# Chapter 8
# Bioinoculants: Understanding Chickpea *Rhizobia* in Providing Sustainable Agriculture

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## 8.1 Introduction

Mass production or high-input agriculture, once believed to be the jewel of economic sustainability, now has become a target of many environmental catastrophes. Pulse crop farming, orchards, rice fields, and cotton farming are the essence of economic stability for many developing countries (Ramarethinam et al. 2005). The agriculture push coupled with increased populations and consumer demands has left many farmers and agriculturalists seeking new methods of providing sustainable growth. To cope with the ever-increasing demand, farmers and horticulturists have turned to biotechnology as a means for creating more applicable fertilizers and creating genetically modified seedlings that can fix to variant environments by introducing selective microorganisms that interact to combat pesticide and rhizobial dwelling pathogens (Nautiyal 2000). These methods brought forth the wake known as the "green revolution" (Khanna-Chopra and Sinha 1998).

Environmental concerns over water availability, alkalinity, and soil health, through traditional methods of chemical applications, had begun to overshadow quality of production. Agrochemicals and pesticide usage became less desirable, opting farmers to push towards a more sustainable and self-sufficient organic control method, encouraging quality with increased production rather than quantity for mass production (Kakde et al. 2005). This has eliminated such traditional methods of chemically taxing harvest areas and therefore opened the gateway for subsidizing biological control agents, one of which is proving to make great strides in providing sustainable agriculture, known as bioinoculants (Kakde et al. 2005; Narain 1998).

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Bioinoculants are defined as the concoctions of microbial entities that are supplemented as biocontrol agents to induce or suppress both biotic and abiotic factors in promoting sustained growth (Gupta et al. 2007). *Pseudomonas* and *Bacillus* spp. are common genera among bioinoculants that interact with diverse rhizobial communities. These bioinoculants undertake interactions between host and surrounding rhizosphere microorganisms by secreting and uptaking nutrients, known as root exudates (Hayat et al. 2010). Through associated, synergistic, and neutralistic interactions, plant growth and nodulation are promoted; however, antagonistic interactions may occur where competition for desired nutrients and production of antibiotic compounds may result in suppressing host characteristics (Nautiyal 2000).

Bioinoculant interactions are very important in low-lying nutrient-deprived soils. They are used in promoting the uptake of nutrients such as nitrogen and phosphorus and are used for their interactive capabilities to promote desired and suppress less desirable rhizospheric microorganisms (Borde et al. 2009). Plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhiza (AM) fungi improve root nodulation and other plant growth parameters, respectively, by mechanisms that increase surface area, improve root and shoot length, encourage sporulation, and eliminate the need for harsh chemical applications such as chemical fertilizers and insecticidal sprays (Wang et al. 2005).

Understanding the complexity of rhizobial interactions is crucial in determining sustainability; however, abiotic factors such as temperature, acidity, and soil composition may be delimiting factors that determine growth. Variant temperatures coupled with limited aeration and pH gradients promote the survival of microbes adapted to conditions suited for such environments (Singh et al. 2011). Regions where water is limited and shade is minimal would have high levels of evapotranspiration, thus requiring deep root and shoot integration. Rhizobial flora, in such an environment, would exist deