempts to manipulate certain rhizobial genes in specific legume rhizosphere niches for improving competition have not produced impressive results (Nambiar et al. 1990; Sitrit et al. 1993).

Biotechnological approaches have limited success upon enhancement of nitrogen fixation and crop productivity under field conditions. For example, recombinant constructs of *R. meliloti* and *B. japonicum* having increased expression of *nif*A and *dct*A genes although showed increase in the rate of nitrogen fixed but under the field conditions, the same constructs were unsuccessful in showing any increase in $N_2$ fixation or agronomically significant yield improvement (Ronson et al. 1990). To improve the bacterial competition by manipulating the common nodulation, genes have usually resulted in either no nodulation, delayed nodulation, or inefficient nodulation (Devine and Kuykendall 1996). Attempts to engineer hydrogen uptake ($Hup^+$) ability by cloning hydrogenase genes into $Hup^-$ strains of *Rhizobium* resulted in experimental successes only in areas where soybeans are cultivated and where the photosynthetic energy is limited. Attempts to develop self-fertilizing crops for nitrogen had also a failure, mainly because of the complexity of the nitrogenase enzyme complex to be expressed in absence of an oxygen protection system in eukaryotes (Dixon et al. 2000).

## 4.7 Conclusions

Nowadays ever-increasing environmental pollution is of great concern due to intensive use of synthetic fertilizers in modern high-input agricultural systems. Therefore, incorporation of nitrogen-fixing plants in crop rotation offers an economically attractive and ecologically sound means for reducing cost of cultivation and improving the quality and quantity of end product. In symbiotic nitrogen fixation, the amount of $N_2$ input is reported to be as high as 360 kg N $ha^{-1}$ when *Rhizobium* species have been used as legume inoculants for converting free atmospheric nitrogen into plant available form. Though, the contribution of plant available $N_2$ by nonsymbionts (associative and free-living) are relatively minor, thus requiring fertilizer N supplementation. Future research regarding the basic mechanisms of the biological nitrogen fixation process facilitates identification of strategies for improvement of nitrogen fixation. In developing countries the field is untapped till date. This triggers implementation and adoption of the known technical know-how in this aspect. Beside symbiotic nitrogen fixation, thrust has to be given for understanding the molecular mechanism of nonsymbiotic nitrogen fixation. Many farmers fertilize crops grown after legumes in crop rotation like nonlegume as preceding crop and treat the legume-derived $N_2$ as a bonus that supports higher yield and protein content than what is normally achieved. Field measurement of biological nitrogen fixation is complex, tedious, and costly, and till date no simplified methods are available for routine on-farm use. Further research is also needed to improve the inputs of organics and BNF in Indian agriculture.

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# Arbuscular Mycorrhizal Symbiosis and Its Role in Plant Nutrition in Sustainable Agriculture

# 5

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**Abstract**

The soil microbiota is associated with the formation and maintenance of the stability of aggregates for the production of proteins and extracellular polysaccharides, and can be a determining factor in controlling the diversity of vegetable and other organisms that live aboveground. Arbuscular mycorrhizal (AM) fungi have yet to be fully utilized in agriculture, because certain aspects of their symbiotic nature (e.g., biogeography, ecology) are not fully understood. This symbiosis is an association formed between the roots of plant species and members of soil fungi belonging to the phylum Glomeromycota. Species of this phylum are obligate biotrophs, which, during symbiosis, establish a source sink for plant photosynthates. In exchange, the symbiont provides, through the hyphae, uptake of inorganic nutrients and water from the soil solution and their translocation to the host plant. Establishment of this symbiosis implies changes in the host plant's primary and secondary metabolism. This reprogramming transcriptome induced by the symbiont results in mycorrhizal plant acclimatization to environmental stresses. This chapter attempts to highlight the contribution that AM symbiosis can play as an ecosystem service provider to guarantee host plant nutrition under abiotic stress conditions, and discusses briefly the exploitation of arbuscular mycorrhiza in sustainable agriculture.

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**Keywords**
Plant-microbe interactions • Abiotic stress • Glomeromycota • Mycorrhiza

## Contents

## 5.1 Introduction

Globally, stresses of abiotic and biotic origin inhibit agricultural production, but abiotic stresses (e.g., drought, salinity, nutrient deficiency, extreme temperatures, and heavy metal (HM) toxicity) are the major factors in plant stress and crop yield losses (Shelden and Roessner 2013). There is a probability that the world's population will exceed the quota of nine billion by 2050 (FAO 2009; Godfray et al. 2010; Shelden and Roessner 2013). By 2025, global agricultural production may need to be improved by ~40% to meet these increasing demands (Pennisi 2008), as well as to provide food security for ~870 million now chronically undernourished people, according to the Organization for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization of the United Nations (FAO) (2012).

The rhizosphere microbiome constitutes the main fraction of plant–microbe symbiosis, also considered to be the second genome of the plant (Coats and Rumpho 2014; Berendsen et al. 2012).The soil is the major microbial reserve that affects host plant morphophysiology, making the plant resistant to stress (Lambers et al. 2008; Lugtenberg and Kamilova 2009; Chaparro et al. 2012; Doornbos et al. 2012).

Plant–microbe symbiosis plays a key role in the development of land ecosystems, and it is believed to have promoted the evolution of terrestrial plants (Zuccaro et al. 2014). In addition, plant–microbe symbiosis (e.g., arbuscular mycorrhizal (AM) interactions) can stimulate host plants, enhance the nutritional supply, improve drought and salinity tolerance (Auge 2004; Porcel et al. 2012; Auge et al. 2015), and trigger resistance to diseases (Pozo and Azcón-Aguilar 2007; Pozo et al. 2009; van Wees et al. 2008; Pozo et al. 2015). However, the genetic mechanisms responsible for increased plant tolerance of stress are not yet understood (Barzana et al. 2015; Ruíz-Lozano et al. 2012; Calvo-Polanco et al. 2014; Saia et al. 2014; Auge et al. 2015; Sanchez-Romera et al. 2015).

## 5.2 General Concepts in Mycorrhizal Status: An Overview

Plant–microbe symbiosis has modeled the origin, organization, and evolution of all organic life on Earth (Coasts and Rhumpho 2014). Symbiosis was an essential evolutionary mechanism in the genesis of the eukaryotic cell (Margulis et al. 2000, 2006). Microorganisms are the most primitive living beings on the planet, whose origin dates back ~3.5 billion years (Burchell 2010). Throughout the evolutionary process, they have acquired characteristics and adaptability for coexistence with other living beings, establishing diverse relationships in form and function (Taylor et al. 2012). Amid the numerous biological relationships existing among all life-forms on Earth, the symbioses between plants and heterotrophic microbes—mycorrhizal symbiosis—is the best known mutualist association between soil fungi and roots (Zuccaro et al. 2014). Approximately 80% of vascular plant species form mycorrhizal associations between their roots and soilborne fungi (Wang and Qiu 2006). AM fungi provide a more extensive absorptive surface than root hairs and thus help in the absorption of immobile ions (e.g., phosphate, copper, and zinc) in the soil, beyond the depletion zones (Pichardo et al. 2012).

This symbiosis may be used as a tool for prediction of sustainability in production systems. Mycorrhizal fungi have been shown to alleviate salinity, drought, and the toxicity of HMs, thus enhancing the host plant growth. To evaluate the potential role of AM fungi in improving soil fertility, research projects on exotic and native AM fungus (AMF) species should be conducted, with the best approaches being applied in sustainable agriculture systems.

### 5.2.1 Mycorrhiza Symbiosis Categories

It is estimated that mycorrhizal symbiosis occurs in about 250,000 species of plants worldwide, including the major arable crops (Smith and Read 2008). Divergent groups in the mycorrhizal symbiosis taxonomy have been accepted by distinct morphology patterns related to the presence of several extraradical or intraradical hyphal structures (Harley and Smith 1983; Bonfante and Perotto 2000). The major types of plant–mycorrhizal fungus interaction are ectomycorrhizas, endomycorrhizas, and ectendomycorrhizas (also called pseudomycorrhizas), according to how the fungal mycelium colonizes the root structure (Brundrett 2004; Smith and Read 2008). Among the endomycorrhizas, the arbuscular mycorrhiza type represents the best known, most studied, and most abundant plant–mycorrhizal association (Balestrini et al. 2015), with great ecological impact and economic importance (Smith et al. 2009).

AM fungi have an obligate life cycle, which receives photosynthates (e.g., carbohydrates) from the host plant, in exchange for supplying the host plant with ion uptake (particularly uptake of phosphorus and potassium in soil solutions) and water (Smith and Read 2008; Smith and Smith 2011; Kumar et al. 2016b). The ions are uptaken from the same soil labile pool from which the roots, via their active extraradical mycelium, improve host plant nutrition in low-nutrient-availability soil conditions (Bago et al. 2003; Smith and Smith 2011). This evolutionary jump was essential for enabling plants to move from an aquatic environment to land habitats, in the soil of which depletion zones rapidly develop after element absorption by organs such as roots (Corradi and Bonfante 2012).

### 5.2.2 Taxonomic Diversity of Arbuscular Mycorrhizal Fungi

Studies on fossilized roots (Remy et al. 1994) and phylogenetic evidence (Schubler and Walker 2011; Redecker et al. 2000b; Brundrett 2002; Wang and Qiu 2006) show that mycorrhizae appeared during the Devonian Period, ranging from 462 to 352 million years ago (Kenrick and Strullu-Derrien 2014). This period was connected with the emergence of land plants. Recently, phylogenetic molecular analyses of small subunit (SSU) ribosomal RNA gene sequences have separated AM fungi from the Zygomycota and placed them into the new phylum Glomeromycota (Schubler and Walker 2011). Conventional taxonomy of AM fungi has been performed on the basis of the morphological features of their spores (Morton and Msiska 2010) and the layers of the cell wall (Goto et al. 2012a, b). The taxonomy of AM fungi based only on these aspects is not sufficient to estimate the actual distribution patterns of Glomeromycota assemblages in all ecosystems (Lee et al. 2013). The latest classification of AM fungi (frequently updated on the website www.amfphylogeny.com) contains four orders, 11 families, and 25 genera (Schubler and Walker 2010; Oehl et al. 2011, 2014; Goto et al. 2012a, b; Błaszkowski et al. 2014).

Recently, major advances in AMF genomics studies (*Rhizophagus irregularis* DAOM197198) have been published (Badri et al. 2016), offering new tools to investigate the biology of this symbiosis (Tisserant et al. 2013). The use of the molecular tool has not yet revealed the real intraspecific diversity within the AMF genome, which still needs to be clearly understood for glomeromycotan species (Behm et al. 2014; Gianinazzi-Pearson et al. 2012).

Recent studies have described aboveground AMF richness, rather than belowground plant richness (Hiiesalu et al. 2012; Kumar et al. 2015, 2016a; Ahmad et al. 2016; Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Jha and Subramanian 2016). This underestimates the plant richness related to dormant plant species, clonal species, and ephemeral plant species (Hiiesalu et al. 2012).

It is estimated that AMF richness is more strongly related to belowground richness than aboveground plant richness because these soil fungi are associated with plant roots. Most importantly, one might expect AMF richness to be more strongly correlated with belowground than aboveground plant richness because these fungi associate directly with host roots (Hiiesalu et al. 2014). It is known that AM fungi can be selective for their host plant species (Gamper et al. 2009; Öpik et al. 2009). Therefore, the greater the value of the plant root richness, the greater the variety of ecological niches or habitats they eventually would potentially have (Hiiesalu et al. 2014).

### 5.2.3 Morphology and Reproduction

In the Glomeromycota, large spores carry out asexual reproduction as a single cell with several hundred or thousands of nuclei (Gianinazzi-Pearson et al. 2012; Jany and Pawlowska 2010). No teleomorphic phase is known for this phylum, but a current transcriptomic analyses of *Rhizophagus irregularis* has revealed that Glomeromycota do possess basic genomic information for sexual reproduction and meiosis (Badri et al. 2016; Tisserant et al. 2013). Species have been described in Glomeromycota usually as morphospecies (Redecker and Schubler 2014). Gianninazi-Pearson et al. (2001) reported that depending on the analytical method and the genome of reference used, the DNA value contents of glomeromycotan fungi vary. However, the use of recognized working standards as controls to calibrate the experimental conditions can avoid these analytical biases (Yu et al. 2015). The scope of intraspecific diversity within the genome still needs to be well determined for glomeromycotan fungus species (Gianinazzi-Pearson et al. 2012).

The genome of glomeromycotan fungi presents a high rate of polymorphism (Sanders and Croll 2010), and the large genomes and asexuality of glomeromycotan fungi remain challenges to understanding of the genome complexity of these organisms (Martin et al. 2008; Gianinazzi-Pearson et al. 2012). Another important point for mycorrhizal research is to provide an acceptable species taxonomy concept for Glomeromycota and laboratory tools to permit easy and fast identification and description of species with functional relevance in relation to symbiosis (Meena et al. 2015a; Priyadharsini and Muthukumar 2016; Jaiswal et al. 2016; Kumar et al. 2017).

### 5.2.4 Development of the Arbuscular Mycorrhizal Association and Root Colonization

AM symbiosis can be separated into distinct steps, which are characterized by the level of progression of fungal hyphae during root colonization (Harrison 2005; Gutjahr and Parniske 2013). The “presymbiotic” stage refers to the mutual recognition, and the “symbiotic” stage, the hyphopodium formation, refers to the appressorium and arbuscule formation (reviewed by Kosuta et al. 2003; Akiyama et al. 2005; Harrison 2005; Akiyama and Hayashi 2006; Genre et al. 2008; Parniske 2008; Kuhn et al. 2010; Maillet et al. 2011; Hocher et al. 2011; Harrison 2012; Wang et al. 2012; Delaux et al. 2013; Genre et al. 2013; Gutjahr and Parniske; 2013; Svistoonoff et al. 2013; Bucher et al. 2014; Etemadi et al. 2014; Favre et al. 2014, Yu et al. 2014; Meena et al. 2015b, f; Dotaniya et al. 2016; Raghavendra et al. 2016; Zahedi 2016).

The next stage of the development of the AM association is called "root colonization" and refers to the changes in plant physiology (Hause et al. 2007). The changes span from alterations in the hormonal balance and transcriptional profile to altered primary and secondary metabolism, and many of the changes are related to defense mechanisms, likely contributing to the plant maintaining control over the symbiotic partner (for further details, see the reviews by Ludwig-Muller and Guther 2007; Liu et al. 2007; Hause et al. 2007; Schliemann et al. 2008; Bari and Jones 2009; Fiorilli et al. 2009; Hause and Schaarschmidt 2009; Pozo et al. 2009; Garcıa-Garrido et al. 2010; López-Ráez et al. 2010; Ludwig-Muller 2010; Chiou and Lin 2011; Hammond and White 2011; Ludwig-Muller 2011; Martın-Rodrıguez et al. 2011; Sato and Miura 2011; Gallou et al. 2012; Aroca et al. 2013; Cosme and Wurst 2013; Wasternack et al. 2013; Fernandez et al. 2014; Gutjahr 2014; Torres-Vera et al. 2014; Martın-Rodrıguez et al. 2015; Pozo et al. 2015) and finally the mycorrhizal network formation (reviewed by Gutjahr and Parniske 2013; Bever et al. 2010; Walder et al. 2016). Following the establishment of all of these steps, the plant has to control the level of fungal invasion within the roots to avoid excessive colonization and carbon drainage, thus maintaining the interaction at mutualistic levels (Jung et al. 2012).

### 5.2.5 Plant Strategy to Acquire Inorganic Phosphate from the Environment

Soil inorganic phosphate (Pi) is essential for proper growth and functioning of all life-forms, so in natural conditions, usually the plant growth and yield are adversely affected when Pi supply is limited (Ullrich-Eberius et al. 1984; Furihata et al. 1992; Schachtman et al. 1998; Epstein and Bloom 2005; Javot et al. 2007; Cordell et al. 2009; Smith et al. 2011; Smith and Smith 2011; Gilbert 2009; Feddermann et al. 2010; Marschner and Marschner 2012; Niu et al. 2013; Lopez-Arredondo et al. 2014; Dominguez-Nunez et al. 2016; Rawat et al. 2016; Yasin et al. 2016).

One strategy is to increase the root–soil interface to maximize access to and uptake of the available Pi (Bates and Lynch 1996; Williamson et al. 2001; Ticconi and Abel 2004; Javot et al. 2007; Ma et al. 2001). A second strategy is to solubilize Pi trapped in mineral–organic complexes (Lefebvre et al. 1990; Duff et al. 1994; Hinsinger 2001; Zakhleniuk et al. 2001; Vance et al. 2003; Johnson and Loeppert 2006; Javot et al. 2007; Nouri et al. 2014; Bahadur et al. 2017; Verma et al. 2017b; Kumar et al. 2017).

#### 5.2.5.1 Regulation of Arbuscular Mycorrhizal Development by Phosphate Availability

At present, the most widely known example of such regulations is the control of AM symbiosis according to phosphorus (P) availability (Johnson 1993; Javot et al. 2007; Smith and Read 2008; Collins and Foster 2009; Balzergue et al. 2011). The AM symbioses are able to uptake Pi by two pathways: directly by root epidermal cells and root hairs, and via AM fungi (Marschner 1995; Liu et al. 1998; Harrison 2005; Bucher 2007; Javot et al. 2007; Liu et al. 2008; Salt et al. 2008; Smith and Read 2008; Christophersen et al. 2009; Nagy et al. 2009; Ramos et al. 2011; Smith et al. 2011; Tian et al. 2013; Carbonnel and Gutjahr 2014; Nouri et al. 2014; Watts-Williams et al. 2015; Meena et al. 2015e, 2016c; Teotia et al. 2016; Bahadur et al. 2016b; Das and Pradhan 2016). Therefore, cracking the code of chemical signaling in AM symbiosis remains a challenging but tangible objective for the near future (Bucher et al. 2009; Nath et al. 2017; Sarkar et al. 2017; Verma et al. 2017a).

## 5.3 Sustainability and Agricultural Sustainability

The impact of xenobiotic compounds employed in intensive agriculture is a serious, complex, controversial issue, widely debated by the entire world scientific community, and has undoubtedly proved to be a dangerous threat to food security, human health, and environment sustainability (Shorette 2012). The consequent problems of contamination (e.g., soil acidification, soil infertility, residues of hormones and antibiotics, poisoning of wildlife, and HM pollution of groundwater, surface water, and the atmosphere) have caused continuing environmental deterioration, affecting human and animal health (Guo et al. 2010; Tian and Niu 2015).

"Sustainable development … seeks to meet the needs of the current generation without compromising the ability of future generations to meet their own needs" (Cerin 2006). Nowadays, undoubtedly there is a tendency to develop agricultural systems that are less aggressive to the environment, based on eco-environmentally sustainable technologies, and less dependent on agricultural inputs (e.g., synthetic fertilizers, herbicides, and pesticides), to reduce impacts on the environment, to conserve and improve soil health, and to guarantee food health and security (Azcón-Aguilar and Barea 2015; Moonen and Bàrberi 2008). To achieve these aims, it is fundamental to promote plant and soil health—properties that are the result of the interactions among physicochemical and biological factors (Altieri 2004; Saha et al. 2016a; Yadav and Sidhu 2016; Meena et al. 2016d; Saha et al. 2016b)—and allow farmers to maintain healthy, productive soil for crops without degrading the agroecosystem and ecosystem.

A subset of the biological components of "soil fertility" is constituted by diverse genetic and functional soil macro-, meso-, and microbiota (e.g., plants, termites and others invertebrates, archaea, nonsymbiotic bacteria and fungi, symbiotic fungi and bacteria, and protozoa). These soil biological components are responsible for a great array of agroecosystem services, including the biogeochemical cycling of plant nutrients, and recycle key nutrient elements by decomposing organic residues (Azcón-Aguilar and Barea 2015). In sustainable, low-input cropping systems, the ecological roles of the microbiota (rhizospheric and nonrhizospheric) in maintaining soil health and biological control of phytopathogens may be more important than they are in traditional agriculture (Johansson et al. 2004).

"Sustainability" in agriculture is difficult to define unequivocally but often involves minimal chemical inputs, efficient nutrient recycling, and enhancement of important microbial-driven processes, such as nutrient acquisition, decomposition, and protection against pathogens (Rooney et al. 2009). The focus and conceptual ideas on sustainable agriculture (e.g., environmental concerns) and food security systems began to appear in the 1950s–1960s (Ward and Dubos 1972). Nonetheless, ideas about life sustainability date back at least to the oldest scribes of ancient Greece, China, Greece, and Rome (Pretty and Bharucha 2014).

In the present day, ~900 million people worldwide are vulnerable to hunger, including those deficient in energy, those with micronutrient deficiencies, and those who are malnourished. Many of them are small farmers, from agricultural settlements. The modern model of agricultural production is facing increasing challenges, such as soil erosion, soil acidification, water scarcity, climate change, phytopathogens, insect pests, market risk, volatility of agricultural products, and a rising risk of production shortfalls. New strategist models need to be adopted to increase agricultural production sustainably, while food security and environmental sustainability are economic opportunities of a futuristic vision of a globalized world (Raja 2013; Verma et al. 2015b; Verma et al. 2014; Meena et al. 2014a; Sharma et al. 2016; Meena et al. 2015d; Singh et al. 2015; Meena et al. 2013b; Bahadur et al. 2016a; Masood and Bano 2016). Currently, sustainability in agricultural models incorporates the concepts of both resilience (the buffer capacity of systems) and persistence (the continuum turnover capacity of systems) and addresses several extensive economic, social, and environmental outcomes (Pretty 2008).

Traditional thinking about several models of agricultural sustainability usually requires imposition of drastic reductions of inputs (i.e., changes from use of fertilizers to use of nitrogen-fixing legumes, changes from use of pesticides to emphasis on natural enemies, and changes from plowing to zero tillage) (Pretty and Bharucha 2014). Against this background, there has recently been impressive progress in reducing negative effects of traditional agriculture on the environment (Pretty and Bharucha 2014; Pretty 2013; Foresight 2011). To put it another way, the increasing pressures on the global food system, caused by the population explosion or the increase in per capita consumption, reveal the major challenge of sustainable agriculture—the production of more food—leading to a plethora of calls all aiming to stimulate, in a rational way, use of environmental resources to increase agricultural productivity, reduce environmental damage, change social politics, alter consumption patterns, and address food losses and waste (Godfray and Garnett 2014). Undoubtedly, the consequences of unsustainable intensification will damage our planet and, in doing so, will undermine its capacity to support the next generations' food production.

## 5.4 Plants Under Abiotic Stress

Adverse physicochemical soil conditions (also called abiotic stresses—e.g., drought, salinity, extreme temperatures, and contamination with HMs) result in soil degradation, and in modern agriculture, they are considered the major cause of crop yield loss worldwide (Wang et al. 2003).

Plant responses to different types of stress are highly complex and involve the activation of molecular cascade events, followed by stress perception, signal transduction, and changes at the transcriptome level (in gene and protein expression and posttranslational modifications of stress-induced proteins), the cellular level, and the physiological level (Hossain et al. 2013; Atkinson and Urwin 2012), which limit damage or facilitate the repair of damaged systems (Potters et al. 2009). Plants possess another mechanism that allows them to tolerate adverse physicochemical conditions, which consists of an association with rhizospheric and nonrhizospheric microbiota that can help them to survive. Among these associations, mycorrhizal symbiosis is the most widely disseminated—so much that in natural ecosystems and agrosystems, this association is considered the rule rather than the exception (Hodge et al. 2009; Verma et al. 2015a).

Mycorrhizal symbiosis has a range of attributes that contribute to tolerance of or resistance to some kinds of abiotic stress in mycorrhizal plants (reviewed by Miransari 2010). These have been recently reported in different papers on metal toxicity (Cicatelli et al. 2014), oxidative stress (Manchanda and Garg 2011), water stress (Pagano 2014; Rapparini and Penuelas 2014), salinity (Abdel Latef and Miransari 2014; Hameed et al. 2014; Hajiboland 2013; Aggarwal et al. 2012; Porcel et al. 2012; Evelinet al. 2009), and soil acidification (Muthukumar et al. 2014). However, despite the ubiquity of AM fungi in extreme environmental conditions and the potential of these symbiotic endophyte fungi to protect plants against several adverse edaphic–climate conditions, there is almost no information available at present concerning the mechanisms implemented by AM fungi themselves to tolerate the deleterious effects induced by stresses (Lenoir et al. 2016; Meena et al. 2013a; Singh et al. 2016).

### 5.4.1 Plants and Salinity Stress

The soil salinity is one of the most catastrophic physicochemical environmental conditions, which causes major losses in crop cultivated land area, crop productivity, and food quality (Yamaguchi and Blumwald 2005; Munns and Tester 2008; Tavakkoli et al. 2010; Yousif et al. 2010; Shahbaz and Ashraf 2013). Usually “salinity” refers to the accumulation of excessive salts in sodic or (alkaline) and saline soils (Abdel Latef and Miransari 2014). This (natural or anomalous) environmental condition represents an array of troubles for plant growth, including poor water quality (salinized water), excessive and toxic amounts of ionic forms of Na and Cl (in soil and water), and low or no availability of Ca (Abdel Latef and Chaoxing 2014; Marschner and Marschner 2012). The major cations mobilized in saline or sodic soils consist of $Na^+$, $Ca^{+2}$, $Mg^{+2}$, and $K^+$, and the main anions are $Cl^-$, $SO_4^{-2}$, $HCO_3^-$, $CO_3^{-2}$, and $NO_3^-$. Other ionic forms that contribute to the increase in this condition in hypersaline substrates and waters include B, $Sr^{+2}$, $SiO^{+2}$, Mo, $Ba^{+2}$, and $Al^{+3}$ (Hu and Schmidhalter 2002; Shrivastava et al. 2016; Velazquez et al. 2016; Meena et al. 2015c; Sindhu et al. 2016; Meena et al. 2014b).

On the basis of adaptive evolution, plants can be separated roughly into two major types: halophytes, which can withstand salinity (Aslam et al. 2011), and glycophytes, which cannot withstand salinity and will eventually die (Xiong and Zhu 2002). Most plants are glycophytes (Abdel Lafet and Miransari 2014).

Plants develop various physiological and biochemical mechanisms (e.g., compatible osmolytes such as proline, glycine betaine, soluble sugars, and sugar alcohols (Ahmad et al. 2011, 2014)) and ultrastructural changes to survive in soils with an excessive salt concentration, which help in averting salt-triggered alterations (Farrant and Ruelland 2015).

Both enzymatic mechanisms, involving the superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (Apel and Hirt 2004) and nonenzymatic mechanisms including the scavenging of reactive oxygen species (ROS), antioxidants, ascorbic acid (AsA), tocopherols, phenols, and thiols in combination with other antioxidants and phytohormones are triggered (Ahmad et al. 2011; Ahmad et al. 2015; Hashem et al. 2015).

#### 5.4.1.1 Effect of Arbuscular Mycorrhizal Fungi on Nutrient Uptake by Salt-Stressed Plants

AM symbiosis has a low affinity for halophyte plants (Brundrett 1991; Juniper and Abbott 1993). Isolation of spores of AM fungi in saline environments has been well documented (reviewed by Zuccarini 2007; Sheng et al. 2008; Evelin et al. 2009; Wang et al. 2008; Abdel Latef et al. 2009; Wilde et al. 2009; Abdel Latef 2010; Abdel Latef 2011a; Abdel Latef 2011b; Aggarwal et al. 2012; Horie et al. 2012; Porcel et al. 2012; Hajiboland 2013; Kapoor et al. 2013; Abdel Latef and Miransari 2014; Hameed et al. 2014; Hazzoumi et al. 2015; El-Nashar 2016).

##### 5.4.1.1.1 Nitrogen

The symbiotic relationship between legumes and nitrogen-fixing rhizobia is susceptible to abiotic factors, such as nutrient deficiency, salinity, drought, acidity, and elevated soil temperature, which induce failure of the infection and nodulation processes (Yinsuo et al. 2004; Ardakani et al. 2009; Tajini et al. 2012; Egamberdieva et al. 2013; Abd-Allah et al. 2015; Egamberdieva et al. 2016; Hashem et al. 2016). This knowledge is important for our understanding of the relationships between AM fungi and endophytic bacteria, their effects on mycorrhizal plants, and the implications for crop management agricultural practices under hostile environmental conditions (Hashem et al. 2016).

##### 5.4.1.1.2 Phosphorus

The different mechanisms that AM fungi use to enhance the salt resistance of host plants include improving nutrient uptake, especially P uptake (reviewed by Marschner and Dell 1994; Ruíz-Lozano and Azcón 2000; Al-Karaki et al. 2001; Feng et al. 2002; Giri et al. 2007; Smith and Read 2008; Evelin et al. 2009; Evelin et al. 2012; Porcel et al. 2012). Furthermore, the higher carbohydrate requirement of AM fungi induces higher soluble sugar accumulation in host root tissues, which is independent of the improvement in the plant P status and enhances resistance to salt-induced osmotic stress in mycorrhizal plants (Feng et al. 2002).

##### 5.4.1.1.3 The $K^+$ to $Na^+$ Ratio

Salt stress affects mainly plant physiology and metabolism through changes in the turgor of ions inside the cells (Blaha et al. 2000; Rus et al. 2001; Rabie and Almadini 2005; Giri et al. 2007; Munns and Tester 2008; Hajiboland 2013). Mycorrhizal plants can reverse the effect of salinity on K and Na nutrition (Juniper and Abbott 1993; Grattan and Grieve 1998; Daei et al. 2009; Evelin et al. 2012; Ruíz-Lozano et al. 2012; Abdel Latef and Chaoxing 2014; Abdel Latef and Miransari 2014). The use of mycorrhizal plants could be a cost-effective and sustainable approach to enhancing the salt tolerance or resistance of crop plants (Evelin et al. 2009; Sarwat et al. 2016).

##### 5.4.1.1.4 Calcium

Mycorrhiza has been reported to alter calcium content in plants under saline stress. Increases in uptake and accumulation of calcium due to AMF have been reported (Cantrell and Linderman 2001; Yano-Melo et al. 2003; Wu et al. 2011; Hart and Forsythe 2012). There is evidence that AM symbiosis affects and regulates several of these mechanisms, but many physiological aspects and the molecular basis of such regulation are almost completely unknown (Ruíz-Lozano et al. 2012). Transcriptomic analysis of roots and AM fungi (Tisserant et al. 2013) is a promising tool, which could provide new data on fungal genes that may also participate in the response of AM symbiosis to salinity stress (Ruíz-Lozano et al. 2012). Recent advances in the field of "omics" technology and advanced microscopy can provide new insight into these mechanisms of plant–AMF interactions (Ruíz-Lozano et al. 2012; Kumar et al. 2015).

### 5.4.2 Plants and Drought Stress

Undoubtedly, the consequences of climate change (e.g., the recent global temperature rise) are not well understood (Vicente-Serrano et al. 2014), and it is expected to cause more drought severity and affect an increasing number of terrestrial biomes (Sheffield et al. 2012). It is no secret that drought stress is gaining importance as human agricultural activities increase to involve low-fertility soil to satisfy the world's growing demands for food (Flexas et al. 2013). These combined effects will lead to water resources being a scarce commodity for fish farming, livestock, agriculture, and other related activities (Rosegrant and Cline 2003). Drought or water deficit has a negative impact on plant growth, development, survival, and crop yield, posing a threat to all sustainable activity (Cattivelli et al. 2008; Boyer 1982). It is widely known that when plants are exposed to stress (e.g., drought stress), the hormone signaling pathways of salicylic acid (SA), jasmonic acid (JA), ethylene (Et), and abscisic acid (ABA) are activated (Alazem and Lin 2015). These metabolic pathways induce essential changes for plant stress acclimation (Pruthvi et al. 2014). Plants stressed by drought accumulate ectoine, glycine betaine, glycerol, trehalose, proline, myoinositol, and others osmolytes inside the plant cells, which help in raising the osmotic pressure and in maintaining turgor pressure and the driving gradient for water uptake (Flexas et al. 2013; Timmusk et al. 2014; Verslues and Juenger 2011; Fleury et al. 2010). The development of crops for enhanced drought resistance is a promising approach to alleviate this crisis (Farooq et al. 2009).

Without any doubt, plant drought resistance depends on species' own strategies and also on the duration and severity of the water scarcity period (Cruz de Carvalho 2008). Drought stress, if prolonged, will inevitably result in oxidative damage due to the overproduction of ROS (Smirnoff 1993).

#### 5.4.2.1 Effect of Arbuscular Mycorrhizal Fungi on Nutrient Uptake by Drought-Stressed Plants

It is generally accepted that mycorrhizal plants under drought stress (Auge and Moore 2005) often show enhanced osmotic adjustment (Morgan 1984; Hoekstra et al. 2001; Ruíz-Lozano 2003; Dodd and Perez-Alfocea 2012), enhanced antioxidase activity and photosynthetic rates (Huang et al. 2011), induction of plant growth hormonal signals (such as those of SA, JA, Et, and ABA) (Yang et al. 2014), accumulation of organic compounds (e.g., sugars and amino acids), and increased levels of ions (e.g., [$Mg^{+2}$], [$K^{+}$], and [$Ca^{+2}$]) (Moe 2013). Other effects that are commonly reported include enriched soil moisture and soil properties (Yooyongwech et al. 2013).

Improved exploitation of soil water due to the hyphal contribution to water uptake and/or induced changes in root morphology and soil structure have been proposed (Auge 2001). Apparently, in mycorrhizal plants, maintenance of root structural integrity during drought was not related to osmoprotection (Auge et al. 1992). Characterization of fungal aquaporin genes profiles has made it possible to support the existence of direct AMF involvement in plant drought tolerance (Li et al. 2013a, b, Maya and Matsubara 2013). Although the role of AM fungi in plant drought tolerance has been well documented in the literature for several plant–AMF combinations (Zhang et al. 2015), the underlying molecular mechanisms involved are still to be elucidated (Chitarra et al. 2016).

### 5.4.3 Plants and Heavy Metals

Unfortunately, beyond natural conditions (e.g., geologic activities), global mechanization, intensive farming, intensive livestock management, and other anthropogenic activities (e.g., mining, urbanization, metallurgy, and industrial activities) have led to the increased release of toxic compounds, such as heavy metals, into the lithosphere (Ma et al. 2016; Mosa et al. 2016), catastrophically altering geochemical cycles and the biochemical balance (Singh et al. 2011). Contamination of soils has received intensive attention in contemporary science (Lutts and Lefevre 2015; Batty and Dolan 2013; Rajkumar et al. 2012). It damages the environment by affecting soil fertility, biomass, and crop yields, with detrimental consequences for ecosystems and global human health (Furini et al. 2015).

HMs are defined as those 38 elements (exhibiting metallic properties) that have a high atomic weight and a density at least five times greater than that of water (Hall 2002; Antonovics et al. 1971). Apart from atmospheric oxygen and soil-derived water, plants typically require 14 essential nutrients, including macronutrients such as nitrogen (N), phosphorus (P), potassium (K), sulfur (S), magnesium (Mg), calcium (Ca), and micronutrients such as sodium (Na), boron (B), manganese (Mn), iron (Fe), zinc (Zn), nickel (Ni), and molybdenum (Mo) (Maathuis 2009). Some of these micronutrients (such as Zn, Fe, Mn, copper (Cu), Mo, and Ni) are required by all life-forms at low concentrations for their metabolic activities, but in excess, they are toxic (Narula et al. 2010). Excessive amounts of both essential heavy metals (e.g., Zn, Cu, Ni, Mn, Mo, and Fe) and nonessential heavy metals (e.g., chromium (Cr), cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg)) may pollute the environment and create problems for agricultural production and for human and animal health (Lin and Aarts 2012; Bothe 2011; Prasad et al. 2011; Abbaspour et al. 2008).

The bioavailability of HMs is often described as being related to their toxicity, which is influenced by physicochemical soil characteristics (e.g., pH, and clay and organic matter content) and biological microbial activities (e.g., those of mycorrhizal fungi) (Berthelin et al. 1995; Leyval et al. 1997; Leyval and Joner 2001; Amir and Pineau 2003). HM toxicity in plants appears to result from generation of ROS (e.g., by the Fenton reaction (Keightley et al. 2004; Kieffer et al. 2008), with prevention of heavy metal uptake (exclusion/amelioration) and physicochemical barriers protecting crucial organs from toxicity (avoidance) (Baker 1987; Baker and Brocks 1989).

#### 5.4.3.1 Metallophyte Plants

Metallophytes are plants that are naturally adapted to grow in heavy metal soils (Bothe 2011; Hildebrandt et al. 2007; Antonovics et al. 1971). In metallophyte plants, metal hyperaccumulation and hypertolerance capacity are naturally selected (Kramer 2010).

#### 5.4.3.2 Phytoremediation

Phytoremediation is a technical type of bioremediation using plants, and comprises two biological components, one involving the rhizosphere microbiota and the other involving the intrinsic characteristics of the plant itself. To date, this has been considered a low-cost, novel, eco-environmental technology for removal of organic and inorganic contamination (Suresh and Ravishankar 2004). Many scientists are currently investigating the phenomenon of metal hyperaccumulation in different species, aiming to determine the mechanisms associated with the accumulation and detoxification of heavy metals and ultimately to use these plants and their rhizosphere-derived microorganisms (e.g., rhizobacteria and mycorrhizal fungi) for decontamination of polluted sites (Furini et al. 2015).

#### 5.4.3.3 Effect of Arbuscular Mycorrhizal Fungi on Nutrient Uptake by Heavy Metal–Stressed Plants

Hyperaccumulation of certain metals/metalloids (such as As, Cd, cobalt (Co), Cu, Mn, Ni, Zn, and Pb) by plants is an extremely rare phenomenon, found in fewer than 0.2% of angiosperms (Regvarand Vogel-Mikuš 2008; Baker and Whiting 2002; Hildebrandt et al. 1999). Bioremediation practices using plants with hyperaccumulating ability or in association with soil microbes (e.g., AM symbiosis) are the most common biological methods of recovery used for soil contaminated by heavy metals (Orłowska et al. 2013; Souza et al. 2013; Guo et al. 2013; Rajkumar et al. 2012; Meier et al. 2011; Miransari 2011; Hua et al. 2009).

AMF spores are found in soil from metalliferous sites, probably resulting from the coevolution process of plant–fungal symbiosis over millions of years (Regvar and Vogel-Mikuš 2008). In AM symbiosis, this process is mainly determined by the type of HM concentration, the dilution of the metal concentration, production of specific proteins (e.g., metallothioneins), plant specifications, and growth conditions (Sun et al. 1999; Turnau and Mesjasz-Przybylowicz 2003; Finlay 2004; Hua et al. 2009; Gohre and Paszkowski 2006; Rivera-Becerril et al. 2005). All of these plant–fungal tolerance mechanisms, as well as specific edaphic conditions (e.g., metal tolerance mechanisms, metal speciation, soil pH, and soil organic matter content) must be taken into account during the evaluation and interpretation of results (Leyval et al. 1997; Leyval and Joner 2001; Audet and Charest 2007).

At specific soil metal concentrations (e.g., high soil Zn concentrations) high rates of root colonization in mycorrhizal plants are reported, which suggests that photoprotective effects should be related to AM-induced biosorption processes that reduce soil metal bioavailability (Audet and Charest 2007). It has also been observed that extraradical hyphae (in the mycorrhizosphere) cause alkalinization of the proximal soil environment (the rhizospheric soil). This soil condition reduces the uptake of HMs into plant cells (in mycorrhizal plants) and may be one of the means that allow metallophytes (Singh et al. 2015) to thrive on heavy metal–polluted sites (Weissenhorn et al. 1995; Leyval et al. 1997; Kaldorf et al. 1999; Khan 2006; Vogel-Mikuš et al. 2006).

In addition, with regard to protection by AM fungi that colonize plant roots on heavy metal–polluted sites, there have been considerably contradictory reports (e.g., it has been reported that AM fungi have no effects or may have negative effects) on the effectiveness of AM fungi for HM uptake by mycorrhizal plants (Pichardo et al. 2012) at high metal concentrations and low pH values (Citterio et al. 2005; Arriagada et al. 2007; Jankong and Visoottiviseth 2008). Not only for financial reasons, but also for practical reasons, considerable scientific efforts are therefore being directed to improvement of the efficiency of phytoremediation using biotechnology tools (Narula et al. 2010; Regvar and Vogel-Mikuš 2008; Vogel-Mikuš et al. 2006). Undoubtedly, phytoremediation mediated by AM symbiosis provides an attractive system to advance plant-based environmental clean-up.

### 5.4.4 Plants and Acidity Stress

Acidic soils are phytotoxic to plant survival, distribution, and interactions with microorganisms (Thomas and Hargrov 1984; Marschner 1995; Marschner and Marschner 2012; Muthukumar et al. 2014). The pH (excessive $OH^-/H^+$ ion concentrations) of the soil solution is influenced by the form of plant ion nutrition because of differences in the cation to anion uptake ratio, ion assimilation, and cellular pH stabilization (Marschner and Marschner 2012). Highly weathered soils (e.g., in the tropics and subtropics, such as Oxisols and Ultisols, as well as many Spodosols and Inceptisols) have pH values below 5.5 (Lynch and Clair 2004). Certain agricultural practices are intensifying soil acidification (e.g., repeated applications of nitrogen) in amounts that exceed crop capacity uptake (Senbayram et al. 2012). In this regard, agriculturally unsustainable systems should be avoided and systematically replaced by practices that are environmentally friendly.

#### 5.4.4.1 Plants and Aluminum Toxicity

In the lithosphere, aluminum (Al) is the third most abundant metal, with a prevalence of ~8.1% (Tian et al. 2013). Al toxicity in acid soils is considered the most important growth-critical factor in crop production, considering that the global arable land area is estimated to be 1.351 billion hectares, and ~50% of this area potentially has acidic soils (for a review, see Kochian et al. 2015). Acidity toxicity and Al toxicity cannot be separated, since Al is soluble only in an acid solution (Kochian et al. 2015). Plants adapted to acid mineral soil may resist Al toxicity by a variety of mechanisms, such as avoidance by Al exclusion, high efficiency of nutrient acquisition, favorable soil microbiota (Marschner and Marschner 2012), and/or tolerance mechanisms (achieved by accumulation) due to the detoxification of Al inside the cells by includer and excluder plants (Inostroza-Blancheteau et al. 2012).

#### 5.4.4.2 Aluminum Toxicity and Arbuscular Mycorrhizal Symbiosis

Previous studies cited in the literature have shown the protective effects of AM symbiosis in plants under Al exposure, but the mechanisms of Al tolerance in mycorrhizal plants are not yet clear (Zhang et al. 2015; Evelin et al. 2009). Still in the field of speculation, it has been proposed that exudation of organic acids (e.g., citrate, malate, and acetate) by AM fungal hyphae (Plassard and Fransson 2009; Toljander et al. 2007; Tawaraya et al. 2006) could attenuate Al toxicity.

#### 5.4.4.3 Plants and Manganese Toxicity

Mn is a plant micronutrient that, depending on its content in the soil and on factors that control its availability—such as pH, redox potential (Eh), organic matter, and microbial activity—can achieve levels that are toxic to plants (Horst 1988) and humans (Marchner 1995). Soils known to cause Mn deficiency in susceptible crops are usually impoverished siliceous and calcareous sandy soils of neutral or alkaline pH, which favor chemical and microbial oxidation and immobilization of plant-available $Mn^{2+}$ (Rengel 2015; Millaleo et al. 2010). In plants, Mn participates in redox processes and is a proteic and enzymatic activator (e.g., Mn superoxide dismutase (MnSOD) is involved in photosystem II (PS II) function and in the metabolic shikimate pathway, leading to biosynthesis of various secondary metabolite compounds (Marschner and Marschner 2012; Marschner 1995), but, when it is in excess, Mn is a toxic element (Kochian et al. 2004).

#### 5.4.4.4 Manganese Toxicity and Arbuscular Mycorrhizal Symbiosis

It has been reported that the negative effects of Mn toxicity are alleviated or accentuated by different light levels, depending on the plant variety, tolerance, and improved nutritional status by mycorrhization (Bethlenfalvay and Franson 1989; Kothari et al. 1991; Nogueira and Cardoso 2003; Nogueira et al. 2002; Nogueira et al. 2007). The exact mechanisms acting in the alleviation of Mn toxicity in mycorrhizal plants should be further investigated (Emamverdian et al. 2015; Viehweger 2014).

### 5.4.5 Importance of Mycorrhizosphere Interactions to Sustainable Agriculture

Agricultural intensification and anthropogenic activities affect soil quality at all levels, including the functions of the soil microbiota (Sudhakaran et al. 2013). The term “rhizosphere”—introduced initially by Hiltner (1904) and cited by Berruti et al. (2016)—is used to describe the proportion of soil that is influenced by roots, but may also be extended to describe root–soil interfaces that can be separated into the ectorhizosphere (rhizosphere soil), rhizoplane (root surface), and endorhizosphere (inner root) (Oburger and Schmidt 2016; Azcón-Aguilar and Barea 2015; Pieterse et al. 2014). Plants and their microbiomes can be considered “superorganisms” in part because of their reliance on soil microbiota for specific functions and traits (van der Heijden et al. 2008; Barea et al. 2005). Mycorrhiza symbiosis induces transformations in the biological and edaphic soil properties in the zone of mycorrhizal influence (Azcón-Aguilar and Barea 2015). In the zone of mycorrhizal influence (the so-called mycorrhizosphere), the ability of AM fungi to control soilborne pathogens is strongly related to their capacity to stimulate establishment of beneficial free-living soil bacteria, referred to as “plant growth-promoting rhizobacteria” (PGPR), for biocontrol of plant pathogen development within the mycorrhizosphere before root infection (Lioussanne 2010). The notion that wild plants harbor a reservoir of genetic traits that aid in maximizing profitable functions of rhizosphere microbiota provides great promise for utilization in plant breeding strategies aimed at optimizing plant–microbiome interactions in crops (Pieterse et al. 2014). Detailed knowledge on how these soilborne supremacy traits are encoded in plant genomes and plant-associated microbiomes will contribute to the design of future crops that, in combination with dedicated microbial agriculture, can sustainably enhance crop production with less input of harmful chemicals (Pieterse et al. 2014).

#### 5.4.5.1 Arbuscular Mycorrhizal Fungus Inoculants

The application of AMF inoculants is seen as being very attractive, since it would substantially reduce fertilizer and pesticide input (Berg 2009). Agricultural and horticultural use of biostimulants will require locally and temporally adapted solutions (Jardin 2015). Bio-inoculum technology is an important agricultural practice, which can be used on a large scale in sustainable agriculture and may be able to improve plant productivity under environmentally adverse conditions (Dash and Gupta 2011). There is no question, that the microbe inoculum technology, being environmentally friendly, is a practicable and promising perspective in the field of sustainable agriculture.

## 5.5 Conclusion and Future Prospects

We currently know that the management of AM fungi and their interactions with ecosystem beneficial microorganisms is a challenge, and we have an opportunity to make use of AM fungi inoculants an innovative, ecologically friendly, and sustainable agricultural practice. This practice will be a valuable tool in lowering energy inputs, hence increasing the efficiency of the cropping system. Breeding for mycorrhiza is another alternative that needs to be considered. Other benefits include minimizing soil erosion, stocking organic carbon, and enhancing soil biodiversity to promote biological activity. Therefore, the application of mycorrhizal inoculation (e.g., AMF bio-inoculum technology) can be used to reduce the use of chemical fertilization and energy, besides promoting healthy plant growth under adverse soil conditions (e.g., soil salinity, soil acidification, and Al and Mn phytotoxicity), hence increasing the efficiency of the sustainability of current cropping systems.

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# *Paenibacillus polymyxa*: A Prominent Biofertilizer and Biocontrol Agent for Sustainable Agriculture

# 6

Kiran Preet Padda, Akshit Puri, and Chris P. Chanway

**Abstract**

Agricultural practice is currently moving from traditional chemical fertilizers and pesticides toward sustainable and environment-friendly biofertilizer and biocontrol agents. *Paenibacillus polymyxa* (previously *Bacillus polymyxa*) is an agriculturally important microbe widely studied for its plant growth-promoting abilities. *P. polymyxa* is an endospore-forming bacterium that could colonize a range of ecological niches. It is commonly found in the agricultural soils, especially in close association with plants, and has been isolated from diverse geographic locations. *P. polymyxa* is renowned for its ability to act as a biocontrol agent against a wide array of plant pathogens. It can produce antibiotic compounds like polymyxin and antifungal compounds like fusaricidin that can suppress the growth of pathogens in both lab and field conditions. Apart from being a potent biocontrol agent, *P. polymyxa* strains are also known widely for their ability to fix atmospheric nitrogen, solubilize phosphate, and produce phytohormones; thus they could be used as effectual biofertilizers in commercial agriculture. The aim of this chapter is to provide an overview about both direct and indirect plant growth promotion accomplished by *P. polymyxa* in a wide variety of agricultural crops, through extensive reviewing of old and recent studies.

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**Keywords**
*Paenibacillus polymyxa* • Sustainable agriculture • Phytohormones • Commercial agriculture

## Contents

## 6.1 Introduction

There has been a dramatic expand in the world's population since the last 150–200 years. According to the United Nations, the world's population is set to reach ~9.7 billion by 2050, which is fourfold the population in 1950 (United Nations 2015). Such drastic increase in population has put a serious pressure on our food resources causing a threat to the food security of many developing countries. Moreover, the environmental crisis like air, water, and soil pollution caused by the growing rate of global industrialization is making things worse for us and our planet (Glick 2015). In agriculture, the overuse of energy-intensive chemical fertilizers to boost crop yield has destroyed our soil ecosystem. Now is the best time to switch from these harmful chemical fertilizers to harmless and sustainable biofertilizers. Biofertilizer is a contraction of the term biological fertilizer, and it is very different from the organic fertilizer. Organic fertilizers contain organic compounds, which directly or indirectly increase soil fertility, whereas biofertilizers contain living organisms that increase the nutrient status of the host plant through their ongoing association with the host plant (Vessey 2003). The biofertilizer is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003; Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2016b).

Apart from chemical fertilizers, the use of chemical pesticides to control the plant pest population is not only destroying our soil and natural environment but also impacting the human health both directly and indirectly. Pesticide is often considered a quick, easy, and inexpensive solution for controlling the pest population, but these benefits are incurred at the cost of our environment (Aktar et al. 2009).

Research on the use of much safer, biological control agents against pests is gaining momentum. "Biocontrol" is a commonly used, abbreviated synonym of "biological control." As defined by Pal and McSpadden Gardener (2006), "Biocontrol refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens." The organism that suppresses the pest or pathogens are referred to as the "biocontrol agent." In this chapter, the term biofertilizer and biocontrol agent are used to signify those bacteria that are involved in promoting plant growth and controlling plant pathogen population, respectively (Kumar et al. 2015, 2016a; Ahmad et al. 2016; Meena et al. 2016a, b; Parewa et al. 2014; Prakash and Verma 2016; Jha and Subramanian 2016).

Plant growth-promoting bacteria (PGPB), as their name signifies, are those bacteria that can promote plant growth either directly, by aiding in nutrient acquisition (biofertilization) and moderating the plant hormone levels, or indirectly by acting as biocontrol agents against harmful plant pathogens (Glick 1995). These PGPB include bacteria that are free-living in the rhizosphere, form symbiotic relationships with plants like *Rhizobia* and *Frankia*, and can colonize interior tissues of plants (known as bacterial endophytes) (Glick 2012). Among the myriads of PGPB thriving in close association with plants, some spore-forming PGPB, particularly the gram-positive bacilli and streptomycetes, have drawn special attention because of their advantages over nonspore formers in product formulation and stable maintenance (Emmert and Handelsman 1999).

Among these, an agriculturally important microbe vital for present and future sustainable agriculture is "*Paenibacillus polymyxa.*" *P. polymyxa* is widely known for its plant growth-promoting (PGP) traits and the ability to thrive in diverse ecological niches. In this chapter, studies are signifying huge potential of *P. polymyxa* as a biofertilizer and biocontrol agent in sustainable agriculture (Priyadharsini and Muthukumar 2016; Kumar et al. 2017; Meena et al. 2015a, b, f; Raghavendra et al. 2016; Zahedi 2016; Dotaniya et al. 2016; Jaiswal et al. 2016).

## 6.2 Brief History of *Paenibacillus polymyxa* (Formerly *Bacillus polymyxa*)

History of *P. polymyxa* dates back to the nineteenth century when Prażmowski (1880) described an organism that closely resembled *Clostridium butyricum* but was able to grow in the presence of air. Prażmowski reported that this organism is slimy, strongly attacks starch and cellulose, and turns some carbohydrates into carbon dioxide gas (Porter et al. 1937). He designated it as "*Clostridium polymyxa*." But, in 1889, Eugéne Macé proposed the species name "*Bacillus polymyxa*" for this bacterium due to its rod-shaped cells (Macé 1889). *Bacillus* means "a rod" and polymyxa means "much slime." Macé reported that these bacteria are rod-shaped, produce spores, and can develop on the cooked slices of beets and turnips when exposed to air, and when grown in liquid media, they form a thick, creamy membrane on the surface (Macé 1889). Since then, this bacterium has been isolated frequently from soil and various plant species (Porter et al. 1937; Nakamura 1987) and has been reported to provide a variety of benefits to plants, like fixed nitrogen (N) (Bredemann 1909; Grau and Wilson 1962; Kalininskaya 1968; Seldin et al. 1984); PGP enzymes, viz., β-amylase (Fogarty and Griffin 1975; Hensley et al. 1980) and 2,3-butanediol (Ledingham and Neish 1954); and pathogen protection by secreting antibiotic polymyxin (Porter et al. 1949, Gordon et al. 1973; Skerman et al. 1980). Nakamura (1987) regarded *B. polymyxa* an agriculturally, industrially, and medically important organism.

Although bacterial species belonging to the genus *Bacillus* have been extensively studied, but it was always acknowledged that the taxonomy of this genus is unsatisfactory. Ash et al. (1991) referring to Bergey's Manual of Systematic Bacteriology, Vol. 2 (Claus and Berkeley 1986), pointed out that genus *Bacillus* is phenotypically and phylogenetically heterogeneous with species showing an extremely wide range of nutritional requirements, growth conditions, metabolic diversity, and DNA base composition. Ash et al. (1991) reported this phylogenetic heterogeneity by comparing and analyzing the small-subunit rRNA sequences of all species (51 species) known at that time. They divided these 51 species into five phylogenetically distinct groups and placed *B. polymyxa* into group 3. Findings reported in this chapter formed the basis for the proposal to reclassify group 3 bacilli comprising of *B. polymyxa* and ten other close relatives into a new genus *Paenibacillus* (meaning: almost a *Bacillus*) under the same family Bacillaceae (Ash et al. 1993). This proposal to form a new genus was officially approved and announced by the International Committee on Systematic Bacteriology (1994) through their official journal. Eventually, genus *Paenibacillus* was reclassified into a separate family *Paenibacilliaceae* and was designated as the family's type genus (Priest 2009; Rawat et al. 2016; Yasin et al. 2016; Meena et al. 2016c; Saha et al. 2016a; Yadav and Sidhu 2016; Meena et al. 2016d; Das and Pradhan 2016; Dominguez-Nunez et al. 2016).

## 6.3 *P. polymyxa* Strains Isolated from Agricultural Crops

The *P. polymyxa* strains inhabit diverse ecological niches including but not limited to rhizosphere and internal tissues of agricultural crops (von der Weid et al. 2000; Gu et al. 2010) and forest trees (Shishido et al. 1995; Bal et al. 2012), fermented food (Piuri et al. 1998; He et al. 2007), and marine environment (Ravi et al. 2007; Ma et al. 2010). Its ability to survive in a range of environmental conditions can be related to its endospore-forming potential. Agriculturally important *P. polymyxa* strains that have been isolated from various agricultural sites over the years are listed in Table 6.1. N-fixing *P. polymyxa* strain B5 was isolated from the rhizosphere of spring wheat (*Triticum aestivum* L.) growing on a research field (Lindberg and Granhall 1984). Since DNA identification techniques were not available at that time, authors identified the isolates using standard biochemical tests and comparing the results with reference strain ATCC 842. Strain B5 reduced significant amounts of acetylene and exhibited nitrogenase activity in vitro, thus indicating that it is a

**Table 6.1** List of important *Paenibacillus polymyxa* strains that have been isolated from agricultural sites

| Strain | Origin | References |
|---|---|---|
| B1, B2 | Wheat rhizosphere | Lindberg and Granhall (1984) |
| 130 different strains | Wheat rhizosphere | Mavingui et al. (1992) |
| CF43 | Wheat rhizosphere | Gouzou et al. (1993) |
| PMD216, PMD230, PMD112, PMD128, PMD66 | Wheat rhizosphere, rhizoplane, and soil | Lebuhn et al. (1997) |
| CM5-5, CM5-6 | Barley rhizosphere | Nielsen and Sørensen (1997) |
| E681 | Winter barley rhizosphere | Ryu and Park (1997) |
| 70 different strains | Corn rhizosphere | von der Weid et al. (2000) and da Mota et al. (2002) |
| GBR-1 | Roots of Korean ginseng | Jeon et al. (2003) |
| BRF-1 | Soybean rhizosphere | Wang et al. (2003) |
| B5, B6 | Peanut rhizosphere | Haggag and Timmusk (2008) |
| BMP-11 | Cucumber rhizosphere | Liu et al. (2008) |
| SQR-21 | Watermelon rhizosphere | Raza et al. (2009) |
| EBL-06 | Wheat phyllosphere | Gu et al. (2010) |
| JSa-9 | Soil | Deng et al. (2011) |
| G-14 | Muskmelon rhizosphere | Shi et al. (2012) |
| SC09-21, SR04-02, SR04-16 | Soil | Xu and Kim (2014) |
| EG2, EG14 | Soil | Górska et al. (2015) |
| APEC128, APEC136 | Apple rhizosphere | Kim et al. (2016a, b) |

potent N-fixing bacterial strain (Saha et al. 2016b; Verma et al. 2014, 2015b; Meena et al. 2014a, 2015e; Teotia et al. 2016; Bahadur et al. 2016b).

Another strain of *P. polymyxa* (CF43) was isolated from the rhizosphere of spring wheat (cv. Castan) growing in a field near Nemours, France (Gouzou et al. 1993). It was determined that population size of strain CF43 present in the wheat rhizosphere ranged from 1 × $10^5$ to 5 × $10^5$ cfu/g dry weight of rhizosphere soil. It was also reported that strain CF43 enhances soil aggregate stability and overall porosity. In another study, bacterial strains were isolated from barley (*Hordeum vulgare* L.) rhizosphere and screened for their ability to produce selected enzymes and antagonize plant pathogenic fungi (Nielsen and Sørensen 1997). Two strains of *P. polymyxa* (CM5-5 and CM5-6) were successful in these screening tests, and according to the authors, these strains could be promising biocontrol agents (Sharma et al. 2016; Verma et al. 2015a; Meena et al. 2013b; Shrivastava et al. 2016; Meena et al. 2016e).

The diversity of *P. polymyxa* strains colonizing the rhizosphere and the surrounding soil is huge. Mavingui et al. (1992) studied the diversity among 130 strains of *P. polymyxa* isolated from rhizosphere soil, non-rhizosphere soil, and rhizoplane of wheat. Phenotypic and genotypic characterization tests revealed that there is higher diversity within *P. polymyxa* strains isolated from non-rhizosphere and rhizosphere soil as compared to the strains isolated from the rhizoplane (Meena et al. 2017). In another study conducted with *Zea mays* plants growing in Cerrado soil, which is found commonly in tropical belts of the world, ~70 different isolates of *P. polymyxa* were harvested from the rhizosphere of corn plants (von der Weid et al. 2000; da Mota et al. 2002). Isolation of two strains designated as B5 and B6 from soil around the peanut (*Arachis hypogaea* L.) roots was performed, and by using genomic identification techniques, strains were identified as *P. polymyxa* (Haggag and Timmusk 2008).

The strains were able to suppress the activity of pathogenic fungus *Aspergillus niger* (causes crown rot disease of peanut) both in vitro and in vivo (greenhouse and field experiments). *P. polymyxa* strains have also been isolated from watermelon (*Citrullus lanatus*) and muskmelon (*Cucumis melo* L.). Fusaricidin-type compound-producing strain SQR-21, exhibiting antagonistic activity against pathogenic fungus, *Fusarium oxysporum* f. sp. *niveum*, was isolated from watermelon plants (Raza et al. 2009). On the other hand, a strain of *P. polymyxa* (G-14) was isolated from soil samples collected from muskmelon fields in Changji, Xinjiang, China (Shi et al. 2012). It was reported that strain G-14 can produce antibiotic compounds that can antagonize the activity of pathogenic bacteria, *Pseudomonas syringae* pv., *Lachrymans*, and *Acidovorax avenae* subsp. *citrulli*, that cause bacterial spot disease in muskmelon. Apart from living in the rhizosphere of a plant, *P. polymyxa* strain has also been reported to inhabit agricultural soils. *P. polymyxa* strain JSa-9 was isolated from soil collected from the farmland of Nanjing (Jiangsu province, China) and was reported to show antagonistic activity against local plant pathogens (Deng et al. 2011; Velazquez et al. 2016; Meena et al. 2014b, 2015c; Sindhu et al. 2016; Singh et al. 2016; Masood and Bano 2016).

Similarly, three strains of *P. polymyxa* (SC09-21, SR04-02, SR04-16) were isolated from soil samples collected from 30 different locations within fields (where Chinese cabbage, garlic, and orrice were cultivated) in Samcheok, Gangwon Province, Korea (Xu and Kim 2014). Isolated strains showed a range of PGP traits both in vitro and in vivo. In another study, two diazotrophic (N-fixing) strains EG2 and EG14 of *P. polymyxa* were isolated from agriculturally used land in Poland (Górska et al. 2015). *P. polymyxa* strain was also isolated from phyllosphere of wheat cultivated in a field located at Tongzhou near Beijing City, China (Gu et al. 2010). A study reviewed in this section clearly proves the potential of *P. polymyxa* strains to inhabit a range of agricultural crops and soil at a variety of locations all around the world (Meena et al. 2013c, 2015d; Singh et al. 2015; Bahadur et al. 2016a).

## 6.4 Complete Genome Sequencing of *P. polymyxa* Strains

Complete genome sequencing determines the complete DNA sequence of an organism's genome at a single time. This technique is crucial to identify the genes that are responsible for different traits of a PGPB. For instance, N-fixing trait of a PGPB is related to the presence of *nif* genes. Genome sequencing helps to link the field/lab observed characteristics of a PGPB with the genes responsible for exhibiting those PGP traits. Although complete genomes of a large number of PGPB have been sequenced, very few studies have reported the complete genome sequence of *P. polymyxa* strains. To date, complete genomes of only six strains of *P. polymyxa* are

**Table 6.2** List of *Paenibacillus polymyxa* strains whose complete genomes have been sequenced to date

| Strain | Origin | Genome size (Mbp) | References |
|---|---|---|---|
| E681 | Winter barley rhizosphere | 5.4 [1 chromosome, 0 plasmid] | Kim et al. (2010) |
| SC2 | Pepper rhizosphere | 5.7 [1 chromosome, 1 plasmid] | Ma et al. (2011) |
| M-1 | Internal root tissues of wheat | 5.8 [1 chromosome, 1 plasmid] | Niu et al. (2011) |
| CR1 | Corn rhizosphere | 6.0 [1 chromosome, 0 plasmid] | Eastman et al. (2014a) |
| SQR-21 | Watermelon rhizosphere | 5.8 [1 chromosome, 0 plasmid] | Li et al. (2014) |
| Sb3-1 | Organically managed soil | 5.6 [1 chromosome, 2 plasmids] | Rybakova et al. (2015) |

available in the NCBI database (https://www.ncbi.nlm.nih.gov), and amazingly all six strains were isolated from agricultural sites and were reported to possess PGP abilities (Table 6.2). Korean researchers were the first to sequence and report the complete genome of a *P. polymyxa* strain (E681) (Kim et al. 2010). *P. polymyxa* E681 is an endospore-forming bacterium that was isolated from the rhizosphere of winter barley in South Korea (Ryu and Park 1997). Based on sequence investigations, Kim et al. (2010) reported that strain E681 possesses genes responsible for synthesizing antibiotic compound polymyxin, antifungal compound fusaricidin, and phytohormone auxin. Subsequently, the complete genome of another *P. polymyxa* strain (SC2) originally isolated from pepper (*Capsicum annuum* L.) (Zhu et al. 2008) was sequenced and reported (Ma et al. 2011). Genome sequencing revealed that this strain possesses genes that are involved in antibiotic biosynthesis like fusaricidin-synthetic gene, polymyxin-synthetic gene cluster, and antibiotic-synthetic gene cluster; thus, it can be concluded that this bacterial strain will exhibit a broad-spectrum antimicrobial activity (Ma et al. 2011). Till now, the one and only endophytic strain of *P. polymyxa* (M-1) whose complete genome has been sequenced (Niu et al. 2011) was isolated from internal tissues of wheat roots (Yao et al. 2008).

In another report, the complete genome of a PGPB (*P. polymyxa* CR1), isolated from corn rhizosphere, was sequenced (Eastman et al. 2014a). Genome sequencing revealed that this strain possesses genes responsible for N fixation (*nif* genes), indole-3-acetic acid (IAA) synthesize, biomass degradation, and antimicrobial production, thus confirming their previous infield observations. Li et al. (2014) and Rybakova et al. (2015) have also reported complete genomes of *P. polymyxa* strains SQR-21 and Sb3-1, respectively. These strains also possess a variety of PGP traits. Eastman et al. (2014b) was the first to conduct a comparative genomic analysis of *P. polymyxa* strains. Their work highlighted that plant growth promotion by *P. polymyxa* is mediated largely through phytohormone production, increased nutrient availability, and biocontrol mechanisms. Similar study comparing the genomes of nine *P. polymyxa* strains and five other *Paenibacillus* spp. isolated from diverse ecological niches and geographic regions was conducted recently (Xie et al. 2016).

Authors concluded that genes relevant to PGP traits, i.e., phosphate solubilization, N fixation, IAA production, and antibiotic synthesis, are well conserved or have evolved with diversity in *P. polymyxa* and its closely related species.

## 6.5 Plant Growth Promotion by *Paenibacillus polymyxa* Strains

Generally, *P. polymyxa* strains promote plant growth either directly by helping in nutrient acquisition (like biological N fixation), producing plant growth regulators (like auxin, cytokinins, gibberellins), controlling plant ethylene levels, and enhancing root permeability and soil porosity or indirectly through biocontrol of major plant pathogens. Primary PGP characteristics of *P. polymyxa* strains have been presented in Fig. 6.1. Various reports about the direct and indirect benefits provided by prominent *P. polymyxa* strains to agricultural crops have been listed in Table 6.3.

### 6.5.1 Biofertilization : Direct Plant Growth Promotion

*P. polymyxa* strains possess many PGP characteristics through which direct plant growth promotion is achieved (Fig. 6.1). This includes biological N fixation by either living in the rhizosphere or internal tissues of the plant; positively affecting the physical structure of rhizosphere soil; enhancing nutrient uptake from the soil, thus increasing the plant length and biomass along with the overall crop yield; and secreting plant growth hormones. Lindberg et al. (1985) observed the N fixation activity of two *P. polymyxa* strains B1 and B2 isolated from field-grown wheat (Lindberg and Granhall 1984) by using acetylene reduction assay (ARA). Results of ARA indicated that strains B1 and B2 can produce 10.6 and 3.3 nmol $C_2H_4$plant$^{-1}$ h$^{-1}$, respectively, thus establishing their N-fixing ability. Besides fixing N, these strains were involved in increasing the seedling biomass and shoot length. To observe the endophytic colonization by these strains in wheat roots, authors used transmission electron microscopy technique and found that these strains can colonize intercellular and intracellular spaces of root epidermal cells. In later studies, production of cytokinin by strain B2 was also assessed using the chromatography techniques (Timmusk et al. 1999). Strains B1 was tagged with GFP to analyze the endophytic colonization ability and pathway through which it enters the plant tissues (Timmusk et al. 2005). Fluorescence microscopy and electron scanning microscopy revealed that this strain colonizes predominantly the root tip, where it forms biofilms and then invade the plant roots. This was the first study in which invasion and colonization of plant roots by a *P. polymyxa* strain were reported in detail. Some studies have implied the physical and microbial approach to analyze the effects of *P. polymyxa* inoculation on soil aggregation. In one such study, *P. polymyxa* strain CF43, isolated from wheat rhizosphere, increased the mass of soil adhering to the wheat roots by 57% when grown in a glasshouse (Gouzou et al. 1993). When aggregate size distribution was compared, it was observed that inoculated rhizosphere soil has a

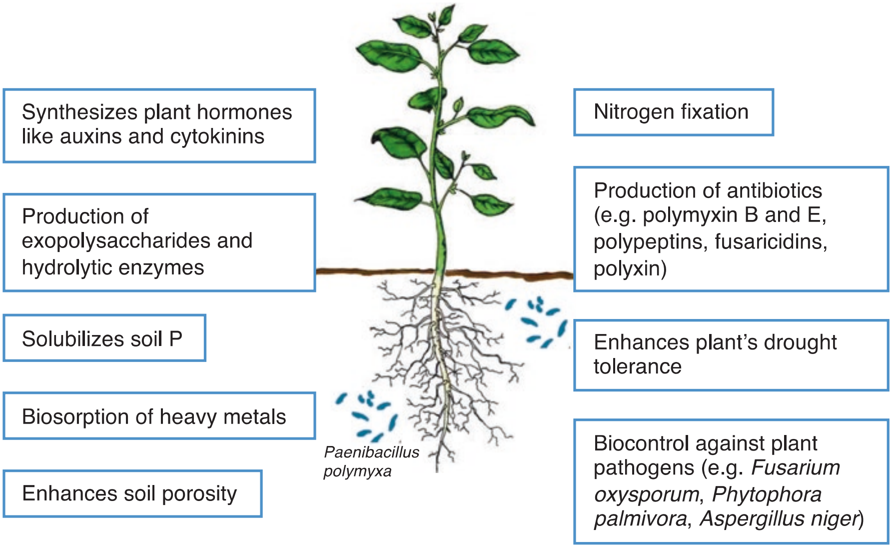

**Fig. 6.1** Primary plant growth-promoting (PGP) characteristics of *Paenibacillus polymyxa* strains

more porous structure as compared to the uninoculated control. Increased aggregation of rhizosphere soil by CF43 was also confirmed by Bezzate et al. (2000) using molecular techniques. Bezzate et al. (2000) hypothesized that levan, which is a fructosyl polymer produced by strain CF43, is responsible for enhanced soil aggregation. A mutant strain, SB03, was constructed by silencing the *sacB* gene (responsible for encoding levan) of strain CF43. Inoculation with CF43 significantly increased the wheat root dry mass and root-adhering soil dry mass as compared to inoculation with SB03 strain, thus signifying the importance of levan produced by the strain CF43 in increasing the soil aggregation. In a recent study, N-fixing ability of two *P. polymyxa* strains (EG2 and EG14) isolated from agricultural soils was assessed (Górska et al. 2015). The genome of these strains was found to carry *nif* genes which encode individual components of the nitrogenase complex. In vitro tests of nitrogenase activity by using ARA revealed that EG2 and EG14 can produce 2.9 and 0.4 nM $C_2H_4ml^{-1}\ h^{-1}$.

Diazotrophic (N-fixing) strains have immense potential as biofertilizers since N is believed to be the most important mineral nutrient required for plant growth and maintenance (Robertson and Vitousek 2009). Bal et al. (2012) isolated an endophytic diazotroph, *P. polymyxa* P2b-2R , from stem tissues of lodgepole pine (*Pinus contorta*). Strain P2b-2R was able to grow on N-free medium [combined carbon medium (CCM; Rennie 1981)] and consistently reduced significant amounts of acetylene in ARA (Bal et al. 2012). By using a more accurate method of determining the amount of N fixed ($^{15}$N foliar dilution assay), Bal and Chanway (2012a), Anand et al. (2013), and Yang et al. (2016) reported P2b-2R's remarkable ability to derive up to 79% of

**Table 6.3** List of prominent *Paenibacillus polymyxa* strains reported to provide a variety of benefits to agricultural crops

| Strain | Host | Benefits | References |
|---|---|---|---|
| B1, B2, B3, B4 | Wheat and *Arabidopsis thaliana* | Nitrogen fixation, cytokinin production, improves abiotic and biotic stress tolerance and antagonizes oomycete plant pathogens | Lindberg et al. (1985), Timmusk and Wagner (1999) and Timmusk et al. (1999, 2009) |
| CF43 | Wheat | Enhance soil porosity | Gouzou et al. (1993) and Bezzate et al. (2000) |
| PMD216, PMD230, PMD112, PMD128, PMD66 | Wheat | Production of auxin and other indolic and phenolic compounds | Lebuhn et al. (1997) |
| CM5-5, CM5-6 | – | Antagonism against pathogenic microfungi | Nielsen and Sorensen (1997) |
| E681 | *Arabidopsis thaliana*, cucumber, red pepper, sesame, and tobacco | Plant growth promotion and suppress the activity of a variety of plant pathogenic fungi both in vitro and in vivo | Ryu and Park (1997), Ryu et al. (2005a, b, 2006), and Lee et al. (2013) |
| Nb | Sugar beet and barley | Plant growth promotion and increase in overall yield | Çakmakçi et al. (1999, 2006) |
| PKB-1 | – | Produce fusaricidin-type antifungal peptides active against *Leptosphaeria maculans* (causative agent of canola blackleg disease) | Beatty and Jensen (2002) |
| B5, B6 | Peanut and *Arabidopsis thaliana* | Inhibitory effect against crown rot disease caused by *Aspergillus niger* pathogen and antagonizes oomycete plant pathogens | Haggag (2007), Haggag and Timmusk (2008), and Timmusk et al. (2009) |
| BRF-1 | Soybean | Control brown stem rot disease caused by the pathogenic fungus *Phialophora gregata* and in vitro antifungal activity against *Rhizoctonia solani* | Zhou et al. (2008) and Chen et al. (2010) |
| SQR-21 | Watermelon | Biocontrol against *Fusarium oxysporum* cause of Fusarium wilt disease and increase plant dry weight | Raza et al. (2009, 2015a) and Ling et al. (2011) |
| JSa-9 | – | Antimicrobial activity | Deng et al. (2011) |
| HKA-15 | Soybean | Antagonism against the phytopathogen *Xanthomonas campestris* pv. phaseoli M-5 | Mageshwaran et al. (2012) |

(continued)

**Table 6.3** (continued)

| Strain | Host | Benefits | References |
|---|---|---|---|
| G-14 | Muskmelon | Suppress the incidence of bacterial spot diseases | Shi et al. (2012) |
| CF05 | Tomato | Suppress Fusarium wilt disease | Mei et al. (2014) |
| SC09-21, SR04-02, SR04-16 | Tomato | Suppress Fusarium crown and root rot disease, enhances a range of plant growth parameters, solubilize phosphate, produce IAA, siderophore, and other phytohormones | Xu and Kim (2014) |
| EBL-06 | Tea | Enhanced tea yield, quantity of water extract, and tea polyphenol levels | Xu et al. (2014) |
| EG2, EG14 | – | Nitrogen fixation | Górska et al. (2015) |
| HT16 | Table grapes | Reduce white rot disease caused by *Coniella diplodiella* | Han et al. (2015) |
| GBR-1 | Korean ginseng | Antagonistic activity against fungal and bacterial pathogens | Kim et al. (2015) |
| P2b-2R, P2b-2R*gfp* | Canola, Corn and Tomato | Nitrogen fixation, increases seedling length, and biomass | Puri et al. (2015, 2016a, b), Padda (2015) and Padda et al. (2016a, b) |
| WR-2 | – | Antifungal compounds that inhibit the growth of *Fusarium oxysporum* | Raza et al. (2015a, b) |
| APEC128, APEC136 | Apple | Biocontrol of anthracnose and white rot diseases in apples | Kim et al. (2016a, b) |
| SC09-21 | Pepper | Induces defensive response against Phytophthora blight (caused by *Phytophthora capsici*) and promotes plant growth | Xu and Kim (2014, 2016) |
| 1465 | Wheat | Increase total root and shoot length, total root, and shoot weight | Yegorenkova et al. (2016) |
| BFKC01 | *Arabidopsis thaliana* | Promote plant Fe assimilation | Zhou et al. (2016) |

N from the atmospheric pool when inoculated into lodgepole pine. In a subsequent report, it was observed that strain possesses *nif* genes required to encode the nitrogenase enzyme (Anand and Chanway 2013c). GFP-tagged P2b-2R strain was constructed to evaluate the endophytic colonization sites in lodgepole pine, and it was reported to colonize both intercellular and intracellular spaces of lodgepole pine interior tissues (Anand and Chanway 2013a).

P2b-2R was able to colonize internal tissues of stem and root of another gymnosperm tree species, western red cedar (*Thuja plicata*), and significantly enhance seedling length and biomass along with fixing considerable amounts of N from the atmosphere (Bal and Chanway 2012b; Anand and Chanway 2013b; Tang et al. 2017; Yang et al. 2017). Puri et al. (2015) hypothesized that strain P2b-2R can provide similar benefits to agricultural crops through rhizospheric and endophytic colonization. Puri et al. (2015) used corn as the model crop to test this hypothesis. P2b-2R colonized rhizosphere and internal root tissues of corn seedlings with a population size of $10^5$ cfu/g dry root or fresh tissue in just 10 days. P2b-2R also fixed up to ~20% of N from the atmosphere and increased seedling length by ~35% and biomass by 30% in 30-day long trials (Puri et al. 2015). P2b-2R successfully colonized an important oilseed crop species, canola (Puri et al. 2016a), and vegetable crop species, tomato (Padda et al. 2016a). Similar benefits were provided by P2b-2R to these crop species indicating that P2b-2R can symbiotically associate and provide benefits to a broad range of hosts (Table 6.4). Padda (2015) reported an astonishing discovery with the GFP-tagged P2b-2R (P2b-2R*gfp*) constructed by Anand and Chanway (2013a). Padda (2015) compared the GFP-tagged strain with the wild-type strain of P2b-2R in terms of their ability to fix N and enhance seedling length and biomass.

P2b-2R*gfp* inoculation significantly enhanced corn seedling growth (length and biomass) as compared to the wild-type P2b-2R inoculation. This was the first report in literature where GFP-tagging of a bacterial strain related to the *Bacillus* (and *Paenibacillus*) genus enhanced its growth-promoting abilities. The ability of P2b-2R*gfp* to perform better than the wild-type strain was also confirmed in canola and tomato (Padda et al. 2016a). Benefits of inoculating this *P. polymyxa* strain and its GFP-tagged counterpart in a long-term trial were also evaluated, and the results were even better than the previous studies (which were of shorter duration) (Puri et al. 2016b; Padda et al. 2016b). In Fig. 6.2, a clear difference can be seen in canola plant growth when seeds were, either inoculated with P2b-2R*gfp* or P2b-2R or not inoculated (controls) (reproduced from Padda et al. 2016b). The increased PGP efficiency of P2b-2R after GFP-tagging is still a mystery, although in an unpublished study, it has been determined that GFP-tagging of P2b-2R leads to overexpression of *nifH*, *nifD*, and *nifK* genes, which play a major role in N fixation activity of a bacterial strain.

Plant hormones, like cytokinins, gibberellins, IAA, ethylene, etc., play a vital role in plant growth and development and in the response of plants to their environment (Glick 2012). PGPB can produce or modulate plant hormone levels, thereby affecting the plant's hormonal balance and its response to abiotic and biotic stress. *P. polymyxa* strains isolated from different proximity to wheat roots were evaluated for production of indolic and phenolic compounds like IAA, indole-3-ethanol, indole-3-lactic acid, indole-3-carboxylic, and benzoic acid (Lebuhn et al. 1997). Authors concluded that the presence of *P. polymyxa* strains at different proximities to roots indicates distinct potentials to produce indolic and phenolic compounds. Phi et al. (2008) reported that a number of genes are involved in the regulation of IAA biosynthesis by *P. polymyxa*, and a change in IAA regulation directly affects

**Table 6.4** Plant growth promotion (seedling length, seedling biomass, and foliar N concentration enhancement) and nitrogen fixation (% nitrogen derived from the atmosphere) by *Paenibacillus polymyxa* strain P2b-2R when inoculated into important agricultural crops

| Host plant | Days after sowing and inoculation | %Ndfa[a] | Foliar nitrogen concentration enhancement[b] | %Seedling length enhancement[c] | %Seedling biomass enhancement[d] | References |
|---|---|---|---|---|---|---|
| Corn | 30 | 19.6 | 10.2 | 35.3 | 30.9 | Puri et al. (2015) |
| | 40 | 15.7 | 17.1 | 24.7 | 28.4 | Padda (2015) |
| | 90 | 30.2 | 27.3 | 51.9 | 52.7 | Puri et al. (2016b) |
| Canola | 40 | 16.2 | 20.0 | 28.4 | 37.1 | Padda et al. (2016a) |
| | 60 | 21.8 | 40.3 | 24.9 | 30.1 | Puri et al. (2016a) |
| | 90 | 27.1 | 11.7 | 70.7 | 100.8 | Padda et al. (2016b) |
| Tomato | 40 | 18.1 | 30.0 | 24.9 | 93.0 | Padda et al. (2016a) |

[a] Percent nitrogen derived from the atmosphere (%Ndfa) by strain P2b-2R
[b] Percent increase of foliar nitrogen concentration after inoculation with strain P2b-2R
[c] Percent increase of seedling length after inoculation with strain P2b-2R
[d] Percent increase of seedling biomass after inoculation with strain P2b-2R

the growth of inoculated plants. In another study, in vitro production of IAA, siderophore, and other phytohormones and phosphate solubilization by *P. polymyxa* strains SC09-21, SR04-02, and SR04-16 isolated from agricultural soil was reported (Xu and Kim 2014). Greenhouse pot trials revealed that inoculation with these *P. polymyxa* strains can enhance tomato shoot and root length, shoot and root fresh weight, shoot and root dry weight, and chlorophyll content. In vitro production of phytohormones like ammonia, cellulase, indole-3-acetic acid, protease, and siderophores and phosphate solubilization by *P. polymyxa* strain SC09-21 was confirmed in a subsequent study (Xu and Kim 2016). Xu and Kim (2016) also inoculated pepper with strain SC09-21 in a 2-week-long greenhouse trial and found that inoculated pepper plants were longer and had more fresh weight, biomass, and chlorophyll content, thus establishing that *P. polymyxa* strain SC09-21 could be an effective biofertilizer which can associate with a variety of agricultural crops. Although regarded as a micronutrient, iron is a major limiting factor in plant growth and development. Microbe-induced iron assimilation in a plant by *P. polymyxa* strain BFKC01 was recently reported (Zhou et al. 2016). Based on their findings, authors proposed a model: "productions of IAA by strain BFKC01 activates auxin-mediated signaling pathways and promotes lateral root formation in Arabidopsis plants, thus plants efficiently absorb iron from the rhizosphere. Strain BFKC01 also regulates plant iron uptake by integrating the mechanisms of both enhancement of iron deficiency responses and increased secretion of iron-mobilizing phenolic compounds."

**Fig. 6.2** *Paenibacillus polymyxa* strain P2b-2R*gfp* inoculated seedling (*left*), *P. polymyxa* strain P2b-2R inoculated seedling (*center*), and uninoculated control seedling (*right*) of canola (*Brassica napus* L.) harvested 3 months after sowing and inoculation. Obvious differences in length, biomass, number of floral buds and pods, and plant health can be seen (Reproduced from Padda et al. 2016b)

## 6.5.2 Biocontrol: Indirect Plant Growth Promotion

Biocontrol of plant pathogens is an effective and environmentally safe alternative to chemical pesticides. *P. polymyxa* can produce two types of peptide antibiotics, one type is only active against bacteria and the other is active against fungi, gram-positive bacteria, and actinomycetes (Beatty and Jensen 2002). This antagonistic potential is the base for effective applications of *P. polymyxa* strains against a wide set of fungal and bacterial plant pathogens. The possible mechanisms that enable *P. polymyxa* strains to control a variety of plant pathogens have been reviewed extensively (Raza et al. 2008). Timmusk and Wagner (1999) used a known PGPB, *P.*

*polymyxa* strain B2, along with other *P. polymyxa* strains (B3 and B4) isolated by Lindberg and Granhall (1984) to assess their abiotic and biotic stress response when tested in *Arabidopsis thaliana*. Challenges by either the pathogenic bacteria, *Erwinia carotovora* (biotic stress), or induction of drought (abiotic stress) revealed that *P. polymyxa* inoculated plants were more resistant than control plants.

Authors also suggested that genes and/or gene classes associated with plant defenses against abiotic and biotic stress may be co-regulated. In another study, strains of *P. polymyxa* (B5 and B6), isolated from peanut rhizosphere, were reported to show in vitro antagonism against pathogenic fungus, *Aspergillus niger* (Haggag 2007; Haggag and Timmusk 2008). *A. niger* causes crown rot disease of peanut, which is the most important disease in Egypt and several other temperate countries (Haggag and AboSedera 2000). Strains B5 and B6 densely colonized the roots of peanut as visualized by scanning electron microscopy and suppressed the activity of *A. niger* in peanut, thereby decreasing the crown rot disease development. It was also reported that these strains increase the activity of plant defense enzymes including β-1,3-glucanase and chitinase, which might be the reason behind the suppression of pathogen activity (Haggag 2007). The results of two field trials indicated that these strains significantly reduce the incidence rate of crown rot disease in peanut; thus, they could be used as an effective biocontrol agent against *A. niger* at farm level (Haggag and Timmusk 2008).

The effectiveness of strains B5 and B6 along with strain B2 was tested against common oomycete plant pathogens, *Phytophthora palmivora* and *Pythium aphanidermatum* (Timmusk et al. 2009). These oomycete pathogens cause one of the most devastating groups of diseases. Almost all plants are susceptible to root rot disease caused by these pathogens, and the disease is difficult to control once the rot has begun. Strains B2, B5, and B6 showed clear antagonism against oomycete pathogens in the in vitro experiment (using agar plates and liquid medium). Using *Arabidopsis thaliana* as the model plant system antagonism against oomycete pathogens was also studied in a soil medium, and it was found that the survival rate of *P. polymyxa* inoculated plants was significantly higher than the control plants. *P. polymyxa* strain E681 isolated from barley in South Korea showed in vitro antagonism against *Rhizoctonia solani*, *P. ultimum*, and *F. oxysporum* f. sp. *Cucumerinum* (Ryu et al. 2005a). When E681 strain was inoculated into cucumber (*Cucumis sativus* cv. Shinpung), incidence of damping-off disease caused by the abovementioned pathogens was significantly reduced (Ryu et al. 2005a).

In another study, Ryu et al. (2006) reported that strain E681 shows in vitro antagonism against nine different pathogens, viz., *P. debaryanum*, *R. solani*, *F. oxysporum*, *Botrytis cinerea*, *B. allii*, *Cladosporium fulvum*, *P. ultimum*, *P. capsici*, and *Aspergillus* sp. Ryu et al. (2006) also established that E681 is an effective biocontrol against damping-off disease caused by pathogens in a month-long field trial conducted with sesame (*Sesamum indicum* L.). In a subsequent study, strain E681 was screened for fusaricidin compounds (Lee et al. 2013). Pre- and posttreatment of a 3-week-old red pepper (*Capsicum annuum* L.) with fusaricidin compound through soil drench or foliar spray application greatly reduced the disease severity caused by

*P. capsici*. Similar effects were reported in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Lee et al. 2013).

Brown stem rot disease of soybean (*Glycine max* L.) caused by the soilborne fungus, *Phialophora gregata*, is one of the most disastrous soybean diseases in the USA and Japan (Bachman and Nickell 2000). Biocontrol effectiveness of *P. polymyxa* strain BRF-1 isolated from the rhizosphere of diseased soybean seedlings was tested (Zhou et al. 2008). The severity of brown rot disease in BRF-1 inoculated and control soybean plants was significantly decreased after 62 days of fungal inoculation, thus establishing its biocontrol efficacy against the *P. gregata* fungus. In vitro antifungal activity by BRF-1 against a range of fungal pathogens was also reported by Chen et al. (2010). Authors also isolated and identified the antifungal peptide of this strain, which is active against a range of pathogens. Raza et al. (2009) isolated *P. polymyxa* strain SQR-21 from the rhizosphere of healthy watermelon plants growing in a heavily wilt-diseased field and evaluated its biocontrol potential against *F. Oxysporum* (the causative agent of *Fusarium* wilt disease of watermelon) in a greenhouse experiment.

Strain SQR-21 combined with organic fertilizer significantly decreased the disease incidence (by 70%) and increased the plant biomass by ~113%. Subsequently, Ling et al. (2011) attempted to understand the plant-microbe communications that take place when watermelon plants are inoculated with either SQR-21 strain or pathogenic fungi *F. oxysporum* f. sp. *niveum*. When treated with SQR-21 watermelon plants produced root exudates that significantly reduced the conidial germination of *F. oxysporum*. Another strain of *P. polymyxa* (WR-2) showed a similar antagonistic effect against *F. oxysporum* f. sp. *niveum* (Raza et al. 2015b). Strain WR-2 inhibited the growth fungal pathogen by ~40% in three different media (agar, sterilized soil, and natural soil), and this inhibitory effect was increased to about 60% when organic fertilizer was added. Raza et al. (2015b) also reported that strain WR-2 produces seven different volatile organic compounds, viz., benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone, and phenol that inhibit the growth of *F. oxysporum*. In another study, *P. polymyxa* strain CF05 showed in vitro antagonism against *F. oxysporum* f. sp. *lycopersici* (the causative agent of Fusarium wilt of tomato).

Greenhouse experiment confirmed this finding, where CF05 suppressed the Fusarium wilt disease by ~78% in tomato. It was also reported that strain CF05 induces systemic resistance in the tomato plant, thereby stimulating the release of plant defense enzymes and protecting the plant from the pathogen.

## 6.6 Field Studies and Commercial Availability of *Paenibacillus polymyxa* Strains

The PGPB inoculated crops signify a minor segment of current global agricultural practice, but lately the interest in the infield usage of PGPB that promotes plant growth and yield has increased. A number of these bacteria are now being used commercially as aides to promote sustainable agriculture. Numerous studies underlining the plant growth-promoting effects of various *P. polymyxa* strains under field conditions have been reported. Ryu et al. (2006) conducted experiments under field conditions to evaluate the antagonistic effect of *P. polymyxa* strain E681 in two types of soilborne diseases: preemergence and postemergence damping-off in sesame seeds. Seed pelleting technique was used in this study to improve the ability of strain E681 as a biocontrol agent. Experiments were conducted at GSNU Research Farm, Daegok, Jinju, where sesame had been cultivated for 2 successive years with serious yield loss. Seed pelleting with strain E681 enhanced emergence rate by ~92%, whereas emergence rate of untreated sesame seeds was less than 30%. Combined treatment of pelleting and strain E681 resulted in a greater percentage of healthy stand (92%) than pelleting alone (40%) or non-pelleted seeds treated with E681 (24%) when evaluated 2 months after sowing. These results suggest that pelleting combined with *P. polymyxa* strain E681 can be used to control damping-off disease caused by complex organisms in the field. The effect of *P. polymyxa* strains B5 and B6 on pod yield and control of crown rot disease in peanut caused by *A. niger* was investigated in two successive field experiments (Haggag 2007; Haggag and Timmusk 2008).

Peanut plants treated with *P. polymyxa* strains displayed decreased incidence of crown rot disease triggered by *A. niger*. Plant growth and yield of seeds treated with strain B5 were found to be significantly higher in comparison to seeds treated with strain B6 and untreated plants. Biocontrol activity of *P. polymyxa* strain G-14 against bacterial spot diseases of muskmelon caused by two pathogens, *Pseudomonas syringae* pv. *Lachrymans* and *Acidovorax avenae* subsp. *citrulli*, was examined under field conditions (Shi et al. 2012). G-14 strain significantly inhibited the development of pathogens and suppressed the incidence of bacterial spot diseases. Inoculation with an N-fixing*P. polymyxa* strain increased sugar beet (*Beta vulgaris* cv. *Loretta*) root yields by 12% and barley seed yields by 15% when evaluated by in-field studies conducted at two different sites in Turkey (Çakmakçi et al. 1999). These results were also confirmed by a subsequent field study (Çakmakçi et al. 2006).

A yearlong field investigation (for three seasons—autumn, spring, and summer) was conducted to determine the effects of *P. polymyxa* strain EBL-06 on the growth of tea (*Camellia sinensis*) plantations (4 years old) in semitropical uplands, Hunan, China (Xu et al. 2014). Inoculation with EBL-06 increased tea plant yield (~17%) and tea quality by enhancing the level of green tea extracts by about 6% and tea polyphenols by ~10%. Thus, it was concluded that this strain could be a successful biofertilizer for tea plants that might be used for organic tea production in the future to enhance tea yield and quality. Field studies play an important role in determining the effects of a particular bacterial strain in actual conditions and open the doors for their use as commercial biological inoculants. Due to numerous field studies that have reported rigorous testing by scientists, many *P. polymyxa* strains are now available commercially in several countries.

## 6.7 Concluding Remark and Future Prospective

Since the first isolation and characterization more than a century ago, significant advances have been made in understanding how *P. polymyxa* affects plant growth. *P. polymyxa* is now known to fix nitrogen, secrete phytohormones, and produce antibiotic and antifungal compounds. The most common antifungal compound produced by *P. polymyxa* is fusaricidin which has been reported to suppress many strains of *Fusaricidin oxysporum* in a variety of plants species both in vitro and in vivo. It is believed that microorganisms with two or more PGP traits which are able to colonize and provide benefits to a wide range of crops may be effectively used for commercial and large-scale agriculture. It is an effective biocontrol agent against a wide range of plant pathogens like *Aspergillus* sp., *B. allii*, *B. cinerea*, *C. fulvum*, *F. oxysporum*, *P. capsici*, *P. debaryanum*, *P. ultimum*, and *R. solani*. Complete genome sequencing indicated that *P. polymyxa* E681 could produce antibiotic polymyxin and antifungal fusaricidin compounds, revealing the genetic evidence behind its ability to antagonize plant pathogens. Another example is *P. polymyxa* P2b-2R, which was reported to fix significant amounts of N directly from the atmosphere in crop species like corn, canola, and tomato. Possibly through N fixation and some other linked mechanisms, strain P2b-2R also promoted plant growth (length and biomass) and crop yield. These PGP properties together with its endospore-forming potential enable it to thrive in a wide range of environmental conditions, making it an important and promising biofertilizer and biocontrol agent for current and future sustainable agriculture. Through continuing research, agricultural scientists are making important inroads to understand the biology and ecology of *P. polymyxa* strains that could ultimately result in more commercially viable and environmentally friendly bio-inoculants for use in agriculture.

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# Part II

# Microbes for Sustainable Crop Protection

# Role of *Pseudomonas* sp. in Sustainable Agriculture and Disease Management

# 7

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## Abstract

Recently the growing population and climate change are the most severe challenge for both farmers and researchers from the last two decades. It is estimated that by 2050 the global population reaches to ~9 billion which demands ~60% extra food from the present, which creates extra pressure on the farmers to achieve the need of food. The farmers currently utilized huge amount of chemical fertilizers and pesticides to enhance the food production and disease management. These chemical fertilizers not only affect the texture and productivity of soil but also the health of plants, humans, and environment. From the last two decades, plant growth-promoting bacteria (PGPB) is one of the best choices as plant and soil inoculants to enhance the plant growth and disease management, due to their less impact on environment and eco-friendly nature. In this chapter, we focused on one of the most broadly used bacterial genus, *Pseudomonas*, which is present in the rhizosphere as well as within the tissue of the plants and used in sustainable agriculture for the enhancement of growth promotion and disease management.

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**Keywords**
*Pseudomonas* • PGPR • Plant growth promotion • Disease management

## Contents

## 7.1 Introduction

Currently the whole world is suffering from two severe concerns: one is the rising population and the other is global warming. It is expected that the population of the world will reach ~9 billion in 2050 (Godfray et al. 2010), which creates extra pressure on the food production, space, and environment in improving life standards. On the other hand, limited availability of lands and depleting natural resources adversely affect the potential for increasing agricultural productivity (Ray et al. 2013). Therefore to fulfill requirements of food production, farmers continuously use huge amount of fertilizers and pesticides in the field. To achieve the need of growing population in limited resources without adversely affecting the environment is the major challenges for both the farmers and researchers (Raaijmakers et al. 2009; Agaras et al. 2015). The use of agricultural by-products as biofertilizers, phytostimulators, and biocontrol agents together with appropriate crop management practices is an attractive choice in sustainable agricultural practices because of eco-friendly nature or possibility to reduce the agrochemical applications (Adesemoye et al. 2009; Carvalho 2006).

Recently, it is seen that during the production and storage of food and food materials, pest causes heavy loss of food grains which result cost incensement. The challenges of disease management are so high that old strategies are not sufficient and should be improved. Plant disease management should strike to ensure food security and social stability by increasing crop productivity, reducing food contamination by microbial toxins, and guaranteeing the supply of diverse and reasonable priced foods (Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015a; Ahmad et al. 2016; Meena et al. 2013a, 2016a; Parewa et al. 2014; Kumar et al. 2016b).

In this context, environment-friendly approach uses plant growth-promoting bacteria (PGPB) as one of the most effective and environmentally safe choice for the enhancement of plant growth and yield and/or for the disease management (Compant et al. 2005; Kumar et al. 2015a, b).

## 7.2 Rhizosphere and Their Interaction with Microbes

Plant growth in agricultural soils is influenced by many biotic and abiotic factors. Bacteria are the most abundant group of microorganism coexisting with fungi, protozoa, and algae in the rhizosphere. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the physiology and competitiveness in root colonization in plants to a greater extent (Glick 2012). These effective colonizing microorganisms are classified according to the interactions with plants; these may be positive, negative, or neutral (Martinz-Viveros et al. 2010). Rhizobacterial species inhabit of plant roots, exert positive effect ranging from direct influence mechanisms to an indirect effect during the plant growth and development. Recently the number of PGPR isolates has been used as plant and soil inoculants for the growth and disease management. These isolates belongs to all the major families; some of the important species are *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *and Serratia* which are broadly used as plant growth-promoting and biocontrol agents (Joseph et al. 2007; Kumar et al. 2016b).

## 7.3 *Pseudomonas* as a PGP Agent in Plant Growth

Nowadays the *Pseudomonas* species get high attention by the researcher in sustainable agriculture because of their contribution in growth promotion and induced systematic resistance (ISR) in plants through the different mode of action like suppression of plant diseases, improved nutrient acquisition and phytohormone production. *Pseudomonas* is an aerobic, gram-negative, *Gammaproteobacteria*, belonging to the family *Pseudomonadaceae* containing ~191 diversified species. Pseudomonads, due to metabolic versatility and genetic plasticity, are ubiquitous in soil ecosystem. They are common inhabitants of the rhizosphere of various agricultural crop plants wherein they play a major role in plant growth promotion and biocontrol agents that are made better suited for the growth, yields, and disease management of the plants (Lugtenberg and Kamilova 2009; Pathma et al. 2011; Jain and Pandey 2016; Kumar et al. 2016b).

In the rhizospheric regions of plants, secretes of root exudates contain large amount of carbohydrates, lipids, and amino acids which act as chemoattractants for the microbes. The chemoattractants play significant role in microbial interaction.

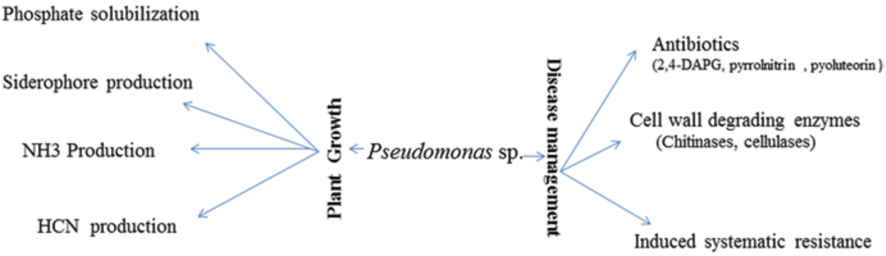

**Fig. 7.1** An overview of *Pseudomonas* in plant growth and disease management

It was concluded from the previous study that microbes showed strong chemoattractants toward particular carbohydrates or amino acids. In case of tomato plants, *P. fluorescens* showed strong chemoattractants toward some amino acids like Cys, Gly, Ile, Lys, Met, Phe, Pro, and Ser which lead to effective root colonization (Oku et al. 2012) (Fig. 7.1).

## 7.4 Mechanism of *Pseudomonas* Action in Plant Growth Promotion

Plant growth-promoting bacteria (PGPB) enhance the growth by making easily soluble phosphorus, nitrogen, and Fe (iron) from the insoluble nondiffusible form present in the soil. But currently plenty of nutrients and minerals are present in the soil. To overcome the problems of insufficient amount of nutrients, farmers are largely dependent upon the application of chemical fertilizers for the requirement of nitrogen and phosphorus in the soil. Continuous utilization of fertilizers adversely affect the texture and productivity of soil and also causes soil and water pollution which not only affect the environment or climate but also affect the health of human beings (Prakash and Verma 2016; Meena et al. 2015a, 2016b; Priyadharsini and Muthukumar 2016; Kumar et al. 2016a, 2017; Jaiswal et al. 2016; Jha and Subramanian 2016).

The mode of action of *Pseudomonas* is also common like other plant growth-promoting bacteria (PGPB). This involves complex mechanisms for the growth and development. Recently many researchers use *Pseudomonas* sp. as plant or soil inoculants to enhance the growth and yields and for the modulation of secondary metabolite production. Kumar et al. (2016b) inoculates *Pseudomonas fluorescens* to enhance the yield and curcumin content of turmeric. Grobelak et al. (2015) utilized PGPR in case of rape and fescue grass for the enhancement in the stem and root length. Hammami et al. (2013) used *Pseudomonas* strain Psf5, to control damping-off disease in tomato seedling caused by *Sclerotinia sclerotiorum* and also found significant increases in the plant stand as well as root dry weight of tomato. Goswami et al. (2013) also reported growth enhancement in chickpea and green gram after inoculation with the *Pseudomonas* sp. In another study, Manikandan et al. (2010) utilized liquid inoculants of *P. fluorescens* Pf1 to enhance the yield of tomato fruit, and many more researchers utilized various strains of *Pseudomonas* sp. to enhance the growth and yields of various plant species as given in Table 7.1. The principal mechanism of growth promotion includes production of growth stimulating hormones and solubilization and mobilization of phosphate, siderophores, ammonia, and HCN production (Whipps 2001; Idris et al. 2007; Richardson et al. 2009; Kumar et al. 2015a, b, 2016a, b).

### 7.4.1 Phosphate Solubilization

Phosphorus is one of the most essential and limiting macronutrients for plant growth. In nature, phosphorus presents in the form of rocks, but due to insoluble forms of phosphate, plants cannot be utilized. To reduce P deficiency and enhance crop production, frequent application of phosphate fertilizers are needed. The continuous application of phosphate fertilizers in the field may cause severe environmental problems and also increased the cost of crop production. Thus, solubilization and mineralization of phosphorus by phosphate-solubilizing bacteria are an important trait in PGPB (Richardson 2001). The solubilization of inorganic phosphorus occurs by the action of low molecular weight organic acids such as gluconic and citric acid, both of which are synthesized by various soil bacteria (Rodriguez et al. 2004; Kumar et al. 2016b; Raghavendra et al. 2016; Zahedi 2016; Meena et al. 2015b, f, 2016c, d; Rawat et al. 2016; Yasin et al. 2016; Saha et al. 2016a; Yadav and Sidhu 2016; Dotaniya et al. 2016).

### 7.4.2 Siderophore Production

Iron is the most common element on earth and is required for the growth and synthesis of plants. In natural form, iron is present in ferric ion or $Fe^{+3}$, which is sparingly soluble; that's why this form is not utilized by both plants and microorganism (Ma 2005). Both microorganisms and plants require a high level of iron and to obtain sufficient amount of iron is more problematic. Bacteria synthesize siderophores molecules which have high affinity for $Fe^{+3}$ act as chelating agents. It binds the $Fe^{+3}$ molecules and made the insoluble form to soluble and facilitates iron uptake by plants and microorganisms (Hider and Kong 2010). Presently there are more than 500 known siderophores of which chemical structures of 270 have been determined (Meena et al. 2017).

Siderophore-mediated competition for iron is one among the mechanism responsible for the antagonistic activity of *Pseudomonas* sp. The secreted iron-chelating compound binds the $Fe^{+3}$ and taken up by microbial cells through specific membrane recognition (receptors), a membrane protein (Srivastava and Shalini 2008).

**Table 7.1** Effect of *Pseudomonas* on plant growth promotion

| Bacteria | Plant | Impact of inoculation on plant | References |
|---|---|---|---|
| *Pseudomonas fluorescens* | Turmeric | Growth and curcumin content | Kumar et al. (2016b) |
| *Pseudomonas* | Wheat | Growth | Shaharoona et al. (2007); Zahir et al. (2009) |
| *Pseudomonas fluorescens* | Wheat | Seed yield and shoot dry mass. | Behn (2008) |
| *Pseudomonas fluorescens* | Mustard | Growth and yield attributes | Aeron et al. (2011) |
| PGPR | Cucumber | Root growth | Bae et al. (2007) |
| *Pseudomonas* | Tomato | Root growth | Belimov et al. (2007) |
| *Pseudomonas fluorescens* | Opium poppy | Morphine, thebaine, codeine | Bonilla et al. (2014) |
| *P. fluorescens*, *Azospirillum brasilense* | Marigold | Shoot fresh weight, root dry weight, leaf number, node number | Cappellari et al. (2013) |
| *Pseudomonas putida* | Maize | Grain yield | Dadnia and Moaveni (2011) |
| *Pseudomonas aeruginosa* | Tomato | Fruit yield | Dashti et al. (2012) |
| *Pseudomonas fluorescens* | Blackberries | Fruit quality | Garcia-Seco et al. (2013) |
| *Pseudomonas putida*, *Pseudomonas fluorescens* | Black henbane (*Hyoscyamus niger*) | Alkaloid yield and content | Ghorbanpour et al. (2013) |
| *Pseudomonas* spp. | Onion | Onion bud | Harthmann et al. (2009) |
| *P. putida*, *P. fluorescens,* | Spinach, Pepper | Plant heights | Hou and Oluranti (2013) |
| *Pseudomonas fluorescens* | *Catharanthus roseus* | biomass yield and ajmalicine production | Jaleel et al. (2007) |
| *Pseudomonas* sp. | Maize | Plant height, dry weight | Jarak et al. (2012) |
| *Pseudomonas putida* | Cherry trees | Fruit set, plant vegetative growth | Karakurt et al. (2011) |
| *Pseudomonas corrugata* | Maize | Grain yield | Kumar et al. (2007) |
| *Pseudomonas aeruginosa* | Sesame | Growth and yield | Kumar et al. (2009a, b) |
| PGPR | Soy bean | Productivity | Salama et al. (2011) |
| *Pseudomonas* sp. | Maize | Compatible solutes, antioxidant | Sandhya et al. (2010) |
| *Pseudomonas* sp., *Burkholderia caryophylli* | Wheat | Growth and yield of wheat | Shaharoona et al. (2007) |
| *Pseudomonas putida*, *Pseudomonas fluorescens* | Rice | Grain iron | Sharma et al. (2013) |
| *Pseudomonas putida* | Tomato | Plant growth, yield | Shen et al. (2012) |

### 7.4.3 Modulating Phytohormone Levels

Phytohormones play an important role in growth and development in plants (Davies 2004). The plants grown under environmental stress often attempt to maintain the levels of their endogenous phytohormones in order to decrease the negative impacts of the environmental stressors (Salamone et al. 2005). IAA, gibberellins, and cytokinins are the most common plant hormones, required for the growth and developments of plants, and their concentrations affect the morphogenesis of plants. PGPB secretes or synthesized significant amount of plant hormones and modulates the growth and synthesis of secondary metabolites (Kumar et al. 2015a, b; 2016b).

### 7.4.4 Indole Acetic Acid

Indole-3-acetic acid (IAA) is the most common and studied auxin, which plays an important role in cell division, differentiation, and germination of seed and tubers, controls vegetative growth, and initiates lateral and adventitious root formation. Florescence affects photosynthesis, pigment formation, and biosynthesis of various metabolites in stress conditions (Spaepen and Vanderleyden 2011; Glick 2014). The response of IAA varies from species to species in the plant. Some of the plant species are sensitive to the IAA; it varies with the genotypes of the plants. In plant root, endogenous IAA may be suboptimal or optimal for growth (Glick 2012).

## 7.5 Role of *Pseudomonas* in Disease Management

Recently it is estimated that huge amount of food commodity/crop loses annually due to the diseases which are caused by the fungus, nematodes, bacteria, etc. Plant diseases are a major factor influencing food production and human societal development over thousands of years (Palmgren et al. 2015; Dun-chun et al. 2016). These efficient PGPB as biocontrol agents have certain advantages over conventional chemical control method, because PGPB are nontoxic naturally occurring microorganism, and their application is sustainable. Another advantage of PGPB is the diverse range of modes of action including antibiotic production, cell wall-degrading enzymes, biosurfactants, and volatiles and also induces systematic resistance in plants (Perez-Montano et al. 2014).

The term biocontrol is used for disease management that occurs during the plant growth stages and also during the storage of food. Studies on biocontrol of pathogens by *Rhizobacteria* usually focused on pathogenic microorganisms like weeds and insects (Flores-Fargas and O'Hara 2006; Siddiqui et al. 2005). Generally cereals are mostly affected by soilborne diseases with respect to crop. The effective controls of diseases by using PGPR have been reported by many workers in different cereals and crops (Whipps 2001; Lucy et al. 2005; Berg and Smalla 2009). Many of the bacterial strains have been identified and used as inoculants, which act a as biocontrol agents in cereals**.** *Pseudomonas* is one of the most widely used

**Table 7.2** The role of *Pseudomonas* in disease management

| Bacterial strain | Plants | Disease/Pathogen | References |
|---|---|---|---|
| *Pseudomonas* | Cucumber | Damping-off | Zohara et al.(2016) |
| *P. fluorescens* | Tomato | *Ralstonia solanacearum* | Raza et al. (2016) |
| *Pseudomonas chlororaphis* | Wheat | Antimicrobial | Jain and Pandey (2016) |
| *Pseudomonas fluorescens* | Mung bean | Root rot | Noreen et al. (2015) |
| *Pseudomonas aeruginosa* | Tomato | Cucumber mosaic virus CMV | Dashti et al. (2012) |
| *P. fluorescens* | Mulberry plant | Root rot disease | Ganeshamoorthi et al. (2008) |
| *Pseudomonas* strains | Cucurbit | Powdery mildew | Garcia-Gutierrez et al. (2012) |
| *P. fluorescens* | Barley | *Fusarium graminearum* | Henkes et al. (2011) |
| *P. fluorescens* | Lupine | *Fusarium solani* | Hewedy et al. (2011) |
| *P. fluorescens* | Chickpea | *Fusarium oxysporum* | Kandoliya and Vakharia (2013) |
| *Pseudomonas rhodesiae* | Pepper | *Xanthomonas axonopodis* | Kang et al. (2007) |
| *P. fluorescens* | Rice | Leaffolder insect and sheath blight disease | Karthiba et al. (2010) |
| *P. fluorescens* | Pigeon pea | Wilt disease | Kumar et al. (2010) |
| *Pseudomonas aeruginosa* | Sesame | *Heterodera cajani* | Kumar et al. (2009b) |
| *Pseudomonas* sp. | Tomato | Blossom-end rot | Lee et al. (2010) |
| *P. fluorescens* | Rice | blast disease | Lucas et al. (2009) |
| *P. fluorescens* | Cucumber | Cucumber root rot | Maleki et al. (2010) |
| *P. fluorescens* | Pigeon pea | *Heterodera cajani* | Meena et al., (2010) |
| *P. fluorescens* | Rice | Leaf folder pest | Saravanakumar et al. (2008) |
| *P. fluorescens* | Rice | Sheath rot disease (*Sarocladium oryzae*) | Saravanakumar et al. (2009) |
| *Pseudomonas sp.* | Tea | Blister blight disease | Saravanakumar et al. (2007) |
| *P. fluorescens* | Sugarcane | Sugarcane red rot | Senthil et al. (2011) |
| *P. fluorescens* | Ground nut | Leafminer insect and collar rot pathogen | Senthilraja et al. (2013) |
| *P. fluorescens* | Tomato | *Bemisia tabaci* | Shavit et al. (2013) |
| *P. fluorescens* | Tobacco | Tobacco mosaic virus | Shen et al. (2014) |

groups of bacteria, which act as biocontrol agents in the different plant species as described in Table 7.2. *Pseudomonas* as biocontrol research can be traced for bacterization studies conducted at several laboratories with *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. syringae*, etc. that produce different antibiotics such as phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), pyoluteorin (Plt), and oomycin A (Ligon et al. 2000; Raaijmakers and Weller 2001).

The genetic analysis of biocontrol resistant of *Pseudomonas* indicated a positive correlation between disease separation and antibiotic production as in the disease of wheat caused by fungus *Gaeumannomyces graminis* var. *tritici* by antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), which suppress soilborne diseases and act as biocontrol agent. *Pseudomonas syringae* strain 01 CE a genetically modified strain protects several crop plants from cold frost. The biocontrol potential of *Pseudomonas* as important agents to combat root and soilborne pathogens has been reported in many crops including chickpea, tomato, and wheat (Grover et al. 2009; Dashti et al. 2012; Perez-Montano et al. 2014).

The earliest mechanism of biocontrol is the suppression of pathogens by secreting compound-like siderophores that efficiently sequester iron and deprive the pathogen from this element (Raaijmakers et al. 2002).The bacterial isolates inhibit the growth of pathogen through different mechanisms like by secreting antibiotics, toxins, and surface-active compounds (biosurfactants), by competition of minerals, and by secreting cell wall-degrading enzyme like chitinase and β-1, 3-glucanase (Haas and Defago 2005; Kumar et al. 2015a, b) and also through the production of metabolites which trigger the induction of systemic acquired resistance (Van Loon et al.1998).

## 7.6 Mechanism of Biocontrol by *Pseudomonas* Strains

### 7.6.1 Production or Synthesis of Antibiotics

During past decades, numerous antibiotics have been isolated from various biocontrol strains representing different bacterial genera. Most of the antibiotics produced by bacterial biocontrol agents have a broad-spectrum activity. Antibiotics are an organic, low molecular weight compounds that are deleterious to the growth or metabolic activities of other microorganisms (Fravel 1988; Raaijmakers et al. 2002). They act against various groups of microorganism. As *Pseudomonas* sp. synthesizes pyrrolnitrinis, DAPG like antibiotics acts against pathogenic fungi bacteria, helminths, etc. (Thomashow and Weller 1995; Raaijmakers et al. 2002).

The production of antibiotic is modulated by both exogenous and endogenous factors; exogenous factors include both the biotic and abiotic factors (Shanahan et al. 1992). Addition of fertilizers, carbon sources, and minerals influences antibiotic production (Duffy and Defago 1999). Addition of glucose significantly enhanced the production of DAPG in *Pseudomonas* strains, whereas supplementation of phosphate fertilizer repressed the DPAG production of *Pseudomonas fluorescens* (Duffy and Defago 1999). It is reported in previous study that bacterization of wheat seeds with *P. fluorescens* strains 2–79 resulted in significant suppression(~ 60%) of diseases in field trials (Weller 2007). *P. fluorescens* strain 2–79 and *P. chlororaphis* 30–84 produce anthranilic acid, and strains 30–84 produce two other phenazines 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine as well as HCN, respectively, which act as a biocontrol agent (Pierson and Thomashow 1992). *P. fluorescens* strain CHA0 was isolated from the rhizosphere of tobacco suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* (Stutz et al. 1986). Strains CHA0 produce DAPG, Plt, Prn, HCN, pyochelin, pseudobactin, and other bioactive metabolites (Voisard et al. 1994).

### 7.6.2 Production of Cell Wall-Degrading Enzymes

One of the mechanisms used by biocontrol agents to control soilborne pathogens involves the production of cell wall-degrading enzymes (Kobayashi et al. 2002). Cell wall-degrading enzymes such as β-1, 3-glucanase, chitinase, cellulase, and protease secreted by biocontrol strains of PGPR exert a direct inhibitory effect on the hyphal growth of fungal pathogens (Bahadur et al. 2016a; Masood and Bano 2016; Meena et al. 2016e; Teotia et al. 2016). Chitinase and β-1, 3-glucanase degrade chitin, an insoluble linear polymer of α-1,4-N-acetylglucosamine (Labuschagne et al. 2010).*P. aeruginosa* and *P. fluorescens* have been found to have chitinolytic activities (Nelson and Sorenson 1999). Cell wall-degrading enzymes of rhizobacteria affect the structural integrity of the walls of the target pathogen (Budi et al. 2000). Someya et al. (2000) reported the chitinolytic and antifungal activities of a potent biocontrol strain of *S. marcescens* B2 against the soilborne pathogens *R. solani* and *F. oxysporum* (Saha et al. 2016b; Verma et al. 2014, 2015a, b; Meena et al. 2013b, 2014a; Sharma et al. 2016; Das and Pradhan 2016; Dominguez-Nunez et al. 2016).

### 7.6.3 *Pseudomonas* in Induced Systematic Resistance (ISR)

*Pseudomonas* strains protect the plants against pathogen through the induction of systematic resistance. Plants have the ability to acquire enhanced level of resistance to pathogens after exposure to biotic stimuli provided by many different PGPR (Singh et al. 2015, 2016; Meena et al. 2013c, 2015d, e). This involves systematic resistance (induced systematic resistance (ISR) and systemic acquired resistance (SAR), which are activated by certain molecules secreted by microorganism referred as elicitors. Elicitors are generally cell wall polysaccharides, salicylic acid, cyclic lipopeptides, siderophores, antibiotics, and the signal molecule N-acyl homoserine lactones (AHLs) (Van loon 2007; Berg and Smalla 2009; Perez-Montano et al. 2014).

### 7.6.4 Regulation of *Pseudomonas* Functions by Signaling Molecules

Many plant growth-promoting rhizobacteria (PGPR) exchange cell-to-cell communication signals between each other and regulate their gene expression in response to changes in population density with other rhizosphere-inhabiting bacteria and fungi in a process called quorum sensing (QS) which is mediated through small diffusible signal molecules, such as N-acyl homoserine lactones(AHLs) (Perez-Montaño et al. 2014). In fluorescent pseudomonads, colonization and biosynthesis of antimicrobial metabolite phenazines QS regulation are AHL based (De Maeyer et al. 2011).

## 7.7 Interaction of *Pseudomonas* with Other Microbe in Rhizosphere

Rhizosphere is the most prominent habitat for the diverse group of microbes. These microbes interacted with each other and have significant effect on the host plants. *Pseudomonas* is the most effective root colonizer present in both rhizospheres as well as within the tissue of plants. In the rhizosphere, microbes can have both beneficial and harmful effect on the plants. Plant species get benefits like phytohormone production, availability of nutrients, and abiotic tolerance through the mutualistic interaction with the microorganism in the rhizosphere. These benefits can be provided by different groups of microorganism. Many studies examined the effect of co-inoculation with different groups of microbes in the soil or plants (Senthilraja et al. 2010; Hol et al. 2013). The co-inoculation of microbial species in the plant or soil may be *Pseudomonas* + fungi, *Pseudomonas* + other bacteria like *Bacillus* and *Azotobacter*, or *Pseudomonas* + different species of *Pseudomonas* (Domenech et al. 2006; Jaderlund et al. 2008).

These multiple agents enhanced the possibility to better response in growth or biocontrol action in the plants due to chance of better survivability of either of the strains in the applied environmental condition. It is also revealed from the previous study that co-inoculation of different microbial species are more effective than single inoculation for biocontrol (Whipps 2001; Hol et al. 2013). Akila et al. (2011) reported combined application of *P. fluorescens* and *Bacillus* sp. more effectively controls the *Fusarium* disease. Co-inoculation of *P. fluorescens* and *Azospirillum* stimulated the root growth in spring wheat (Combes-Meynet et al. 2011). The most common inoculation of fungi with *Pseudomonas* is the mycorrhizal fungi, used to improve the plant growth and nutrition. Senthilraja et al. (2011) reported combined use of *P. fluorescens* with *Beauveria bassiana* more effective than single application.

In case of interaction of *Pseudomonas* with other PGPR, *Bacillus* is the most common species. Garcia-Gutierrez et al. (2012) reported that single or combined application of *P. fluorescens* with *Bacillus* is equally effective against bacterial and fungal pathogen. It is concluded from the previous study that combined introductions of *Pseudomonas* and other PGPR have been more beneficial in growth promotion and in biological control (Shrivastava et al. 2016; Velazquez et al. 2016; Sindhu et al. 2016; Meena et al. 2014b, 2015c; Bahadur et al. 2016b).

## 7.8 Concluding Remarks

In the current scenario, plant growth-promoting bacteria (PGPR) are one of the most suitable choices for plant growth promotion and in disease management over the use of chemical fertilizers and pesticides. Rhizosphere is a large reservoir of bacteria, in which *Pseudomonas* genus is most commonly found. The exudates of plant roots largely interact the *Pseudomonas* species and help in root-colonizing activity. The beneficial effect of *Pseudomonas* includes plant growth promotion and biological control in the host plants. Besides this *Pseudomonas* strains have been broadly utilized in detoxifying certain organic and inorganic water pollutants (Wasi et al. 2011) and also in the bioremediation of heavy metals and pesticides (Lamire et al. 2010; Singh et al. 2011). The broad-spectrum rhizobacterial and endophytic strains of *Pseudomonas* can provide an effective, economical, and practical way of growth promotion, plant protection, and also managed the environment from soil, air, and water pollution. Sustainable agriculture is the need of the world in recent times because of the adverse effect of chemicals used during the agriculture. From the current study, it is recommended that *Pseudomonas* is one of the best choices as plant and soil inoculants and have been implemented as growth promotion and in disease management.

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# Role of Nutrients in Controlling the Plant Diseases in Sustainable Agriculture

# 8

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**Abstract**

The importance of sustainable agriculture can be understood as an ecosystem approach toward the integrated agricultural management practices. It is capable of enhancing soil and environmental quality with conserving natural resources. Therefore, in recent years, it has become a component of the modern agricultural practices. However, at the same time, yield loss of field crops due to diseases is causing bottlenecks toward the sustainable agricultural production systems worldwide. The conventional method for disease management has caused degradation of environment, land resources, and water bodies, developed pesticide resistance in pathogens, and contaminated the food with toxins. These have evolved crave for the alternative disease management practices, which are economic, eco-friendly, and sustainable approach for farmers. To be precise, rate of development of diseases can be reduced by an adequate and balanced mineral nutrition in crops. The plant nutrients determine its resistance or susceptibility to disease, its histological or morphological structure or properties, and the ability of pathogens to survive on the host. The disease symptoms frequently reflect the altered nutritional status of the plant, and many factors that influence this response are not well understood. This article summarizes some of the most

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recent developments regarding the effect of macronutrients (e.g., N, P, K), secondary nutrients (e.g., Ca, Mg), and micronutrients (e.g., B, Mn, Zn, Fe, Cu, and Si) on disease resistance/tolerance and susceptibility and their use in sustainable agriculture.

**Keywords**
Disease management • Plant nutrients • Sustainable agriculture

## Contents

## 8.1 Introduction

Nowadays, the importance of sustainable agriculture has risen to become one of the most important issues in agriculture from the last two decades. Although numerous definitions of sustainable agriculture exist, most agree on the three basic, overlapping components: ecological, economic, and social sustainability (Kaur 2013; Pilgeram 2013). The word is most appropriately used with the agricultural practices that need to be sustainable to fulfill the requirements of the increasing population and of the future generation without having its effect on the factors of the ecosystem which must be utilized, maintaining its diversity, productivity, liveliness, and capacity to function (Lewandowski et al. 1999; Heslin 2015). Meanwhile, incidence of plant diseases poses major limiting factor toward sustainable agricultural production systems worldwide, especially in tropics and subtropics. To manage plant diseases, farmers often apply agrochemicals (i.e., pesticides, insecticides, fungicide, herbicides etc.) in doses excess of their recommended dose that have raised serious concerns about food safety, soil, environmental quality, and pesticide resistance, which have dictated the need for alternative pest management techniques (Dordas 2008; Kaur 2013). Moreover, the continuous use of many pesticides and biocides has developed pesticide-specific resistance in pathogens.

Sustainable agriculture attempts to provide long-term sustained yields through the use of ecologically sound management technologies such as crop diversification, recycling of plant nutrients, and biological methods of pest control (Heslin 2015; Srivastava et al. 2016). The sustainability of agriculture has faced many significant challenges in recent years. The major challenges include (1) the rapid growth of the human population and the increased demand for agricultural land and resources (Godfray et al. 2010), (2) increased resistance of pests and diseases toward pesticides (Lucas et al. 2015), (3) overdependence on fossil energy and the increased monetary and environmental costs of nonrenewable resources (Srivastava et al. 2016), (4) global climate change (Srivastava et al. 2016), and (5) globalization (Pilgeram 2013). These dominant issues are challenging agriculturists to develop more sustainable management systems, like never before, in history. To meet the food and nutritional requirements of a growing population, agriculture will need to move beyond the past emphasis on productivity to encompass improved public health, social well-being, and a sound environment (Hanson et al. 2007). Thus, it becomes important to find alternatives to manage plant diseases which do not harm the environment and at the same time increase yield and improve product quality (Bahadur et al. 2014; Maurya et al. 2014; Ahmad et al. 2016; Meena et al. 2016a; Kumar et al. 2016).

To control pests and diseases, the farmers have several options which can be combined in the integrated pest management approaches like (1) genetics, the cultivation of crops, which are less susceptible or resistant to pests and diseases; (2) biological control, referring to utilization of biological agents and predators; (3) chemical control, through organic/inorganic fungicides and pesticides; (4) cultural practice, to create optimal growth conditions of the cultivated crops and/or to eradicate those conditions, which are favorable for multiplication of pests and diseases; and lastly (5) plant nutrition. When it comes to host resistance to diseases, it is often observed that the plant nutrition and health is being overlooked. However, rate of development of diseases may be reduced by adequate knowledge of balanced mineral nutrition in many crops. There are 18 nutrient elements required to grow crops. Among them, 15 nutrients are taken up from the soil and are usually grouped as primary nutrients, secondary nutrients, and micronutrients (Bhaduri et al. 2014).

Plants uptake the following mineral nutrients for a healthy growth: the primary macronutrients, nitrogen (N), phosphorus (P), and potassium (K); the three secondary macronutrients, calcium (Ca), sulfur (S), and magnesium (Mg); and the micronutrients/trace minerals, boron (B), chlorine (Cl), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo), and nickel (Ni). Nutrients are important factors in disease resistance and control and they are also important for growth and development of plants (Datnoff et al. 2007). Some nutrients have a greater impact on plant diseases than others. However, it should be considered that a particular nutrient may have opposite impacts on different diseases and in different environments, i.e., the same nutrient may increase the incidence of one disease but at the same time decrease the incidence of others (Agrios 2005). Application of fertilizers is not a substitute for pesticides, but an important component in integrated pest management, allowing reductions in the pesticide doses and, thus, decreasing pesticides and hazardous residues in food crops. There are two types of primary resistance mechanisms that mineral nutrition can affect either by formation of mechanical barriers, primarily through the development of thicker cell walls, or synthesis of natural defense compounds, such as phytoalexins, antioxidants, and flavanoids, that provide protection against pathogens (Bhaduri et al. 2014; Prakash and Verma 2016; Meena et al. 2015a, 2016b; Priyadharsini and Muthukumar 2016; Kumar et al. 2017). Moreover, addition of nutrients indirectly enhances the pathogen inactivity, thus increasing the yield of crops.

## 8.2 Role of Nutrients in Reducing Disease Severity

Nutrition has been a primary component of disease control and management; still the importance of plant nutrient is unexplored. The effect of mineral nutrients on disease has been based on (1) the observed effects of fertilization on a specific disease's incidence or severity, (2) the comparison of mineral concentrations in healthy or resistant tissues compared with diseased or susceptible tissues, or (3) conditions influencing the availability of a specific nutrient with disease. All of these observations can generally be correlated for a particular nutrient and disease interaction, although growth stage of the plant, environmental conditions, and biological activity can influence the outcome (Meena et al. 2017). Mineral nutrition has an important role in this system, and its management can affect not only the yield but also plant health and the environment (Katan 2009). The summarized influence of the essential nutrients on disease resistance or severity is shown in Table 8.1 and also discussed in the following subsections.

### 8.2.1 Nitrogen (N)

Nitrogen is the most commonly used fertilizer and is essential for the synthesis of many cellular components (Havlin et al. 2009). It is absorbed by plants in either a reduced or an oxidized form. The rapid rate of nitrification in many cultivated soils provides nitrate ($NO_3^-$) for plant uptake which is internally reduced to amino acids prior to utilization by cells (Marschner 1995; Havlin et al. 2009). The two forms of nitrogen (i.e., $NO_3^-$ and $NH_4^+$) absorbed by the plant are assimilated differently and can have a profound effect on diseases. Despite the fact that abundance of N in plants is one of the most important factors influencing disease development, there are several reports of the effect of N on disease development that remain elusive and contradict each other, and the facts for this elusiveness remained poorly understood

**Table 8.1** Effect of plant nutrients on disease resistance, tolerance, or susceptibility

| Nutrient | Disease | Causal organism | Crop | Presence of nutrient | References |
|---|---|---|---|---|---|
| *A. Primary nutrients* | | | | | |
| Nitrogen | Gray mold | *Botrytis cinerea* | Tomato | Resistance increased with N | Hoffland et al. (1999) |
| | Early blight, blight | *Alternaria solani* | Potato, tomato, cotton | Severity of the infection decreases with high N supply | Blachinski et al. (1996) |
| | Bacterial speck, wilting | *Pseudomonas syringae, Oidium lycopersicum* | Tomato | Susceptibility increases with N | Hoffland et al. (2000) |
| | Gummy stem blight | *Didymella bryoniae* | Water melon | Severity increased with increasing dose of N | Santos et al. (2009) |
| | Foliar disease | *Rhynchosporium secalis, Drechslera teres, Cochliobolus sativus* | Barley | Increasing rate of N had no effect | Turkington et al. (2012) |
| | Stripe rust | *Puccinia striiformis* f. sp. *tritici* | Wheat | Severity of the infection decreases with N supply | Devadas et al. (2014) |
| Potassium | Leaf blight | *Pyrenophora tritici-repentis* | Wheat | Resistance increases with K | Sharma et al. (2005) |
| | Leaf rust | *Puccinia triticina* | Wheat | Resistance increased with K | Sweeney et al. (2000) |
| | Sheath blight | *Rhizoctonia solani* | Rice | Resistance increased with high K | Schurt et al. (2015) |
| | Pod and stem blight | *Diaporthe sojae* | Soybean | Susceptibility increases with low K | Snyder and Ashlock (1996) |
| | Rust | *Phakopsora pachyrhizi* | Soybean | Resistance increased with K | Pinheiro et al. (2011) |

(continued)

**Table 8.1** (continued)

| Nutrient | Disease | Causal organism | Crop | Presence of nutrient | References |
|---|---|---|---|---|---|
| Phosphorus | Root disease | *Rhizoctonia* | Wheat | Resistance increases with P | Kirkegaard et al. (1999) |
| | Powdery mildew | *Sphaerotheca fuliginea* | Cucumber | Reduction in disease severity with P application | Reuveni et al. (2000) |
| | Root rot | *Rhizoctonia solani* | Faba bean | Severity of the infection decreases with P supply | Mousa and El-Sayed, (2016) |
| *B. Secondary nutrients* | | | | | |
| Magnesium | Brown spot | *Bipolaris oryzae* | Rice | Resistance increased with increased Mg | Moreira et al. (2015) |
| | Leaf blight | *Helminthosporium maydis* | Maize | Susceptibility increases with Mg | Taylor (1954) |
| Calcium | Phytophthora stem rot | *Phytophthora sojae* | Soybean | Resistance increased with Ca | Sugimoto et al. (2011) |
| | Clubroot | *Plasmodiophora brassicae* | Crucifer crops | Reduction in incidence in soil with sufficient Ca | Campbell et al. (1990) |
| | Apple decay | *Gloeosporium perennans* | Apple | Severity of the infection decreases with Ca supply | Krauss (1971) |
| | Fruit rot | – | Fleshy fruits | Ca treatment before storage prevents physiological disorders and rotting | Dordas (2008) |
| Sulphur | Potato scab | *Streptomyces scabies* | Potato | Possibility of reduction in severity by S application | Huber (1980) |

| | | | | | |
|---|---|---|---|---|---|
| *C. Micronutrients* | | | | | |
| Boron | Eutypa dieback | *Eutypa lata* | Grapevine | Resistance increased with B | Rolshausen and Gubler (2005) |
| | Tobacco mosaic virus, tomato yellow leaf curl virus | TMV, TYLCV | Bean, tomato | Reduction in disease with B application | Graham and Webb (1991) |
| | Powdery mildew | *Blumeria graminis* | Wheat | Decreases with applied B | Marschner (1995) |
| | Blue rot of orange | *Penicillium digitatum* | Orange | Reduction with B treatment before storage | Tarabih and El-Metwally (2014) |
| Zinc | Root rot | *Rhizoctonia solani* (AG 8) | Bur clover | Resistance increases with Zn | Streeter et al. (2001) |
| | Banana wilt | *Fusarium oxysporum* f. sp. *cubense* | Banana | Resistance increases with Zn | Sanjeev and Eswaran (2008) |
| | Pythium rot | *Pythium deliense* | Cucumber | Resistance increases with increased Zn | Kucukyumuk et al. (2014) |
| | Root rot | *Fusarium solani* | Wheat | Application of Zn increases tolerance | Khoshgoftarmanesh et al. (2010) |
| Iron | Apple canker | *Sphaeropsis malorum* | Apple, pear | Resistance increases with Fe application | Graham (1983) |
| | Black shank | *Phytophthora parasitica* var. *nicotianae* | Tobacco | Presence of Fe favors growth | Hendrix et al. (1969) |
| | Wilt | *Fusarium* spp. | Tomato | Severity reduced with decreased Fe availability | Scher and Baker (1982) |

(continued)

**Table 8.1** (continued)

| Nutrient | Disease | Causal organism | Crop | Presence of nutrient | References |
|---|---|---|---|---|---|
| Manganese | Brown spot | *Helminthosporium oryzae* | Rice | Resistance increased with Mn | Kaur and Padmanabhan (1974) |
| | Take-all | *Gaeumannomyces graminis* var. *avenae* | Bent grass | Resistance increases with Mn | Carrow et al. (2001) |
| | Black leaf mold | *Pseudocercospora fuligena* | Tomato | Control of disease with Mn | Heine et al. (2011) |
| Copper | Powdery mildew | *Blumeria graminis* f. sp. *tritici* | Wheat | Suppression with applied Cu | Graham (1980) |
| | Bacterial canker | *Clavibacter michiganensis* subsp. *michiganensis* | Tomato | Decreases with Cu application | Bastas (2014) |
| | Ergot | *Claviceps* sp. | Wheat | Reduction with Cu application | Evans (2007) |
| | Sheath blight | *Rhizoctonia solani* Kuhn | Paddy | Decreased with application of Cu compounds | Khaing et al. (2014) |
| Chlorine | Stripe rust | *Puccinia striiformis* f. sp. *tritici* | Wheat | Controlled in presence of Cl | Graham and Webb (1991) |
| | Root rot | *Cochliobolus sativus* | Barley | Reduction with Cl fertilizer | Timm et al. (1986) |
| | Leaf rust | *Puccinia recondite* | Wheat | Suppression with Cl fertilizer application | Elmer (2007) |

| | | | | | |
|---|---|---|---|---|---|
| *D. Other nutrients* | | | | | |
| Silicon | Powdery mildew | *Erysiphe cichoracearum* | Cucumber | Resistance increased with Si | Miyaki and Takahashi (1983) |
| | Anthracnose | *Colletotrichum gloeosporioides* | Capsicum | Resistance increases with Si | Jayawardana et al. (2016) |
| | Angular leaf spot | *Xanthomonas malvacearum* | Cotton | Resistance increases with Si | Oliveira et al. (2012) |
| | Phytophthora blight disease | *Phytophthora capsici* | Bell pepper | Resistance increases with Si | French-Monar et al. (2010) |
| | Pythium root rot | *Pythium ultimum* | Cucumber | Resistance increased with Si | Cherif and Belanger (1992) |
| | Blast | *Magnaporthe oryzae* | Paddy | Resistance increased with Si fertilization | Sun et al. (2010) |
| | Brown spot | *Bipolaris oryzae* | Rice | Resistance increased with Si | Dallagnol et al. (2014) |

(Marschner 1995; Hoffland et al. 2000; Dordas 2008; Bahadur et al. 2017; Verma et al. 2017b; Kumar et al. 2017).

These differences may be due to the form of N absorbed by the host plant (Harrison and Shew 2001; Celar 2003) or the type of pathogen, viz., obligate or facultative under consideration (Buschbell and Hoffmann 1992; Marschner 1995). In addition, most of the contradiction in reports of the effect of N on diseases may result from a failure to recognize the different effects of the various forms of nitrogen (Huber and Thompson 2007). For example, with high N supply, there is an increase in severity of the infection caused by the obligate parasites, e.g., *Puccinia graminis* (Howard et al. 1994), *Erysiphe graminis* (Buschbell and Hoffmann 1992), and *Oidium lycopersicum* (Hoffland et al. 2000). On the other side, when the disease is caused by facultative parasites like *Fusarium oxysporum* (Woltz and Engelhar 1973), *Alternaria solani* (Blachinski et al. 1996), and *Xanthomonas* sp. (Chase 1989), high N supply decreases the severity of the infection. Such difference in reports between the obligate and facultative parasites is mainly due to the nutritional requirements of these parasites. Obligate parasites require assimilates supplied directly from living cells. In contrast, facultative parasites are semi-saprophytes, which prefer senescing tissue or which release toxins in order to damage or kill the host plant cells (Meena et al. 2015b; Meena et al. 2016c; Saha et al. 2016; Meena et al. 2016d; Dotaniya et al. 2016; Meena et al. 2015f). Therefore, the factors, which influence the metabolic activities of the host cells and which delay senescence of the host plant, can increase resistance or tolerance to facultative parasites (Agrios 2005). Thus, the effect of N is quite variable in the literature, which is mainly due to the different response depending on the type of the pathogens. A trend of increased severity with increasing doses of N was observed by Santos et al. (2009) in gummy stem blight (*Didymella bryoniae*) of water melon and by Devadas et al. (2014) in stripe rust (*Puccinia striiformis* f. sp. *tritici*) of wheat, with a decreasing trend in yield. In general, there is a higher growth rate during the vegetative stage with the increased rate of N and the proportion of the young to mature tissue shifts in favor of the young tissues, which are more susceptible (Dordas 2008). At the same time, plant metabolism changes due to high N rates, as some key enzymes of phenol metabolism have lower activity. The content of the phenolics decreases and the lignin content may be lower, and all these are part of the defense system of plants against infection (Meena et al. 2014a; Shrivastava et al. 2016; Meena et al. 2015c; Bahadur et al. 2016b; Das and Pradhan 2016; Nath et al. 2017; Sarkar et al. 2017; Verma et al. 2017a).

Thus, the main reason for the increased susceptibility of the host plants toward high N rates is the various anatomical and physiological changes. On contrary, Last (1962) observed that N increased the level of infection of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) as well as the grain yield of the infested plant. Last (1962) argued that the more vigorous growth of the plant due to application of more N supplied more assimilates to the plants which lowered the competitive effect of the pathogen. Hofer et al. (2016) demonstrated that N fertilization restricts *Fusarium* grain infection of barley by influencing canopy characteristics and possibly plant physiology. They concluded that N may act differently on infection caused by different species of the *Fusarium* complex or other fungal genera attacking barley, because of differences in life cycles, infection strategies, or production of secondary metabolites. These differences should be kept in mind in incorporation of N fertilization into an integrated pest management strategy against *Fusarium* in spring barley. Similarly Turkington et al. (2012) reported that increasing the N rate from 50 to 100% had no effect on leaf disease levels in barley.

The different forms of N supply can have a major impact on the activity of root-borne diseases, which can be sensitive to pH. The use of ammonium-based fertilizers can increase the incidence of some diseases (e.g., *Phytophthora* root rot, *Fusarium* wilt, and *Fusarium* crown and root rot), whereas nitrate-based fertilizers generally have the reverse effect (McGovern 2015). The situation becomes more complex for soilborne pathogens, as on the root surface, there are many more microorganisms than in the bulk soil (Bhaduri et al. 2014). Interaction between nitrogen and other nutrients is also found to be influencing disease resistance. For example, $NO_3^-$ nutrition stimulates $K^+$ uptake, whereas $NH_4^+$ uptake competes with $K^+$ uptake (Marschner 1995). Proper K nutrition has been found to protect crops from diseases and pests. Therefore, uptake of nitrogen as $NO_3^-$ imparts disease resistance to some extent over $NH_4^+$ uptake. In addition, there is a decrease in Si content with the application of high levels of N, which can also affect the disease tolerance.

### 8.2.2 Phosphorus (P)

After N, phosphorus is the most widely applied plant nutrient and its deficiency in soils significantly reduces crop yields (Havlin et al. 2009). P is an essential element of the building blocks of life, the ribonucleic acids (RNA), as well as being required for many additional biochemical and physiological processes including energy transfer, protein metabolism, and other functions (Marschner 1995; Prabhu et al. 2007).

However, in the field of agriculture, P has been extensively used to prepare the formulations of fertilizers for enhancing yield of the crops and as fungicides, bactericides, and nematicides for controlling the harmful pathogens. Regarding disease resistance, the role of P is inconsistent and seemingly unpredictable. For example, P application increased resistance in certain crops as in tomato to *Fusarium* and tobacco to *Pseudomonas tabaci*, whereas in some crops, it showed decrease in resistance like in tobacco to *Tobacco mosaic virus* and in cucumber to *Cucumber mosaic virus* (Kiraly 1976). P has been found to be the most effective when it is applied to control fungal diseases of seedlings, where faster root development allows plants to escape disease (Huber and Graham 1999). Similarly, in corn, P application can reduce root rot, especially when it is grown on soils deficient in P (Huber and Graham 1999).

A number of studies have shown that P application could reduce the diseases like potato scab, peanut rust, bacterial leaf blight in rice, leaf curl virus disease in tobacco, pod and stem blight in soybean, brown stripe disease in sugarcane, blast disease in rice, and cowpea anthracnose and *Rhizoctonia* root rot disease in faba bean (Davis et al. 1976; Mayee 1983; Adebitan 1996; Reuveni et al. 1998, 2000; Huber and Graham 1999; Kirkegaard et al. 1999; Mousa and El-Sayed 2016). Contrary to these, some other studies reported that application of P may increase the severity of diseases caused by rust in sugarcane, *Sclerotinia* in many garden plants, and flag smut in wheat (Huber 1980). Application of adequate amounts of P increased *Fusarium* wilt in tomato at pH 6.0, but suppressed it at pH 7.0–7.5 and reduced the severity of *Rhizoctonia* disease in soybean, emphasizing the importance of balanced and adequate nutrition (Katan 2009). Foliar sprays of P can confer local and systemic protection against some foliar pathogens, e.g., powdery mildews in cucumber, grape, mango, apple, wheat, and peppers; rust on maize; and others (Reuveni and Reuveni 1998; Katan 2009). Soils deficient in adequate P may also induce the severity of diseases in plants suffering from P starvation. For example, Zhao et al. (2013) found a 35% reduction in P content in citrus trees affected by Huanglongbing (HLB) disease (Candidatus *Liberibacter asiaticus*) compared to healthy trees. They further demonstrated that application of P solutions to the affected plants significantly reduced HLB symptoms and improved fruit yield in citrus.

### 8.2.3 Potassium (K)

Potassium is required by the plant for various vital biochemical and physiological functions (Marschner 1995; Havlin et al. 2009; Wang et al. 2013). Deficiency symptoms of potassium such as thin cell walls, weakened stalks and stems, smaller and shorter roots, accumulation of sugar in the leaves, and accumulation of unused N encourage disease infection (Graham 1983). Each of these factors minimizes the ability of the plant to resist entry of infection by fungus, bacteria, and virus. K fertilization is widely reported to affect the insect infestation and disease incidence in many host plants (Marschner 1995). Adequate nutrient serves two major roles as in protecting the plant from metabolic stress and disease resistance. Nevertheless, K increases the resistance of host plants up to the optimal level, beyond which, there is no further increase in resistance with increasing supply of K and its contents in plants (Huber and Graham 1999). Perrenoud (1990) reviewed 2449 references and found that the use of K significantly decreased the incidence of fungal diseases by ~70%, bacteria by ~69%, viruses by ~41%, insects and mites by ~63%, and nematodes by ~33%. Meanwhile, K increased the yield of plants infested with fungal diseases by 42%, bacteria by ~57%, viruses by ~78%, insects and mites by ~36%, and nematodes by ~19%. It has been observed that the intensity of several infectious diseases of obligate and facultative parasites can be reduced by the application of K fertilizer (Table 8.2).

In potato, K fertilization was found to decrease the incidence of several diseases, such as late blight (*Phytophthora infestans*), dry rot (*Fusarium* sp.), powdery scab (*Spongospora subterranean*), and early blight (*Alternaria solani*) (Marschner 1995). A classical example is presented by Ismunadji (1976) who showed that the degree of damage caused by rice stem rot (*Helminthosporium sigmoideum*)

**Table 8.2** Effect of K level on disease severity of several diseases

| S. No. | Pathogen or disease | Low K | High K | References |
|---|---|---|---|---|
| 1 | *Puccinia graminae* | Increase | Decrease | Lam and Lewis (1982) |
| 2 | *Xanthomonas oryzae* | Increase | Decrease | Chase (1989) |
| 3 | Tobacco mosaic virus | Increase | Decrease | Ohashi and Matsuoka (1987) |
| 4 | *Alternaria solani* | Increase | Decrease | Blachinski et al. (1996) |
| 5 | *Fusarium oxysporum* | Increase | Decrease | Srihuttanum and Sivasithamparam (1991) |
| 6 | *Pyrenophora tritici-repentis* | Increase | Decrease | Sharma et al. (2005) |
| 7 | *Erysiphe graminis* | Increase | Decrease | Menzies et al. (1992) |

Adopted from Dordas (2008)

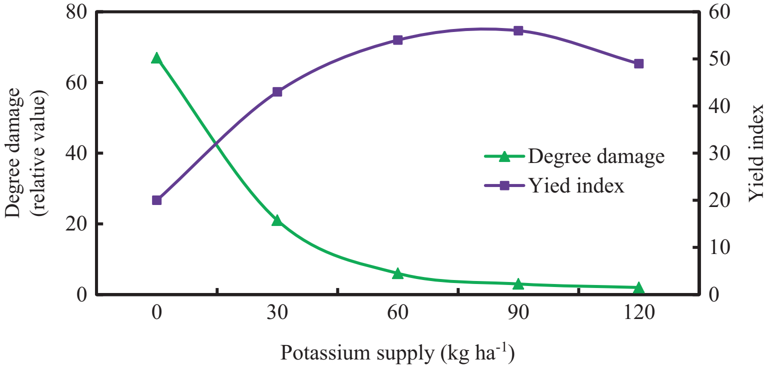

**Fig. 8.1** Effect of potassium supply on grain yield of wetland rice and incidence of stem rot (*Helminthosporium sigmoideum*). Basal dressing of nitrogen and phosphorus constant at 120 and 60 kg ha$^{-1}$, respectively (Adopted from Ismunadji 1976)

decreased gradually with concomitant increase in K fertilization rate (Fig. 8.1). A study reported the supplementary dose of potassium (400 kg ha $^{-1}$) represented an alternative for the control of anthracnose in tomato along with the probiotic (Luengas-Gómez et al. 2012; Meena et al. 2014b; Meena et al. 2015d; Bahadur et al. 2016a; Meena et al. 2015e).

Potassium phosphate was found to be ~70% effective against powdery mildew disease in barley (Mitchell and Walters 2004). Similarly, Liljeroth et al. (2016) observed that potassium phosphite in combination with fungicides was used against late blight disease of potato. They detected higher efficacy of this compound probably due to its combined effects on inducing defense reactions in the plant as well as inhibiting effect against sporulation of *Phytophthora*. Phosphate salts of K have good control efficacy compared to conventional fungicides when used in field conditions with lower risks for human health and environment (Kromann et al. 2012). Various studies have been reported the use of K fertilization in reducing *Alternaria* leaf spot disease in cotton on a soil low in K (Miller 1969; Hillocks and Chindoya 1989; Bhuiyan et al. 2007). It has been demonstrated that spraying with KCl

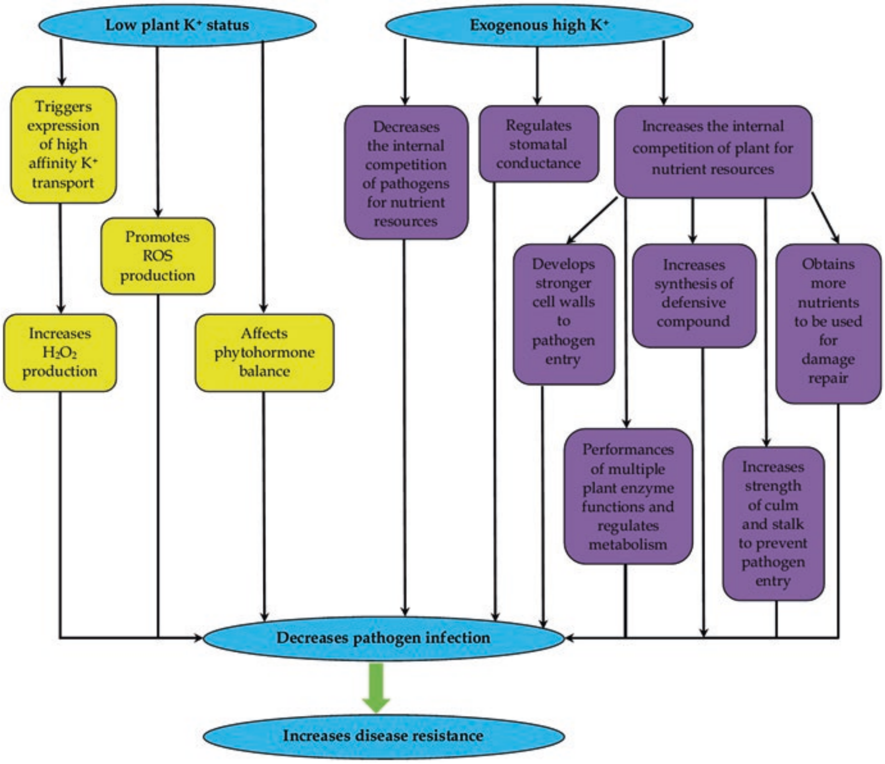

**Fig. 8.2** Mechanism of K in disease resistance in host plants (Adopted from Wang et al. 2013)

suppresses infection of powdery mildew in wheat and septoria leaf blotch due to an inhibition of fungal spore germination (Mann et al. 2004). High concentrations of KCl were applied in this field trial; the effects were attributed to the high osmolarity of the treatment solutions (Masood and Bano 2016; Meena et al. 2016e).

Higher $K^+$ concentrations decrease the internal competition of pathogens for nutrients (Fig. 8.2). Another benefit of high $K^+$ concentration is that it makes the stronger cell wall less prone to lodging, thus improving airflow through crop canopy. Some pathogens (airborne), such as bacteria and virus, enter the plant via the stomata, and perception of pathogenic elicitors, or pathogen-associated molecular patterns, by the stomatal guard cell induces a full closure of the pore (Melotto et al. 2006). However, K-deficient plants are defective in stomatal regulation. This means that this first line of defense against airborne pathogens is likely to be leaky in such plants (Zörb et al. 2014). Potassium regulates the metabolite pattern of higher plants by increasing the performances of multiple plant enzyme functions (Marschner 1995; Wang et al. 2013).

The high susceptibility of K-deficient plants to various parasitic diseases is directly related to these metabolic functions. In deficient plants, the synthesis of high-molecular-weight compounds is severely impaired, and low-molecular-weight organic compounds accumulate, indispensable for feeding pathogens and insects (Marschner 1995; Amtmann et al. 2008; Zörb et al. 2014). Insufficient K causes a pale leaf color that is particularly attractive to aphids, which transmit viruses at the same time (Marschner 1995; Oosterhuis et al. 2014). Cracks, fissures, and lesions that develop at K deficiency on the surface of leaves and fruits provide easy access, especially for facultative parasites (Krauss 2001). In addition, the accumulation of inhibitory amino acids, phytoalexins, phenols, and auxins is dependent on the level of K (Perrenoud 1990), while K deficiency results in inorganic N accumulation, due to poor translocation, and phenol (with fungicidal properties) break down (Kiraly 1976). On contrary, some circumstantial evidence points to a decreased infestation of K-deficient plants by insects and necrotrophic pathogens (Amtmann et al. 2008). K deficiency also induces the jasmonate signaling network (Troufflard et al. 2010). Jasmonates act as systemic trigger of defense responses toward necrotrophs and insects (Wasternack 2007). It has thus been concluded that a temporary K limitation may be imposed to protect crops from herbivory and from necrotrophic fungi (Amtmann et al. 2008).

### 8.2.4 Calcium (Ca)

Imbalance in Ca, one of the secondary plant nutrients, is also a cause of various diseases in plants. In general, the pathogens release enzymes to dissolve the middle lamella, which is strongly opposed by the presence of Ca (Marschner 1995). Also the deficiency of Ca leads to leakage of metabolic products that stimulates pathogen infections easily (Spann and Schumann 2010).

There is an increased susceptibility to various fungi when Ca content drops, which preferentially invade the xylem leading to wilting type of symptoms (Dordas 2008). Ca also affects the incidence of various bacterial diseases. First, Ca plays an important role in the formation of rigid cell walls. Secondly, adequate Ca also inhibits the formation of pectolytic enzymes produced by fungi and bacteria. The role of bacteria and fungi is to dissolve the middle lamella, allowing penetration and infection. Ca deficiency triggers the accumulation of sugars and amino acids in the apoplast, which attracts disease-causing organisms and lowers disease resistance.

Low level of Ca in fruit tissue results in less resistant to various parasitic diseases and physiological disorders (Singh et al. 2007; Shafiee et al. 2010). Postharvest disorders such as decay, delay in fruit ripening, and decreased postharvest fruit weight loss were recorded when they used pre- and postharvest application of Ca (Lara et al. 2004; Hernandez-Munoz et al. 2006). Garcia et al. (1996) found that Ca prevented fruit softening during storage and delayed total soluble solids decrease, especially when it was combined with 45 °C hot-water treatment. Similarly, Shafiee et al. (2010) also found that there were less weight loss and decay and higher firmness when strawberry fruits are dipped in $CaCl_2$ solution. Madani et al. (2014) observed the effect of preharvest $CaCl_2$ applications on postharvest anthracnose disease (*Colletotrichum gloeosporioides* Penz.) incidence in papaya. Six preharvest foliar $CaCl_2$ sprays significantly decreased spore germination and mycelial growth of the pathogen (Dordas 2008, Huber et al. 2012). Hence, combination of B and Ca is more effective in stabilization of cell wall in comparison to Ca alone (Liebisch et al. 2009).

The sufficient amount of Ca at neutral to slightly alkaline soils can reduce clubroot disease in crucifer crops, e.g., broccoli, cabbage, and turnips (Campbell and Arthur 1990). Ko and Kao (1989) reported that reduction of damping-off disease in various crops is caused by *Pythium* sp. after amending the soil with Ca. Ca also confers resistance against the *Rhizoctonia, Sclerotinia*, and *Botrytis* (Graham 1983; Huber 1980). Ghani et al. (2011) found that Ca can increase anthracnose resistance in dragon fruit. Similarly, deficiency of boron (B) coupled with Ca can also cause damages on the plant's surface. The activity of polygalacturonase and pectate transeliminase decreases substantially with increasing Ca content of beans, resulting in a higher resistance to soft rot disease, *Erwinia carotovora* (Platero and Tejerina 1976). The lower infestation of lettuce with gray mold, *Botrytis cinerea*, at higher Ca contents (Krauss 1971) or the decreasing incidence of apple decay caused by *Gloeosporium perennans* at increasing Ca contents can also be related to the control of pectolytic enzymes by Ca.

### 8.2.5 Magnesium (Mg)

Magnesium plays a major role in plant photosynthesis being a central atom of chlorophyll that captures the light energy (Marschner 1995). Mg plays a vital role in transporting the phloem export of photosynthates; however, in the deficient conditions, the products like sucrose and amino acids get deposited in the leaves which make conducive environment for various disease-causing pathogens to attack (Huber and Jones 2013). Thus, the factors, which govern Mg availability in soils and its uptake by plants, may influence Mg-induced resistance and/or susceptibility in host plants. For example, Mg deficiency is quite common in K-rich micaceous soils (due to antagonistic interaction), and consequently, Mg deficiency could be aggravated when K fertilizers are applied (Debnath et al. 2015). The effect of Mg has been investigated in some studies in reducing the disease severity in crops like rice, wheat citrus, potato, poppy, and peanut (Moreira et al. 2015).

### 8.2.6 Micronutrients

Micronutrients are equally important as with primary and secondary nutrients in controlling the plant diseases. Micronutrients play a role on reducing the severity of different diseases due to the involvement in physiology and biochemistry of the plant because many of the important micronutrients are involved in many processes in plants and that can affect response of plants to pathogens (Marschner 1995). Micronutrients inhibit the pathogen from penetrating by affecting the cell wall rigidity and also the physical integrity of the membrane structure (Graham and Webb 1991; Marschner 1995; Huber et al. 2012). In addition, micronutrients can also affect disease resistance indirectly, as nutrient-deficient plants not only exhibit an impaired defense mechanism but may also become more suitable for feeding as many metabolites such as sugars and amino acids leak out from cell (Huber et al. 2012). However, micronutrients are also known to reduce the severity by inducing the resistance within the plant also called as systemic acquired resistance (SAR) (Dordas 2008).

Boron (B), an essential micronutrient, has been used in the direct application to soil and foliar sprays to overcome its deficiency (Christensen 2001). B has a beneficial effect on reducing disease severity (Marschner 1995; Dordas 2008). B promotes rigidity of the cell wall and, therefore, supports the shape and strength of the plant cell (Marschner 1995; Dordas 2008; Broadley et al. 2012). Furthermore, B is possibly involved in the integrity and permeability of the plasma membrane (Marschner 1995; Dordas 2008; Broadley et al. 2012). Under B deficiency, structural integrity of cell membranes is substantially impaired causing membranes to become leaky, which can result in massive release of organic compounds (e.g., carbohydrates, amino acids, etc.) from cells to outside, representing a very suitable feeding medium for pathogens and their spreading (Huber et al. 2012). By affecting phenolics and lignin synthesis, B can also suppress penetration of pathogens (Table 8.3).

B has been widely used in controlling wood decay fungi and *Heterobasidion annosum* that causes infection in conifers (Smith 1970; Schultz et al. 1992). Infection of B-deficient wheat plants with powdery mildew is several times greater than in B-sufficient plants (Schütte 1967; Stangoulis and Graham 2007), which may be due to increased leakage through the plasma membrane under B deficiency

**Table 8.3** Effect of preharvest calcium chloride treatments and days in storage on anthracnose disease incidence and severity (%) in papaya fruits during storage

| Calcium chloride concentrations (%) | 7 days | 14 days | 21 days | 28 days | 35 days |
|---|---|---|---|---|---|
| Incidence | | | | | |
| 0.0 | 0.0 a,[a]A[b] | 24.9 a,C | 100.0 a,D | 100.0 a,D | 100.0 a,D |
| 0.5 | 0.0 a,A | 0.0 b,A | 41.6 b,C | 58.3 ab,C | 95.8 a,D |
| 1.0 | 0.0 a,B | 0.0 b,B | 21.0 bc,A | 49.9 b,C | 83.3 a,D |
| 1.5 | 0.0 a,A | 0.0 b,A | 13.0 c,AC | 24.9 b,C | 45.8 b,D |
| 2.0 | 0.0 a,C | 0.0 b,C | 0.0 c,C | 21.0 b,C | 45.8 b,D |
| Severity | | | | | |
| 0.0 | 0.0 a,B | 8.7 a,A | 25.4 a,C | 96.2a,D | 100.0 a,D |
| 0.5 | 0.0 a,C | 0.0 b,C | 14.6 b,C | 57.9b,D | 84.2 a,D |
| 1.0 | 0.0 a,B | 0.0 b,B | 10.2 bc,A | 22.9c,C | 46.7 b,D |
| 1.5 | 0.0 a,C | 0.0 b,C | 3.4 cd,C | 8.7c,C | 23.7 c,D |
| 2.0 | 0.0 a,C | 0.0 b,C | 0.0 d,C | 6.1c,C | 17.3 c,D |

Adopted from Madani et al. (2014)

[a] Lower case letters in columns (a, b, c, d) show the mean comparison among concentrations of calcium chloride. Means with the same letter are not significantly different according to the Waller-Duncan *k*-ratio *t*-test ($p = 0.05$)

[b] Capital letters in rows (A, B, C, D) show the mean comparison among days in storage. Means with the same letter are not significantly different according to the Waller-Duncan *k*-ratio t-test ($p = 0.05$)

**Table 8.4** Average number of lesions per flag leaf (±SE) induced by *D. tritici-repentis* at three different growth stages (booting stage, heading stage, and milk stage) of winter wheat, after foliar application of different micronutrients

| | Average number of lesions per flag leaf | | | | |
|---|---|---|---|---|---|
| Treatments | Booting stage | Heading stage | Milk stage | LA | DS |
| Control | 10.6 ± 0.71 c | 14.7 ± 0.59 c | 20.4 ± 0.53 c | 25.9 ± 0.36 b | 0.86 ± 0.03 c |
| Boron | 5.2 ± 0.42 a | 9.9 ± 0.38 a | 18.4 ± 0.43 b | 26.2 ± 0.38 b | 0.77 ± 0.02 b |
| Manganese | 8.0 ± .059 b | 12.8 ± 0.53 b | 16.2 ± 0.37 a | 27.0 ± 0.35 ab | 0.65 ± 0.02 a |
| Zinc | 6.3 ± 0.45 ab | 10.6 ± 0.46 a | 19.8 ± 0.46 bc | 27.6 ± 0.39 a | 0.75 ± 0.02 b |

Adopted from Simoglou and Dordas (2006)

LA is the leaf area of the flag leaf at the milk stage, and DS is the disease severity which is the number of lesions per $cm^2$ of the leaf area recorded at the milk stage

Numbers followed by the same letter in a column are not significantly different (Tuckey's HSD test, $p = 0.05$)

(Huber et al. 2012). During booting and milk stages, B significantly affected the number of lesions per leaf (Table 8.4). In general, micronutrients reduce disease severity by involving in physiology and biochemistry of the plant. Application of nutrients such as Mn, Cu, and B activates SAR mechanisms by releasing $Ca^{2+}$ cations from cell walls, which further interact with salicylic acid and activate defense mechanism in plants. B strongly inhibited spore germination, germ tube elongation, and mycelial spread of *Botrytis cinerea* in culture medium (Qin et al. 2010). The effects of copper (Cu) and boron (B) were evaluated by using the foliar applications on fungal diseases of rice and found that the application of Cu and B reduces fungal disease infestation in MR219 rice cultivar and also increases rice yield (Liew et al. 2012). Use of boric acid (1%) and jojoba oil (0.1%) treatment gave maximum reduction in mycelial growth of *Penicillium digitatum* and *P. itelicum* (blue and green rot of orange, respectively) as well as disease infection (Tarabih and El-Metwally 2014).

Iron (Fe) is an essential micronutrient required by most living organisms and pathogens (Kieu et al. 2012; Aznar et al. 2015). However, iron can catalyze the formation of deleterious reactive oxygen species, and hosts may use iron to increase local oxidative stress in defense responses against pathogens. Due to this duality, iron plays a complex role in plant-pathogen interactions. Plants' defense against pathogens and response to iron deficiency share several features, such as secretion of phenolic compounds, and use common hormone signaling pathways (Aznar et al. 2015). Several plant pathogens *Colletotrichum musae*, *Sphaeropsis malorum*, *Olpidium brassicae*, and *Fusarium oxysporum* have been suppressed by the application of iron (Graham 1983; Dordas 2008). Nevertheless, Fe has both positive and negative effects on the host and in host disease resistance. Graham (1983) observed foliar application of Fe can increase resistance of apple and pear to *Sphaeropsis malorum* and cabbage to *Olpidium brassicae*. In other cases, Fe in nutrient solution did not suppress take-all of wheat and *Colletotrichum* sp. in bean (Graham and Webb 1991). On contrary, Scher and Baker (1982) and Jones and Woltz (1970) suggested that reduced Fe availability through Fe competition could reduce wilt severity on tomato caused by *Fusarium* sp. Fe supply may have an indirect effect on the metabolic activity of the plant and also helps in production of antimicrobial compounds (Aznar et al. 2015). For example, Kieu et al. (2012) observed that symptom severity, bacterial fitness, and the expression of bacterial pectate lyase-encoding genes of two soft rot-causing plant pathogens (*Dickeya dadantii* and *Botrytis cinerea*) were reduced in iron-deficient plants.

In certain plant-fungus interactions, Fe is reported to enhance pathogen growth. Hendrix et al. (1969) reported that growth of *Phytophthora parasitica* var. *nicotianae* was enhanced by $Fe^{3+}$ when added to a synthetic glucose asparagine medium. In root-infecting fungi, the role of Fe is in *Pseudomonas*-mediated biological control (Scher and Baker 1982). Pseudomonads are adapted to produce iron-chelating agents called the siderophores in Fe-deficient soils which, in turn, suppress the growth of fungal pathogens by starving them for iron (Chet and Inbar 1994; Calvent et al. 2001). Siderophores are also involved in the synthesis of some volatile antibiotic compounds (Thomashow 1996). Depending on the host, the mechanism of defense activation by siderophores involves either their Fe scavenging property or receptor-mediated recognition as in the case of pattern-triggered immunity (Aznar and Dellagi 2015). Furthermore, the mechanisms used by plants to perceive local Fe depletion induced by siderophores and translate it into a defense response remain to be elucidated (Aznar et al. 2015) and are not only mechanisms to limit the growth of parasitic organisms (Dordas 2008).

Zinc (Zn) plays an important role in activating enzymes involved in various metabolic pathways, especially in protein and starch synthesis, and therefore, a low zinc concentration induces accumulation of amino acids and reduces sugars in plant tissue (Marschner 1995; Graham and McDonald 2001). Zinc is also involved in the maintenance of the integrity of biomembranes (Marschner 1995; Huber et al. 2012). Zn deficiency might lead to increased membrane leakage of low-molecular-weight compounds, the presence of which becomes more suitable feeding substrate for the pathogens (Graham and Webb 1991; Marschner 1995; Huber et al. 2012). For example, with Zn deficiency, leakage of sugars onto the leaf surface of *Hevea brasiliensis* increases the severity of infection with *Odium* (Bolle-Jones and Hilton 1956). On the other side, application of Zn had a positive effect on the tolerance of wheat to *Fusarium solani* root rot (Khoshgoftarmanesh et al. 2010). A balanced Zn application was found to increase the phenol contents of plant and to reduce the severity of rice sheath blight (Singh et al. 2010; Khaing et al. 2014). Addition of Zn to the soil reduced infection of crown root rot disease in wheat (Grewal et al. 1996; Singh et al. 2010; Khaing et al. 2014). Zinc is found to be most potential in reducing the disease severity caused by *Macrophomina phaseolina* (Pareek 1999). Wadhwa et al. (2014) found that soil application with Zn at 20 mg $kg^{-1}$, as soil-nutritive agent, played an important role in defense mechanism and provided resistance in cluster bean seedlings against *Rhizoctonia* root rot by enhancing the activity of

**Table 8.5** Effect of different levels of zinc on the incidence of root rot, specific activity of antioxidative enzymes in roots of cluster bean seedlings uninoculated (UI) and inoculated (I) with *Rhizoctonia* species at 15 days after sowing

| Treatments | Application rate (mg $kg^{-1}$ soil) | Disease incidence (%) | Polyphenol oxidase ($\times 10^2$, units[a]) | | Peroxidase ($\times 10^2$, units[a]) | |
|---|---|---|---|---|---|---|
| | | | UI | I | UI | I |
| Control | 0 | 68 | 2.81 | 5.38 | 10.17 | 25.52 |
| Zinc | 10 | 41 | 4.16 | 4.78 | 16.50 | 27.97 |
| Zinc | 20 | 27 | 4.26 | 6.87 | 29.54 | 34.73 |
| $SE_m$ | | 0.19 | 0.06 | 0.14 | 0.48 | 1.24 |
| $p = 0.05$ | | 0.55 | 0.16 | 0.41 | 1.36 | 3.46 |

Adopted from Wadhwa et al. (2014)

[a] 1 unit = change in 0.01 O.D. $min^{-1}$ $mg^{-1}$ protein

antioxidative enzymes, which helps in fungal invasion (Table 8.5). The results suggest that the addition of Zn plays an important role in disease tolerance.

Manganese (Mn) is the most studied micronutrient for its effect on diseases and its importance in the development of resistance in plants (Graham and Webb 1991; Huber and Graham 1999). Mn application can affect disease resistance, but the use of Mn is limited, which is mainly due to the ineffectiveness and poor residual effect of Mn fertilizers on most soils (Dordas 2008). Mn plays an important role in biosynthesis of lignin and phenol compounds (Graham and Webb 1991; Marschner 1995; Broadley et al. 2012). Due to such kind of roles, the capacity of roots to restrict penetration of fungal hyphae into the root tissue is impaired in Mn-deficient hosts (Graham and Webb 1991). Plants with proper Mn nutrition subdue the synthesis of aminopeptidase that renders essential amino acids required for fungal growth and a fungal enzyme, namely, pectin methylesterase, which breaks down the host cell walls.

Manganese is useful in controlling a number of pathogenic diseases (Huber and Graham 1999; Heckman et al. 2003). Mn is responsible for the lignifications of cell wall that serve as a barrier against take-all disease in bent grass (Carrow et al. 2001). Suppression of take-all disease by soil application of Mn fertilizers is possible under field conditions (Brennan 1992a), but foliar Mn sprays are not effective in suppression of root pathogens because of the poor phloem mobility of Mn (Huber et al. 2012). Cacique et al. (2012) reported that high Mn concentration on leaf tissues was found to decrease blast symptoms (*Pyricularia oryzae*) in rice. Heine et al. (2011) observed that Mn can also contribute to the control of black leaf mold disease (*Pseudocercospora fuligena)* in tomato. The authors concluded that Mn-induced activation of plant peroxidases in apoplast was responsible for this enhanced disease resistance. The micronutrients may control the diseases in the influence of each other as well. In a study reported by Abd El-Hai et al. (2007), the nutritional elements (ferrous, zinc, calcium. and manganese) were promising in controlling both rust and chocolate spot diseases in faba bean (*Vicia faba* L.).

Copper (Cu) is a component of many enzymes (polyphenol oxidase, diamine oxidase, etc.) important for the synthesis of lignin that imparts strength and rigidity

**Table 8.6** Stem melanosis (caused by *Pseudomonas cichorii*) in wheat grown on a soil with low Cu availability with and without different forms of Cu application

| Treatment | Cu rate (kg Cu $ha^{-1}$) | Disease (%) | Grain yield (kg $ha^{-1}$) |
|---|---|---|---|
| Control | – | 92 | 294 |
| $CuSO_4$, banded | 10 | 76 | 511 |
| $CuSO_4$, incorporated | 10 | 34 | 2016 |
| $CuSO_4$, foliar spray | 10 | 6 | 2116 |
| Cu-chelate, foliar spray | 2 | 7 | 2505 |

Adopted from Malhi et al. (1989)

of the cell wall (Marschner 1995; Broadley et al. 2012). Increased incidence of disease is observed due to reduced lignification in plants with low Cu. Copper deficiency also alters lipid structure in cell membranes that is essential for the resistance to biotic stress (Broadley et al. 2012). Stem melanosis, take-all root rot, and ergot infection can occur in Cu-deficient small grains (Marschner 1995). Copper fertilization had decreased the severity of a wide range of fungal and bacterial diseases associated by cell wall stability and lignification in plants (Marschner 1995; Broadley et al. 2012). The best evidence of an effect of Cu on host plant resistance to disease is when Cu is applied to soil and it depresses leaf infections, for example, powdery mildew in wheat and ergot (*Claviceps* sp.) in wheat (Evans et al. 2007), or the control of stem pathogens by foliar application of Cu (Table 8.6). Application of Cu compounds and their mixtures with addition of fungicides like mancozeb has been found to reduce the severe canker symptoms and fruit spotting in North Carolina (Shoemaker 1992). In various studies, Cu compounds and their different combinations were found to decrease diseases like sheath blight (*Rhizoctonia solani* Kuhn.) in rice (Khaing et al. 2014) and bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) in tomato (Bastas 2014).

### 8.2.7 Other Nutrients

Although silicon (Si) is not considered as an essential nutrient for plants, it stands out for its potential to decrease disease intensity in many crops belonging to the families Poaceae, Equisetaceae, and Cyperaceae (Marschner 1995; Huber et al. 2012; Pozza et al. 2015). Grasses, in general, and rice, in particular, are Si accumulator plants. As Si is translocated in the xylem favorably to mature leaves, the blast infection in rice occurs mainly in young leaves; the increased Si supply strongly reduces the number of lesions on young leaves, indicating the increase of the resistance to the disease, particularly at high N supply (Osuna-Canizales et al. 1991). The mode of action on the control of plant diseases and its function in several pathosystems are not yet fully understood.

However, there is evidence that the silicates induce the host defense responses that are involved in strengthening of the cell wall structures via increased lignification or activation of phytoalexin production and pathogenesis-related protein synthesis (Fawe et al. 2001, Oliveria et al. 2012). Si accumulated mainly in epidermal cells and exclusively on endodermal cells in roots, creating a physical barrier to penetration of the roots by fungal hyphae (Najihah et al. 2015). Si is rapidly deposited around the infected area (Heath and Stumpf 1986). In wheat and barley, the Si accumulates at the sites of hyphal penetration (Leusch and Buchenauer 1988) within 20 h, and this accumulation is 3–4 times higher around unsuccessful infection sites than around successful ones (Carver et al. 1987). A continuous supply of Si is required for the accumulation of Si at the point of pathogen penetration from the roots (Samuels et al. 1991). Although the Si accumulation at the sites of penetration causes inhibition of hyphal invasion and formation of haustoria in plant cells, the protective effect is not due to Si alone. It was further reported that the presence of soluble Si appears to facilitate the rapid deposition of phenolics at the sites of infection, which is a general defense mechanism to pathogen attack (Menzies et al. 1991). However, the mechanism by which Si induces accumulation of phenolics at the infection sites remains unclear (Huber et al. 2012). Si application also increases the activity of antioxidative enzymes involved in plant defense such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase (Shetty et al. 2011; Polanco et al. 2012), which are considered as chemical barriers to pathogen entry in host plant (Pozza et al. 2015).

It has been suggested that Si fertilization could be integrated as a sustainable and environment friendly practice for the management of fungal brown spot (*Bipolaris oryzae*) disease in rice (Ning et al. 2014). Increased brown spot resistance in response to silicon fertilization was observed to be less in knockout mutant compared to its wild-type counterpart cultivar Oochikara (Dallagnol et al. 2014). Si content in rice straw and husks was proportional to the amount of Si applied to the soil, and the severity of blast on panicles was inversely proportional to the amount of the Si in rice tissues (Kawashima, 1927).

Grain discoloration, caused by a complex of fungal species such as *Bipolaris oryzae*, *Curvularia* sp., *Phoma* sp., *Microdochium* sp., *Nigrospora* sp., and *Fusarium* sp., is another important constraint for irrigated and upland rice production worldwide. Prabhu et al. (2001) showed that the severity of grain discoloration in several irrigated and upland rice genotypes decreased linearly as the rates of $SiO_2$ in the soil increased. Chang et al. (2002) reported a significant reduction in lesion length of bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) of 5 to 22% among four rice cultivars following Si application. The reduction in lesion length was positively correlated with a decrease in the content of soluble sugar in leaves of plants amended with Si. A recent study (Khaing et al. 2014) showed that Si fertilization was significantly more effective than Cu and Zn treatments in minimizing rice sheath blight (*Rhizoctonia solani* Kuhn.) severity and yield loss (Table 8.7). Reduced severity in rice sheath blight disease was attributed to the increased lignin content and enhanced activities of antioxidative enzymes in rice leaves with Si addition. Silicon, in the form of silicic acid, acts locally by inducing defense reactions in elicited cells and also contributes to systemic resistance by enhancing the production of stress hormones (Alexander et al. 2010). Cacique et al. (2012) observed that addition of Si to the nutrient solution medium significantly reduced the lesion size and area under blast progress curve caused by blast (*Pyricularia oryzae* Cooke) in rice.

**Table 8.7** Effect of sheath blight inoculation-fertilizer treatment combinations on sheath blight incidence, highest relative lesion height (HRLH), and rice grain yield

| Inoculation | Treatment | Incidence (%) | HRLH (%) | Grain yield (g pot$^{-1}$) |
|---|---|---|---|---|
| Non-inoculated | Non-treated | 8.3 | 3.2 | 32.9 |
| Inoculated | Non-treated | 51.4 | 40.0 | 22.2 |
| Inoculated | Si | 39.2 | 30.5 | 39.5 |
| Inoculated | Cu | 42.0 | 32.2 | 31.9 |
| Inoculated | Zn | 44.5 | 33.9 | 31.1 |

Adopted from Khaing et al. (2014)

Therefore, Si application can contribute in the management of plant diseases, among other practices. The potential of Si as a crop protectant has been investigated and found promising against several patho-systems such as Asian soybean rust, tomato bacterial wilt, melon bacterial blotch, passion fruit bacterial spot, and wheat bacterial streak (Brancaglione et al. 2009; Ferreira 2009; Lima et al. 2010; Silva et al. 2010). Resende et al. (2013) reported that sorghum grown in nutrient solution with Si (2 mmol L$^{-1}$) had severity of anthracnose (*Colletotrichum sublineolum*) around 20%, while the control was 93%, at 10 days after inoculation. There are also reports of reduction of disease severity in sugarcane rust (*Puccinia melanocephala*) (Naidoo et al. 2009) and control of *Blumeria graminis* f. sp. *tritici* on wheat (Belanger et al. 2003) and *Erysiphe graminis* in barley (Carver et al. 1987) with Si application. Cucumber plants inoculated with *S. fuliginea* and grown in nutrient solution supplemented with sodium silicate showed a reduction in spore germination and the number and area of colonies per leaf compared with the control plants without Si (Menzies et al. 1991). Plants in nutrient solution supplemented with Si (2 mmol L$^{-1}$) had higher incubation period compared with plants without Si (Polanco et al. 2012). Therefore, knowing its effects in disease reduction, it can be included in disease management plans, not as the only method able to solve disease-related problems but as an important component of the integrated management of diseases, that is, it can contribute.

## 8.3 Control of Diseases Through Sustainable Nutrient Management

Disease suppression or management through nutrient manipulation has been reported by many investigators, which were achieved by either modifying the nutrient availability or modifying nutrient uptake (Huber and Graham 1999). Fertilizer application affects the development of plant diseases under field conditions directly through the nutritional status of the plant and indirectly by affecting the conditions which can influence the development of the disease such as dense stands, changes in light interception, and humidity within the crop stand (Dordas 2008). It is a general assumption that balanced nutrition leads to a healthy plant, which reduces the disease susceptibility and infection. Thus, it is important to provide a balanced nutrition at the time when the nutrient can be most effectively used for disease control.

Not only fertilization can affect the disease development but also any management practice that affects the soil environment such as pH modification through liming or gypsum application, tillage, seedbed firmness, site for nursery, moisture control through irrigation, and manures. For example, liming does not directly affect clubroot of crucifers, but at pH > 7.0, germination of clubroot spores is inhibited. Alternatively, liming of soils to near-neutral pH increases the incidence of diseases such as potato scab and take-all infection in wheat (Havlin et al. 2009). Thus, the incidence of potato scab (caused by *Streptomyces scabies*) could be suppressed either by lowering soil pH or by application of Mn (Thompson and Huber 2007). The suppressive effect on Mn is perhaps due to (1) increased resistance of the tuber tissue to the pathogen and (2) inhibition of the vegetative growth of *S. scabies* before the onset of infection.

The root rot disease of wheat and barley (take-all) caused by *Gaeumannomyces graminis* (take-all) is capable of seriously limiting grain production in many regions of the world, but disease severity can be effectively controlled by nutrition of the host plant (Graham and Webb 1991; Thompson and Huber 2007). The fungus has a growth optimum at pH 7 and is very sensitive to low pH (Römheld 1990); liming of acid soils therefore increases the risk of root infections and yield losses by take-all. Mn availability in the rhizosphere and Mn concentration of root tissues play a key role in root infection and severity of take-all, as well as other soilborne fungal diseases (Graham and Webb 1991; Thompson and Huber 2007). The severity of take-all in wheat is increased not only by Mn deficiency but also by deficiency of N, P, or Cu (Brennan 1992b). The decrease in severity with application of N (especially of $NH_4^+$) and P fertilizer to deficient plants is most likely due to a greater tolerance by more vigorous growth rather than an increase in physiological resistance (Huber et al. 2012).

Botrytis fruit rot (gray mold), caused by *Botrytis cinerea*, is the most devastating disease of strawberry, which causes great losses and assumes serious concern with the increasing environmental temperature (Singh et al. 2007; Fernandez-Ortuno et al. 2014). Increasing the Ca and B content in the cell wall of fruit tissue can help to delay tissue softening (by affecting phenolics and lignin synthesis) and mold growth (by suppressing penetration of fungal hyphae) and thus can decrease the gray mold incidence and other physiological disorders (Table 8.8). This study suggested that a combined foliar spray of Ca + B was more effective in reducing incidence of gray mold, albinism, fruit malformation, and higher fruit yield and quality parameters than either with Ca or B alone. Therefore, in other words, the conjoint use of nutrients could serve better than nutrients alone, with respect to disease suppression.

*Verticillium* wilt, caused by *Verticillium albo-atrum* and *V. dahlia*, is one of the most devastating diseases of vegetables, ornamentals, fruits, herbs, field, and forage crops. *Verticillium* wilt can be controlled by resistant cultivars, careful crop rotation, sanitation, soil fumigation, and sufficient nutrient additions such as N, P, and K which have the potential to reduce the disease (Huber and Graham 1999). Methods like soil fumigation and nitrification inhibitors maintain $NH_4^+$ in the soil; increase

**Table 8.8** Effect of foliar application of Ca and/or B on quality parameters, physiological disorders, gray mold, and fruit yield of strawberry

| Treatment | TSS (%) | Acidity (%) | Albinism incidence (%) | Fruit malformation (%) | Gray mold (%) | Fruit yield (g plant$^{-1}$) |
|---|---|---|---|---|---|---|
| Control | 7.8 b | 0.99 a | 15.1 a | 12.4 a | 5.2 a | 149.3 a |
| Ca spray[a] | 7.1 a | 1.15 b | 6.7 b | 10.9 a | 1.3 b | 168.4 c |
| B spray[b] | 7.7 b | 1.02 a | 14.8 a | 3.4 b | 4.8 a | 161.3 b |
| Ca + B spray | 7.2 a | 1.12 b | 6.5 b | 3.1 b | 1.2 b | 179.2 d |

Adopted from Singh et al. (2007)

Means within the column with the same letter are not significantly different by Duncan's multiple range test at $p \leq 0.05$

[a] As $CaCl_2$ at 2.0 kg Ca ha$^{-1}$ spray$^{-1}$

[b] As boric acid at 150 g B ha$^{-1}$ spray$^{-1}$

Mn, Cu, and Zn; and reduce *Verticillium* wilt in tomato. *Fusarium* wilt (*Fusarium oxysporum*) is an important disease in crops covering different vegetables and fruits which is favored by warmer climate and acidic soil reaction. Application of $NO_3^-$ fertilizers and lime, which reduces the availability of Mn and Fe and increases soil pH, results in the reduction of the pathogen (Dordas 2008).

Manipulating the various interactions of the host, pathogen, and environment over time can also influence most diseases through (1) the level of genetic resistance of the plant (systemic induced or acquired resistance); (2) the nutrient availability relative to plant needs (deficiency, sufficiency, or excess); (3) the predominant form and biological stability of a nutrient that is applied or available (oxidized or reduced); (4) the rate, time, and method of nutrient application; (v) the nutrient balance and associated ions in plants; and (6) the integration of fertilization with other crop production practices (crop rotation, intercropping, manuring, tillage, etc.) (Huber and Haneklaus 2007). For example, higher bacterial blight disease (caused by *Xanthomonas axonopodis* pv. *punicae*) severity in pomegranate plants grown on high pH soil was mainly associated with lower concentration of Mg, Ca, Mn, and Cu and higher concentration of N in leaves (Maity et al. 2016). In another case, the take-all infection of spring wheat was high without N fertilization and was further increased by application of ammonium in the autumn, leading to yield depressions because of increased disease severity (Table 8.9). In contrast, the same amount of ammonium N sprayed in spring season suppresses take-all, and high grain yields were obtained. Ammonium N applied in the autumn is rapidly nitrified and nitrate intensifies take-all in non-suppressive soils. The use of timed ammonium fertilizer application is therefore a practical approach to suppress take-all, and variations in suppression between years and locations (Christensen et al. 1987) are probably related to the rate of nitrification prior to N uptake by the crop. Therefore, various effects of nutritional status and of fertilizer application on diseases are of direct relevance to disease and pest control by pesticides and other chemicals. Fertilizer application may substitute, or at least reduce, the demand for chemical disease control in some cases but may increase the demand in others (Huber et al. 2012).

**Table 8.9** Take-all (*Gaeumannomyces graminis*) root infection and grain yield of winter wheat at different times and rates of ammonium N fertilizer application

| Time of application | Rate of application (kg N ha$^{-1}$) | Take-all infection (%) | Grain yield (kg ha$^{-1}$) |
|---|---|---|---|
| 0 | 0 | 1.9 | 2610 |
| Autumn | 83 | 2.8 | 1740 |
| Spring | 83 | 0.1 | 5290 |
| Autumn + Spring | 83 + 28 | 1.9 | 2350 |

Adopted from Huber (1989)

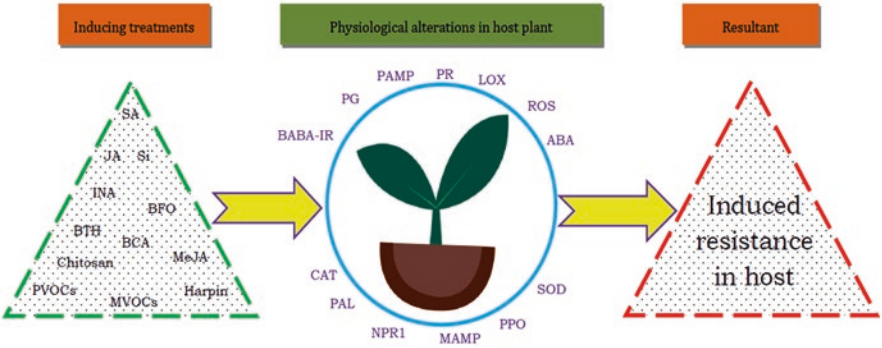

**Fig. 8.3** Treatments that can induce resistance in host plants and the mechanisms or enzymes involved. *SA* salicylic acid, *JA* jasmonic acid, *Si* silicon, *INA* 2,6-dichloronicotinic acid, *BFO* Burdock fructooligosaccharide, *BTH* benzothiadiazole, *BCA* biocontrol agents, *MeJa* methyl jasmonate, *MVOCs* microbial volatile organic compounds, *PVOCs* plant volatile organic compounds, *PG* polygalacturonase, *BABA* β-aminobutyric acid, *PR* pathogenesis-related proteins, *MAMP* microbe-associated molecular pattern, *LOX* lipoxygenase, *ROS* reactive oxygen species, *CAT* catalase, *ABA* abscisic acid, *PPO* polyphenol oxidase, *PAL* phenylalanine ammonia lyase, *PAMP* pathogen-associated molecular pattern, *SOD* superoxide dismutase, *NPR1* non-expressor of pathogenesis-related genes 1 (Plant image source http://hostted.com/plants-clip-art)

## 8.4 Systemic Induced or Acquired Resistance

Various biotic inducers (e.g., fungi, bacteria, viruses, phytoplasma, insects, etc.) and abiotic stresses (e.g., chemical and physical inducers) can trigger resistance in plants, which is known as “induced resistance” (Pieterse et al. 2014). These can produce rapid expression of defense responses (Fu and Dong 2013). Induced resistance, produced by an array of treatments that elicit a cloud of defense responses (Fig. 8.3), is of two types in plants: systemic acquired resistance (SAR) and systemic induced resistance (SIR). Both of these mechanisms can induce defenses that confer long-lasting protection against a broad spectrum of microorganisms and are mediated by phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). SAR requires the signal molecule SA and is associated with accumulation of pathogenesis-related (PR) proteins, which are believed to contribute to resistance (Durrant and Dong 2004). Instead, the SIR pathway functions independently of SA, while it is dependent on JA and ET (Van Wees et al. 1999). This induced resistance does not directly activate plant defense responses but activates the plant to a state of "alertness," so that a future pathogen attack will be strongly and efficiently recognized and responded to. This phenomenon is also known as the "priming effect," and one of the most known priming effects is root colonization by plant-growth-promoting rhizobacteria (PGPR), which induce plant development and SIR-mediated resistance (Verhage et al. 2010).

Systemic acquired resistance (SAR) is the most adapted response of plants to infection by pathogens. The concept of SAR has been widely recognized and studied for the past 100 years in relation to increasing resistance to fungal, bacterial, and viral pathogens of economically important crop plants. SAR occurs in many plant species and is effective against a broad range of pathogens, and it can last for several weeks to months after its induction. SAR is associated with the coordinate expression of a suite of genes (Ward et al. 1991), some of which confer resistance to specific pathogens when the genes are individually and constitutively expressed in transgenic plant (Alexander et al. 1993). SAR and microbial biological control are examples of emerging alternatives for disease management that are of interest because of the current focus on making farming systems more environmentally safe. Such alternatives can be expected to be used in combinations and as a result may interact in unexpected or unpredicted ways. Therefore, ecological research is needed to understand whether and how disease management methods interact or interfere. Such research also offers new opportunities to increase our understanding of plant and microbial ecology at the molecular level. The term chemically induced resistance introduced by Wiese et al. (2003) is used to describe the systemic resistance after application of synthetic compounds. This resistance is due to the formation of structural barriers such as lignification, induction of pathogenesis-related proteins, and conditioning of the plants (Graham and Webb 1991). Nearly nine gene families were induced in uninfected leaves of inoculated plants; these gene families are now known as SAR genes (Ward et al. 1991).

Several of these SAR gene products have direct antimicrobial activity or are closely related to classes of antimicrobial proteins. These include β-1,3-glucanases, chitinases, cysteine-rich proteins related to thaumatin, β-l,3-glucanase, and PR-1 proteins (Anfoka and Buchenauer 1997). The characterization of pathogenesis-related proteins (PRs) greatly helped to reveal the induced proteins involved in the regulation of $Ca^{2+}$ on SA-induced resistance to *Botrytis cinerea*.

Linlin et al. (2016) have found that the combination treatment of $CaCl_2$ and SA, the defense response, and antioxidative protein were clearly upregulated much more than SA alone or the control treatment by the method of proteomics and real-time PCR. They have suggested that susceptible tomato cultivars treated by the combination treatment of $CaCl_2$ and SA might possess a more sensitive SA signaling system or effective pathway than SA treatment alone. In addition, their results indicated that SA could coordinate other cellular activities linked with photosynthesis and metabolism to facilitate defense response and recovery, indicating that the self-defense capability of tomato was improved by the combination treatment of $CaCl_2$ and SA.

Systemic induced resistance (SIR) is often reported to be induced by foliar applications of many essential elements like nitrogen, potassium, and phosphorus. Due to SIR, an immunity signal has been found to be synthesized at the elicitation site of the induced leaves. Further, the signal is systemically transferred to the affected leaves, where it is assumed to activate the defense mechanisms that put a barrier to the pathogen attack. Many researchers indicated that salicylic acid (SA) could be the possible immunity signal synthesized in the induced leaves; moreover, its foliar spray induces host resistance and can trigger the production of PR proteins, which typically accompany SIR (Dordas 2008). Therefore, stimulation of SIR with application of nutrients/SA could become an indispensable component of disease reduction strategies in agricultural production systems. For example, very high levels of systemic resistance against powdery mildew (*Sphaerotheca fuliginea*) can be induced by a single foliar spray of phosphate in cucumbers. Similarly, foliar spray with phosphate induced systemic resistance against common (*Puccinia sorghi*) and northern leaf blight (*Exserohilum turcicum*) in maize (Dordas 2008).

Micronutrients inducing systemic resistance may also play an important role in the host plant susceptibility to fungal or bacterial infection. Foliar application of B as $H_3BO_3$, Cu as $CuSO_4.5H_2O$, Mn as $MnCl_2$, or $KMnO_4$ has been found to impart systemic protection against powdery mildew (*Sphaerotheca fuliginea*) in cucumbers. Similarly, Simoglou and Dordas (2006) found that foliar spray of Mn, Zn, and B separately induced the systemic protection of plants to tan spot disease in wheat. Foliar application of trace elements like B, Cu, and Mn synergistically interacts with $Ca^{2+}$ and can increase its concentrations in plants, which further interacts with SA and can trigger the activation of SIR in the host plants (Dordas 2008). These reports indicate that the inherent resistance of the host plants could be induced by foliar spray with simple inorganic chemicals, and this induced resistance against the virulent pathogens is not pathogen specific.

Further, many commercially usable products such as acibenzolar-S-methyl, better known as “Actigard,” are available that trigger the same systemic resistance as much as SAR in plants. Till date, in addition to SA, derivatives of isonicotinic acid (INA) and benzothiazoles (BTH) have also been used to induce SAR in plants against a wide range of pathogens. However, BTH is reported to be used as commercially. When these three compounds, namely, Actigard, INA, and BTH, were applied separately to reduce powdery mildew in barley, these compounds induced systematic resistance in barley by inducing expression of a number of defense response genes, including the genes encoding $Ca^{2+}$-binding protein, fatty acid desaturase, acid phosphatase, lipoxygenase, serine proteinase inhibitor, thionin, and several other proteins whose specific functions in disease resistance have not been assessed yet. Out of these chemicals, the latter two were reported to be better inducers of gene expression and subsequent disease resistance in plants. Apart from these chemical compounds, many fungicides such as triazoles, fosetyl-Al, and metalaxyl reported to have shown resistance-inducing activity to some extent. Probenazole, a fungi-bactericide, is little toxic in vitro but is reported to induce various defense responses in submerged paddy. These defense responses include the appearance of reactive oxygen species (ROS) due to an oxidative burst and significant accumulation of fungitoxic factors such as unsaturated fatty acids. Likewise, several other compounds and many microbes have also been tried for their potentiality to induce SIR and/or SAR in field crops, but so far, their significant efficacy has not been proved (Agrios 2005).

## 8.5 Disease Resistance Through Sustainable Practices

### 8.5.1 Soil Organic Matter/Amendments

Soil health and quality, as affected by soil organic matter (SOM) content, is a major component of raising holistic and preventive approach to sustainable agricultural ecosystems. SOM is an important aspect of fertility in soils, sustainable agricultural production systems, crop yield, and productivity, and there is increased concern about the declining levels of SOM in many soils around the world, with its potent relation to global warming. Long-term experimental results from the Rothamsted (since 1843) render the most farsighted data sets on the effect of soils, crops/cropping systems, manuring, and other field management practices on changes in SOM contents, under temperate climate (Johnston et al. 2008). The contents of SOM depend primarily on the quantity and quality of organic inputs, its rate of decomposition, C/N ratio, existing microbial communities, clay content, and climate. Organic residues such as crop residues, green manures, cover crops, and other organic wastes can affect the soilborne pathogens and ability to infect the hosts, and their field application mostly affects the availability of nutrients to the crops (Stone et al. 2004).

Hu et al. (1997) observed that addition of sphagnum peat to soil was able to inhibit the disease caused by *Pythium* spp. Similarly, *Phytophthora* root rot was also reported to be suppressed by addition of various organic amendments in a number of species (Spencer and Benson 1982; Szczech et al. 1993; Hu et al. 1997). Soil amendment with paper mill residue (PMR), in a composted form, suppressed the symptoms of bacterial speck (*Pseudomonas syringae* pv. *tomato*) in tomato plants compared with plants grown in fields without composted PMR or non-amended soils (Vallad et al. 2003). Further, the authors attributed the enhanced resistant to the disease mainly due to systematic activation of plant defenses, comparable to SAR. Wu et al. (2009) experimented with a biofertilizer, which is composed of the microorganisms *Paenibacillus polymyxa* and *Trichoderma harzianum* for the control of *Fusarium* wilt in watermelon. The microbial combination was able to reduce the infection of *Fusarium* wilt, which was induced due to the increase in the defense-related enzyme activities like peroxidase, catalase, SOD, and β-1,3-glucanase in the affected leaves of watermelon. Recent study reports have shown that biofertilizer application may lead, with time, to reduction of banana *Fusarium* wilt disease caused by *F. oxysporum* f. sp. *cubense* (Fu et al. 2017) and point to the sustainability of biofertilizer, as a soil amendment.

Organic amendments encompass a wide range of products, from crop residues and wastes and animal manures to solid wastes and various rural/urban composts. Till date, most of the research has often been concluded that addition of organic amendments/wastes to fields has a beneficial effect on the disease suppression. Composts or manures have been successfully applied for the enhancement in soil health and quality, crop yield/productivity, nutrient and SOM contents, plant growth, and also the suppression of diseases caused by soilborne pathogens (Mays and Giordano 1989; Janvier et al. 2007; Mehta et al. 2013). Lewis et al. (1992) found that 3–4 years of compost treatment improved cotton stand and also significantly reduced the inoculum population of *Rhizoctonia solani* in soil. Szczech (1999) also reported that application of vermicompost to a conductive pot culture resulted in suppression of *Fusarium* wilt (*F. oxysporum*) in tomato. Compost-based suppression of germination of *S. rolfsii* sclerotia was studied by Danon et al. (2007). Suppressive germination of the sclerotia on plates was detected, when an intermix of sewage sludge and yard waste (mature biosolids compost) was used as a medium. This intermix was also found to suppress the disease development in bean (*Phaseolus vulgaris* L.) plants. *Fusarium*, a soilborne pathogenic fungus, is the causal organism for common root rot, stem rot, and wilt diseases in many field/vegetable crops, and there are several reports available on compost-based suppression of wilt caused by this fungus (Reuveni et al. 2002; Postma et al. 2003). In these studies, suppression of pathogens by the application of compost was reported to be about 20–90%, and enhanced beneficial microbial activities in soil played the major role in suppression. The critical role of organic wastes/residues is that they are rich source of energy for the soil microorganisms due to the presence of easily assimilable C substrates, and moreover, these can contain microorganisms that are antagonistic to the soil pathogens (Janvier et al. 2007).

In addition, recent advances have indicated that anaerobic soil disinfestation (ASD) could serve as a better approach for suppression of the soilborne diseases in relation to organic amendments. ASD is based on the fact that application of organic residues/composts, or other labile sources of C in conjunction with irrigation, and followed by covering of the surface soil with polythene mulch, can create microbial-mediated anaerobic soil environments, which could suppress the soilborne pathogens and even the weeds due to anoxia (Momma et al. 2013). To be precise, ASD is developed based on the experience that submerged paddy-arable cropping system was found to suppress soilborne pathogens at a greater extent compared to arable cropping systems. In addition to anoxia, the suppression in the inoculums of fungal pathogens by ASD has also been ascribed to many changes in the soil microenvironment including high temperature, synthesis of fungitoxic organic acids (acetic acid, *n*-butyric acid) and volatile organic compounds, and increased solubility and availability of trace metals like $Fe^{2+}$ (Momma and Kobara 2012; Momma et al. 2013). For example, ASD in conjunction with 1% ethanol (C source) was found to strongly suppress tomato *Fusarium* wilt in Japan (Momma et al. 2010).

### 8.5.2 Intercropping

The holistic approach of practices to control plant diseases is inclined to subdue pesticides consumption, and intercropping is, by far, one of such practices. Intercropping is a practice of growing two or more crops within a field in order to derive the benefits of unutilized inter-row spaces, complementation, and competition among the crops (Boudreau, 1993). The most potent goal of this practice is to harness a greater yield from a selected piece of field by maximizing the potential use of resources that is normally not utilized by a single field crop, otherwise. Prior to intercropping, proper planning is required by taking into account of the soil conditions, climatic parameters, type of crops, varieties, and compatibility. Special emphasis is always given on growing crops that do not compete with each other for solar radiation, space, nutrient accession, and water. Therefore, the most common intercropping strategies adopted include either growing deep-rooted crops with shallow-rooted crops or planting crops together which are different with respect to their heights. The success of intercropping primarily depends on understanding the physiology of the crops to be planted together, their growth patterns, canopy and root architecture, nutrient and water requirement, and the extent of utilization (Gómez-Rodríguez et al. 2003). In general, plants used to compete for sunlight in phyllosphere, while for nutrients and moisture in rhizosphere; therefore, competition involves a combination of radiation and edaphic factors in time and space. Further, different crop species struggle for various resources at different times in their life cycle, and, for example, the competition might end up in shade issue that begun with nutrient competition among the crops.

Intercropping leads to the efficient utilization of resources over monoculture and thus usually attracts more resources. This cuts down the accessibility of worthy resources like water and nutrients to the weeds, and the resulting underutilization of resources is declined (Zimdahl 1993). Numerous theoretical and experimental studies demonstrated the effectiveness of intercropping in disease management (Bouws and Finckh 2008). For example, a series of experiment indicated that intercropping was able to reduce the incidence of pests and diseases by 53%, while some of the studies reported increment in them by 18% (Francis 1989). The possible causes for this increase in pests and diseases are attributed to the reduced tillage operations and light penetration and increased humidity, generating a microclimate favorable for pests and pathogens and their associated alternate hosts. Further, the crop residues remaining on the fields during zero tillage practice could serve as a hibernating place for the pathogen inoculums in the absence of appropriate hosts. However, at the same time, intercropping resulted in the enhancement of nutrient availability such as increasing N from legumes or may increase the uptake of many nutrients like K and P (Anil et al. 1998).

Intercropping watermelon with upland rice suppressed *Fusarium* wilt in rice (Ren et al. 2008). They argued that the differences in the compounds, released as root exudates between watermelon and aerobic rice, are responsible for *Fusarium* wilt suppression in the aerobically grown rice. In addition to the differences in the contents of sugars and amino acids in the root exudates of rice and watermelon,

**Table 8.10** Effect of rice monoculture and rice-water chestnut intercropping systems on sheath blight and blast diseases in rice. Values are means ± SE

| Disease parameter | Disease | Rice monocropping | Rice-water chestnut intercropping |
|---|---|---|---|
| Growth rate (%) | Sheath blight | 64.81 ± 0.96 | 25.39 ± 0.72 |
| | Rice blast | 62.54 ± 0.58 | 39.26 ± 0.23 |
| Lesion length (cm) | Sheath blight | 4.36 ± 0.32 | 2.06 ± 0.37 |
| | Rice blast | 6.76 ± 1.46 | 5.28 ± 0.74 |
| Lesion width (cm) | Sheath blight | 0.89 ± 0.13 | 0.53 ± 0.02 |
| | Rice blast | 0.63 ± 0.03 | 0.51 ± 0.05 |
| Disease level[a] | Sheath blight | 1.15 ± 0.19 | 1.00 ± 0.17 |
| | Rice blast | 5.94 ± 0.15 | 5.25 ± 0.18 |
| Disease index[b] | Sheath blight | 20.53 ± 0.65 | 6.04 ± 1.21 |
| | Rice blast | 54.07 ± 0.30 | 33.35 ± 0.61 |

Adopted from Qin et al. (2013)

[a] Disease level rated visually using a 0–9 scale, where 0 = no lesion and 9 = lesions covering the whole rice leaves

[b] Disease index <4 moderately resistant, 5–6 moderately susceptible, and 7–9 highly susceptible

*p*-coumaric acid was only detected in root extracts of upland rice. Later, Hao et al. (2010) observed that exogenous application of *p*-coumaric acid was able to decline sporulation and spore germination with increasing concentrations over the non-treated spores.

A likewise observation was also recorded when water chestnut was intercropped with paddy in relation to the suppression of rice sheath blight and blast (Qin et al. 2013), which is shown in Table 8.10. They detected that antifungal activity of water chestnut extracts and root exudates played a critical role in restricting the expansion of fungal lesions. Nevertheless, the extent of these inhibitions was found to be limited by time and accumulation of root exudates. Thus, paddy-water chestnut intercropping system could be a potent environmentally sound and sustainable practice for the control of these diseases in submerged rice field. Moghaddam et al. (2014) reported that intercropping of fenugreek in cumin had positive effect on *Fusarium* wilt disease control because of the physical barrier established by the fenugreek. On contrary, the incidence or severity of *Fusarium* wilt remained unaffected, when tomato intercropped either with cucumber, leek, or basil, which is ascribed to the absence of allelopathic activities between these vegetables (Hage-Ahmed et al. 2013).

### 8.5.3 Crop Rotation

Crop rotation refers to the practice of growing different type of crops in a piece of land in successive seasons in a calendar year. This practice particularly helps in increasing soil fertility and crop production, maintaining the soil health, and reducing nutrient depletion and soil erosion. Growing any particular crop in a piece of land for successive years (i.e., monocropping) disproportionately exhausts the soil fertility and nutrient availability.

With rotation, a crop that leaches the soil of one kind of nutrient is followed during the next growing season by a different crop that returns that nutrient to the soil or uptakes a balanced ratio of soil nutrients. Further, it eliminates the chances of accumulation of soil borne-pathogen inoculums and pests, unlike what is observed in monocropping. Moreover, differences in the biomass from varied root structures of the crops in a crop rotation may also result in better soil structure and its stability against the erosive forces. Rotating crops allows soil to "rest," that is, to replenish its vital nutrients, microbial activity, and other important components. This practice can increase the availability of N in soil when rotated with legumes and, subsequently, can affect other nutrient availability, which can also affect the severity of many disease incidences (Huber and Graham 1999; Reid et al. 2001).

Research findings indicate that Mn availability is strongly affected by crop rotation, and Graham and Webb (1991) reported that crop rotation with lupins increased soil Mn availability. Cerkauskas (2005) observed that growing paddy followed by tomato can suppress *Fusarium* wilt in tomato. The broccoli residues are reported to have suppressive effect on soilborne pathogens, and effective suppression of strawberry *Fusarium* wilt (*Fusarium oxysporum* f. sp. *fragariae*) can be achieved when rotated with broccoli, which may serve as non-host to the fungi (Njoroge et al. 2008). Pathogenic inoculums of *Fusarium* wilt (*Fusarium oxysporum* f. sp. *niveum*) in a long-term watermelon monocropping and its subsequent disinfection in soil by land fallowing were studied by Wu et al. (2013). The authors investigated the fields with a continuous 5 years of watermelon production with subsequent 3 years of land fallowing. The results of this study indicated a sharp reduction in culturable fungal pathogen recovered from soil by about 20, 40, and 50% in the first, second, and third years of land fallowing. Surprisingly, fungal inoculums of other *Fusarium* species like *F. merismoides* and *F. fusariodes* also reduced. However, bacterial communities increased as a proportion of the gross microbial population as the period of land fallowing increased.

Crop rotation is likely to affect the incidence and severity of soilborne diseases by increasing the soil buffering capacity, restraining the virulent pathogen from the access to host plant by growing of a non-host crop species, and affecting the contents of inorganic N forms by influencing the rate of nitrification in soil (Graham and Webb 1991; Huber and Graham 1999). Rotation of field out of the host crops can be a strategic and relatively cost-effective approach for management of many diseases in field crops. Crop rotation without hosts is a well-tested strategy of integrated pest management (IPM) that is indispensable for suppression of many soilborne diseases (Fang et al. 2012; Koike and Gordon 2015). To be precise, the success of crop rotation for managing diseases lies within the understanding of the epidemiology of the pathogens. In general, crop rotation is aimed for managing diseases by growing non-host crop species until the eradication of pathogens from soil, or its inoculums are declined to such extent, which can cause least yield reduction. Successful management of diseases with crop rotation mainly depends on the knowledge of the following factors: (1) the associated plants (weeds and other crops) serving as alternate hosts that it can hibernate up on, (2) the persistence pathogen in the absence of hosts, (3) how the pathogen can be reintroduced into a land, (4) other ways of survival between the susceptible host crop species, and (5) management of other pathogen sources (Davis and Nunez 1999; Smith and McSorley 2000). For example, windblown airborne pathogens, which can also survive in soil, may not be effectively controlled by crop rotation, if infected plants occur in the nearby areas, with possible chances of spore dispersal to the cultivated crop species.

## 8.6 Concluding Remarks and Future Perspective

It is essential to fulfill the need of ~9 billion people, the estimated global population by 2050. The demand has to be supplied; however, system and method must be adopted for sustainable agricultural production systems. This can be achieved by developing high-yielding crops, pathogen-resistant varieties, and biofortified crops with minimum addition of synthetic fertilizers and nutrients, thus maintaining the integrity of the ecosystem.

Disease resistance of any plant is mainly genetically controlled but has a close association with the nutritional status of the plants or pathogens; and thus, nutrient management has always been an important regulator for plant diseases. There is a dynamic interrelation between the nutritional status of plants with pathogen and abiotic environment, and hence, proper management of nutrients in cultivated crops can effectively decline the severity of most diseases. Further, with nutrient management, the decrease in the severity of diseases is more pronounced, when the crops are undernourished. The morphological or histological characteristics of the host plants are also governed by their nutritional status, which, in turn, regulates the pathogen entry, its penetration, and spreading of infection to the unaffected plant parts. Healthy plants with optimum nutrition can suppress diseases to a permissible level or to a level which can further be controlled by pesticides or other conventional practices that are more successful, cost-effective, and environment friendly. Nevertheless, knowing the effects of plant nutrients in disease reduction, it should be included in disease management plans, strictly not as the only method but as an integral aspect of IPM practices.

This chapter has discussed many studies which mostly report that foliar spray of nutrients or fertilizer application has either declined the severity of diseases or increased resistance in many crops. Changes in the physiological or biochemical processes, as affected by the plant nutrients, are perhaps responsible for this elevated tolerance or resistance mechanisms of the host plant. However, more intensive studies are required to investigate the use of plant nutrients to alleviate the plant diseases vis-a-vis enhancing the soil fertility that can sustain crop productivity for our future generations. It is of paramount importance to understand the physiological or biochemical mechanisms involved in disease suppression with the application of nutrients. To lessen the use of extra nutrients, genetically engineered crops with better nutrient use efficiencies and biofortified crops should be included in sustainable agricultural production systems to maintain soil fertility and health. In addition, there is an utmost need for inclusion of varieties with disease resistant or tolerance in IPM practices that can effectively be combined with specific nutrient management schedules for managing plant diseases and preserve environmental quality, at the same time.

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# Integrated Mechanisms of Plant Disease Containment by Rhizospheric Bacteria: Unraveling the Signal Cross Talk Between Plant and Fluorescent *Pseudomonas*

# 9

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## Abstract

Being the universal green factories on earth, plants harbor many microbes in the rhizosphere arena. Majority of such free-living microorganisms have a positive effect on plant, known as plant growth-promoting rhizobacteria (PGPR). The PGPR are the extensively studied bacteria which elicit the plant probiotic traits and disease resistance through its competitive dominance in rhizosphere and production of secondary metabolites which act directly or indirectly on plant signaling mechanism. *Bacillus* and *Pseudomonas* are the major rhizobacterial members of PGPR group; both are known to exert direct and indirect means of growth promotion on host plant. Although *Bacillus* have an additional advantage of spore forming nature, majority of the biocontrol negotiators are made of vast *Pseudomonas* group. *Pseudomonas* beholds numerous qualities that enable them well suited to function as biocontrol and plant growth-promoting agents in agriculture. The direct mechanism is majorly by phosphate solubilization and release of major growth-promoting hormones. Indirect mechanisms of growth promotion are complex diverse mechanisms, which work individually or together, resulting in imparting probiotic traits. The present review portrays a broad updated understanding of principal mechanisms of *Pseudomonas*-induced probiotic traits associated with systemic resistance signaling.

## Keywords

PGPR • Fluorescent *Pseudomonas* • Growth promotion • Disease resistance

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## Contents

## 9.1 Introduction

The rhizosphere is the narrow zone of soil which is directly influenced by the root. It is one of the great frontiers and the most active portion in which the biogeochemical processes influence a host of landscape and global scale processes (McNear 2013; Venturi and Keel 2016). As quoted by Leonardo da Vinci "We know better the mechanics of celestial bodies than the functioning of the soil below our feet" (Badri et al. 2009). The interactions in the rhizosphere are affected by many different regulatory signals, of which only a few have been identified. It is the soil region adjacent to roots with dense population of diverse microorganisms including fungi, bacteria, protists, nematodes, and invertebrates. In addition, it is the battlefield, where microflora and microfauna interact with the soilborne pathogens (Meena et al. 2013a, 2016a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015a, 2016b; Ahmad et al. 2016; Parewa et al. 2014). The physicochemical changes taking place in the rhizosphere arena determines the rhizosphere composition. This selection can happen either by favoring the active recruitment of microbes that may support plant growth, development, and resistance enhancement toward environmental stress or by favoring the growth of opportunistic microbes which are already adapted to specific physiochemical environment (Philippot et al. 2013; Lopes et al. 2016).

The rhizosphere is colonized by rhizobacteria that can, individually or in cooperation with mycorrhizal fungi, improve plant fitness. Rhizobacteria bring in qualitative and quantitative alterations by continuously metabolizing the various organic compounds from the root exudates which significantly influence the nutrient supply (Bais et al. 2006; Doornbos et al. 2011; Ahemad and Kibret 2014). Hence, they play a key role in maintaining the nutrient turnover in the soil (Ahemad and Khan 2011; Gupta et al. 2015; Khan et al. 2016). They can also influence plant growth directly by releasing a variety of compounds and indirectly by producing phytohormones or antimicrobial compounds (de Vleesschauwer et al. 2006; Lee et al. 2011; Negi et al. 2015; Patil et al. 2016).

Plant roots secrete a spectrum of primary (comprising organic acids, carbohydrates, amino acids, etc.) and secondary (e.g., alkaloids, terpenoids, and phenolics) metabolites which are believed to shape, signal, interfere with, or in some way affect the rhizospheric microflora. These exudates come at a significant cost of carbon and nitrogen for the plant, with the ultimate benefit of attracting and promoting beneficial microorganisms while combating pathogenic or otherwise harmful ones (Lareen et al. 2016).

## 9.2 PGPR in Disease Containment

Innumerous pathogens result in the emergence of new complex plant diseases which have led to the introduction of new chemical agents to protect plants. This has redirected the crop scientists to find alternative, natural, strategies to manage diseases ending up in natural allelopathic chemicals from antagonizing microbes. A realistic alternative or supplement to chemical fungicide is the use of soilborne nonpathogenic bacteria that inhibits fungal phytopathogens (Saraf et al. 2014; Negi et al. 2015; Ng et al. 2015; Patil et al. 2016).

The term plant growth-promoting rhizobacteria (PGPR) was coined by Joe Kloepper in late 1970s and later defined by Kloepper and Schroth (1978) as "the soil bacteria that colonize the roots of plants following inoculation on to seed and enhance plant growth." About 2–5% of the rhizospheric bacteria belong to the group PGPR (Prashar et al. 2014). Various bacterial genera such as *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Actinomyces*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, and *Serratia* fall into this category (Hariprasad et al. 2014; Perez-Montano et al. 2014).

In a broad overview, application of PGPR in plants can be in three contexts, as biofertilizers, phytostimulants, and biocontrol agents (Bloemberg and Lugtenberg 2001; Prashar et al. 2014). Some PGPR strains directly regulate plant physiology through molecular mimicking through synthesis of various hormones, whereas others help in nutritional supply through mineralization and nitrogen fixation. In addition, volatile organic compounds (VOCs) synthesized by the PGPR act as a signal to regulate plant–microbe interaction (Kumar et al. 2015a, b). PGPR reaches the root surfaces by dynamic motility accelerated by flagella and are directed by chemotactic responses (Nihorimbere et al. 2011) indicating their competency in a given environment. Rhizosphere competence of biocontrol agents involves effective root colonization along with their ability to survive and proliferate with growing plant roots over a longer period of time, in the presence of the native rhizosphere microflora (Haas and Defago 2005; Buddrus-Schiemann et al. 2010; Mendes et al. 2013; Podile et al. 2014). However, different plants exhibit substantial genotypic and phenotypic diversity in the varying rhizosphere and different soil types.

Abiotic factors reportedly have a considerable impact on the effectiveness of PGPR (Duca et al. 2014). Therefore, the number, diversity, and action of rhizospheric bacteria vary with the host plant and the environment (Vacheron et al. 2013). Effects of PGPR on plant growth advancement and against pathogens depend on multiple elements comprising plant species, genotype, pathogen, and various abiotic conditions (Hol et al. 2013). These efficient PGPR confer an enhanced defensive capacity to the host plant against a comprehensive spectrum of fungal, bacterial, and viral diseases through induced systemic resistance (ISR), which is maintained for prolonged periods after induction (Haas and Defago 2005; Garcia-Gutierrez et al. 2012; Pieterse et al. 2014; Venturi and Keel 2016).

Although the concept of PGPR is known since ~40 years (Kloepper and Schroth 1978), the molecular characteristics that define PGPR remain vague, especially in *Proteobacteria*, due to two major reasons. First, PGPR may harbor different microbial habitats which range from saprophytic free-living soil bacteria to endophytes. Second, several bacteria display alternate ecological niches and at times may function as PGPR (Bruto et al. 2014; Prakash and Verma 2016; Meena et al. 2015a, 2016b; Priyadharsini and Muthukumar 2016; Kumar et al. 2016a, 2017; Jha and Subramanian 2016).

PGPR promote plant growth in direct and indirect ways (Fig. 9.1). Direct plant growth promotion can be by facilitating fixation of nitrogen and phosphorus solubilization (Sulochana et al. 2013; Khan et al. 2016) or by modulating phytohormones, viz., auxins, gibberellins, cytokinins, and nitric oxide (Lenin and Jayanthi 2012; Fierro-Coronado et al. 2014; Goswami et al. 2016). Indirectly it can be through iron sequestration by siderophore, niche competition, ISR, and biosynthesis of stress-related phytohormones like salicylic acid and jasmonic acid (Doornbos et al. 2011; Sorokan et al. 2013; Jayakannan et al. 2015; Pieterse et al. 2016), 1-aminocyclopro pane-1-carboxylate (ACC) deaminase, and cadaverine (Glick et al. 1998; Blaha et al. 2006; Shahzad et al. 2010; Yadav et al. 2013; Gontia-Mishra et al. 2014; Nadeem et al. 2016).

In this wide group of PGPR, species of *Pseudomonas* and *Bacillus* are the most investigated and are widely applied as field vehicles in the current agriculture scenario (Babu et al. 2015). With its wide metabolic diversity, fluorescent *Pseudomonas* is universally accepted as a best candidate for biological control. Some of the species of *Pseudomonas*, viz., *P. putida*, *P. aeruginosa*, and *P. fluorescens*, which fluoresce under UV radiation, are referred to as fluorescent pseudomonads or fluorescent *Pseudomonas* (Haas and Defago 2005; Khan et al. 2016).

### 9.2.1 *Pseudomonas*: The Supreme Diverse Group

*Pseudomonas* is a rod-shaped, gram-negative, uni- or multi-flagellate, motile, aerobic, and non-endospore-forming bacteria belonging to the family proteobacteria (Yadav et al. 2013). It is the most ubiquitous bacteria in rhizosphere, which have the property to be well suited as PGPR (Haas and Defago 2005; Hol et al. 2013; Sivasakthi et al. 2014). *Pseudomonas* comprehends the most diverse and ecologically significant group as they are specifically involved in plant disease suppression and growth promotion (Spiers et al. 2000; Haas and Defago 2005; Couillerot et al. 2009; Saraf et al. 2014; Khan et al. 2016). Although the number of reported isolates in the genus *Pseudomonas* varied from time to time, the latest molecular diversity analysis depicts 144 species and continues to remain as the largest genus (Raghavendra et al. 2016; Zahedi 2016; Meena et al. 2015b, 2016c; Rawat et al. 2016; Yasin et al. 2016; Jaiswal et al. 2016).

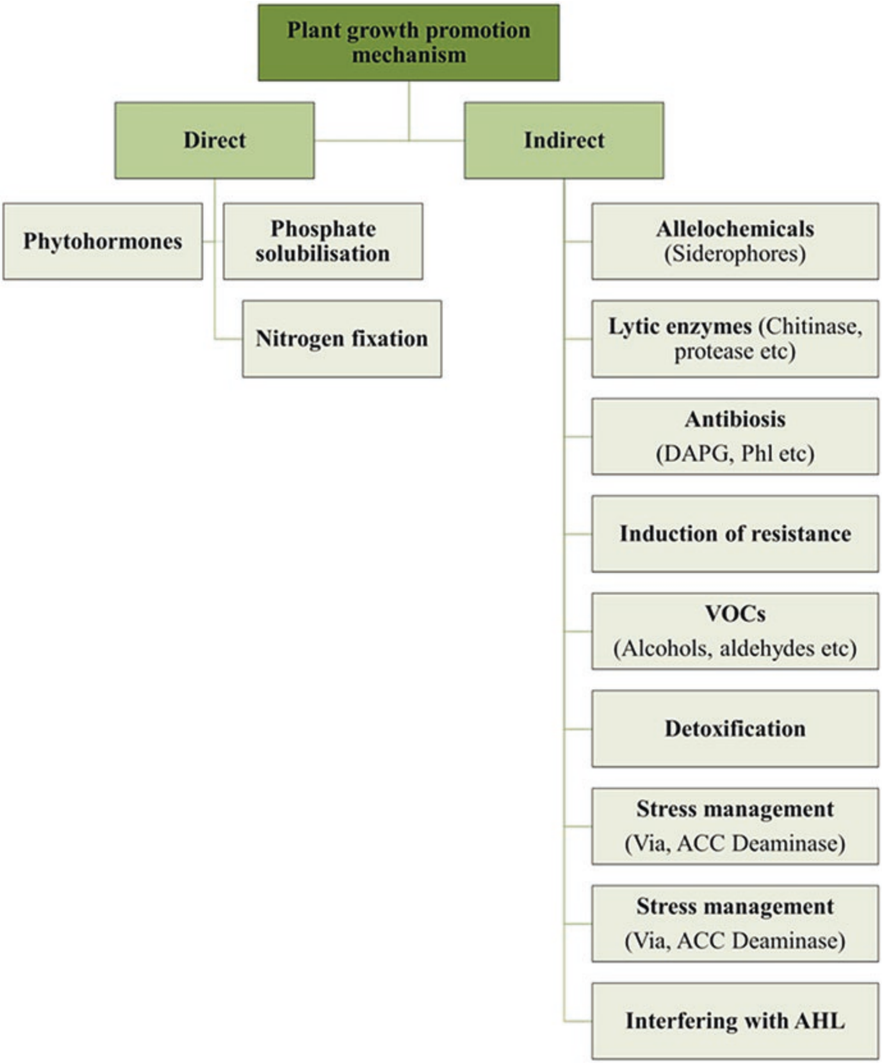

**Fig. 9.1** An overview of direct and indirect growth promotion mechanism induced by PGPR on host plant systems (*DAPG* 2,4-diacetylphloroglucinol, *Phl* phloroglucinol, *VOCs* volatile organic compounds, *AHL* N-acetyl homoserine lactones, *ACC* 1-aminocyclopropane-1-carbxylic acid)

*Pseudomonas* sp. beholds numerous qualities that enable them well suited to function as biocontrol and plant growth-promoting agents in agriculture (Saharan and Nehra 2011). This comprises rapid utilization of seed and root exudates, rapid colonization, production of a wide range of bioactive compounds (i.e., antibiotics, VOCs, siderophores, and growth-promoting substances), aggressive rhizosphere competence, and stress adaptation. In addition, *Pseudomonas* is a major player as a natural suppressor of specific soilborne fungal pathogens (Saharan and Nehra 2011; Hol et al. 2013; Khan et al. 2016).

Cho and Tiedje (2000) observed strong endemicity of fluorescent *Pseudomonas* genotypes in diverse soil samples from four different continents which suggests that these heterotrophic bacteria are not globally mixed. Through whole genome sequencing, it is estimated that ~6% of *P. fluorescens* Pf-5 genome (~ 400 kb) is dedicated only to secondary metabolite production, which shows the direct link between genetic elements and secondary metabolite production (Paulsen et al. 2005; Gross and Loper 2009).

## 9.2.2 *Pseudomonas* as Plant Growth-Promoting Agents

### 9.2.2.1 Direct Phyto-augmentation Mechanisms

These efficient growth promotion traits exerted directly on plants by *Pseudomonas* are mainly driven by three mechanisms – production of phytohormones, nitrogen fixation, and mineral solubilization. Many *Pseudomonas* spp. are well known to produce phytohormones (auxins and cytokinins) and secondary metabolites such as 2, 4-diacetylphloroglucinol (DAPG) and nitric oxide which interfere with the auxin pathway (Vacheron et al. 2013). *Pseudomonas*-synthesized auxins control almost all the aspects of plant development. The most crucial and common auxin produced by *Pseudomonas* is indole-3-acetic acid (IAA) which plays a major role in plant growth (Duca et al. 2014). It is reported that ~80% of rhizospheric microorganisms carry the ability to synthesize and release auxins. (Lugtenberg and Kamilova 2009; Bhattacharya and Jha 2012; Fierro-Coronado et al. 2014). IAA produced by *Pseudomonas* likely interferes with the physiological processes of plants by changing the plant auxins. However, recent studies suggest that IAA also serve as a signal for biofilm formation which is a critical stage in rhizosphere competence (Spaepen et al. 2007; Hu et al. 2010; Kerkar et al. 2012).

IAA is a reciprocal signaling molecule in plant–microbe interactions which evolved through millions of years of coevolution, with a sustaining symbiotic relationship (Spaepen et al. 2007; Malhotra and Srivastava 2009; Choudhary et al. 2016). Functionally, IAA affects cell division, extension, and differentiation; stimulates seed and tuber germination; increases xylem and root growth; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity, and florescence; affects photosynthesis, pigment formation, and biosynthesis of various metabolites; and imparts resistance to the plants (Meena et al. 2017). Production of IAA varies among the strains of bacteria. The amino acid tryptophan released from plant root exudates are the major substrate for IAA synthesis for the rhizobacteria. Tryptophan-dependent IAA synthesis occurs through three major pathways, indole-3-pyruvic acid, indole-3-acetamide, and indole-3-acetonitrile pathways (Saha et al. 2016a; Yadav and Sidhu 2016; Meena et al. 2014a, 2015f, 2016d; Saha et al. 2016b; Verma et al. 2015b; Verma et al. 2014; Dominguez-Nunez et al. 2016; Dotaniya et al. 2016).

First reported use of *Pseudomonas* isolate for growth promotion of potato seed tubers resulted in significant growth promotion (Kloepper and Scroth 1981). Since then, thousands of reports have been published on role of *Pseudomonas* on plant growth promotion (Table 9.1). Anitha and Kumudini (2014) have reported the use of fluorescent *Pseudomonas*, which are positive for production of IAA, siderophore, hydrogen cyanide, and ammonia and exhibit significant growth-promoting characters in tomato plants under greenhouse condition. A similar study by Vaikuntapu et al. (2014) tested different *Pseudomonas* isolates on different plants such as tomato, sorghum, groundnut, and chickpea to analyze the host-specific growth promotion traits. Results indicated that majority of tomato rhizosphere isolates showed growth promotion in all the plants.

Studies on *Arabidopsis* with *P. fluorescens* WCS417r priming improved lateral root growth formation and root hair development through auxin production ethylene signaling (Pieterse et al. 2014). Generally, IAA produced by soil rhizobacteria affects developmental processes in the plant. This is due to the imbalance created in the endogenous IAA pool of plants due to the increase in bacterially synthesized IAA (Ahemad and Kibret 2014).

Cytokinins (especially zeatin), another major plant growth hormone, have been studied in several PGPR such as *Bradyrhizobium japonicum*, *Arthrobacter giacomelloi*, *Paenibacillus polymyxa*, *Bacillus licheniformis*, *Pseudomonas fluorescens*, and *Azospirillum brasilense* (Hussain and Hasnain 2009). Cytokinins are hormones which encourage plant cell division, induce proliferation of root hairs, control root meristem differentiation, and at the same time inhibit lateral root formation and primary root elongation (Pieterse et al. 2014). Inoculation of plants with cytokinin-producing *Pseudomonas* has been shown to accelerate shoot growth and reduce the root to shoot ratio in the host plant (Vacheron et al. 2013). Root colonization and growth promotion of wheat, maize, and rice using *P. aurantiaca* SR1 have been reported by Rosas et al. (2009).

Ethylene is another fundamental volatile phytohormone, commonly induced by wounding in plants, inhibits root elongation, and promotes senescence, auxin transport, abscission, and senescence of various plant organelles (Glick et al. 2007). In addition, ethylene is acknowledged as a key signal in the ISR signaling process (Pieterse et al. 2014). Higher levels of ethylene in plants are known to have an inhibitory effect on root elongation in plant seedling. ACC deaminase can efficiently lower ethylene level by deamination of the immediate ethylene precursor, ACC, and hence help in sustaining plant growth (Yadav et al. 2013).

Earlier evidences suggested that *Pseudomonas* do not contribute significantly to the nitrogen fixation in rhizosphere zone. Hence *Pseudomonas* was considered as putative nitrogen fixers or non-nitrogen fixers. However, in recent years, several *Pseudomonas* strains are identified as nitrogen fixers (Khan et al. 2016). *P. stutzeri* CMT.9.A and *P. stutzeri* A15 are the two strains of *Pseudomonas* which were recently identified as strong nitrogen fixers (Khan et al. 2016). In addition, recent studies identified that denitrification is an important mechanism in rhizosphere competence. *P. fluorescens* with impaired nitrite reductase activity exhibited poor rhizosphere colonization traits (Redondo-Nieto et al. 2012).

**Table 9.1** *Pseudomonas*-mediated disease resistance against various pathogens with different plant systems

| Strain | Host | Pathogen/disease | Observed mechanism(s) | References |
|---|---|---|---|---|
| Fluorescent *Pseudomonas* EM85 | Cotton | *Rhizoctonia solani* | Hydrogen cyanide | Pal et al. (2000) |
| | | Siderophore | | |
| | | Fluorescent pigments | | |
| | | Antifungal antibiotics | | |
| Fluorescent *Pseudomonas* spp. | Ragi | *Magnaporthe grisea* | Mycolytic enzymes | Patil et al. (2016) |
| *P. fluorescence* Pf30 | Ragi | *M. grisea* | In vitro antifungal activity | Negi et al. (2015) |
| | | | Chitinase | |
| | | | PR proteins | |
| *P. fluorescens* UMAF6031, 6033 | Melon | *P. syringae* pv. *lachrymans* | ISR | Garcia-Gutierrez et al. (2012) |
| *P. fluorescence* EBC5, EBC6 | Chilli | *Pythium aphanidermatum* | ISR | Muthukumar et al. (2010) |
| *P. fluorescence* F113 | Potato | *Erwinia* carotovora | Antibiotics | Cronin et al. (1997) |
| *P. fluorescence* PICF7 | Olive | *Verticillium dahliae* | Host defense compounds | Prieto et al. (2009) |
| | Olive | *Pseudomonas savastanoi* | ISR | Maldonado-Gonzalez et al. (2013) |
| *P. fluorescence* CHO | Barley | *Fusarium graminearum* | Systemic interaction | Henkes et al. (2011) |
| *P. fluorescence* CHA0 | *Arabidopsis* | *Peronospora parasitica* | Systemic interaction | Lavicoli et al. (2003) |
| *P. fluorescens* HC1-07 | Wheat | *Rhizoctonia solani* | Cyclic lipopeptides | Yang et al. (2014) |
| *P. fluorescens* BREN6 | Pea | *Sclerotium rolfsii* | PAL | Tonelli et al. (2011) |
| | | | Ethylene | |
| | | | Glucanase | |
| *P. fluorescence* WCS374 | Radish | *F. oxysporum* | Iron competition | Leeman et al. (1996) |
| | | | ISR | |
| | | | Siderophore | |
| | | | Lipopolysaccharide | |

| | | | | |
|---|---|---|---|---|
| *P. fluorescence* and *putida* | Sugarcane | *Colletotrichum falcatum* | ISR | Viswanathan and Samiyappan (2002) |
| *P. aeruginosa* 7SK2 | Tomato | *Pythium* spp. | ISR | De Vleesschauwer et al. (2006) |
| | Rice | *M. grisea* | Antibiotics | |
| | | | Siderophores | |
| *P. aeruginosa* UPMP1 | Rice | *M. grisea* | Increased peroxidase | Ng et al. (2015) |
| | | | Polyphenol oxidase | |
| | | | PAL | |
| *P. chlororaphis* PCL 1391 | Tomato | *F. oxysporum* | Root colonizer | Chin-A-Woeng et al. (2000) |
| | | | Antibiotics | |
| *P. chlororaphis* O6 | Cucumber | *Corynespora cassiicola* | ISR | Kim et al. (2004) |
| | Tobacco | *Erwinia carotovora* | 2R,3R butanediol | Han et al. (2006) |
| | | Tobacco mosaic virus | Antiviral peptides | Lee et al. (2011) |
| | Tomato | Root-knot nematode | Nematicidal compounds | Park et al. (2005) |
| | Tobacco | *Cucumber mosaic* virus | ISR | Ryu et al. (2007) |
| *P. pseudoalcaligenes* AVO110 | Avocado | *Rosellinia necatrix* | Competition for nutrition | Pliego et al. (2008) |
| *P. putida* BTP1 | Bean | *B. cinerea* | Iron-regulated metabolite | Ongena et al. (2008) |
| | Tomato | *B. cinerea* | ISR | Akram et al. (2008) |
| *P. putida* IsoF | Tomato | *Alternaria alternata* | Homoserine lactones | Schuhegger et al. (2007) |
| | | | Salicylic acid | |
| | | | Ethylene | |
| *P. putida* PCI2 | Tomato | *Fusarium oxysporum* | Salicylic acid | Pastor et al. (2016) |
| | | | Jasmonic acid | |

*ISR*, induced systemic resistance, *PAL* phenyl ammonia lyase, *SAR* systemic-acquired resistance

#### 9.2.2.2 Indirect Phyto-augmentation Mechanisms

##### 9.2.2.2.1 Production of Siderophores

Although iron is plentiful in soil, it does not normally ensue in its biologically relevant ferrous form under aerobic conditions, as the ferrous ion is unstable in nature (Bhattacharya 2010). These efficient microorganisms overcome scarcity of iron via two routes: iron acquisition by cognate receptors using low molecular weight iron chelators (siderophores) and receptor-mediated iron acquisition from host proteins. Siderophores are nonprotein non-porphyrin compounds that bind to iron, whose synthesis is repressed when iron is plentiful in the surrounding environment (Jenifer et al. 2013), a pathway active even in pathogenic bacteria (Sharma et al. 2016; Verma et al. 2015a; Meena et al. 2013b, 2015e; Shrivastava et al. 2016; Velazquez et al. 2016; Bahadur et al. 2016b; Das and Pradhan 2016).

Microbial siderophores may stimulate plant growth by increasing the obtainability of iron in the soil surrounding the roots and by competitively inhibiting the growth of plant pathogens with less efficient iron uptake system (Upadhyay and Srivastava 2010). Microbes producing siderophores in the rhizosphere soils are believed to play a central role in heavy metal extraction (Perez-Monta et al. 2014). Currently, there are almost 500 compounds which are identified as siderophores (Bhattacharya 2010). Siderophore compounds such as pseudobactin produced by the fluorescent *Pseudomonas* spp. demonstrated considerable roles in the biological control of soilborne phytopathogens (Aznar and Dellagi 2015; Khan et al. 2016). Contemporary reports also show that the plant growth-promoting *Pseudomonas* with hydroxamates can be used as major criterion for “selective shortlisting” of best isolates (Subramanian and Satyan 2014). Although various bacterial siderophores differ in their abilities to sequester iron, they deprive pathogenic fungi of iron, since the fungal siderophores have lower affinity (Saharan and Nehra 2011; Meena et al. 2014b, 2015c, d; Sindhu et al. 2016; Singh et al. 2016; Teotia et al. 2016).

### 9.2.3 Biofilm Formation and Bacterial Signal Cross Talk

The most important characteristic of *Pseudomonas*, one of the PGPR for field application, is their ability to establish on host roots and move along with their growth (Dutta and Podile 2010). Therefore, available nutrients also affect the ability of an introduced isolate to colonize roots and carry out their activity (Miethling et al. 2000). Expression of several characteristics in bacteria, such as bioluminescence, biofilm formation, motility, and production of virulence factors, exoenzymes and antibiotics, is often a cell-density-dependent phenomenon which is also mediated by cell-to-cell communication, allowing the bacteria to monitor their population density in response to environmental signals (Schuster et al. 2013). Biofilms can change the nature of bacterial social interactions, which can lead to a densely packed bacterial population. This microbial colonization with considerable potential for cooperation and competition between cell populations is formed by biofilms (Nadell et al. 2009; Lopes et al. 2016). Communication between bacteria is enabled in a cell density-dependent manner, which controls the expression of certain functions, termed as quorum sensing. This communication is made possible through complex chemical signaling, in gram-negative bacteria mediated by N-acetyl-L-homoserine lactone (N-AHL) molecules which allows bacteria to monitor their population density by responding to the concentration of quorum sensing molecules they produce (Schuster et al. 2013).

The N-AHL molecule produced by many gram-negative rhizosphere bacteria plays a major role in their biocontrol activity (Perez-Montano et al. 2014). Some strains of *P. fluorescens* are unable to synthesize AHLs, but they carry a cognate receptor, which may even recognize a plant compound that later triggers the expression of genes coding for biocontrol properties (Subramoni et al. 2011). The study of wheat and tomato rhizosphere microbiome showed that signal cross talk between species using the same AHL or even a structurally similar AHL can occur in natural habitats (Steidle et al. 2001). It is shown that during quorum sensing, bacterial cells produce molecular signals which are released outside the cell, traverse in the extracellular environment, and are perceived by other microbial cells in proximity or by the producer cells themselves which typically form biofilm or microbial mat (Decho et al. 2010). AHL release has a good connection with its secondary metabolite release, particularly antibiotics, subsequently leading to antagonistic action and enabling them to perform as efficient biocontrol agents (McClean et al. 2004).

ISR is defined as the enhanced defense on whole plant body by PGPR against a broad range of pathogens and insect herbivores (Pieterse et al. 2014; Pieterse et al. 2016; Venturi and Keel 2016). Here the innate immunity of the plant is triggered by rhizomicrobes by exogenous signal molecules called priming (Venturi and Keel 2016) which further leads to activation of the phenylpropanoid pathway (Alvarez et al. 2016; Choudhary et al. 2016). ISR induction is usually initiated by priming which is physiological alert state of plant, following a stimulus enabling to produce a faster and more robust defense response (Venturi and Keel 2016). Under natural conditions, rhizobacteria is the well-known agent for inducing priming. A study by Sumayo et al. (2013) induced ISR in tobacco plants against soft rot disease by *Pectobacterium carotovorum* sub sp. *carotovorum*. In order to determine exact factor for induction of ISR, study used PGPR strain *Ochrobactrum lupini* KUDC1013 cell suspension, along with fraction of heat-killed cells, culture supernatant, crude lipopolysaccharide, and flagella. Both lipopolysaccharide and flagella from KUDC1013 were effective in eliciting ISR in tobacco plants which shows the significance of PGPR motility. Bacterial flagellins and pili play a crucial role in colonization of PGPR (Dutta and Podile 2010) and recognizing host and nonhost plants for beneficial bacteria (Che et al. 2000). Study on the root exo-metabolites of tomato plants and *Pseudomonas* strains indicated that the antifungal activity of PGPR in plant rhizosphere depends on sugar and organic acid composition of root exudates (Kravchenko et al. 2003). This shows significance of biofilm formation and rhizosphere colonization in biocontrol process.

### 9.2.3.1 Detoxification and Degradation of Virulence Factors

There are very few reports on the detoxification process exerted by PGPR. A major report on the detoxification mechanism has been through production of a protein that can reversibly bind to the toxin in *Klebsiella oxytoca* and *Alcaligenes denitrificans* (Saraf et al. 2014). Several rhizospheric microorganisms can also hydrolyze fusaric acid, a picolinic acid derivative which can act as a phytotoxin and is usually produced by various species of *Fusarium* (Saraf et al. 2014). Nagarajkumar et al. (2005) reported an oxalic acid degrading *P. fluorescens* strain PfMDU2 in rice, which reduced the severity of sheath blight disease caused by *Rhizoctonia solani* up to ~75% on seed treatment.

### 9.2.3.2 Through Reduction of Biotic and Abiotic Stress

Biotic and abiotic stress alleviation is a major mechanism of rhizobacteria. Ethylene is a volatile hormone which enhances root initiation and growth in lower concentrations. However, at higher levels, ethylene can act as a stress factor by inhibiting root elongation (Glick 2014). This stress can be mitigated by action of *Pseudomonas* through cleaving ACC into ammonia and α-ketobutyrate by its ACC deaminase activity (Parray et al. 2016). ACC deaminase is a member of a large group of enzymes which utilizes pyridoxal 5′-phosphate (vitamin B6) as a cofactor (Glick 2014). Usually the level of ACC in the plant and ACC deaminase in the rhizobacterium is low. Any biotic stress promotes the induction of ACC oxidase in the plant so that there is an increased flux through ACC oxidase resulting in the peak of ethylene which induces the expression of specific genes. When ACC deaminase-producing rhizobacteria are present, ethylene levels are dependent upon the ratio of ACC oxidase to ACC deaminase due to greater affinity of ACC toward ACC oxidase. Simultaneously bacterial ACC deaminase is induced by the increased ACC, so that the ethylene peak is decreased (Glick 2014; Singh et al. 2015; Meena et al. 2013c; Bahadur et al. 2016a; Masood and Bano 2016; Meena et al. 2016e).

It has been hypothesized that ACC deaminase increases the symbiotic performance of many PGPR strains in the rhizosphere (Choudhary et al. 2016; Khan et al. 2016). Ali et al. (2013) report the identification of a new ACC deaminase-producing fluorescent *Pseudomonas* strain enabling the drought-tolerant host plant. Mayak et al. (2004) hypothesized that PGPR are endemic to sites where rainfall is limited and are capable of protecting plants than those from water abundant conditions. Since then, many reports have shown strong plant-supporting behavior of PGPR from drought-hit regions (Arshad et al. 2008; Shakir et al. 2012), and most of them were induced through bacterially synthesized ACC deaminase. Similarly in the case of salinity stress, reports have shown that the fluorescent pseudomonads isolated from normal soil conditions induced growth promotion in tomato under salt stress (Nishma et al. 2014; Nadeem et al. 2016).

ACC deaminase-producing bacteria have been extensively utilized for promoting plant growth both under stress and normal conditions. Moreover, their use also protects plants from the deleterious effects of ethylene, which is synthesized due to various environmental stresses including heavy metals (Varsha and Kumudini 2016), flooding and water logging (Grichko and Glick 2001; Barnawal et al. 2012), phytopathogens (Toklikishvili et al. 2010; Rani and Reddy 2012; Patil et al. 2016) (Table 9.1), drought (Mayak et al. 2004; Arshad et al. 2008; Belimov et al. 2009, Govardhan and Kumudini 2016), and under saline conditions (Saravanakumar and Samiyappan 2007; Jalili et al. 2009; Siddikee et al. 2011; Anitha and Kumudini 2014; Nadeem et al. 2016).

Ethylene acts as a messenger during plant–microbe interactions, and all types of diseases caused by fungi, bacteria, viruses, and nematodes show an enhanced ethylene response. Some of the researchers have reported that treatment of plants with PGPR having ACC deaminase activity helps in conferring stress-ethylene generated by biotic stress (Toklikishvili et al. 2010). Robison et al. (2001) developed transgenic tomatoes expressing the *acdS* gene from *Enterobacter cloacae* UW4 activated by any of the promoters: 35S CaMV, rolD, or prb-1b. These plants were studied for their response toward wilt caused by *Verticillium dahliae*. Remarkable reduction was observed in the symptoms of verticillium wilt in rolD- and prb-1b-propelled *acdS* transformants due to reduced ethylene synthesis. These observations suggest that tolerance to diseases caused by various phytopathogens can be achieved through engineering plants for lower disease-related ethylene synthesis (via *acdS* gene expression). Recent studies showed that the synergistic action of ACC deaminase and IAA results in both direct and indirect ways of plant growth promotion in the environment (Glick 2014).

#### 9.2.3.3 Production of Mycolytic Enzymes

Disease management strategy through PGPR treatment is a host-targeted approach, where in plants acquire disease resistance through natural microbial signal elicitors (Biswas et al. 2012). There are numerous reports on mycolytic enzymes such as chitinase, cellulase, and protease production by rhizobacteria which results in cell wall degradation of fungal–bacterial pathogens and promote plant growth (Illakkiam et al. 2013; Tailor and Joshi 2014; Khan et al. 2016). The expression of these enzymes ends up in digestion/deformation of fungal cell wall made of chitin, cellulose, hemicellulose, proteins, etc., resulting in direct pathogen suppression. This strategy is being exploited extensively for crop disease management using rhizobacteria (Saraf et al. 2014).

Chitinases constitute the second largest group of antifungal proteins found in a large group of organisms, whose production can be constitutive or inducible. Studies by Velusamy et al. (2011) demonstrated that the antifungal activity of *Pseudomonas* sp. A3 against *Fusarium* was by the production of chitinase. Overall, mycolytic enzymes can exert a direct impact on biocontrol mechanisms. In addition, *Pseudomonas* are shown to be excellent producers of other mycolytic enzymes such as proteases, lipases, β-glucanases, and cellulases (Gonzalez-Sanchez et al. 2010).

#### 9.2.3.4 Secretion of Chemical Volatiles

VOCs are low weight lipophilic compounds which are derived from the microbial biosynthetic pathways, which vaporizes into atmosphere (Helman and Chernin 2014). In 2003, Ryu and his co-workers first described the volatile-mediated induction of ISR. This report identified two volatile components 2,3-butanediol and acetoin which help in the growth promotion of *Arabidopsis*. Further, many experimental evidences clearly suggested that the volatile signals synthesized by a large group of microbes in the soil arena communicate efficiently with the host plants. This gained particular attention because of its long distance and inter-kingdom communication (Helman and Chernin 2014). However, until recent times, such experiments were restricted to controlled conditions, due to experimental limitations (Kanchiswamy et al. 2015).

Most common VOCs of bacterial origin with an evident impact on plant systems are pentanone, 4-heptanone, 2-heptanol, 2-undecanone, 2tridecanone, and 2-pentadecanone (Kanchiswamy et al. 2015). A study by Elkahoui et al. (2015) identified the endophytic *Pseudomonas* P2 strain which produces multiple VOCs along with production of siderophores and protease against *R. solani*. The responsible VOCs were identified as dimethyl disulfide and dimethyl trisulfide. Herbivore-induced volatile indolic compounds were shown to prime maize plants, which subsequently showed the best systemic resistance even in neighboring plants (Erb et al. 2015). Recent study shows that the rhizobacterial VOCs are below 300 Da and lipophilic in nature with low boiling point (Kanchiswamy et al. 2015).

## 9.3 Mechanism of Antibiosis

Antibiosis is a condition in which one or several metabolites excreted by an organism have a detrimental effect on other organisms (Haas and Defago 2005). Antibiosis operation of microbes require appropriate colonization of microbial strains, whereas, for induced resistance mechanism, a reduced number of cells for limited period are adequate to obtain an effective host plant response (Paulsen et al. 2005). Antibiotic production by fluorescent *Pseudomonas* has been well documented; they produce many antibiotic substances, such as DAPG, amphisin, oomycin A, hydrogen cyanide, phenazine, pyoluteorin, pyrrolnitrin, and cyclic lipopeptides (Companta et al. 2010). The type of antibiotics produced depends on the age of the host plants, which is well supported by Picard et al. (2000) which reports that the young plant root exudates did not produce DAPG, while it was induced by older plant root exudates. This powerful diversity of antimicrobial compounds by *Pseudomonas* is considered as part of an indirect strategy to promote plant growth and their ability to induce systemic resistance in plants (Glick 2014).

**Phenazines** First clear-cut experimental demonstration that a *Pseudomonas* antibiotic can suppress plant disease in an ecosystem was made by Thomashow and Weller (1988). They identified a phenazine antibiotic, phenazine-1-carboxylic acid (PCA), as a biocontrol factor produced by *P. fluorescens* 2-79. This strain, originally isolated from wheat rhizosphere, was found to suppress take-all disease caused by fungal pathogen *Gaeumannomyces graminis* var. *tritici* on wheat. PCA, phenazine-1-carboxamide, hydroxyphenazines, and pyocyanin are the common phenazines produced by different *Pseudomonas*. They exhibit unique redox properties (Mavrodi et al. 2006), which exists in different forms by conversion of basic PCA structure through genetic manipulations. *P. fluorescens* produces only the yellow-pigmented PCA, while other species or strains can produce one or more additional forms, viz., orange-dark red hydroxyphenazines, green-pigmented phenazine-1-carboxamide, and blue-green pyocyanin (5-methyl-1-hydroxyphenazine) as reviewed by Mavrodi et al. (2006).

Production of phenazines is regulated by the Phz operon which encompasses *phzA*, *B*, *C*, *D*, *E*, *F*, and *G* genes which are highly conserved among *Pseudomonas* species. The first step in the biosynthesis of phenazines requires the accumulation of chorismic acid (catalyzed by *PhzC*), which is then sequentially modified by *PhzE*, *PhzD*, *PhzF*, *PhzB*, and *PhzG* to produce PCA. Additional genes involved in modification of PCA to other forms of phenazines are present either adjacent to the operon or elsewhere in the genome. *PhzO* (hydroxylases) and *PhzS* convert PCA into hydroxyphenazines, while a methyltransferase (*PhzM*) acts with PhzS to produce pyocyanin and *PhzH* (asparagine synthase) converts to pyocyanin.

**Phloroglucinols** DAPG is most widely reported in *Pseudomonas* and known to have a broad antiviral, antibacterial, antifungal, and anthelminthic property (Feunte et al. 2008). DAPG alters the metabolism of glucose to fructose by inhibiting the aldose reductase enzyme, thereby regulating the phytopathogens. DAPG is shown to activate *eir1* signaling instead of *NPR1 or JAR1*, in *Arabidopsis* plants (Roman et al. 1995). Evidences on *Arabidopsis* mutants clearly showed that the DAPG producing *P. fluorescens* CHA0 activates ISR response against *Peronospora parasitica* infections (Lavicoli et al. 2003).

**Pyrrolnitrin** Pyrrolnitrin (3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole) is a potent antifungal compound, first isolated from *Pseudomonas pyrrocinia* from *Burkholderia* by Arima et al. (1964). Studies on *P. aureofaciens* showed that tryptophan is the precursor for pyrrolnitrin formation in many steps, the details of which have not been elucidated. A 6.2 kb DNA fragment was cloned and characterized in *P. fluorescens* BL915 containing four genes (*prnABCD*), which are involved in biosynthesis of pyrrolnitrin. It is synthesized from tryptophan by chlorination of pyrrole and benzene rings (*PrC* and *PrA*, respectively), rearrangement of the rings and decarboxylation (*PrnB*), and oxidation of amino group to nitro by *PrnD*. Pyrrolnitrin was found to be active against diseases caused by *R. solani*, *Pyrenophora tritici*, *Aphanomyces cochliodes*, *Gaeumannomyces graminis* var. *tritici* (Ggt), and *Botrytis cinerea* and not against *P. ultimum*.

**Pyoluteorin** Pyoluteorin (4,5-dichloro-2-(2′,6′-dihydroxybenzoyl)-pyrrole) is a chlorinated aromatic polyketide produced by most of the *Pseudomonas* species. Pyoluteorin is produced by addition of three acetyl groups to proline and further oxidation and chlorination of the molecule (Fuente et al. 2008). Well-known *P. fluorescens* CHA0 strain produces 2,4-DAPG and pyoluteorin, which has been shown to interfere with the growth of numerous pathogens and exhibit disease suppression (Khan et al. 2016).

## 9.4 Host Specific Signal Variations Associated with Microbial Priming

Fluorescent *Pseudomonas* can elicit a range of defense-responsive activities in plants including activation of antioxidant status by reprogramming defense-specific enzymes, modulation of quorum sensing, and activation of the phenylpropanoid pathway leading to phenolic production, lignin deposition, and trans-generational defense response to combat the pathogen challenge (Choudhary et al. 2016). Production of the reactive oxygen species (ROS) is the first line of plant defense in response to any fungal infection on the host, which is followed by the activation of pathogenesis-related (PR) proteins.

Biochemical studies on plants showed that the ROS, such as superoxide anion ($O_2^-$) and $H_2O_2$, are involved in the host defense response. Excess amount of ROS can lead to oxidative stress, resulting in reduced root growth, stimulation leaf abscission and desiccation, peroxidation of membrane lipids, and damage to proteins, carbohydrates, and nucleic acids which is generally mitigated by the action of antioxidative enzymes (Gill and Tuteja 2010; Das and Roychoudhary 2014; Lehmann et al. 2014; Camejo et al. 2016). This detoxifying machinery is regulated by ascorbate peroxidases (APX), glutathione, superoxide dismutases (SOD), and catalases (CAT). Major enzymatic antioxidant pathway in plants comprises CAT, APX, monodehydroascorbate reductase, guaiacol peroxidase (GP), SOD, glutathione reductase (GR), and dehydroascorbate reductase. Though considered as a hazardous byproduct earlier, it is now proved that ROS is a cell signal in activation of defense genes, apoptosis, and cell signaling cascade against various environmental stresses (Das and Roychoudhary 2014; Lehmann et al. 2014). A study on tomato showed that the nonhost pathogen *Magnaporthe grisea* induced callose deposition and hypersensitive cell death. Biochemical response on tomato showed increased activity of $H_2O_2$, hydroxyl radicals, superoxide radicals (superoxide dismutase and guaiacol peroxidase), and NADH peroxidase (Uma and Podile 2015). Studies showed that priming with *Pseudomonas* can efficiently lower the oxidative stress mechanism during biotic (Rammoorthy et al. 2002; Jain et al. 2013) and abiotic stress encounter (Mishra et al. 2008; Varsha and Kumudini 2016).

The major change that occurs in the host plant during ISR is the upregulation of defense-specific genes such as *PAL*, *LOX*, and *GST* (Aznar and Dellagi 2015). Tonelli et al. (2011) studied that the pea plants inoculated with two strains of PGPR, *Pseudomonas*- (BREN6) and *Bacillus* (CHEP5)-induced ISR in host plant along with significant variation in chlorophyll content, β-1,3 glucanase, peroxidase, and phenylalanine ammonia lyase (PAL). This was the first report on positive ISR response on pea plants, which was supported by a significant elevation of ethylene level, leading to induction of resistance in the host plant.

Phenylpropanoids are a diverse group of compounds derived from phenylalanine which play a major role in plant defense, structural support, and survival (Vogt 2010; Fraser and Chapple 2011). Phenylalanine is an end product of the Shikimate pathway, which also gives rise to the aromatic amino acids, tyrosine and tryptophan.

Lignin biosynthesis starts from phenylalanine through a set of structural modification in the side chains (Hatfield and Vermerris 2001). In higher plants, the Shikimate pathway directly leads to phenylpropanoid pathway (Fraser and Chapple 2011; Zhang et al. 2015; Choudhary et al. 2016). They are assigned to various defense-specific functions, such as cell wall reinforcement, antimicrobial activity (as modulators of plant hormones in defense signaling), and ROS scavenging (Kogovsek et al. 2016). Accumulation of PR proteins depends on three variables, the pathogen, the host plant genotype, and the physiological conditions. A large number of PR proteins are mainly enzyme linked with activation of ISR, such as peroxidase, lipoxygenase (LOX), β-1, 3-glucanase, and chitinase (Waewthongrak et al. 2014).

Jasmonic acid (JA) is synthesized from the precursor α-linoleic acid, a natural hormone-regulating plant development and responses against wounds and pathogens. This is converted to 13-hydroperoxy linolenic acid, which is again converted to 12-oxo-phytodionic acid, in chloroplast. 12-Oxo-phytodionic acid is transported to peroxisomes by ATP-binding cassette and subsequently reduced to 3-oxo-2 (2 pentenyl) cyclopentane-1-octanoic acid, which is transformed into JA through a series of $\beta$-oxidation (Lyons et al. 2013). Upon intrusion of pathogens, JA is synthesized and regulates a signaling cascade which further leads to various defense responses. Traditionally, necrotrophic fungi are the primary activators of JA-dependent defense in the host. Synergistic or antagonistic action by JA, salicylic acid, and ethylene finally leads to the alteration in the plant immune system (Antico et al. 2012; Pieterse et al. 2014). Interestingly, even exogenous application of salicylic acid and JA imparted resistance on host plants against stem borer *Chilo partellus* (Hussain et al. 2013).

LOX is a major enzyme which is involved in plant growth and developmental signal processes. It additionally takes part in the synthesis of regulatory metabolic signals which help in defense responses (Choudhary et al. 2016; Alvarez et al. 2016). LOX incorporates molecular oxygen to fatty acids to generate fatty acid hydroperoxides. These hydroperoxides act as substrates for allene oxide synthase leading to the production of jasmonates, which is a well-known signal in plant defense mechanisms (Lavanya et al. 2012). Previous studies such as Mariutto et al. (2011) showed that induction of resistance is followed by transcriptional activation of two LOX gene isoforms, *TomLoxD* and *TomLoxF*, on priming with *P. putida* BTP1 in tomato. This transcriptional activation of two LOX isoforms leads to high linolenate consumption, resulting in the rapid accumulation of 13-hydroperoxy-octadecatrienoic and 13-hydroxy-octadecatrienoic acids in host plants in response to PGPR-mediated ISR (Fraser and Chapple 2011). Study of Akram et al. (2008) showed activation of LOX and accumulation of phytoalexin while using *P. putida* strain against *Botrytis cinerea* infection in tomato. Another study by Jain et al. (2013) used microbial consortium (*Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Trichoderma harzianum* strains) as biocontrol agent in pea plants against *Sclerotinia sclerotiorum*. Time course biochemical analysis depicted significant alleviation of stress in consortium-treated plants during pathogen encounter.

## 9.5 Conclusions

Rapidly evolving phytopathogens and climate changes brings drastic changes in the crop yield and food security. Consequently, the amount of biotic and abiotic stress challenged by the plants is fluctuating which threaten the existence the life. Hence, the primary aim of scientists in agriculture has been to reestablish food security by maintaining the plants in a low-stress environment by using alternative strategies. Undoubtedly, the secondary characteristics of rhizo-microbiota provide sufficient opportunities to use them as a part of sustainable agriculture. The fluorescent *Pseudomonas*, widely recognized as a model organism for studies on plant–microbe interaction, is highly unpredictable in the environment. Biological controls of phytopathogens using species of fluorescent *Pseudomonas* have been documented with many findings and showing strong correlation with its secondary metabolic traits. However, its efficiency varies with strain and environment. Recent trends such as consortium-based biocontrol approach rectified many of such limitations of using a single biocontrol strain. Hence, identification of any new potential rhizobacterial isolate and conceptual understanding of its intriguingly complex and dynamic ways of imparting resistance in the otherwise susceptible plants is a contribution to sustainable agriculture which may benefit to reform the multi-decade-old mainstream pesticide-based disease management approaches.

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# Toward Plant Defense Mechanisms Against Root Pathogens

# 10

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## Abstract

As we consider the importance of the plant microbiome for sustainable agriculture, we note that some of these organisms are beneficial to plants, others are commensal, and the third category includes pathogens. Root pathogens, such as *Fusarium oxysporum*, are of particular interest as these can be controlled by effective plant defense mechanisms or by biocontrol microbes. This chapter reviews recent findings of plant defense mechanisms against root pathogens. This process can be greatly assisted by microbes that prime the plant, leading to induced systemic resistance. These microbes prepare the plant to mount a faster and stronger response against root pathogens at a very low energetic cost to the plant. New strategies to develop resistance come from the knowledge that agriculturally important root pathogens often hijack the wrong defense pathway in plants. An interesting emerging area is the plant defense mechanism that leads to the production of root exudates which can result in the recruitment of beneficial microbes that assist the plant in developing resistance. Future work may focus on the selection of plants whose defense responses against pathogens may be better assisted by agriculturally important beneficial microbes.

## Keywords

Biocontrol microbes • Disease resistance • Root defense • PGPR • Plant beneficial microbes • Plant defense signaling

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## Contents

## 10.1 Introduction

As agricultural plant productivity has immensely intensified in the past few decades, the demand for agrochemicals by many agricultural industries has also increased to provide reliable methods for crop protection against various destructive diseases (Jan et al. 2011). The increased use of fertilizers, pesticides, herbicides, and fungicides has led to the development of biological alternatives to these established chemicals (Meena et al. 2013a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015, 2016b; Ahmad et al. 2016). This has alarmed the public concern about the environmental problems caused by the direct or indirect use of these agrochemicals (Glick et al. 2007). Therefore, a more economically and environmentally friendly method is required to provide reliable and sustainable crop protection against emerging plant diseases in the future. This has opened up the opportunity to further develop research in the area of biological control of crops using beneficial microbes as soil inoculants to sustain plant health and growth promotion (Arora and Mishra 2016; Patel et al. 2016; Nath et al. 2017; Sarkar et al. 2017).

## 10.2 The Beneficial Plant Microbiome

It is well studied that there are a large and diverse number of bacterial populations associated with plants, and over two dozen genera of bacteria are characterized as either having biocontrol or plant growth-promoting activities (Kim et al. 2011). This special group of bacteria is popularly termed as plant growth-promoting rhizobacteria (PGPR), first coined by Kloepper and Schroth (1978), to describe soil bacteria that colonize the root's rhizosphere and directly or indirectly enhance plant growth. The term rhizosphere is used to define the soil region that is affected by the presence and activity of roots. Intensive research on PGPR has opened up a diversity of opportunities to further improve the production of food crops and also to provide a new global trend toward the use of a more sustainable and environmentally friendly method for controlling widespread plant pathogens (Arora et al. 2010; Verma et al. 2017b).

This improvement is governed by the enhancement of plant growth directly or indirectly, by means of biological control of plant diseases (Kim et al. 2011; Arora et al. 2010; Laslo et al. 2012). This dual mode of action by PGPR makes them suitable for diverse agricultural applications such as biofertilizers, phytostimulators, and biopesticides, or a more commonly used term, biocontrol agents (Parewa et al. 2014; Prakash and Verma 2016; Priyadharsini and Muthukumar 2016; Meena et al. 2015a, 2016a, b; Raghavendra et al. 2016; Kumar et al. 2016a, 2017).

Direct enhancement of plant growth is mediated by the role of PGPR as biofertilizers and phytostimulators. Some rhizobacteria promote plant growth in the absence of pathogen pressures (Lugtenberg and Kamilova 2009). These biofertilizers supply plants with nutrients, such as nitrogen, iron, and phosphorus. Nitrogen-fixing bacteria, including *Rhizobium* and *Bradyrhizobium*, form nodules on leguminous plant roots, where they convert $N_2$ into bioavailable ammonium (Li et al. 2016; Ramírez-Bahena et al. 2016; Spaink et al. 1998; Vanrhijn and Vanderleyden 1995). Meanwhile, limitations of soluble phosphorus can also limit the growth of plants.

Some PGPR can solubilize phosphate from either organic or inorganic phosphates, thereby facilitating plant growth (Lipton et al. 1987; Vassilev et al. 2006). The biofertilization of crops with these PGPR is definitely considered to be a promising alternative to chemical fertilization (Garcia-Fraile et al. 2012). In addition, some PGPR can also play a major role as phytostimulators, as several symbiotic and free-living rhizobacterial species are reported to produce phytohormones, such as indole-3-acetic acid (IAA) and gibberellic acid in the rhizosphere soil, and therefore significantly increase the root surface area and number of root tips in many plants (Bhattacharyya and Jha 2012; Han et al. 2005). In regard to the biological control of plant diseases, PGPR also exhibit an indirect enhancement of plant growth by increasing competition with pathogens for nutrients, niche exclusion, induced systemic resistance, and production of antimicrobial metabolites (Bloemberg and Lugtenberg 2001; Zahedi 2016; Meena et al. 2015b, f, 2016c; Rawat et al. 2016; Yasin et al. 2016; Saha et al. 2016a; Dotaniya et al. 2016; Jaiswal et al. 2016; Jha and Subramanian 2016).

These biocontrol agents can be used as composite inoculum to suppress diseases and to increase chlorophyll content, total number of leaves, and shoot height, leading to a higher overall crop yield and productivity (Bhattacharyya and Jha 2012). In view of this, the research area of plant-microbe interactions not only allows us to better understand direct and indirect effects of PGPR on plant health promotion, but it also increases our knowledge on the complex interactions through metatranscriptomics, genetic studies, and also microbial biodiscovery of novel bioactive compounds for biocontrol (Schenk et al. 2012; Kumar et al. 2011; Verma et al. 2017a).

## 10.3 Dynamics of Plant-Microbe Interactions

Plants possess mechanisms that can modify the physicochemical properties and biological composition of the rhizosphere through different methods, which include acidification via proton extrusion and the release of root exudates (Jones et al. 2009; Li et al. 2016; Richardson et al. 2009). Therefore, changes in soil pH and increased nutrient availability indirectly affect interactions with soil microorganisms. Clearly, plant-microbe interactions at the rhizosphere are complex, involving diverse soil microbial communities that function in very varied environments (Giri et al. 2005). These complex interactions can be beneficial, pathogenic, and neutral to the plants. Beneficial microorganisms can directly or indirectly promote plant growth by nutrient acquisition, production of growth regulators such as plant hormones, and suppressing plant diseases through the production of pathogen-inhibiting compounds (Schenk et al. 2012; Verma et al. 2017b). These microorganisms include root-colonizing rhizobacteria, mycorrhizae, endophytes, and PGPR (Fig. 10.1).

On the contrary, pathogenic microorganisms provide a much broader scope of detrimental interactions that lead to major problems in agricultural industries and food crops worldwide. They consist of pathogenic fungi, oomycetes, bacteria, and viruses that can potentially cause destructive infections on plants (Yadav and Sidhu 2016; Saha et al. 2016b; Verma et al. 2014, b; Meena et al. 2015e, d; Bahadur et al. 2016b; Das and Pradhan 2016; Dominguez-Nunez et al. 2016). These pathogens can be further classified into biotrophic, necrotrophic, and hemibiotrophic pathogens, based on different pathogenic lifestyles and mechanism of infection (Table 10.1).

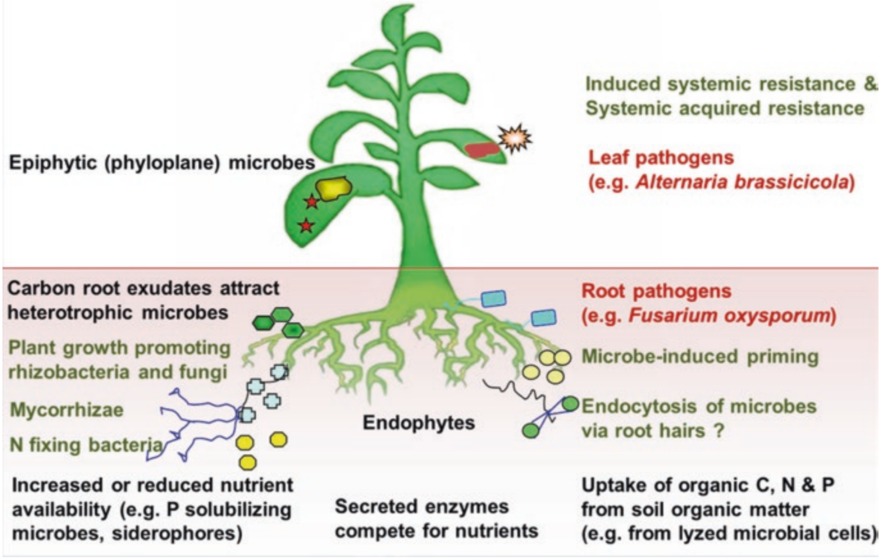

**Fig. 10.1** An overview of the multiple plant-microbe interactions including beneficial, neutral, and pathogenic microorganisms

**Table 10.1** Examples of different classes of pathogenic lifestyles from different plant pathogens, commonly encountered in agriculture (adapted from Oliver and Ipcho (2004)

| Mode of nutrient acquisition | Pathogen | Plant disease |
|---|---|---|
| Biotrophic | *Fusarium oxysporum*, *Cladosporium fulvum* | Fusarium wilt, tomato mold |
| Necrotrophic | *Botrytis cinerea*, *Phytophthora cinnamomi, Alternaria brassicicola* | Gray mold, root rot, black spot |
| Hemibiotrophic | *Colletotrichum* spp., *Phytophthora infestans*, *Pseudomonas syringae* | Anthracnose, potato late blight, bacterial speck |

## 10.4 Emerging Agricultural Plant Pathogens

The number of recognized fungal plant pathogens has more than quadrupled over the last 15 years (Fisher et al. 2012). This cannot be attributed to better detection methods alone (by comparison the number of animal pathogens has not increased accordingly) but appears to be a direct result of our crop cultivation and landscape management practices (Meena et al. 2017). These are characterized by a narrow genetic diversity and large monocultures with low or no crop rotation. This provides an ideal breeding ground for the emergence of virulent pathogens, as, unlike in biodiverse natural landscapes, pathogens do not get penalized by damaging their host and conversely, receive the same crop repeatedly over many years (Sharma et al. 2016; Verma et al. 2015a; Meena et al. 2013b, 2014a; Shrivastava et al. 2016; Velazquez et al. 2016; Teotia et al. 2016).

As mentioned previously, plant pathogens can be classified into biotrophs, necrotrophs, and hemibiotrophs according to their nutrient uptake behavior. Biotrophic infections, like rusts, powdery mildews, or downy mildews, require living plant cells to derive their nutrients without producing toxins and harmful compounds. The establishment of this parasitic relationship depends on manipulation and suppression of plant defense responses by the pathogen. On the contrary, necrotrophic pathogens, such as *Alternaria brassicicola* or *Phytophthora cinnamomi*, damage tissues by toxins and cell wall-degrading enzymes, and the degraded tissues serve as nutrient sources (Barna et al. 2012). For example, previous studies have demonstrated that *A. brassicicola* produces a host-specific toxin (HST), namely, AB toxin, which is a known low molecular weight secondary metabolite (Sindhu et al. 2016; Meena et al. 2014b, 2015c, 2016e; Singh et al. 2016; Masood and Bano 2016). However, knowledge on the pathogenesis mechanisms of *A. brassicicola* or other necrotrophic pathogens has been limited. On the other hand, hemibiotrophs are a group of pathogens that have both characteristics of biotrophs and necrotrophs. Hemibiotrophs like *Colletotrichum* sp. or *Phytophthora infestans* have an initial period of biotrophy followed by necrotrophic hyphae (Barna et al. 2012). This poses significant challenges to plants that have to mount the appropriate defense signaling at the right time (Thatcher et al. 2016; Singh et al. 2015; Meena et al. 2013c, 2015d; Bahadur et al. 2016a).

## 10.5 Plant Disease and Resistance

Plant health can be disrupted by a wide variety of living agents (biotic), like viruses, bacterial, and fungal pathogens, or by environmental (abiotic) factors including nutrient shortage, drought stress, oxygen deficiency, extreme temperature, ultraviolet radiation, or pollution. In order to respond to the vast array of potential biotic stresses, plants have evolved a multilevel defense system that includes both constitutive and inducible defenses. Constitutive (continuous) defense is the first layer that plants use to defend themselves from infection by potentially harmful organisms. This layer consists of various preformed barriers, including cell walls, waxy epidermal cuticles, and bark (Hematy et al. 2009; Reina-Pinto and Yephremov 2009). These physical barriers play an important role in protecting plants from invasion as well as providing strength and rigidity. For pathogens to pass these barriers requires either possessing specific mechanisms that allow them to enter the plant tissue such as physical pressure (e.g., appressoria) or by producing enzymes that can degrade the cell wall.

The second layer that plant cells use to stop pathogen entry is inducible defenses that include chemical defense mechanisms. After a pathogen penetrates the cell wall, plants not only produce toxic chemicals and enzymes that can degrade pathogens, they may also initiate hypersensitive response (HR)-mediated programmed cell death in order to isolate the pathogen from the rest of the plant (VanEtten et al. 1994). This strategy is highly effective against biotrophic or hemibiotrophic pathogens if recognized early but works to the plant's disadvantage if applied against necrotrophic pathogens (Thatcher et al. 2016).

## 10.6 The Plant Immune System

A vast biodiversity of microorganisms surrounds plant roots in the rhizosphere. These microorganisms can be either harmful or beneficial to plants (Whipps 2001). Thus plants have developed basal resistance, also known as innate immunity. There are two layers of sophisticated surveillance used to detect potential pathogens: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). PAMPs include microbial cell wall components or specific pathogen proteins (such as bacterial flagellin). PTI is activated when PAMPs of potential pathogens have been recognized by PAMP receptors in plants (Nicaise et al. 2009). PAMP receptor activation leads to reactive oxygen species (ROS) production (Lamb and Dixon 1997) as well as activation of the MAP kinase signaling cascades which then lead to increased ion flux, phytoalexin, hormone production, and defense gene expression (Nicaise et al. 2009).

On the other hand, pathogens have evolved proteins known as effectors that can suppress PAMP-triggered immunity and allow the pathogens to colonize inside the plant. Plants might respond by recognizing these effector proteins and activating the second line of defense known as effector-triggered immunity (ETI) that may lead to

HR at the infection site. If pathogens overcome ETI as well, the plants must respond by evolving new resistance (*R*) genes that are able to recognize these effectors and again recruit a successful defense response to protect the plant (Bent and Mackey 2007). Alternatively, if dealing with slow-growing pathogens, plants may simply try to outgrow the infection. It seems that plants and pathogens are in constant competition to develop new strategies to outsmart one another.

## 10.7 Plant Defense Hormones

The plant hormones most commonly associated with plant defense include salicylic acid (SA), jasmonate (JA), and ethylene (ET). The former is commonly produced in response to biotrophic pathogens, while the latter two are often associated with a response to necrotrophic pathogens (Glazebrook 2005). SA plays a role in mediating the systemic acquired resistance (SAR) pathway that results in the systemic activation of defense mechanisms, such as PR (pathogenesis-related) gene expression, like *PR1* and *PR5* (Uknes et al. 1993). The JA-dependent defense response is mediated by jasmonoyl isoleucine (JA-ile) and leads to the induction of defense-related genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) and *THIONIN2.1* (*THI2.1*) (Penninckx et al. 1996).

In addition to its involvement in plant defense, the ET pathway is involved in a variety of plant growth and developmental processes, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Johnson and Ecker 1998). The JA, ET, and SA pathways interact with each other, and this cross talk can be quite complex. Generally, the JA and SA pathways are said to be antagonistic to each other via NPR1, but at low concentrations, they can also be synergistic (Schenk et al. 2000). For example, SA has been stated to stop not only JA biosynthesis but also JA-responsive gene expression (Penninckx et al. 1996), whereas JA has been shown to suppress the SA-signaling pathway. Defense hormone cross talk is controlled by transcription factors (TFs) that can activate target genes of one pathway while inhibiting those of another pathway.

The three closely related *Arabidopsis* basic leucine zipper (bZIP), TFs, TGA2, TGA5, and TGA6, are examples of TFs that are required to activate SA-regulated defense genes and at the same time repress the JA pathway (Zander et al. 2010). Apart from SA, JA, and ET, the abscisic acid (ABA) pathway can play a role in plant defense. Although generally this pathway is upregulated following abiotic stress (e.g., drought, high/low temperature, salinity, etc.), the pathway leads to stronger cell walls which also provide structural defenses against invading pathogens.

It is interesting to note that this pathway is antagonistic to the JA pathway via MYC2 (Dombrecht et al. 2007). Also noteworthy is the RNAi pathway that acts against plant viruses and possibly other pathogens. It degrades double-stranded RNA which can occur during viral replication, leaving small interfering RNA (siRNA) molecules that provide specific sequence-based resistance against RNA molecules with homology to siRNAs (Fig. 10.2). Typically, the SA pathway can provide resistance against biotrophic or hemibiotrophic pathogens by mounting an

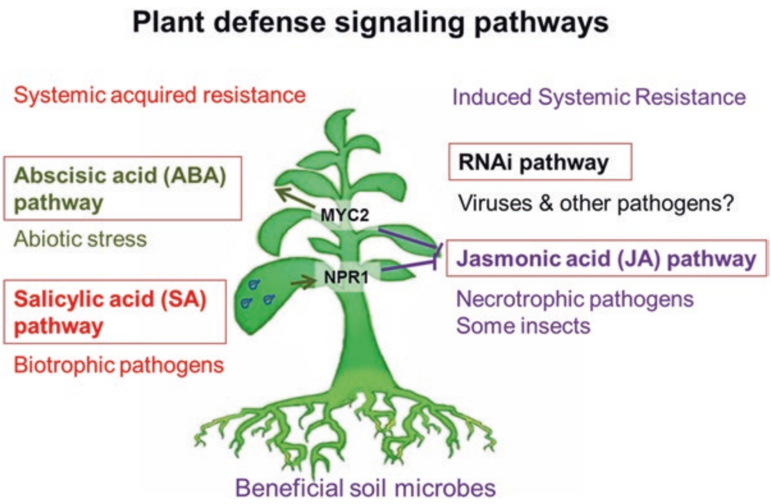

**Fig. 10.2** Overview of plant defense signaling pathways

HR via ROS production that results in programmed cell death and consequently an isolation of the pathogen. This pathway also leads to SAR. Conversely, the JA pathway suppresses programmed cell death but mounts a number of other defense responses that can act directly against necrotrophic pathogens or insects. Interestingly a branch of this pathway is also used by beneficial soil microbes that consequently lead to a state of priming or induced systemic resistance (ISR) that enables the plant to respond faster and stronger to invading pathogens. As an example, the following paragraphs focus on defense responses of the root-infecting fungal hemibiotrophic pathogen *Fusarium oxysporum* (Thatcher et al. 2016).

## 10.8 Defense Against the Root Pathogen *Fusarium oxysporum*

Root pathogens are particularly difficult to control, as they cannot easily be reached by pesticides and they often persist in the soil for many decades. Plant defense against one of these soil-borne pathogens, *Fusarium oxysporum*, has been studied extensively and is described in more detail. More than 100 different plant species are infected by the root pathogen *F. oxysporum,* including cotton, tomato, banana, and *Arabidopsis* (Thatcher et al. 2016). Being a root-infecting pathogen poses some unique challenges in studying the plant-pathogen interaction.

However, a number of scientific studies have established some general methods to study the early infection process and response of the plant, in tomato (Beckman et al. 1989; Gao et al. 1995; Olivain et al. 2006), cotton (Shi et al. 1991; Rodriguezgalvez and Mendgen 1995), banana (Vandermolen et al. 1987; Visser et al. 2004) and *Arabidopsis* pathosystems (Czymmek et al. 2007; Thatcher et al. 2016).

These investigations have uncovered some strategies that the host plant uses to resist the pathogen.

The infection of *F. oxysporum* typically starts from the root toward the vascular tissue, where it proliferates and causes the plant to wilt. The disease symptom progression mainly relies on the extent to which the xylem is blocked or not. Several studies that conducted electron microscopy have indicated that a variety of physical alterations occur in response to infection. For example, the contact parenchyma cells (parenchyma cells adjacent to *F. oxysporum*-infected xylem cells in resistant tomato plants) displayed an expanded cytoplasm opposite the infection (Beckman 2000). In addition, Mueller et al. (1994) have reported that these contact cells deposit apposition layers made of callose to secure the cell wall. According to these studies, the plants can sense the presence of the pathogens in the xylem; therefore, callose-containing apposition layers are synthesized in the nearby cells to inhibit the lateral spread of the pathogen.

Moreover, plants possess numerous armaments to block off longitudinal extension of the fungus. Shi et al. (1992) have showed that after cotton plant infection, the contact cells play an important role to secrete a combination of gels and gums to block off the vasculature. Tyloses can be produced as well to impede the infection. Tyloses are parenchyma cells that balloon out into the lumen through the xylem pits (Vandermolen et al. 1987). Also, a wide range of phenolic compounds is released to support both tyloses and gel-gum secretions. The phenolic compounds oxidize in the xylem and polymerize together to form stable lignified barriers (Beckman et al. 1974). Nevertheless, it is not known if all plants can produce defense mechanisms, such as tyloses or gum secretions, to block *F. oxysporum* infection. Furthermore, more research is needed to determine the signaling pathway that is required to trigger these defense responses.

## 10.9 Defense Against *F. oxysporum* in *Arabidopsis*

Investigating the plant defense signaling network is a worthy alternative approach to understand *F. oxysporum* resistance. These signaling processes can be modified to study which genes may be required for resistance or susceptibility to pathogens. *Arabidopsis* is a good model system to study these defense signaling pathways, as numerous genes encoding mutants of known defense regulators have been studied to assess their involvement in plant defense (Thatcher et al. 2016). SA defense signaling-compromised mutants are typically more susceptible to *F. oxysporum*. For example, the *sid2* mutant, impaired in SA biosynthesis, is more susceptible to *F. oxysporum* f. sp. *conglutinans* (Berrocal-Lobo and Molina 2004; Diener and Ausubel 2005). In addition, treatment of SA to *Arabidopsis* leaves resulted in a partial increase in resistance in these plants (Edgar et al. 2006).

Three *Arabidopsis* R genes, resistance *to F. oxysporum* (*RFO1, RFO2, RFO3*), have also been successfully cloned. Diener and Ausubel (2005) discovered the *RFO1* gene by using a cross between the moderately resistant ecotype Columbia-0 (Col-0) and the susceptible ecotype Taynuilt-0 (Ty-0). *RFO1* encodes a cell wall-associated kinase-like kinase 22 (WAKL22). *RFO1* is an atypical R gene in that it provides broad-spectrum resistance to three dissimilar types of *F. oxysporum* as opposed to a single race. Furthermore, the resistance provided by *RFO1*, *RFO2*, and *RFO3* is quantitative and hence provides only partial resistance.

However, *RFO3* only confers specific resistance to *F. oxysporum* f. sp. *matthioli* and provides no resistance to two other species; *F. oxysporum* f. sp. *conglutinans* or *F. oxysporum* f. sp. *raphani*. A more recent study has shown that *RFO2* provides strong pathogen-specific resistance (Shen and Diener 2013). The extracellular leucine-rich repeats (eLRRs) in RFO2 and the related receptor-like protein (RLP) 2 protein are interchangeable for resistance and very similar to eLRRs of the receptor-like kinase PSY1R, which perceives tyrosine-sulfated peptide PSY1 (Shen and Diener 2013). This study also found reduced infection in the *psy1r* mutant.

Collectively, these findings suggest that *F. oxysporum* produces an effector that prevents the negative feedback regulation of PSY1R, which stabilizes PSY1 signaling and induces susceptibility. On the other hand, RFO2, which acts as a decoy receptor of PSY1R, is also stabilized by the effector and instead confers resistance (Shen and Diener 2013). The *RFO* R genes are unique in that they provide protection to multiple formae speciales of *F. oxysporum* and provide only partial resistance in some host-pathogen combinations. Nonetheless, the process by which these genes provide *F. oxysporum* resistance is still unknown.

Interestingly, there is mounting evidence that plants insensitive to JA are more resistant to *F. oxysporum*. MYC2, a basic helix-loop-helix transcription factor, is a positive regulator of insect defense, wound responses, flavonoid metabolism, and oxidative stress tolerance during JA signaling (Dombrecht et al. 2007). In contrast, MYC2 is thought to act predominantly as a negative regulator of JA-/ET-associated pathogen defense, but the JA signaling-compromised *myc2* mutant shows increased resistance to *F. oxysporum* and has increased SA defense gene expression which could potentially explain the enhanced resistance to *F. oxysporum*. Similarly, the *coi1* mutant that is deficient in JA signaling has also been shown to possess increased resistance to *F. oxysporum* (Thatcher et al. 2009). COI1 (CORONATINE INSENSITIVE1) is an integral part of the SCF (Skp-Cullin-F-box) type E3 ubiquitin ligase complex that acts as co-receptor with the JAZ repressor for JA-ile (Thines et al. 2007; Sheard et al. 2010).

Mutation of *COI1* abolishes JA function, including the expression of JA-associated defense genes, such as *PDF1.2* (Penninckx et al. 1996). Therefore the increased resistance of the *coi1* mutant further suggests that functional JA signaling is required for susceptibility to *F. oxysporum* and that activation of this susceptibility program may overwhelm any benefit of increased JA defense gene expression. Grafting studies performed with the *coi1* mutant showed that the root section is required for resistance/susceptibility to *F. oxysporum*. A *coi1* rootstock grafted to a WT scion resulted in almost complete resistance, suggesting that roots play a significant role in plant defense against *F. oxysporum*.

Similarly, Kidd et al. (2009) found that the MEDIATOR25 (MED25) subunit of the plant mediator complex which is also known as *PFT1* (*PHYTOCHROME AND FLOWERING TIME1*) is required for jasmonate-dependent defense gene expression and resistance to leaf-infecting necrotrophic fungal pathogens. A MED25 overexpressing line is revealed to be highly susceptible to *F. oxysporum*, while *med25* mutants display reduced defense gene expression, yet increased resistance to *F. oxysporum.* Collectively these findings suggest that *F. oxysporum* hijacks the non-defensive aspects of JA signaling leading to wilt disease symptoms and plant death in *Arabidopsis*.

Pantelides et al. (2013) showed that mutant receptor 1 ethylene (*etr1-1*) plants exhibited less *Fusarium* wilt symptoms compared to wild-type Col-0 plants. Quantitative PCR analysis correlated the reduction in symptom severity in *etr1-1* plants with reduced vascular growth of the pathogen, indicating the activation of defense mechanisms in *etr1-1* plants against *F. Oxysporum* in the roots. Hence, this study suggested that *F. oxysporum* also requires ETR1-mediated ethylene signaling to promote disease development in plants.

## 10.10 Progress on the use of PGPR as Biocontrol Agents Against Root Pathogens

Soil and rhizosphere microbes potentially offer solutions to soil-borne pathogens. As discussed in the previous section, PGPR possess a variety of mechanisms to act as biocontrol agents against phytopathogens and therefore also indirectly stimulate plant growth (Patel et al. 2016). This allows them to potentially be used as biocontrol inoculants in agriculture. Intensive biotechnological research showed that they possess additional biochemical features such as metabolic versatility, excellent root colonization, outcompeting pathogens for nutrients and iron, and also the ability to produce a diversity of antimicrobial peptides and secondary metabolites. However, probably the most important drawback is that the practical application of PGPR in general has been inconsistent in agricultural field conditions (Arora and Mishra 2016).

There are many scientific publications regarding the effective experimental use of PGPR as biocontrol agents under laboratory and other environmentally controlled conditions, but only a few showed consistencies in efficiency under field conditions. In addition, timing seems to be critical, as they typically are not effective under conditions of high pathogen abundance and disease pressure. Previous studies have shown that plant-root inhabiting bacterial groups from the genera *Bacillus*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Serratia, Azotobacter*, *Burkholderia*, *Enterobacter*, and *Xanthomonas* are effectively involved in plant disease suppression and play an important role in improving overall plant health (Whipps and Lumsden 2001). Some of the best-studied biocontrol agents are from the *Pseudomonas* species (Ahmad et al. 2008).

Multiple biocontrol strains of *Pseudomonas* have proven to produce antifungal metabolites, such as phenazines, pyrrolnitrin, and pyoluteorin. Research carried out by Ahmad et al. (2008) has demonstrated that these compounds prevent further infection in sugar beet by *Pythium ultimum* (Laslo et al. 2012). The research also examined the antimicrobial effect of 47 bacterial isolates against various plant pathogens, such as *Alternaria* sp. and *Fusarium* sp. The results showed varied levels of pathogen inhibition against both fungi. For 16 soil bacterial strains, the inhibition rate was over ~80%. The highest detected antifungal activity was reported for bacterial isolates originating from the rhizosphere (Laslo et al. 2012).

The increasing demand for production of agricultural crops and food in response to the ever-increasing human population has led to further development of biocontrol agents to sustain crop productivity and plant health. Therefore, in the past decade, PGPR have been used commercially as biocontrol agents to a limited extent, in a number of different agricultural, horticultural, and environmental systems worldwide (Glick et al. 2007). However, this limited progress of commercial use is due to the variability and inconsistency of results between laboratory, greenhouse, and field trials. In addition, other limitations include the recent multifactorial basis for biocontrol in plants. Studies carried out by Kim et al. (2011) have demonstrated that such active biocontrol bacteria do not act individually in disease suppression but instead perform combined antagonistic actions as multiple bacterial populations. In view of this, formulations of combinations of different biocontrol agents are carefully being tested to ensure optimal impact of these products when used in large-scale agricultural crops. At present, many products based on biocontrol agents are available (Table 10.2).

However, commercial developments of products must fulfill several requirements, including safety, production costs, and large applicability to major crops effective against a broad range of pests or pathogens. Hence, a prerequisite to successful biocontrol of plant pathogens is knowledge of the regulation of antimicrobial and antifungal metabolite production by antagonists in biocontrol organisms in response to environmental factors including other microorganisms. In addition, a comprehensive profiling of the population dynamics of bacterial rhizosphere

**Table 10.2** Examples of current commercial biocontrol products available in the market for use against plant pathogens (adapted from Jan et al. (2011)

| Product | Biocontrol | Crops | Target | Manufacturer |
|---|---|---|---|---|
| AtEze | *Pseudomonas chlororaphis* | Ornamentals and vegetables | Wilt diseases, stem and root rots | Turf Science Laboratories |
| Bio-save 10LP | *Pseudomonas syringae* | Pome fruits, citrus, cherries, and potatoes | *Botrytis cinerea*, *Penicillium* sp., *Mucor piriformis*, *Geotrichum candidum* | JET Harvest Solutions |
| Frostban A, B, C, D | *Pseudomonas fluorescens*A506 and 1629RS<br>*Pseudomonas syringae* 742RS | Almond, potato, tomato | Frost-forming bacteria | Frost Technology Corporation |
| Kodiak | *Bacillus subtilis* GB03 | Cotton, legumes | *Fusarium* sp., *Alternaria* sp., *Aspergillus* sp. | Gustafson, Inc. |
| Mycostop | *Streptomyces griseoviridis*K61 | Ornamentals and vegetables | *Fusarium* sp., *Alternaria brassicola*, *Phytophthora* sp. | AgBio, Inc. |

communities that trigger efficient root colonization is needed (Jan et al. 2011). An important emerging area for sustainable agriculture is to develop metabolites and additives for bioformulation that provide stability of biocontrol agents for more consistent and reliable performance (Arora and Mishra 2016).

## 10.11 Induced Systemic Resistance (ISR) as a Mechanism of Disease Suppression by Rhizobacteria

One particular focus in phytopathological studies is to understand how plant-associated microbes can link to the three main molecular defense pathways in plants characterized by their signaling molecules SA, JA, and ET. As discussed in previous sections, PGPR possess multiple biocontrol activities and may suppress soil pathogens through antagonistic interactions and/or by inducing a systemic resistance against above- and belowground pathogens (Patel et al. 2016). The generally unspecific properties of induced resistance can establish basal resistance against several pathogens simultaneously, which is useful under natural conditions when plants can be under attack by multiple pathogens at the same time. Several *Pseudomonas* strains induce systemic resistance in carnation, cucumber, radish, tobacco, and *Arabidopsis*, where they enhance plant defense capabilities (Van Loon and Bakker 2005).

Some bacterial strains can equally induce resistance in different plant species, while others are specific, suggesting specific recognition between plants and bacteria at the root surface. For example, the O-antigenic side chain of the bacterial lipopolysaccharide (LPS) can be recognized by the plant in *Arabidopsis*, and pseudobactin siderophores have also been linked to signaling leading to resistance in tobacco and *Arabidopsis*. Similarly, pseudomonine, another siderophore, has been associated with SA-mediated induction of resistance in radish. Although SA signaling leads to similar phenotypes as SAR, it is not a requirement for ISR by most rhizobacterial strains. Instead, rhizobacteria-mediated ISR requires JA- and ET-signaling pathways. *Arabidopsis* leaves expressing ISR are primed to express JA/ET, but not SA-responsive genes, while leaves expressing SAR after pathogen challenge exhibit a primed expression of SA, but not JA-/ET-defense signaling. Interestingly, a combination of ISR and SAR can enhance resistance against pathogens that are defended through both pathways and further extend protection to a wide spectrum of pathogens (Van Loon and Bakker 2005; Pieterse et al. 2000).

## 10.12 Conclusions and Future Directions

Plants have evolved sophisticated mechanisms to defend themselves against soil-borne pathogens. Although roots have not been studied as well, recent studies indicate that plant defense mechanisms easily cross the root-shoot interphase, and those secondary metabolites, structural alterations, and root exudates are the main defense mechanisms. Root pathogens, such as *F. oxysporum*, on the other hand seem to actively alter defense responses in their favor. For example, hemibiotrophic *F. oxysporum* induces the JA pathway during its early interaction, therefore suppressing successfully the SA pathway that otherwise would have provided an effective strategy for disease resistance to the plant via ROS production, HR, and programmed cell death. The notion that plants can specifically attract beneficial "soldier" microbes to assist roots with plant defense has been recently proposed by Carvalhais et al. (2013, 2015).

A better understanding of the processes underlying this specific microbial recruitment to the rhizosphere may provide future opportunities for controlled microbiome engineering. These "plant-optimized microbiomes" may not only assist in biocontrol of soil-borne diseases but may also provide the other well-known growth-promoting functions from PGPR. Another important untapped area is the breeding of crops that are better adapted to benefit from rhizosphere microbes. Future research may focus on the identification of genetic markers in crops that are linked to beneficial interactions. This would enable breeding programs toward "microbe-optimized plants" which may not only have higher yields but may also display higher resilience to changing environmental conditions.

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# Attempts for Biological Control of *Ralstonia solanacearum* by Using Beneficial Microorganisms

# 11

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**Abstract**

Bacterial wilt pathogen *Ralstonia solanacearum* causes catastrophic loss in different plants across the genera and climatic conditions. It has a huge genetic diversity which affects tropical, subtropical, and warm temperate region. Apart from solanaceous plants, it affects a vast array of many other plant species. Wide host range and its survival capacity in various environments such as irrigation water and soil make it difficult to control *R. solanacearum*. Host resistance breakdown due to high genotype and environment interactions was frequently encountered. Therefore, integrated approach combining host plant resistance and cultural and biological control measures seems effective. Although excellent attempts have been made in management of *R. solanacearum*, still there is great opportunity to contribute to this problem for a stable solution. Varied chemical, cultural, agronomical, biological, biotechnological approaches, etc. have been used in addressing problem of *Ralstonia* with different levels of success. Biocontrol of *R. solanacearum* by different microorganisms has great potential. Microbes like *Bacillus*, *Pseudomonas*, *Azotobacter*, *Streptomyces*, etc. have been found suitable in suppressing bacterial wilt. This chapter focuses on different approaches of *R. solanacearum* biocontrol like the use of arbuscular mycorrhizal (AM) fungi, bacterial endophytes, bacteriophages, bacterial volatile compounds, chitosan, silicon, etc. in detail. It also briefs about present scenario of *R. solanacearum* control with future potential to be achieved.

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**Keywords**

*R. solanacearum* • Biocontrol • AM fungi • Bacterial endophytes • Silicon • Chitosan

## Contents

## 11.1 Biological Control of Plant Pathogens

Nowadays the agriculture is experiencing one of the very serious hurdles to increase production with declining/degrading resources. The commercialization in agriculture has brought about detrimental impacts like environmental pollution, resistance buildup in pests, high cost of cultivation, ecological imbalance in food chain, health hazards, etc. (Srivastava et al. 2014) by excessive use of agrochemicals. The use of these harmful chemicals can be replaced by suitable biocontrol agents. This approach has many successful paradigms (Guo et al. 2004; Yang et al. 2012; Feng et al. 2013; Maji and Chakrabartty 2014; Raza et al. 2016). Replacement by these biocontrol agents (BCAs) at economically viable ratio, a lot of chemicals can be precluded from pouring into the food chain and ecosystem. The farmers too are getting aware of this kind of practices as there are visible losses in crop production due to degrading resources.

The biological control is the use of biological agents such as microbial antagonists for suppression of diseases and the use of predatory insects, entomopathogenic microorganisms, nematodes, etc. for management of insect pests (Pal and Gardener 2006). It is also recognized as a crucial part of integrated pest management (Patro et al. 2013) and plant disease management (Harman 2006). In ecosystem, biological control is a result of complex interactions among the organisms like mutualism, competition, commensalism, etc. (Chisholm et al. 2006); varying atmosphere also adds to the complexity of the scenario. Biocontrol approach is utilizing naturally occurring interactions by microbes and its ecosystem. Losses from plant pathogens make a great hurdle in crop production; shrinking resources and degrading environment add to the difficulty. A global loss of ~20–40% in agricultural productivity comes from pathogens, weeds, and animals (Teng and Krupa 1980; Oerke 2006). Therefore, a strong but ecologically sustainable control measure is need of the hour. In crop improvement programs, crop protection is one of the major concerns along with improving production to reach the goal of food quality and quantity (Strange and Scott 2005). Serious attention is required in improving biocontrol efficiency of BCAs to attain the quality and quantity in crop production.

Biological control comprises of conserving, manipulating, and introducing natural enemies like dragonflies, coccinellids, spiders, chrysopids, wasp (Modi 2012), and microbial agents like bacteria (*Bacillus*, *Pseudomonas*, *Burkholderia*, *Streptomyces*) and fungal (*Metarhizium*, *Trichoderma*, *Ampelomyces*, *Beauveria*, *Paecilomyces*, *Dactylella*, *Gliocladium*, etc.) genera (Pal and Gardener 2006). This bacterial wilt caused by *Ralstonia solanacearum* is a devastating disease of solanaceous crops (Kelman 1998). Various methods have been developed, but controlling this pathogen is still a problem. The biological control in integration with other control measures can be a useful solution for controlling bacterial wilt disease (Sinha et al. 2012; Pieterse et al. 2014). This strategy is helpful in developing alternative management plans in integrated manner for effective disease management of *Ralstonia* wilt at field level. This approach will also be useful in reducing the environmental pollution caused by excessive use of harmful agrochemicals.

## 11.2 *Ralstonia solanacearum*: Distribution and Classification Systems

### 11.2.1 Introduction and Distribution

*R. solanacearum* (Smith 1896; Yabuuchi et al. 1995) is a gram-negative, β-proteobacteria, nonsporulating, aerobic, rod-shaped, soilborne, motile bacterium with a polar tuft of flagellum. It causes wilt diseases in plants by invading through xylem vessels (Genin 2010). Mode of infection is through roots and natural openings (Vasse et al. 1995). It colonizes the root surface and then invades xylem vessels by degrading the cell wall and produces ample quantities of exopolysaccharides which in turn block water flow causing chlorosis, wilting of plants, and eventually plant death. Its ability to persist in soil and infest plant parts adds to the difficulty in elimination of inocula (Genin and Denny 2012).

It occurs widely in tropical, subtropical, and warm temperate parts of the globe (Liu et al. 2009) and causes catastrophic loss in major crops like tomato, eggplant, potato, tobacco, pepper, banana, peanut, ginger, etc. (Peeters et al. 2013). It has a powerful and tissue-specific tropism in host plants, specifically invading and rapidly multiplying in the xylem vessels. It has an extensive host range of ~450 crop species (Swanson et al. 2005) across 54 families (Wicker et al. 2007). In addition, *R. solanacearum* Biovar 3 also found affecting woody perennials including cashew in Indonesia (Shiomi et al. 1989) and custard apple (Mayers and Hutton 1987) and Alexandra palm (Akiew and Hams 1990) in Australia. This bacterium has got unusually wide host range which offers a unique opportunity for researchers in analyzing strong and generalized range of virulence factors. It can easily survive in soil in crop debris when host plant is not available (Granada and Sequeira 1983); thereby it provides an efficient system in investigating functions which are responsible for adaptation to such ecological niche.

It has a 5.8 megabase (Mb) genome which consists of a megaplasmid (2.1 Mb) and chromosome (3.7 Mb). The presence of mosaic structure in genome indicates occurrence of horizontal gene transfer. This acts as model system in dissecting molecular determinants which govern pathogenicity.

### 11.2.2 Classification Systems

There is huge genetic diversity in *R. solanacearum* (formerly *Pseudomonas solanacearum*, Yabuuchi et al. 1995) across the globe, and thus it is termed as *R. solanacearum* species complex (RSSC). These marked differences in geographical distribution suggest separate evolutionary development. The variability in *R. solanacearum* species complex is classified by different workers on the basis of different criteria like host range, utilization of different carbon sources, phylogenetic relationship, etc. (OEPP 2004).

#### 11.2.2.1 Based on Host Range: Five Races (Buddenhagen et al. 1962; He et al. 1983)

- *Race 1*: Found in tropical areas and have high temperature optima of 35 °C. It is pathogenic to broad range of hosts, including tomato, tobacco, peanut, etc.
- *Race 2*: Found in tropical region of South America, have high temperature optima of 35 °C, and causes disease in banana and *Heliconia* (Moko disease) in South America and Bugtok disease in the Philippines.
- *Race 3*: Found in high altitudes of tropic, subtropical, and warm-temperate region and have low temperature optima of 27 °C. It affects tomato, potato, *Pelargonium zonale*, aubergine, capsicum, and few other plants including weeds which often harbor *R. solanacearum* asymptomatically (Pradhanang et al. 2000).
- *Race 4*: Found in tropical areas, have high temperature optima of 35 °C, and devastating pathogen of ginger.
- *Race 5*: Found in tropical areas and have high temperature optima of 35 °C. It infects mulberry plant (*Morus alba*).

#### 11.2.2.2 Based on Utilization/Oxidation of Certain C Sources

This classification is based on utilization of three carbohydrates (cellobiose, maltose, and lactose) and oxidation of three alcohols (dulcitol, sorbitol, and mannitol). It divides *R. solanacearum* species complex into six biovars (Hayward and Hartmann 1994; Xue et al. 2011; Chandrashekara et al. 2012). Biovar 1 is predominant in America and not found in most parts of Asia. Biovar 3 is nutritionally most versatile and predominant in Asia.

#### 11.2.2.3 Based on Phylogenetic Analysis

Various genetic techniques have been used to investigate the diversity and interrelatedness within the RSSC (Denny 2006). Based on multiplex polymerase chain reaction and large-scale DNA sequence analyses, the RSSC is grouped into four major subdivisions, and they are denoted as phylotypes (Fegan and Prior 2005).

The “phylotypes” correspond roughly to the strains of geographic origin:

- *Phylotype I*: Asia
- *Phylotype II*: America
- *Phylotype III*: Africa
- *Phylotype IV*: Indonesia

Phylotype II has two clearly recognizable sub-clusters (IIA and IIB) (Prior and Fegan 2005; Villa et al. 2005). These phylotypes are not based on host range, so all phylotypes are capable of causing disease in crops like tomato, potato, eggplant, pepper, etc. (Lebeau et al. 2011).

#### 11.2.2.4 Based on Endoglucanase (Egl) Gene Sequence

Phylotypes of *R. solanacearum* is further subdivided into sequevars (Fegan and Prior 2005; Prior and Fegan 2005). This genetic fingerprinting on the basis of *egl* gene sequence has identified 51 sequevars so far (Xu et al. 2009).

#### 11.2.2.5 Based on RFLP and Other Genetic Fingerprinting Studies

There are two divisions (Hayward 2000):

- *Division I*: Contains Biovars 3, 4, and 5; Asian origin
- *Division II*: Contains Biovars 1, 2A, and 2T; South American origin

#### 11.2.2.6 New Classification into Three Species (Genin and Denny 2012)

- *Ralstonia sequeirae* (Phylotype I and III)
- *R. solanacearum* (Phylotype II)
- *R. haywardii* (Phylotype IV, *R. celebensis* and *R. syzygii*)

### 11.2.3 Losses Due to *Ralstonia solanacearum*

*Ralstonia solanacearum* is one of the most destructing plant pathogens due to rapid and fatal wilting in host plants (Kelman 1998). A wider host range coupled with vast genetic variability contributes in its extensive distribution throughout the world and causes serious damage across wider genera and ecological niches with catastrophic economic impact (Yao and Allen 2006). Sources of inoculum spread are irrigation water, agricultural tools and equipment, weeds, infested soil, contaminated and latently infected planting material, insects, nematode, etc. In infected field, pathogen moves from roots of infected plants to roots of healthy plants (Stevenson et al. 2001; Ji et al. 2004).

Entry occurs through wounded parts and places of lateral root emergence (Agrios 1997). It multiplies and spreads to the entire plant via large xylem vessels. It then enters to parenchyma and pith. After dissolving the cell wall, it makes slimy pockets of bacteria and cell debris. Excessive production of polysaccharides increases the viscosity of xylem, and as a result, plugging of xylem vessels occurs (Shew and Lucas 1991). Finally it withers the plant and kills them. Yield losses depend on host plant, cultivation practices, humidity, soil, cropping pattern, weeds, strain and virulence of pathogen, etc. *R. solanacearum* causes yield losses up to 90% in tomato, 33–90% in potato, 10–30% in tobacco, 80–100% in banana, and 20% in groundnut (Elphinstone 2005). The difficulties in controlling *R. solanacearum* are also associated with its abilities to grow as endophyte, survive in deep layers of soil and latent infections in weeds, and ease of traveling along with water (Wang and Lin 2005).

### 11.2.4 Chemical Control Measures

Different chemicals were tested against bacterial pathogens of tomato. The use of antibiotics like streptomycin and oxytetracycline has shown promising results in effectively protecting tomato fruits against *Pseudomonas syringae* pv. tomato causing bacterial speck (Jardine and Stephens 1987). Control of bacterial speck and bacterial spot diseases in tomato was also attempted using copper-related chemicals (Saad and Abul Hassan 2000). Despite being harmful for the environment, there is no much success in eradicating *R. solanacearum* (Saddler 2005; Denny 2006). This has led to the search for alternatives for control of bacteria such as antimicrobial plant extracts and defense activator. Although varied attempts had been taken for chemical control of other bacterial diseases of tomato, there are only a few success stories for appropriate control measures of bacterial wilt disease that are available.

As per the findings of Enfinger et al. (1979), fumigation by compounds like chloropicrin was efficient in controlling *Ralstonia* wilt of tomato. Some defense activator compounds including acibenzolar-S-methyl found enhancing disease resistance against bacterial wilt in tomato (Pradhanang et al. 2005). Vitamin B2 (riboflavin) is recently identified as a plant defense activator for *R. solanacearum* infection in tobacco (Liu et al. 2010). The use of pesticides is at its incipient stage in case of bacterial wilt. As compared to 14 pesticides approved for controlling bacterial spot, only one (dazomet) was used for bacterial wilt in South Korea till 2010. Therefore, the loss occurring at field level is huge (Liu et al. 2010).

In geranium plants, drenching with phosphorous acid salts is found effective for protection against infection by *R. solanacearum* (Norman et al. 2006). Few more chemical control measures were found efficient in controlling bacterial wilt at greenhouse and up to some extent at field level are Actigard (acibenzolar-*S*-methyl), phosphorous acid (Pradhanang et al. 2005; Norman et al. 2006; Ji et al. 2007), and soil fumigation using methyl bromide, Vapam, chloropicrin (Enfinger et al. 1979), etc. Preplant soil fumigation with plant-derived volatile compound like thymol was reported to reduce incidence of bacterial wilt effectively on tomato (Ji et al. 2005).

Dannon and Wydra (2004) reported significant reduction in bacterial wilt incidence by silicon amendment in tomato. There was a negative correlation between silicon content in roots and number of *R. solanacearum* in midstems of tomato genotypes "Hawaii 7998" and "King Kong 2." This indicates importance of silicon in inducing resistance. There were different chemical measures tested against bacterial wilt of tomato, but for more efficient control, it may be achieved by using integrated disease management approach.

## 11.3 Biological Control of *R. solanacearum*

Biological control approach is not only helpful in suppressing disease but also reduces harmful agrochemical load and avoids environmental pollution. Biological control of *Ralstonia solanacearum* is still in research phase with few success stories (Van Overbeek et al. 2002; Messiha et al. 2007), and improvement is required for successful integrated disease management. Several antagonistic biocontrol agents have been evaluated against *Ralstonia solanacearum* but with variable success. Microbial antagonists like *Candida ethanolica* have shown its efficacy in controlling bacterial wilt at research level (Lwin and Ranamukhaarachchi 2006). Suppressive effect of few antagonists on *R. solanacearum* was also reported (Toyota and Kimura 2000). Furthermore, field experiments by Ciampi-Panno et al. (1989) concluded that antagonistic microbes were effective in suppressing bacterial wilt caused by *R. solanacearum*.

However, the application of commercial mixture containing effective microorganisms (EM) was found suppressing bacterial wilt pathogen by Lwin and Ranamukhaarachchi (2006). Many efficient microorganisms have been identified in further studies having potential of suppressing bacterial wilt, but evaluation of their effectiveness is yet to be proven in the field (Hoang et al. 2004). These EMs are a mixture of microorganisms (photosynthetic bacteria, *Azotobacter*, *Streptomyces*, and *Lactobacillus* sp.) for plant growth promotion (PGP) activities which increase the crop yield and protect from pathogens (Higa 1996). These EMs enhance yield by improving photosynthesis, $N_2$ fixation, and suppression of soilborne diseases and accelerate lignin degradation in the soil (Hussain et al. 1993; Javaid et al. 2000; Nguyen and Ranamukhaarachchi 2010).

### 11.3.1 Bacteria-Mediated Biological Control of *R. solanacearum*

Apart from antagonism, induced systemic resistance (ISR) is a well-proven defense mechanism caused by microorganisms for protection against plant pathogens (Pieterse et al. 2014). Various other effects like fixation of $N_2$, P solubilization, secretion of growth hormones, siderophore production, etc. add to the protection of plants (Persello-Cartieaux et al. 2003). Studies indicated greater possibilities of biocontrol of race 3 of *R. solanacearum* by the use of microbes like *Pseudomonas fluorescens*, *Bacillus subtilis*, *Bacillus pumilus*, *Paenibacillus macerans*, and *Serratia marcescens* (Kurabachew et al. 2007; Alyie et al. 2008). *Bacillus subtilis* has been reported as an effective biocontrol agent of *R. solanacearum* race 1 Biovar 3 (Sinha et al. 2012).

As per the findings of Kurabachew and Wydra (2013), they characterized 13 plant growth-promoting rhizobacterial (PGPR) isolates as antagonist to *R. solanacearum* which were identified as *Pseudomonas* sp. *Serratia marcescens* and *Bacillus cereus*. Four promising isolates, viz., *B. cereus* BC1AW, BC2BA, BC3AW, and BC4SS and *Pseudomonas putida* PP3WT, were further tested in pot experiment for disease suppression. Two of these isolates, BC1AW and PP3WT significantly reduced the incidence of bacterial wilt (~47 and 45%, respectively, in moderately resistant genotype; ~34 and 30%, respectively, in susceptible genotypes) in tomato.

Antagonism of different bacterial isolates was assessed in greenhouse experiment against *R. solanacearum* causing wilt of four ginger isolates, viz., *Bacillus subtilis* strain 1JN2, *Myroides odoratimimus* 3YW8, *Bacillus amyloliquefaciens* 5YN8, and *Stenotrophomonas maltophilia* 2JW6, that were showing ~50% biocontrol efficiency against ginger wilt (Yang et al. 2012). Endophytic bacteria which confer a wide range of benefits to plants, like biological nitrogen fixation enhance nutrient uptake (Malinowski et al. 2000), production of anti-herbivory substances (Sullivan et al. 2007), plant growth promotion (Kang et al. 2007), induce ISR, reduce disease severity (Kloepper et al. 2004), etc. The use of these endophytic microorganisms has also been reported to control *R. solanacearum*, but field level results are inconsistent (Almoneafy et al. 2012). There are many literature describing potential uses of endophytes in the form of plant growth and health managers (Hoffland et al. 1995; Sturz and Nowak 2000; Berg 2009).

It was reported that endophytic colonization enhances the ability of microbes to act as biocontrol agent (Timmusk et al. 2005). It was also evident from a study that there is a correlation between diversity of endophytic bacteria and plant resistance (Sturz et al. 1999; Araujo et al. 2002; Feng et al. 2013). However, detailed mechanism behind this is yet to be explored. There are detailed reports available on endophyte-mediated biological control of *R. solanacearum* (Almoneafy et al. 2012; Feng et al. 2013). Ramesh and Phadke (2012) reported that talc formulation of endophytic bacteria belonging to genera *Pseudomonas* and *Bacillus* was effective in suppressing eggplant wilt caused by *R. solanacearum*. As per the finding of Wang and Liang (2014), 35 endophytic bacterial isolates from peanut are screened against peanut bacterial wilt caused by *R. solanacearum.* Out of eight antagonists, one endophyte *Bacillus amyloliquefaciens* BZ6-1 showed highest antimicrobial activity.

In pot experiment, the addition of 10 mL inocula containing $10^8$ CFU $mL^{-1}$ has got disease incidence of ~24% as compared to ~85% in control. Production of antimicrobial substances surfactin and fengycin was confirmed by HPLC analysis in BZ6-1.

However, the suitability of exogenous application of PGPR was also observed in controlling bacterial wilt in tomato (Xue et al. 2009; Nguyen and Ranamukhaarachchi 2010). Xue et al. (2009) tested two potential bacterial strains Xa6 (*Acinetobacter* sp.) and Xy3 (*Enterobacter* sp.) against *R. solanacearum* under field condition. In two field experiments, biocontrol efficacy of Xy3 was ~65% in both field trials, and the yield increases caused by Xa6 were ~32 and 41%, respectively, in the two trials. This is the first report of an *Acinetobacter* sp. strain used as a BCA against *Ralstonia* wilt of tomato.

There are also reports on controlling *R. solanacearum* in tomato by addition of *Bacillus amyloliquefaciens*-fortified organic fertilizer (Wei et al. 2011). This bioorganic fertilizer was found suppressing bacterial wilt in greenhouse as well as in field condition, although the latter was dependent on crop season. The suppressive effects were more on spring crop than on autumn crop. Several bacterial agents were found impressive like, *Bacillus* sp. (Guo et al. 2004), *Pseudomonas* sp. (Chakravarty and Kalita 2012; Maji and Chakrabartty 2014), *Serratia* sp. (Guo et al. 2004), avirulent mutants of *R. solanacearum* (Frey et al. 1994), *Acinetobacter* and *Enterobacter* sp. (Xue et al. 2009), *Stenotrophomonas maltophilia* (Messiha et al. 2007a), and actinomycetes (Tan et al. 2006), but most of the studies were either lab based or greenhouse based.

### 11.3.2 Fungi-Mediated Biological Control of *R. solanacearum*

Although less studies are available for control of bacterial wilt by arbuscular mycorrhizal (AM) fungi, in the Philippines, increased yield of tomatoes and reduced infection by *R. solanacearum* were reported. AM fungus *Glomus versiforme* showed some proficiency against *Ralstonia* wilt disease. In plants, defense system was found activated by a number of other fungal populations against the soilborne plant pathogen (Shoresh et al. 2010), and also microbial antagonism by competition, parasitism, and production of antimicrobial substances also plays a major role in suppressing the pathogens (Benhamou et al. 1999; Cameron et al. 2013).

“Q” strains of *Trichoderma* (*Gliocladium) virens* were effective as biocontrol agent for cotton seedling disease caused by *Rhizoctonia solani* in both greenhouse and field studies (Howell 2003; Vinale et al. 2008). *Trichoderma harzianum* is also inducing systemic resistance in plants (Hoitink et al. 2006; Alizadeh et al. 2013) apart from direct confronting pathogens by parasitism and antibiosis (Howell 2003).

In plants like tomato, sugar beet, and wheat, *Pythium oligandrum* induces disease resistance against different fungal pathogens, but it is nonspecific in affording higher protection (Benhamou et al. 1997; Takenaka et al. 2003). Induction of resistance to pathogens is characterized in tomato, and two elicitor molecules were reported. Oligandrin, a ~10 kDa elicitin-like protein secreted by *P. oligandrum*, acts as elicitor (Picard et al. 2000).

Oligandrin induces resistance against *Phytophthora parasitica* by triggering cytological and biochemical modification in tomato cells (Picard et al. 2000). Signal transduction in oligandrin-induced resistance still needs to be exemplified (Takenaka et al. 2003). Two major proteins POD-1 and POD-2 were extracted as a novel elicitin from cell wall of *P. oligandrum*. Cell wall protein elicitin has a major role in *P. oligandrum* mediated inducing systemic resistance. Treatment of tomato roots with mycelial homogenate of *P. oligandrum* reduced severity of bacterial wilt caused by *R. solanacearum* (Hase et al. 2006). The mycelia homogenate acts as elicitor in activating defense response against *R. solanacearum* in tomato by activating ethylene-, salicylic acid-, and jasmonic acid-dependent signaling pathways (Hase et al. 2008). This was confirmed by the finding that salicylic acid non-accumulator *NahG* mutant does not show any such effects on *R. solanacearum*.

### 11.3.3 Biotechnological Approaches for Biological Control of *R. solanacearum*

Lytic phages were also tested for biocontrol of *R. solanacearum*. Fujiwara et al. (2011) applied three phages (ϕRSA1, ϕRSB1, and ϕRSL1) in different combinations. A rapid decline in host bacterial cell density after infection with ϕRSA1 and ϕRSB1was reported. The penetration, growth, and movement of the pathogen inoculated in roots were drastically limited in tomato seedlings pretreated with ϕRSL1, and these plants have not shown any symptoms of wilting. The untreated plants wilted by 18 days of inoculation. The phage ϕRSL1 was relatively stable in soil even at higher temperatures of 37–50 °C, and active particles have been recovered from soil after 4 months of inoculation. This may be one of the strategies for biocontrol of bacterial wilt at field level.

The use of wild relatives is a great source of genetic variability for disease resistance. A set of introgression lines (ILs) developed by Cornell University using *Solanum habrochaites* accession LA1777 were crossed to CLN2498E line (resistant to bacterial wilt) to make IL hybrid. Performance of the hybrid (Hchk) was found highly significant in increasing tomato yield in Taiwan and Thailand (Hanson et al. 2007).

Few more approaches suggested for biocontrol of bacterial wilt by using genetically engineered *Hrp* mutants of *R. solanacearum* and other antagonistic bacteria (Park et al. 2007). Avirulent mutant of *R. solanacearum* (*Hrp*$^{-}$) has also been used to control virulent strains in tomato and potato, but the appropriate success at field level is still awaited (saddler 2005). The source of genetic resistance to *R. solanacearum* was found to be polygenic, and QTLs have been identified in tomato, tobacco, and eggplant (Qian et al. 2013; Lebeau et al. 2013). Despite this, there is difficulty of transferring more number of genes by breeding approach which may be linked to some non-desirable traits (Denny 2006). In tomato, strain-specific polygenic bacterial wilt-resistant cultivar Hawaii 7996 (Wang et al. 2000) may also be helpful in phylotype-specific resistance (Carmeille et al. 2006). Understanding of induced systemic resistance against wilt pathogen may also be beneficial in preventing disease outbreak. Some of the effectors of *R. solanacearum* has been identified (Deslandes and Genin 2014); some of them have shown hypersensitive response (HR) in *Arabidopsis*, tobacco, and eggplant (Sole et al. 2012; Williams et al. 2014; Nahar et al. 2014). Study and manipulation in these effectors can be a useful tool in identifying new sources of resistance for controlling *R. solanacearum* (Vleeshouwers and Oliver 2014). Stacking of R-genes will also help in durable resistance against *R. solanacearum* (Dangl et al. 2013).

Usually resistance to *R. solanacearum* is a polygenic trait, but single R-gene can also trigger immunity against it. A pair of R-gene, RRS1 and RPS4, has been found triggering immunity against bacterial wilt (Narusaka et al. 2009; Williams et al. 2014). Transferring these genes from *Arabidopsis thaliana* to tomato conferred immunity to bacterial wilt caused by *R. solanacearum* (Narusaka et al. 2013).

### 11.3.4 Management of *R. solanacearum* by Biofumigation Approach

Biofumigation is one of the successful method of integrated pest management which works on the principle of exploiting the natural biocide compounds from high glucosinolate (GSL)-containing plants (Kirkegaard et al. 1998) to reduce the levels of several soil pathogens, including bacterial wilt of tomato (Ji et al. 2005). The hydrolyzed products of isothiocyanates (ITC) are known to have broad-spectrum biocidal property like insecticidal, nematicidal, fungicidal, antibiotic, etc. (Brown and Morra 1997). Glucosinolates are commonly found in all vegetative and reproductive parts of *Brassica* (Buskov et al. 2002). The efficacy of biofumigation depends on the specific glucosinolate hydrolysis products formed during tissue breakdown. Therefore, different biofumigant crops have different capacities of pathogen control (Motisi et al. 2009).

Akiew et al. (1996) and ACIAR (2000) reported that tissues of mustards (*B. juncea*, *B. nigra*, and *B. carinata*) were more suppressive than those of other *Brassica* sp. tested and suggested that the 2-propenyl GSL is the active compound. This compound is also present in kale, and cabbage residues may also have activity against bacterial wilt. Incorporation of some *Brassica* fumigants has reduced bacterial wilt by ~50–60% in three of the four experiments of ACIAR in 2002 compared to non-*Brassica* controls.

Effect of biofumigation with thymol and palmarosa oil on *R. solanacearum*-infected soil was studied by Ji et al. (2005). Artificially infected fields were inoculated with thymol and palmarosa oil at concentration of 0.7% and sealed with plastic mulch for 1 week. In this treated plot, tomato seedlings were transplanted.

In untreated plot, 92.5% of plants were wilted as compared to 33.1 and 48.1% in thymol- and palmarosa oil-treated plots, respectively.

Raymond et al. (2014) reported that raw seeds of mustards contain the highest levels of glucosinolates. Allyl isothiocyanate was found as the major breakdown product by reaction catalyzed by exogenous myrosinase enzyme, and it has antimicrobial effect. Elizabeth (2012) conducted an in vitro analysis and showed that crude extract of cabbage suppresses the growth of *R. solanacearum*, and field experiment also revealed that incorporation of cabbage plants reduces the incidence of bacterial wilt of tomato and potato. Pablo Velasco et al. (2013) reported that gluconapin and its ITC varieties had an antibacterial effect on *Xanthomonas*, and the effect was strongly dependent on the concentration applied. Extracts from *B. rapa*, containing glucosinolates and phenolic compounds, inhibited the growth of tested rhizobacteria.

Biofumigation potentially provides a sustainable disease control option for integrated bacterial wilt management system while simultaneously enhances the soil health (soil fertility). The incorporation of biofumigants into the soil provides valuable organic matter, possibly reducing the dependence on organic fertilizers. Other benefits of biofumigation are improved soil texture, higher water holding capacity, and improved microbial community structure (Harvey and Sams 2001; Wheeler 2016).

Biological soil disinfection is also a technique used for biocontrol of *R. solanacearum.* In this technique, the anaerobic condition is induced in soil by applying fresh organic amendments and covering it with polythene to stop the air re-circulation in soil. This practice results in 92.5–99.9% reduction in *R. solanacearum* inoculum and also degrades potato tubers left in the field which would otherwise have served as a source of inoculum for the next crop (Messiha et al. 2007b). This can be adopted in endemic infection areas.

### 11.3.5 Silicon-Mediated Resistance Against *R. solanacearum*

Resistance against this pathogen is described as quantitative or polygenic (Wang et al. 2000). In quantitative resistance, cell wall characteristics or modifications play a key role, directly as barriers against entry of pathogens or indirectly by their effect on enhancing other active compounds (Prell 1996). Many soil elements found in cell walls have been reported to influence the susceptibility or resistance of plants to pathogen attack, silicon, among them as constituent of the cell wall, considered to be a “beneficial” element for plants and higher animals (Epstein 2001). In nature, silicon is found in the form of silica ($SiO_2$), aluminum silicates, iron silicates, calcium silicates, etc. and is absorbed by the plant as monosilicic acid ($Si(OH)_4$) (Chen et al. 2000). Ma et al. (2001) had classified plant species into silicon accumulator species, intermediate types, and silicon non-accumulators. The uptake mode in the first group is active, for the second passive, and rejective for the third.

Enhancing host resistance using elicitors, such as silicon in the form of silicic acid, monosilicic acid, orthosilicic acid, aluminum silicate, iron or calcium silicate, silicon dioxide, potassium silicate, and so on, can be an effective control strategy. Si is known as a multifunctional element which increases plant tolerance significantly against biotic stresses (pests and diseases) and abiotic stresses (toxic metal concentrations, drought and frost, etc.) (Ma 2004). Beneficial effects of silicon against various pathogens were reported in Si accumulator plants like cucumber (Fauteux et al. 2005), and rice (Rodrigues et al. 2007). Also in silicon non-accumulator plants like tomato, the effect of silicon on bacterial wilt has been studied (Dannon and Wydra 2004). Concerning its different modes of action in disease resistance, Si is reported to have a mechanical role by cell wall reinforcement and accumulation at the infection sites (Kim et al. 2002). Si is shown to act as a modulator which influences the expression of plant defense responses where it interacts with key components of plant stress signaling systems by binding to hydroxyl groups of proteins involved in signal transduction leading to resistance induction (Fauteux et al. 2005).

A number of studies have indicated that Si can enhance the resistance of plants to various diseases. Exogenous application of silicon was found enhancing the resistance against tomato bacterial wilt (Dannon and Wydra 2004; Diogo and Wydra 2007), sheath blight in rice (Datnoff et al. 2001), *Pythium* and *Sphaerotheca fuliginea* in cucumber (Fawe et al. 2001), etc. Silicon-treated plants show higher resistance toward pathogen penetration as $Si(OH)_4$ accumulates and polymerizes in host cell walls. Si may also activate a series of biochemical defense responses to enhance host resistance and increase activities of antioxidant enzymes and production of antifungal compound.

In tomato, the beneficial effects of silicon in prohibiting *R. solanacearum* development were also studied. Recent studies showed that Si could induce the production of defense-related signal molecules, peptides, signal transduction, etc. in plants infected with *R. solanacearum*. Dannon and Wydra (2004) reported significant reduction in bacterial wilt incidence by silicon amendment in tomato. Bacterial wilt incidence was reduced by 38 and 100% in silicon-treated plants of the moderately resistant tomato genotype King Kong 2 and resistant genotype Hawaii, respectively. Immunohistochemical analysis of stem cell walls treated with Si indicated changes in the structure of pectic polysaccharide. Following infection, in non-silicon-treated plants, homogalacturonan with non-block wise degradation of methyl esters was enhanced in vessel walls, but not in silicon-treated plants. It may be due to the action of pathogen pectin methyl esterase (Rodrigues et al. 2007).

However, the application of silicon resulted in a more stable expression of the three housekeeping genes (phosphoglycerate kinase genes, α-tubulin, and actin) in tomato, showing alleviation of the biotic stress imposed by the *R. solanacearum* (Ghareeb et al. 2011a). Ghareeb et al. (2011b) observed induced expression of defense marker genes generally upon challenging the silicon-treated plants with *R. solanacearum* and reached its highest levels at 72 h postinoculation.

Ayana et al. (2011) reported that silicon fertilizer significantly reduced the bacterial population, average wilt incidence, and disease severity index in moderately resistant tomato variety (King Kong 2). Trees treated with silica gel showed significantly lower necrotic buds and leaves with similar results like conventional treatment with Bordeaux mixture. However, minor differences in *P. syringae* like population levels were observed (Barranquero et al. 2012).

As per the findings of Kurabachew et al. (2013), a higher number of defense-related genes were regulated in the plant treated with Si than those treated with *B. pumilus*. Wang et al. (2013a) showed that exogenous 2.0 mM Si treatment reduced the disease index of bacterial wilt by ~19–53% compared with non-Si-treated plants. Si supply to soil has significantly increased urease and acid phosphatase activity under pathogen-inoculated conditions. Si amendments also increased the amount of bacteria and actinomycetes and reduced fungi/bacteria ratio by ~54% in soil. Application of Si and *B. pumilus* reduced bacterial wilt incidence in King Kong 2 genotype (moderately resistant) by ~51 and 27%, respectively, and in L390 genotype (susceptible) by ~31 and 22%, respectively. Insignificant increases of peroxidase (PO) and phenylalanine ammonia lyase (PAL) activity were observed in either silica, *B. pumilus*- or pathogen-inoculated plants. Therefore, separate application of each agent is recommended as the best strategy for inducing systemic resistance against tomato bacterial wilt (Kurabachew and Wydra 2013). Chen et al. (2015) reported that ~62% of the genes were altered in silicon-amended plants inoculated with *R. solanacearum*. Five proteins were grouped as defense-responsive proteins, and four of which were membrane-associated proteins.

### 11.3.6 Chitosan-Mediated Disease Resistance Against *R. solanacearum*

Enhancing host resistance using elicitor such as chitosan can be an effective disease control strategy. Chitosan is a natural, nontoxic homopolymer of β-1, 4-linked 2-amino D-glucose units (Yin et al. 2010). Chitosan is obtained from chitin by deacetylation and acts as elicitor of plant resistance (Yin et al. 2010). Oligochitosan is shown to inhibit pathogen invasion and induce phytoalexin production and expression of defense-related genes in different plants like, rice, rapeseed, tobacco, and grapevine (Lin et al. 2005; Yin et al. 2008). In rapeseed, upregulation of PDF1.2 gene (jasmonic acid/ethylene pathway), phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, and lipoxygenase was observed by oligochitosan application (Yin et al. 2008). In *Brassica napus*, the generation of reactive oxygen species (ROS) such as nitrogen dioxide and hydrogen peroxide is reported after oligochitosan treatment (Zaninotto et al. 2006).

Combined application of Si and chitosan (Chi) showed higher efficiency of suppressing *R. Solanacearum* in tomato. Microarray analysis revealed that the genes for chitinases and peroxidases were upregulated in combined Si and Chi treatment. Bacterial wilt incidence was reduced by ~40 and 57% in Si and Chi treatment, respectively, in King Kong 2 and by ~27 and 33% in Si and Chi treatment, respectively, in L390, and by ~75% in King Kong 2 and ~47% in L390 after combined application of Si and Chi (Kiirika et al. 2013).

### 11.3.7 Antimicrobial Volatile Organic Compounds (VOCs) for the Management of *R. solanacearum*

A good biocontrol agent has the ability to efficiently colonize plant roots with diverse modes of action that can hinder the pathogen with multiple weapons like the production of a diverse array of antibiotics, hydrolytic enzymes, and VOCs. Among the different modes of action of biocontrol agents, the production of VOCs has its own importance. The VOCs are low molecular weight compounds with high vapor pressure; therefore they tend to evaporate (Schulz and Dickschat 2007). These compounds not only affect rhizosphere but also travel long distance because of volatile nature. The VOCs produced by soil microbes have not only been reported to display antifungal, antibacterial, and nematicidal activity but also have plant growth promoting and systemic resistance inducing ability in plants (Audrain et al. 2015). Yuan et al. (2012) reported ~35% growth inhibition and complete inhibition of spore germination in *Fusarium oxysporum* f. sp. *cubense* by application of *Bacillus amyloliquefaciens* NJN-6 producing VOCs. Park et al. (2013) reported that C13 VOC from *Paenibacillus polymyxa* E681 induces systemic resistance against *Pseudomonas syringae* in plants. Raza et al. (2016) examined the effect of VOCs produced by *P. fluorescens* strain WR-1 on the growth and virulence traits of tomato wilt pathogen *R. solanacearum*. Bacteriostatic effect and inhibition of virulence were observed by VOCs produced by *P. fluorescens* WR-1 on the growth of *R. solanacearum* on agar medium as well as in infested soil.

Raza et al. (2016) investigated the effects of volatile organic compounds (VOCs) produced by *Bacillus amyloliquefaciens* SQR-9 on growth and virulence of *R. solanacearum*. The VOCs of SQR-9 significantly inhibited the growth of *R. solanacearum* on agar medium and in soil. Inhibition of virulence traits such as motility, production of antioxidant enzymes and exopolysaccharides, biofilm formation, root colonization, etc. was also observed. The proteomics analysis showed that the VOCs of SQR-9 downregulated *R. solanacearum* proteins related to virulence. More studies on composite impact of different VOCs are required to better understand the mode of action in natural environment.

### 11.3.8 Other Approaches for Control of *R. solanacearum*

Various control measures like host plant resistance (Dalal et al. 1999), transgenic resistance (Park et al. 2007), altering cropping system, crop rotation (Saddler 2005; Adebayo et al. 2009), use of soil amendments (Ayana et al. 2011), integrated control approach (Adebayo et al. 2009), and biological control (Raza et al. 2016) have been developed for controlling *R. solanacearum*. Some of the recent attempts have been listed in Table 11.1. Organic amendment is also an approach being used for

**Table 11.1** Different control strategies applied for bacterial wilt pathogen *R. solanacearum*

| Strategy | Agent | Crop | References |
|---|---|---|---|
| Constitutive expression of foreign antimicrobial proteins | Potato pseudothionin-St1, lactoferrin, bovine lactoferricin, Cecropin B | Tobacco, potato | Alan and Earle (2002) |
| Tolerance through transgenic having constitutive expression of stress response factor | *TSRF1* gene | Tobacco and tomato | Zhang et al. (2004) |
| Induced systemic resistance | *Bacillus* sp. | Tomato, sugar beet, bell pepper, tobacco, and other crops | Kloepper et al. (2004) |
| Resistant cultivar | Surya, Swetha, Haritha, and one F1 hybrid Neelima | Eggplant | Gopalakrishnan et al. (2005) |
| Use of clean, high-quality seed material | On-farm seed-plot | Potato | Kinyua et al. (2005) |
| Cultural practice | Crop rotation with several grasses | Sweet potato | Lemaga et al. (2005) |
| Cultural practice | Shift cropping dates, soil amendments | Tomato, potato, tobacco | Lemaga et al. (2005) |
| Cultural practice | Solarization | Potato | Saddler (2005) |
| Disease tolerance | Grafting on resistant rootstocks | Eggplant and tomato | Nakaho et al. (2000) and Gousset et al. (2005) |
| Biological soil disinfestation | Fresh organic amendments (grass or potato haulms) | Potato | Messiha et al. (2007b) |
| Jasmonic acid signaling | *Pythium oligandrum* | Tomato | Hase et al. (2008) |
| Biocontrol | *Bacillus subtilis* | Tomato | Sinha et al. (2012) |
| Biocontrol | *B. subtilis*, *P. aeruginosa*, *T. viride* | Tomato | Sarkar and Chaudhuri (2013) |
| Biocontrol | Soilborne antagonists | Tomato and pepper | Nguyen and Ranamukhaarachchi (2010) |
| Biocontrol | Botanicals | Tomato | Sangoyomi et al. (2011) |
| Polygenic resistance | QTL mapping | Tomato | Wang et al. (2013b) |
| Polygenic resistance | QTL mapping | Tobacco | Qian et al. (2013) |
| Polygenic resistance | QTL mapping | Eggplant | Lebeau et al. (2013) |
| R-gene-mediated resistance | RRS1 and RPS4 | Arabidopsis, tomato | Narusaka et al. (2013) and Williams et al. (2014) |

suppression of *R. solanacearum.* Soil amendment with compost (Schönfeld et al. 2003), pig slurry (Gorissen et al. 2004), sewage sludge (Ghini et al. 2007), etc. was found enhancing suppression of *R. solanacearum.*

Antibacterial substance β-D-glucogallin synthesis in leaves and its translocation to other plants have shown suppression of bacterial wilt (Kawaguchi et al. 1981). In tobacco, the use of somaclones had shown higher resistance to *R. solanacearum* as compared to their parents (Daub and Jenns 1989). Three bacterial wilt-resistant varieties (Surya, Swetha, and Haritha) and one F1 hybrid (Neelima) were developed by Kerala Agriculture University in eggplant (Gopalakrishnan et al. 2005). It was observed that these cultivars were having small and tightly packed root cortical cells and high phenolic content in root region which could prevent the pathogen infection. A successful attempt was taken by a team of scientists from Indian Institute of Horticultural Research by developing a triple gene-resistant F1 hybrid which is resistant to ToLCV, bacterial wilt, and early blight. This variety is named as "Arka Rakshak." Arka Alok, Arka Ananya, Arka Samrat, and Arka Abha are also tolarant to bacterial wilt (Thomas et al. 2014). Arka Rakshak has given excellent protection against these three diseases at field level. In China, tomato cultivar "Drica" was recommended as resistant cultivar against Biovar 3 infestation (Neto et al. 2002). Practice of grafting on a resistant rootstock like eggplant (IC-354557, IC-11105) can be a source of resistance in tomato. They can also be used for reducing bacterial wilt. *Solanum torvum* was reported as tolerant rootstock for bacterial wilt (Gousset et al. 2005). Intercropping can also be used as a measure for reducing inoculum load from field (Ratnadass et al. 2012). Potato intercropping with bean in Burundi (Autrique and Potts 1987), potato with corn and cowpea in the Philippines (Kloos et al. 1987), and growing tomato with sugarcane were helpful in reducing wilt incident.

Soil amendment with a chemical mixture, namely, S-H mixture, has been used for broad-spectrum suppression of a number of soilborne diseases including *R. solanacearum* wilt (Sun and Huang 1985). Application of S-H mixture at 0.5–1.0% (w/w) for a week before transplanting was found effective in suppressing bacterial wilt at greenhouse. Soil amendment with urea and mineral ash was also found comparable as S-H mixture in controlling bacterial wilt. Similarly in Surinam, mixing of seashell grit with sandy or clay soil infected with bacterial wilt has reduced bacterial wilt incident (Power 1983). Application of urea at 200 kg $ha^{-1}$ and CaO at 5000 kg $ha^{-1}$ was found beneficial in suppressing bacterial wilt in Taiwan (Michel et al. 1997). High soil temperatures and moisture make a conducive environment for bacterial wilt development (Kelman 1953). This can be minimized by manipulating date of sowing to avoid the most conducive phase for the pathogen and to grow in seasons less favorable for wilt disease development. In northwestern and northeastern hills in India, the incidence of bacterial wilt in the autumn is not found, and losses due to wilt of the potato grown at this season are avoided.

Apart from all other approaches, integrated control of *R. solanacearum* is a better option. Integrated approach by crop rotation and intercropping significantly reduced the soil population of *R. solanacearum*. Monocropping of *Crotalaria* and *Mucuna* and intercropping of cassava/*Mucuna* significantly reduced *R. solanacearum* population as compared to natural grass fallow system (Adebayo et al. 2009).

## 11.4 Challenges in *R. solanacearum* Biocontrol

Despite massive efforts in controlling *R. solanacearum*, it is still difficult to manage at field condition. The huge genetic diversity of pathogen often overcomes host resistance (Denny 2006) and other identified control measures by frequently occurring higher genotype × environment interactions (Nguyen and Ranamukhaarachchi 2010). Also, having wide genetic diversity makes it distributed all over the world (Genin and Denny 2012). It can successfully survive in the absence of a host as it has got an extensive host range of 450 crop species (Swanson et al. 2005) across 54 families (Wicker et al. 2007). This huge array of host helps it in lucrative survival. The difficulties in controlling *R. solanacearum* can also be addressed to its abilities to grow as endophyte in various plants, surviving in the deep layers of soil, and ease of traveling with water (Wang and Lin 2005). Alternate hosts also add fury in the fire, especially latently infected weeds which are possibly sites of overwintering in temperate regions. *R. solanacearum* also has the ability to persist in soil for longer periods along with fallen infested debris of plants and, therefore, is difficult to eradicate (Swanson et al. 2005).

*R. solanacearum* also has the ability to survive in a nutrient-poor environment for extended periods (Grey and Steck 2001). It can be disseminated by farm implements, irrigation water, aquatic weeds (Lopez and Biosca 2005), contaminated planting materials, insect transmission as in the case of Moko disease of banana (Kelman 1953), and many other means. Being soilborne and systemic in nature, it causes quick and lethal wilting of plants (Kelman 1998; Sangoyomi et al. 2011) and eliminates the chances of recovery. The climate with high temperature and humidity is conducive for disease development; therefore, it is a very serious problem of crops grown in tropical, subtropical, and warm-temperate areas (Hayward 2000). All these reasons make it a devastating pathogen across the globe and make its management a mammoth task.

## 11.5 Future Potential for Biocontrol of *R. solanacearum*

In the future, an intense approach is needed to address the problem of *R. solanacearum*, and it will not be a merely generalized statement if we call it a need for the integrative approach. The pathogen is sound enough in its smart survival strategy, genetic diversity, multiple dissemination opportunity, complex physiology and virulence, quick and fatal infection, and many more. Therefore, we need an out-and-out approach which can integrate expertise of a soil scientist and chemical scientist (who knows how to make conditions less conducive for pathogen?); a microbiologist and plant pathologist (they can better understand the bacterial cytology, physiology, and epidemiology to find any such phenomenal behavior which can be a prime target in reducing virulence and controlling it); a geneticist, biotechnologist, and bioinformatician (who can track the diverse pathogenic genes and survival strategies to come up with a solution in silico, in vitro, and finally in vivo as a stable resistant cultivar); and other related agricultural, horticultural, forestry sciences, etc. which can add a brick to build a strong wall against *R. solanacearum*. The use of endophytic microorganisms, chemicals like Si, metabolic inhibitors, bacteriophages, biotechnological approaches, etc. can be one of the key approaches in the future for controlling *R. solanacearum* infection at field.

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# Prospect and Potential of *Burkholderia* sp. Against *Phytophthora capsici* Leonian: A Causative Agent for Foot Rot Disease of Black Pepper

## 12

Khairulmazmi Ahmad and Tijjani Ahmadu

**Abstract**

Foot rot disease is a very destructive disease in black pepper in Malaysia. It is caused by *Phytophthora capsici* Leonian, which is a soilborne pathogenic protist (phylum, Oomycota) that infects aerial and subterranean structures of many host plants. This pathogen is a polycyclic, such that multiple cycles of infection and inoculum production occur in a single growing season. It is more prevalent in the tropics because of the favourable environmental conditions. The utilization of plant growth-promoting rhizobacteria (PGPR) as a biological control agent has been successfully implemented in controlling many plant pathogens. Many studies on the exploration of beneficial organisms have been carried out such as *Pseudomonas fluorescens*, which is one of the best examples used for the control of *Fusarium* wilt in tomato. Similarly, *P. fluorescens* is found to be an effective biocontrol agent against the foot rot disease in black pepper. Nowadays there is tremendous novel increase in the species of *Burkholderia* with either mutualistic or antagonistic interactions in the environment. *Burkholderia* sp. is an indigenous PGPR capable of producing a large number of commercially important hydrolytic enzymes and bioactive substances that promote plant growth and health; are eco-friendly, biodegradable and specific in their actions; and have a broad spectrum of antimicrobial activity in keeping down the population of phytopathogens, thus playing a great role in promoting sustainable agriculture today. Hence, in this book chapter, the potential applications of *Burkholderia* sp. to control foot rot disease of black pepper in Malaysia, their control mechanisms,

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plant growth promotion, commercial potentials and the future prospects as indigenous PGPR were discussed in relation to sustainable agriculture.

**Keywords**

*Phytophthora capsici* Leonian • Foot rot disease • Black pepper • Plant growth-promoting rhizobacteria (PGPR)

## Contents

## 12.1 Introduction

Black pepper (*Piper nigrum* L.) known as “the king of spices” is a historic, traditional spice and one of the most important agricultural produce in Malaysia (Anon 2003). The ~80% of pepper is processed as black pepper, and the remaining ~20% is processed as white pepper. Despite the substantial contribution made by this crop to the socio-economy of Malaysia and other parts of the world, production potentials of the crop are on the trend of decline due to the activities of pests and diseases. For example, a disease known as foot rot caused by *Phytophthora capsici* Leonian is a major obstacle in black pepper production in Malaysia and worldwide. The pathogen was first isolated and identified under *P. palmivora* (Holiday and Mowat 1963) in Malaysia, but later further investigations by Kuch and Khuthubutheen (1985) identified the pathogen as *P. capsici* Leonian (Fig. 12.1a–d). Foot rot is considered as the most serious disease of black pepper causing yield reduction that

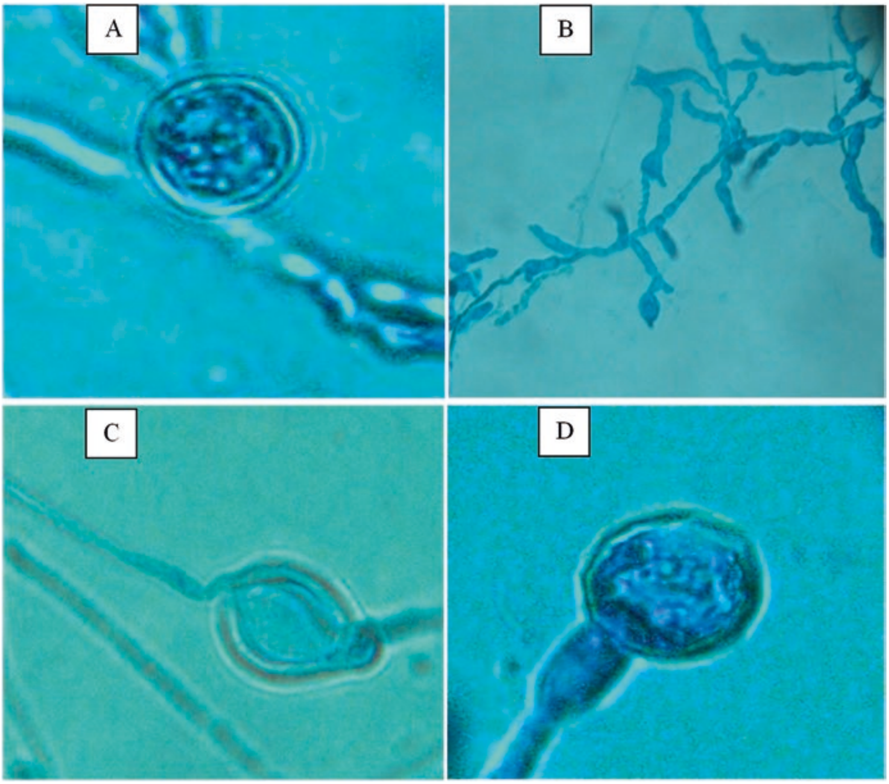

**Fig. 12.1** Typical morphological characteristics of *Phytophthora capsici* Leonian isolated from infected black pepper root: chlamydospore (**a**), torulose hyphae (**b**), lemon-shaped sporangium with long pedicel (**c**) and globose oogonia with paragynous antheridia (**d**)

ranges from ~20 to 80 and up to 95% for individual farmers (Manohara et al. 2004). The fungus is a soilborne pathogen that causes infections on roots, leaves and fruits of black pepper (Fig. 12.2a–c) and other crops (Fig. 12.3a–c) in most of the tropical countries. Efforts have been made to check these incessant problems caused by this fungus. Today, the primary means of controlling the disease is through synthetic fungicides applications which have been effective but found to be associated with some drawbacks. Among the drawbacks are their high cost, carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and possible side-effects on human health through the food (Wang et al. 2011; Meena et al. 2013a, 2016a; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2015; Kumar et al. 2015, 2016b; Ahmad et al. 2016; Parewa et al. 2014).

These drawbacks coupled with public concern have increased interest in developing further alternative control methods, particularly those that are eco-friendly, biodegradable, feasible to the farmers, non-toxic to human and animals, specific in their actions and have a broad spectrum of antimicrobial activity (Abhishek et al. 2013). Thus, indigenous plant growth-promoting rhizobacteria (PGPR) have been found to

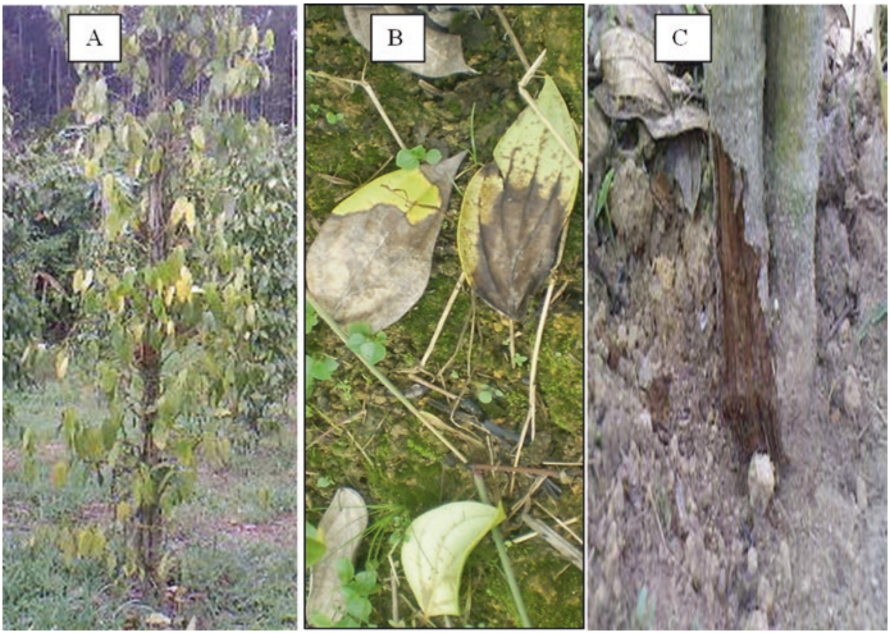

**Fig. 12.2** Yellowing symptoms of foot rot disease observed on black pepper foliage (**a**), leaves defoliation (**b**) and collar rot (**c**) symptoms on infected black pepper in Sarawak

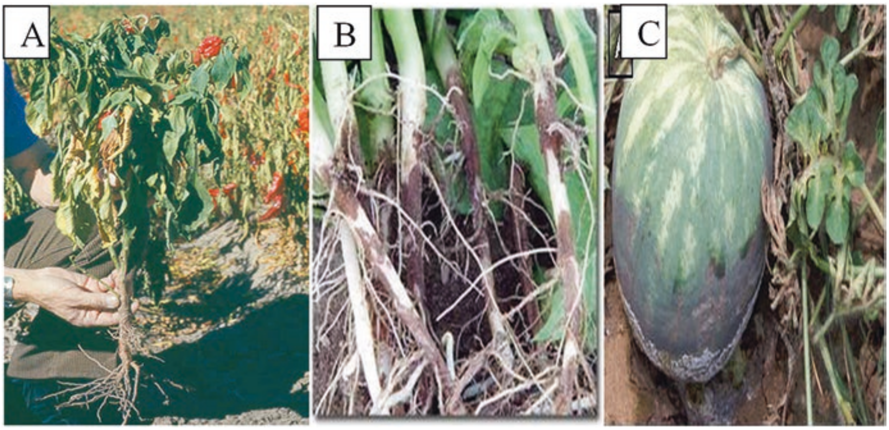

**Fig. 12.3** Yellowing symptoms of foot rot disease observed on chili pepper (**a**), infected root of potatoes (**b**) and infected fruit of watermelon (**c**) (Source: Wharton et al. 2007; Sanogo 2003)

play a major role in keeping down the population of pathogen to a low level and can therefore be used as an alternative to synthetic chemicals. Some PGPR, such as *Burkholderia* sp., *Pseudomonas* sp. and *Bacillus* sp., have been found to perform these functions by inducing systemic resistance in plants and showing biological traits like antibiosis and lysis (Eberl and Vandamme 2016; Rahamat Bivi et al. 2010; Prakash and Verma 2016; Priyadharsini and Muthukumar 2016; Kumar et al. 2017; Meena et al. 2015a, 2016b; Jaiswal et al. 2016, 2016a; Jha and Subramanian 2016).

The soil system is a natural body called "pedosphere" that served as a habitat for a quantum of endophytic and rhizospheric microorganisms which in turn modify the complex matrices of the soil especially in the root zone. Recent studies on microbial plant-related interactions revealed that bacterial communities called PGPR belonging to the genus *Burkholderia* are associated with the development of plants and are responsible for a range of physiological activities. In addition to their beneficial features as promoters of plant growth, they also protect plants against pests and pathogens (biocontrol agents) and increase plant fitness by nitrogen fixation, production of phytohormones and antimicrobial substances and induction of systemic resistance (Eberl and Vandamme 2016; Lodewyckx et al. 2002). Additionally, they are involved directly in the plant growth through biofertilization, stimulation of root growth, control of plant stress through host adaptation to environmental stress, sequestration of iron, phosphate solubilization (Raghavendra et al. 2016; Zahedi 2016; Meena et al. 2015b, 2015f, 2016c; Rawat et al. 2016; Yasin et al. 2016; Saha et al. 2016a; Dominguez-Nunez et al. 2016; Dotaniya et al. 2016), ACC deaminase activities and quinolinate phosphoribosyltransferase activity (Barrett and Parker 2006; Janssen 2006; Balandreau and Mavingui 2007; Compant et al. 2008) without conferring pathogenicity (Lugtenberg and Kamilova 2009; Compant et al. 2010; Eberl and Vandamme 2016).

These efficient bacteria are found in the "rhizosphere" which is defined as any volume of soil specifically influenced by plant roots and/or in association with root hairs and plant-produced materials (Dessaux et al. 2009; Silveira et al. 2012; Ahemad and Kibret 2014). The rhizosphere has been identified to consist of three major separates but interacting components that include rhizosphere (soil), the rhizoplane and the root itself. The rhizosphere is the soil zone influenced by roots through the release of substrates that affect microbial activity. The rhizoplane is the root surface that consists of strongly adhering soil particles, and the root itself is a component of the system, where the tissues are colonize by many microorganisms (like endophytes) (Barea et al. 2005; Ahemad and Kibret 2014).

Colonization of the rhizoplane and rhizosphere differs from one another (Kloepper et al. 1991), in that microbial colonization of rhizoplane is termed as root colonization, whereas rhizosphere colonization is microbial colonization of the adjacent volume of soil under the influence of the root (Kloepper 1994; Barea et al. 2005). Hiltner (1904) discovered that the rhizosphere is much richer in bacteria than the surrounding bulk soils, with composition of 10–1000 times higher than that in bulk soil.

The bacteria covered a small part of the root surface (Rovira 1956), and the most popular sites for bacterial growth are junctions between epidermal cells and areas where side roots appear. The effect of rhizosphere is caused by substantial amount of the carbon fixed by the plant, ~5–21% (Marschner 1995), which is secreted mainly as root exudates. In addition to facilitating water and nutrient uptake and providing mechanical support to the plants, a diverse array of compounds is synthesized, accumulated and secreted by plant roots (Walker et al. 2003).

The compounds secreted by the roots are generally referred to as root exudates. They act as attractants on diverse number of active microorganisms in the soil. In addition, they change the physical features and chemical compositions of the soil, therefore, restructuring the microorganisms in the area of the root (Eberl and Vandamme 2016; Dakora and Phillips 2002). They also repel microorganisms, promote symbiosis and control the growth of other unwanted plant species (Nardi et al. 2000). Kang et al. (2010) reported that the compositions of these exudates depend on the species of plants, their physiological status and microorganisms present.

PGPR are species of bacteria collectively found growing around plant tissues in the rhizosphere that enhanced the growth of plant by a number of mechanisms (Vessey 2003; Lemaire et al. 2015). They are distinctively characterized by some inherent features that include the following: they must (i) colonize the surface of the root effectively; (ii) promote plant growth; (iii) be able to survive and multiply, at least for sometimes to exert their protection and growth-promoting activities; and (iv) be able to compete well with other rhizosphere microbes for nutrients secreted by the root and for sites that can be occupied on the root (Kloepper 1994; Lugtenberg and Kamilova 2009). Certain species in this extremely versatile group are capable of causing disease in humans and plants (Eberl and Vandamme 2016), while others are very effective as biological control agents, bioremediation and promotion of plant growth (Perin et al. 2006).

Nowadays, rigorous research are carried out globally with greater aim to explore a vast number of PGPR having novel characteristics that could serve as biocontrol agents (Eberl and Vandamme 2016; Hynes et al. 2008; Joo et al. 2005; Russo et al. 2008) alongside with normal growth promotion characteristics like biofertilization (Tank and Saraf 2010; Ahemad and Khan 2012b), ACC deaminase (1-aminocyclopropane-1-carboxylate), production of ammonia and nitrogenase activities (Khan 2005; Glick 2012), siderophore (Tian et al. 2009; Jahanian et al. 2012), solubilization of phosphate and potentials in heavy metal detoxification (Ma et al. 2011; Ahemad and Khan 2012b), salinity tolerance (Tank and Saraf 2010; Mayak et al. 2004) and pesticide degradation (Ahemad and Khan 2012a). Typical examples of rhizobacteria that showed marvellous plant growth beneficial traits and potential as biological control agents against various root pathogenic microbes that are today used globally as bioinoculants in promoting growth and development of plant under different stresses such as heavy metals (Wani and Khan 2010), herbicides (Ahemad and Khan 2010, 2011a), insecticides and fungicides (Ahemad and Khan 2011b, 2011c, 2012c) and salinity (Mayak et al. 2004) include *Agrobacterium* sp., *Arthrobacter* sp., *Azotobacter* sp., *Azospirillum* sp., *Azomonas* sp., *Bacillus* sp., *Caulobacter* sp., *Chromobacterium* sp., *Erwinia* sp., *Flavobacterium* sp., *Micrococcus* sp., *Pseudomonas* sp., *Serratia* sp., *Allorhizobium* sp., *Azorhizobium* sp., *Bradyrhizobium* sp., *Mesorhizobium* sp., *Rhizobium* sp., *Micromonospora* sp., *Streptomyces* sp., *Streptosporangium* sp., *Thermobifida* sp., *Klebsiella* sp. and *Burkholderia* sp.

The name of the genus *Burkholderia* was derived from "Walter H. Burkholder" who described *Phytomonas caryophylli* (Burkholder 1942) as the first *Burkholderia* sp. which was later known as *Pseudomonas caryophylli*. Burkholder (1950) again described another species named "cepacia" named after onion, which was later called *Pseudomonas cepacia*. Species of *Burkholderia* were included for years in the genus of *Pseudomonas*, but with the advent of molecular rRNA-DNA hybridization analysis, considerable diversity in the genotype was noticed between the genus members (Compant et al. 2008), and as a result they were grouped into five rRNA groups (Palleroni et al. 1973). Later, genomic analysis had shown that five groups are related to one another. Recently, considerable numbers of species are included in the genus of *Burkholderia* (Coenye and Vandamme 2003) known as *Burkholderia cepacia* and representing complex of closely related genotypic species as confirmed by numerous taxonomic studies (Coenye et al. 2001; Vandamme et al. 2003; Vermis et al. 2004; Eberl and Vandamme 2016). The group is called as the *Burkholderia cepacia* complex and recently consists of a total of nine species that include *Burkholderia cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *Burkholderia cenocepacia* (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *Burkholderia dolosa* (genomovar VI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII) and *Burkholderia pyrrocinia* (genomovar IX). The first discovery of *B. cepacia* by W.H. Burkholder had today led to the identification of many other species of *Burkholderia*.

Currently, the genus *Burkholderia* includes more than 50 species that are found in various ecological niches, rather than in bulk soil (Coenye and Vandamme 2003; Luvizotto et al. 2010), most of which interact with plants in different ways resulting in beneficial effects to the intimate associating hosts. Finally, the potentials of PGPR should not be overemphasized as their application under both normal and stressed conditions has increased the health and productivity of different plant species and decreased global huge reliance on synthetic chemical pesticides that pollute the ecosystem (Yadav and Sidhu 2016; Saha et al. 2016b; Verma et al. 2014, 2015b; Masood and Bano 2016;Teotia et al. 2016; Meena et al. 2015e, 2016d, 2016e; Bahadur et al. 2016b; Das and Pradhan 2016. Therefore, in this book chapter, the potential application of indigenous PGPR (*Burkholderia sp.)* to control foot rot disease of black pepper in Malaysia, their control mechanism and plant growth promotion, the commercial potential application and the future prospects for sustainable agriculture were discussed.

## 12.2 General Mechanisms of Action for PGPR as a Biological Control Agent

These PGPR generally mediated plant growth promotions in rhizosphere as biocontrol agents by reducing the inhibitory effects of various pathogenic microbes on plant growth and development (Glick 2012), and their utilization to control diseases as biocontrol agents is an eco-friendly approach (Lugtenberg and Kamilova 2009).

The following are the mechanisms that can be distinguished in PGPR as a biocontrol agent.

### 12.2.1 Competition for Nutrients

The first step in pathogenesis of soilborne microbes is the colonization of rhizosphere and rhizoplane (Lugtenberg et al. 2001; Compant et al. 2010; Eberl and Vandamme 2016). As it is widely believed that root colonization is an important aspect of biocontrol, therefore, PGPR have to be highly competitive to successfully colonize the narrow root zone of the plant to be protected and also be able to exhaust the available nutrients against other microorganisms (Lugtenberg and Kamilova 2009; Compant et al. 2010; Shehata et al. 2016). The roots produce what is known as root exudates which consist of food nutrients that are essentially required by rhizosphere microbes that include sugars, amino acids, organic acids and numerous compounds including enzymes, sterols, vitamins, fatty acids, putrescine, nucleotides, osmoprotectants and signal molecules. In general, PGPR acted by displacing and suppressing the growth and development of pathogens through competition for the nutrients, space and essential elements ( Sharma et al. 2016; Verma et al. 2015a; Meena et al. 2013b, 2013c, 2014a, 2015d; Shrivastava et al. 2016;Singh et al. 2015; Bahadur et al. 2016a).

As a mechanism, some of the PGPR secreted siderophores and lytic enzymes that deter the growth of the phytopathogens present in the rhizosphere and rhizoplane. However, some secreted antibiotics that offer them a better chance for rhizosphere and rhizoplane colonization (van Loon and Bakker 2006; Shehata et al. 2016) and typical examples of the antibiotics secreted include 2,4-diacetylphloroglucinol (DAPG), rhamnolipids, hydrogen cyanide, zwittermicin A, oligomycin A, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, thiotropocin, tropolone, cyclic lipopeptides, kanosamine and xanthobaccin, as well as many others (Takeshita et al. 2015; Nielsen et al. 2002; Raaijmakers et al. 2002; de Souza et al. 2003; Compant et al. 2010). Fan et al. (2011) reported that successful colonization of seedlings root was achieved via root dipping in the suspension of *Bacillus* strain (FZB42) before transplanting. The biocontrol ability of *Bacillus* can be understood by the reports of Chen et al. (2009) and Malfanova et al. (2011) that *Bacillus* produces cyclic lipopeptides (cLPs) that are involved in the biological control through ISR (Ongena et al. 2007), in a mechanism that requires rhizosphere colonization only (Dekkers et al. 2000).

### 12.2.2 Signal Interference

Signal interference is a biocontrol mechanism employed by some PGPR to break the sensing ability of some virulent and/or pathogenic microbes. This is specifically seen in bacteria toward their ability to sense the production level of exoenzymes (cell wall-degrading enzymes) regulated by quorum sensing (QS) molecules such as homoserinelactones (AHLs) (Lugtenberg et al. 2013; Bassler 1999). Inactivation of the molecule called homoserinelactones (AHLs) needed for the production of exoenzyme is one way of controlling the activities of pathogens that can be achieved through signalling interference mechanism (Dong et al. 2004). Lactone ring-hydrolyzing enzymes, AHL lactonases, and the amide linkage-breaking enzymes, AHL acylases, are the two main types of AHL-inactivating enzymes that have been identified (Uroz et al. 2009; Lugtenberg et al. 2013).

Typical example of signal interference mechanism is the production of AHL lactonases by *B. thuringiensis* strains which hydrolyse the lactone ring and/or AHL acylases that break the amide link in the pathosystem (Lugtenberg et al. 2013). Volatile organic compounds (VOCs) produced by rhizospheric strains *P. fluorescens* B-4117 and *S. plymuthica* IC1270 have been demonstrated to be involved in the suppression of crown gall disease in tomato plants caused by *Agrobacterium* (Dandurishvili et al. 2011). Also VOCs produced these strains, which are capable of causing a noticeable decrease in the transcription of phzI and csaI genes capable of AHL synthesis (Chernin et al. 2011; Velazquez et al. 2016; Sindhu et al. 2016; Meena et al. 2014b, 2015c; Singh et al. 2016).

### 12.2.3 Induced Systemic Resistance (ISR)/Systemic Acquired Resistance (SAR)

The phenomenon induced systemic resistance (ISR) is an activated response immunity by plant that is mediated by some rhizobacteria living on or interacting with roots of host plants (Pierterse et al. 2009, 2014), mediated by the signalling pathway of jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Van Wees et al. 2000; Pierterse et al. 2014) within the plant resulting in the host plant's defence responses against a number of bacteria, fungi, viruses, nematodes and insects (Beneduzi et al. 2012; Glick 2012). Labuschagne et al. (2010) showed that PGPR elicited the ISR in the host plants by increasing the mechanical and physical strength of the cell wall of the host plant as well as changing the physiological and biochemical reactions of the host plant. The successes of ISR rely on the plant species or cultivar (van Loon and Bakker 2006) and require only rhizosphere colonization as a competitive mechanism (Dekkers et al. 2000; Lugtenberg et al. 2013).

It is important to note that ISR is not associated with the activation of pathogenesis-related proteins (PRs) as was the case in systemic acquired resistance (SAR). Various individual bacterial-derived compounds were reported to induce ISR, such as bacterial molecules like lipopolysaccharides and salicylic acid; organelles such as flagella; metabolites like siderophores, cyclic lipopeptides and biosurfactants; volatiles such as 2,3-butanediol and acetoin; phenolic compounds; antibiotics; and the signal molecule or quorum sensing molecules (Ahemad and Kibret 2014; Lugtenberg et al. 2013; Beneduzi et al. 2012; Perez-Garcıa et al. 2011).

The term SAR describes a salicylic acid-defendant induced resistance caused by a localized infection (Vleesschauwer and Hofte 2009). Ryals et al. (1996) defined SAR as a defence mechanism activated in the plant following the primary infection

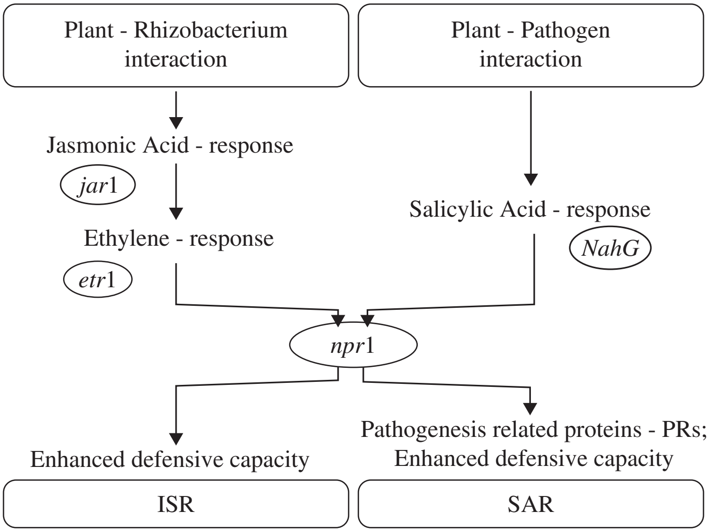

**Fig. 12.4** Transduction signal pathways leading to rhizobacteria-mediated induced systemic resistance (*ISR*) and pathogen-induced systemic acquired resistance (*SAR*) in *A. thaliana* (Source: Van Loon et al. 1998)

by pathogens, mediated through the accumulation of salicylic acid signalling pathway (Beneduzi et al. 2012). The plant resists further attacks after the first infection that predisposes the host to subsequent attacks. The defence capacity is linked with the accumulation of PRs. This, therefore, suggested the relevance of these PRs in their contribution to increased defence ability of the infected or induced tissue (Beneduzi et al. 2012). The most important feature of SAR is the activation of SAR genes especially those encoding the PRs that are usually taken as molecular markers for the state of induced resistance attained (Vleesschauwer and Hofte 2009; Mandal and Ray 2011; Shaikh et al. 2016).

Typical examples of PRs that served as hallmarks in several plant species and which have also shown to contribute in the inducement of resistance are 1,3-glucanases and chitinases that are effective in hydrolyzing fungal cell walls. Pieterse et al. (1996) reported that in an experiment conducted on *Arabidopsis* plants inoculated with *Pseudomonas syringae* pv. tomato and/or sprayed with salicylic acid, it developed PRs (PR-1, -2, and -5 mRNAs), and with this conclusion, it could be made that PRs are dominantly associated with induction of SAR (Beneduzi et al. 2012; Meena et al. 2017). Both ISR and SAR can act together in conferring resistance to host against pathogens and exert a protection better than each system alone (Van Wees et al. 2000). Salicylic acid transduction signal needs the activator (regulatory) protein NPR1 which works in the terminal signalling pathway of the SAR, and NPR1 takes part in the defence responses mediated by various signalling ways that act beyond the expression of pathogenesis-related genes, showing ISR and SAR meet at the end of the signalling pathway (Van Loon et al. 1998; Beneduzi et al. 2012). The transduction signal pathways leading to ISR (rhizobacteria) and pathogen-induced SAR in *Arabidopsis thaliana* are shown below (Fig. 12.4).

### 12.2.4 Siderophores Production to Compete for Ferric Ions

The term siderophores is a ferric ion ($Fe^{3+}$)-chelating compound produced by many rhizobacteria in an attempt to overcome the conditions under $Fe^{3+}$ limitations (Lugtenberg et al. 2013). Virtually, all living organisms essentially need $Fe^{3+}$ for a variety of functions such as synthesis of ATP, formation of heme, reduction of ribotide precursors of DNA and for growth (Saraf et al. 2011; Lugtenberg et al. 2013; Sermwan et al. 2015). The need for iron to support the growth of organism became a challenge to the organisms in a situation of shortage supply. Therefore, survival of the fittest became the rule to survive. As a mechanism of biocontrol agents, siderophore-$Fe^{3+}$ complex is formed by continuous binding to $Fe^{3+}$ limitation receptors, and the $Fe^{3+}$ ion is subsequently conveyed into the cell of bacterial where it becomes active as $Fe^{2+}$. Those bacteria that secrete siderophores effectively good enough to bind $Fe^{3+}$ to a level that fungal pathogens can no longer grow anymore under iron limitation can act as biological control agents (Leong 1986). Pyoverdine is a good example of a siderophore (Lugtenberg et al. 2013), and examples of bacteria that produced siderophore include *P. fluorescens* strains, *Bacillus*, *Alcaligenes*, *Bradyrhizobium*, *Rhizobium* and *Enterobacter* (Shaikh et al. 2014; Shaikh and Sayyed 2015). *Burkholderia cepacia* was reported to produce siderophore called deferoxamine mesylate salt equivalent. A concentration of $0.64\ \mu g\ mL^{-1}$ is sufficient to inhibit 91.1 ± 0.5% of phytopathogen growth on mango (Santos Villalobos et al. 2012). In short, increased concentration siderophore production by the PGPR bacteria could trigger inhibition of phytopathogens due to the starvation of iron.

### 12.2.5 Antibiosis

The term antibiosis is an antagonistic association between organisms and is the productions of metabolic substances by one organism which is detrimental to the other. In addition to siderophore production, majority of rhizosphere bacteria produced metabolites with antifungal properties which are known in controlling fungal diseases (Shehata et al. 2016; Opelt et al. 2007). These AFMs are also known as antibiotics which are compounds that deter the metabolic processes or growth of other microorganisms (Beneduzi et al. 2012; Duffy et al. 2003). Generally, PGPR produced one or more antibiotic as a mechanism which gave them ability to play the role of antagonism against pathogens (Beneduzi et al. 2012; Glick et al. 2007). Better understanding of the phenomenon of antibiosis as the activity of biocontrol has come to the domain of its peak in the last two decades (Lugtenberg and Kamilova 2009).

The possible mechanisms of action for most of these compounds are discussed by Haas and Defago (2005). Majority of the antibiotics have been isolated and studied, and a great diversity has been observed in their mechanisms to prevent synthesis of pathogen cell walls and inhibit the formation of initiated complexes on the small subunit of the ribosome (Maksimov et al. 2011). The antibiotics best known to involve in biological control by PGPR include bacillomycin D, phenazines, pyocyanin, pyrroles, pyoluteorin, pyrrolnitrin, volatile hydrogen cyanide (HCN), oomycin A, iturins, fengycins, surfactin, mupirocin, bacillomycin, zwittermicin, 2-hexyl-5-propyl resorcinol (Sindhu et al. 2009; Akhtar and Siddiqui 2010; Ahanger et al. 2014; Mabood et al. 2014; Shaikh and Sayyed 2015), volatiles 2,3-butanediol (Ryu et al. 2003), d-gluconic acid (Kaur et al. 2006), 2-hexyl-5-propyl resorcinol (Cazorla et al. 2006), 6-pentyl-α-pyrone (Lorito et al. 2010) and polymyxin, circulin and colistin (Maksimov et al. 2011). Some researchers have proved this through analysis (mutational) followed by studies like complementation studies (Lugtenberg et al. 2013). Majority of these antibiotics were produced by the group of bacteria known as *Bacillus* sp. These antibiotics are found to be effective against phytopathogenic fungi *Aspergillus flavus*, *Fusarium oxysporum*, *Alternaria solani*, *Botryosphaeria ribis*, *Phomopsis gossypii*, *Helminthosporium maydis*, *Colletotrichum gloeosporioides*, etc. (Maksimov et al. 2011).

Nowadays, a detailed investigation has been carried out on the class of antibiotics secreted by numerous species of bacteria, including *Bacillus* known as cyclic lipopeptides (cLPs). The cLPs consist of three major families, namely, the iturins, surfactins and the fengycins. Their mechanisms of beneficial action depend on direct antibiosis of phytopathogens (Borriss 2011; Perez-Garcıa et al. 2011). Several reports have been presented as evidences for the involvement of cLPs in biocontrol activity as exemplified by the fengycins' activity in biological control of *B. cinerea* on apple which was traced in the infected parts of apple at some level of concentrations (Toure et al. 2004). Zeriouh et al. (2011) recently proved the involvement of iturins in the control of *Xanthomonas campestris* and *Pectobacterium carotovorum*. Similarly, Yanez-Mendizabal et al. (2012) observed and reported the involvement of fengycins in the inhibition of peach brown rot disease with mutational analysis. Henry et al. (2011) also enumerated that fengycins combined with surfactins affect defence pathways in tomato and bean. Furthermore, cLPs are involved in biofilm formation, cell differentiation and cannibalism (Lopez et al. 2009).

### 12.2.6 Bacteriocins Production

Bacteriocins are proteinaceous toxins produced by some bacteria to inhibit the growth of similar or closely related bacterial strains which were first discovered and called colicine in 1925 by A. Gratia because it killed *Escherichia coli* (Gratia 2000). Bacteriocins are narrow in their action and toxic mostly to bacteria related to the producing species, and this is the main difference between bacteriocins and antibiotics (Riley and Wertz 2002). Typical examples of bacteriocins secreted by some bacteria especially gram negative that are lethal to related strains include cloacins derived from *Enterobacter cloacae*, pyocins from *P. pyogenes*, colicin from *E. coli*, megacins from *B. megaterium* and marcescens from *Serratia marcescens* (Beneduzi et al. 2012; Cascales et al. 2007). Abriouel et al. (2011) reported that bacteriocins from *Bacillus* sp. have a broad spectrum against gram-positive species, gram-negative bacteria and fungi or yeast.

### 12.2.7 Interference with the Activity in Survival, Multiplication, Germination, Sporulation and Spread of the Pathogen

Many bacterial strains have been harnessed and used as biocontrol agents to interfere with growth of some soilborne fungal pathogens. Majority of these strains are from fluorescent pseudomonads including *P. fluorescens*, *P. putida*, *P. aeruginosa and P. aureofaciens* that suppressed soilborne pathogens through antibiosis, rhizosphere competition and iron chelation by siderophores production (Jianbin et al. 2010). *Pseudomonas* strains, *P. fluorescens* WCS365 and *P. putida* PCL1760, have been reported to suppress tomato foot and root rot (TFRR) in stone wool, and their characteristics are well known and documented (Kamilova et al. 2006; Validov et al. 2009). Studies on the control of tomato *Fusarium* root rot disease with the biological control agent *P. fluorescens* strain WCS365 have indicated a positive result through a series of activities that interfere with the cyclic events in the growth of the pathogen including germination, sporulation, multiplication, survival and spread of the pathogen (Lugtenberg et al. 2013).

In the process of biocontrol, the hyphae of the fungus secreted fusaric acid (FA) which is believed to attract the cells of the strain *P. fluorescens* WCS365 with subsequent extensive colonization of hyphae, leading to the formation of biofilms or microcolonies (Lugtenberg et al. 2013; de Weert et al. 2004). Colonization of hyphae and subsequent formation of biofilms make the fungus ineffective and inhibit its growth, reproduction and survival. In a situation where there is nutrient scarcity (nutrient deprivation), biocontrol strain *P. fluorescens* WCS365 used the hyphae as a food source through hyphal colonization with subsequent spore germination inhibition (Kamilova et al. 2008). This conclusively showed that in the presence of *P. fluorescens* WCS365, spore formation will be reduced, and, therefore, this will also reduce pathogen spread, thus, serving as a biocontrol agent (Kamilova et al. 2008; Validov et al. 2009).

### 12.2.8 Cell Lysis and Degradation

Most of the PGPR produce enzymes such as chitinases, cellulases, glucanases and proteases that hydrolyse polymeric compounds like chitin, cellulose, proteins, hemicellulose and DNA. This will help in the inhibition of phytopathogens (Shaikh et al. 2016). Mabood et al. (2014) reported that these enzymes are known to cause degradation and lysis of cell walls which help in the control of phytopathogens. For example, chitinases and β-1,3-glucanase-producing PGPR such as *B. subtilis* BSK17, *B. suly*, *Paenibacillus illinoisensis*, *P. illinoisensis* KJA-424, *Pseudomonas* sp., *Enterobacter ammrenus*, *Pantoea dispersa* and *Pythium ultimum* are reported to demonstrate some potentials in biocontrol activity (Shaikh et al. 2016). Dubbey et al.

(2014) reported that chitinases and β-1,3-glucanase are produced by *B. subtilis* BSK17 that assist in their root zone competition and antagonistic activity. Similarly, severity of *Fusarium* infections produced under greenhouse conditions is reduced through chitinase production by *B. suly* (Hariprasad et al. 2011). Biocontrol activity by *Paenibacillus illinoisensis* has also been demonstrated against *Phytophthora capsici* causing blight in pepper by the secretion of chitinase (Jung et al. 2005).

## 12.3 Biological Control Mechanisms in *Burkholderia* sp. Against Phytopathogens

*Burkholderia* species are considered beneficial in the ecosystem in that they can be used for biological control of diseases caused by fungi in plants, plant growth promotion and bioremediation (Perin et al. 2006; Compant et al. 2008). Several *Burkholderia* species have shown the ability to use different mechanisms such as competition and secretion of allelochemicals, including antibiotics and siderophores known with antimicrobial activity, competition for nutrients, induced systemic resistance (ISR), antagonism as well as hyphal colonization. All these are good features of potential biocontrol agents against phytopathogenic fungi (Baldani et al. 2000; Welbaum et al. 2004; Compant et al. 2005b; Kang et al. 1998; Hu and Young 1998). The efficacy of these *Burkholderia* species as biocontrol agents has been shown by *B. cepacia*, *B. ambifaria*, *B. pyrrocinia*, *B. cenocepacia*, *B. vietnamiensis* and *B. phytofirmans* strains against *Fusarium* sp., *P. capsici*, *Pythium ultimum*, *P. aphanidermatum*, *B. cinerea* and *R. solani* (Compant et al. 2008; Ait Barka et al. 2002; Cain et al. 2000; Parke and Gurian-Sherman 2001; Singh et al. 2006). Several reports have proved these potentials as exemplified by the report of Cuong et al. (2011) by the colonization activity of hyphae-colonizing *Burkholderia* sp. against *R. solani* causing sheath blight in rice. Some traits of *Burkholderia* sp. strains have been shown to encompass antifungal genes which enable members of the group to produce a wide range of secondary metabolites active against *R. solani*. Examples of the metabolites are pyrrolnitrin, phenazine, cepaciamide A (Cartwright et al. 1995; Rosales et al. 1995; El-Banna and Winklemann 1998; Jiao et al. 1996; Mao et al. 2006) and some unknown compounds (Mao et al. 2006).

Bevivino et al. (1994) reported that *Burkholderia* sp. produced very efficient low-molecular-weight iron-chelating compounds known as siderophores which are shown to be involved in antibiosis mechanism against plant pathogens through iron competition under iron-limiting conditions. Ornibactins, cepaciacheline and cepabactine are the predominant siderophores produced by *Burkholderia* strains (Meyer et al. 1995; De Meyer et al. 2015). Recently, it has been reported that 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing endophyte belonging to *Burkholderia* sp. exhibited antagonistic activity against *R. solani* and *Sclerotinia sclerotiorum* (Pandey et al. 2005).

## 12.4 PGPR as a Plant Growth Promoter

Generally, plant growth-promoting mechanisms exhibited by PGPR were categorized into two main groups, i.e. direct and indirect mechanisms. In the past, more emphasis has been laid on direct interaction rather than indirect interaction. Direct mechanism may involve nitrogen fixation, phosphate solubilization ability, siderophore production and production of plant growth regulators. On the other hand, indirect mechanisms may include suppression of phytopathogens and enhancement of mutualisms between host plants and other symbionts (Kloepper et al. 1989).

### 12.4.1 Nitrogen Fixation

Nitrogen-fixing microbes are generally categorized into two main groups (a) symbiotic $N_2$-fixing bacteria and (b) non-symbiotic bacteria. Diazotrophs are a PGPR that fix $N_2$ in nonleguminous plants (Glick et al. 1999). Basically, biological nitrogen fixation (BNF) is restricted to prokaryotic organisms. Currently, hundreds of bacterial species were identified, covering most of the different biotrophic energy systems such as photosynthetic bacteria (e.g. *Rhodospirillum rubrum*), anaerobic bacteria (e.g. *Clostridium* sp.), microaerobic (*Burkholderia* sp.) and aerobic bacteria (e.g. *Azotobacter*). Biological nitrogen fixation usually takes place at mild temperatures (Raymond et al. 2004), so that the fixation process can occur everywhere on the earth (Table 12.1). The genus *Burkholderia* was documented as one of the richest $N_2$-fixing bacteria. Among them *B. vietnamiensis* was the first known $N_2$-fixing species of this genus and was isolated from the rhizosphere of rice plants in Vietnam. This bacterium has attracted interest of many researchers because of its abilities to fix $N_2$, promote rice plant growth and enhance grain yield.

### 12.4.2 Phosphate Solubilization

The search for an ecologically safe and economically reasonable option for improving crop production in low-phosphorus soils becomes the ultimate outcome in soil fertility research. In this context, phosphate-solubilizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with phosphate from sources otherwise poorly available by various mechanisms (Zaidi et al. 2009). Excellence examples of phosphate-solubilizing bacteria are *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* (Bhattacharyya and Jha 2012). These bacteria were reported to solubilize inorganic phosphorus through synthesization of the low-molecular-weight organic acids in the soil (Zaidi et al. 2009). The mineralization of organic phosphorus occurs through the synthesis of a variety of different phosphatases, catalysing the hydrolysis of phosphoric esters (Glick 2012). Most importantly, both phosphate solubilization and mineralization can coexist in the same bacterial strain (Tao et al. 2008).

**Table 12.1** Distribution of biological nitrogen fixation system

| Ecosystem | Nature of BNF | Type | Occurrence |
|---|---|---|---|
| Natural | Symbiotic | Root and stem nodule | Legumes |
| | | *Rhizobium*, *Frankia* | Actinorhizal trees/ shrubs |
| | | Mosses, lichens, pteridophytes | Soil, rock, tree surface |
| | | Insects | Gut of termites |
| | | *Gunnera-Nostoc* | Base of leaves, cycad root |
| | Non-symbiotic | Free-living saprophytes (numerous species, aerobes, microaerobes, anaerobes) | Soil and plant root rhizosphere bacteria on litters |
| | | Photosynthetic, *Anabaena*, *Nostoc*, etc. | On plant surfaces cyanobacteria (blue-green algae) |
| | | Photosynthetic bacteria, *Rhodospirillum rubrum*, etc. | Aquatic and marine bacteria |
| Agriculture/forestry | Symbiotic | Nodulated legumes | Annual, perennial, rotation crops, green manure |
| | | Actinorhizal, angiosperms | Plantation system |
| | | Miscellaneous symbiotic | Pioneer uses, *Azolla*, sugar cane, etc. |
| | Non-symbiotic | Free-living saprophytes (numerous species, aerobes, microaerobes, anaerobes) | Rice paddies |
| | | Photosynthetic, *Anabaena*, *Nostoc*, etc. | |
| | | Photosynthetic bacteria, *Rhodospirillum rubrum*, etc. | |

Adapted from Kennedy and Cocking 1997

### 12.4.3 Phytohormone Production

Microbial synthesis of the phytohormone, namely, auxin (indole-3-acetic acid/ indole acetic acid/IAA), was reported a long time ago. Apart from IAA, PGPR are also capable of synthesizing other plant hormones, such as gibberellins (GAs) and cytokinins (CKs) or affecting plant hormone biosynthesis (homeostasis) *in planta* (Kurepin et al. 2014). IAA plays crucial role in bacteria-host interactions (Spaepen and Vanderleyden 2011). It is well known that IAA affects plant physiological processes such as cell division, extension and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; and affects photosynthesis, pigment formation, biosynthesis of various metabolites and resistance to stressful conditions. *Burkholderia phytofirmans* strain PsJN was reported capable of inducing biomass growth of several crops including potato. This report showed massive root growth increases after inoculation, and this was associated with a twofold to threefold increase in IAA and CK (trans-zeatin or tZ) levels (Kurepin et al. 2015).

### 12.4.4 Harmonizing Ethylene Production

Many studies show ethylene gas is a crucial growth regulator of numerous aspects of plant development and physiology (Merchante et al. 2013) such as germination, seedling growth and morphology, fruit ripening, organ senescence and stress/defence response (Khalid et al. 2006; Broekgaarden et al. 2015). However, under usual condition the ethylene gas production is always in low concentration. This is due to the biosynthesis of this compound which depends on transcriptional and post-translational mechanisms that regulate the activity levels of the biosynthetic enzymes (Booker and DeLong, 2015). On the other hand, if ethylene present is in high concentration, it may inhibit physiological activities in plant-like root elongation. In this case, PGPR are needed in harmonizing the level of ethylene in plant by converting 1-aminocyclopropane-1-carboxylate (ACC) into ammonia and α-ketobutyrate (Nascimento et al. 2014). Currently, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Acinetobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* were reported to have ability to harmonize ethylene gas production in plant (Kang et al. 2010; Zahir et al. 2008, 2009). *Burkholderia phytofirmans* PsJN is one of the best-studied *Burkholderia*. This strain was reported to inhabit the rhizosphere and endosphere of plant, thus promoting growth and enhancing stress adaptation in selected herbaceous and woody plant species (Da et al. 2012; Fernandez et al. 2012; Kim et al. 2012; Naveed et al. 2014). According to Poupin et al. (2013) and Zuniga et al. (2013), *B. phytofirmans* PsJN showed excellent capability of promoting growth and accelerating the whole life cycle of *Arabidopsis thaliana*. Moreover, this strain also induces primary root growth and root hair development and promotes aerial growth increasing the epidermal cell size (Poupin et al. 2013) and induces salt stress tolerance (Pinedo et al. 2015) in *A. thaliana*.

## 12.5 Commercial Potentials of PGPR in Malaysia

Malaysia was the largest pepper-producing country in the world. However, after 1980, Malaysia lost its top position to India and Indonesia (Azmil 1993). Currently, Malaysia is ranked sixth in terms of world pepper production (IPC 2012). Approximately 45,000 families and more than 115,000 workers are involved in the pepper industry in Malaysia. This crop generates about one third of Sarawak's agriculture export earnings, and Sarawak is the main black pepper export producer in

Malaysia. Currently, production of black pepper in Sarawak showed a declining trend. One of the main factors is due to pests and diseases infestation. Foot rot disease is considered the most devastating disease in black pepper.

At present, no effective control measure is available to effectively manage this disease in the world. Application of PGPR might be one of the alternative solutions to chemical control of the disease in the field. Attempt was made to find potential indigenous PGPR strain to control foot rot disease in vitro and in vivo. We found promising PGPR strains that are able to induce systemic resistance in black pepper as well as showing biological control traits like producing antibiotics which caused lysis of the mycelial cells of *P. capsici*. The tested PGPR strains were also found to promote the growth of the treated plants. The use of PGPR should be a preferable method as they are internal colonizers and more efficient to compete in the vascular systems. Thus, this will certainly deprive *P. capsici* in terms of nutrient uptake and space for their proliferation. Based on dual culture test, these three PGPR strains, BPA011, BPA040 and BPA025, exhibited high percentage of inhibition on radial growth with recorded PIRG values as ~81, 83 and 81%, respectively. Furthermore, in culture filtrate test, all the three strains exhibited 100% PIRG (Fig. 12.5 and Table 12.2). These potential strains were successfully identified using GC-FAME as *B. cepacia*, *B. cenocepacia* and *Bacillus alcalophilus*, respectively.

Results from in vivo test demonstrated that application of PGPR resulted in disease suppression and delayed disease onset on treated plant. The present study showed that there were significant differences in terms of disease incidence (DI) and disease severity index (DSI) as compared with control treatment. Our findings showed treated plant with *B. cenocepacia* showed the lowest DSI (1.67%) in the first month, and the severity index was increased gradually in the second month (5.85%), and finally the DSI remained steady at ~10% along the assessment period. A similar trend was observed at *B. cepacia* and *B. alcalophilus* treatments.

Assessment on production of inducible compounds by the host plant was also conducted. Our findings revealed an increased in enzymatic activity of peroxidase (PO), total phenolic content (TPC) and hydrogen peroxidase ($H_2O_2$) in the treated plants. Significant amount of inducible compounds was expressed in root, stem and leaf parts of the treated plants. Our findings indicated that the systemic protection was offered to the host plant by the tested PGPR strains. This event resulted in limiting and preventing the phytopathogens activities, even at foliar infection by the *P. capsici*. Moreover, the positive effects of PGPR on plant growth are always correlated with a remarkable increase in the root morphology such as lateral root length, root hair number and also shoot length and yield. In our study, we found root, stem and leaf biomass were significantly increased in the treated plants, and this is generally assumed that these developmental responses are triggered by phytohormones such as auxins, cytokinins and gibberellins produced by the PGPR strains.

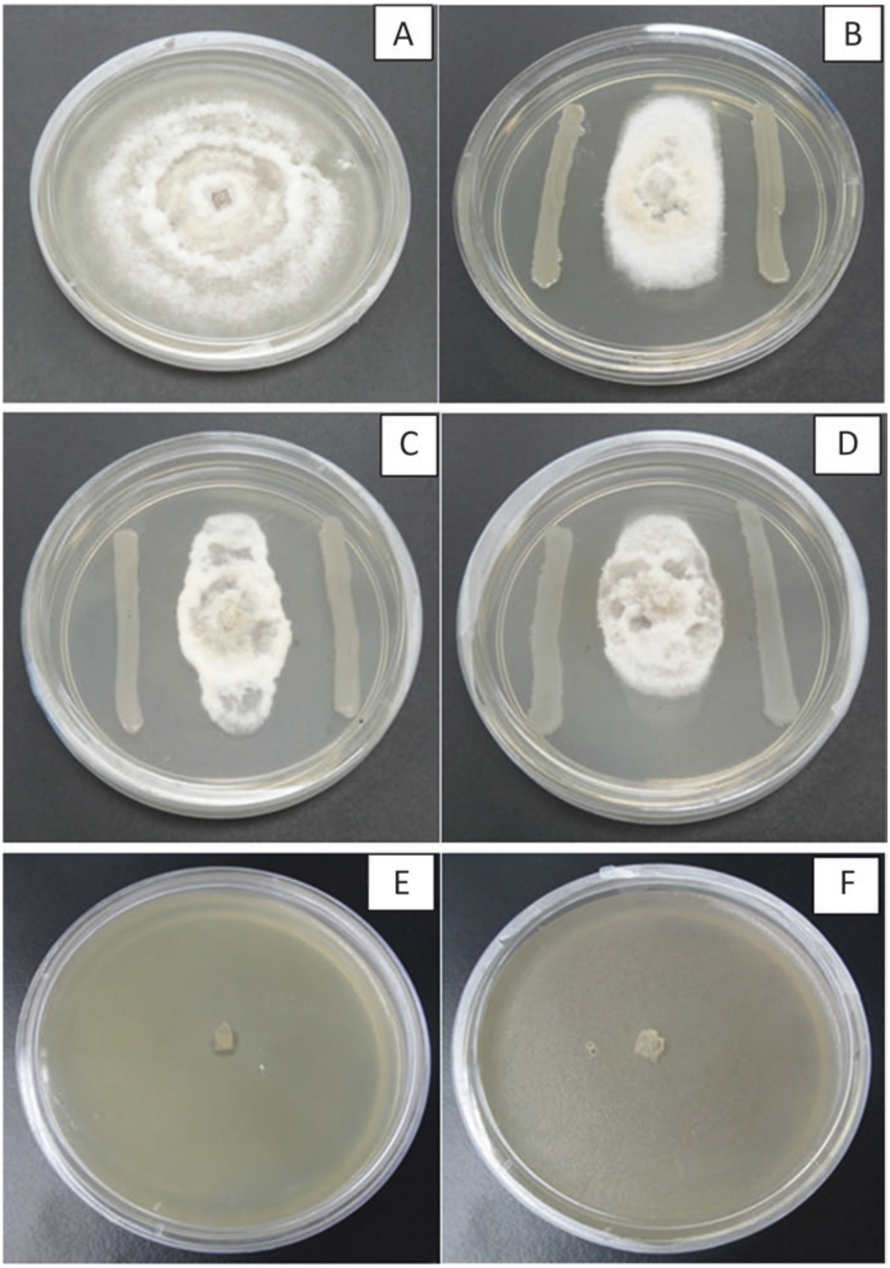

**Fig. 12.5** The effect of endophytic bacteria on mycelial growth of *P. capsici* in dual culture and cultural filtrate tests at 7 days after incubation. Pure culture of *P. capsici* in control plate (**a**), BPA011 (**b**), BPA040 (**c**), BPA025 in dual culture test (**d**) and BPA011 and BPA040 in culture filtrate test (**e** and **f**), respectively

**Table 12.2** Potential candidates of PGPR were tested using dual culture and culture filtrate tests against *P. capsici* in vitro

| Bacteria code | Dual culture test (% PIRG)* | Culture filtrate test (% PIRG) | Identification by CG-FAME |
|---|---|---|---|
| BPA011 | 81.04 ± 0.59[a] | 100 ± 0[a] | *Burkholderia cepacia* |
| BPA025 | 80.83 ± 0.09[a] | 100 ± 0[a] | *Bacillus alcalophilus* |
| BPA040 | 82.97 ± 0.47[a] | 100 ± 0[a] | *Burkholderia cenocepacia* |

Means in the same column with different alphabet(s) are significantly different ($p \leq 0.05$) according to DNMRT

[a]Percentage inhibition of radial growth (PIRG) of *P. capsici* was assessed at 7 days after incubation

## 12.6 Future Prospect

Currently, majority of black pepper farmers in Malaysia rely extensively on chemical fungicides to control foot rot disease in black pepper. Heavy reliance on chemical fungicides may lead to numerous biohazards such as environmental pollutions, residual effect in food and pathogen resistance and may be hazardous to beneficial microorganisms. From crop management perspective for sustainable agriculture, the control of foot rot disease in black pepper using PGPR (*Burkholderia*) would best be achieved by combining these two techniques: (i) disease control through the use of biocontrol agents native to black pepper farms involving continuously inoculation of PGPR inoculum to increase their populations and (ii) disease control through application of antifungal metabolites responsible for effectiveness of the biocontrol agent-developed product usually more effective and easier to be used by farmers. Meanwhile, diminishing the biohazards is inherent in the use of intact microbial cells (and the associated potential risk to human health). Application of green technology in agriculture in Malaysia has become more evident in recent year. Implementation of National Green Technology Policy since 2009 contributed huge impact in research and development as well as in agriculture practices in Malaysia. One of the biggest impacts is the ability to achieve reduction in the greenhouse gas intensity of gross domestic product (GDP) of 35% in 2015. Even though many incentives and funds were given by Malaysian government in developing new and effective formulations for effective delivery of PGPR, the process is still very slow. Formulation of biopesticides with PGPR-like *Burkholderia* sp. is a big challenge in practical agriculture especially in the tropical regions where the environmental conditions are favourable for the pathogen to grow. Hence, improvement in the formulation of biopesticides is the key to the success in the development of sustainable agriculture.

In plant protection perspective, integrated pest management (IPM) programme is now adopted widely by commercial planters and farmers. With this regard, PGPR strains tested in this study are showing promising outcomes to be used for sustainable and environmentally friendly horticultural production system. The prospect and potential of manipulating PGPR by direct cell inoculation to increase crop yield and reduce disease pressure have shown considerable promise in laboratory and greenhouse studies. However, this technique is not really successful under field conditions. This might be due to climatic variations, and the soil itself is an unpredictable environment, and an intended result is sometimes difficult to achieve. Hence, development of new formulation biofungicide is urgently needed to overcome the above-mentioned limitations as well as to effectively control phytopathogens in field condition. As reported by many authors, biofungicides are safe or have very small residues and harmless to beneficial organisms, and the most important biofungicides are cost-effective to control many pests and diseases in the field.

## 12.7 Conclusions

*Burkholderia* sp. are promising biological control agents against the causal agent of foot rot disease, *P. capsici* Leonian, through the production of antifungal metabolites, induction of disease resistance and promoting plant growth. These results support the potential use of *B. cepacia* or its antifungal metabolites as a microbial alternative to control phytopathogens involved in high losses of agricultural production, diminishing the environmental problems caused by current practices. Government involvement by introducing specific policies or long-term programmes which is associated with "green technology" in order to monitor and protect clean environment is highly recommended for sustainable agriculture.

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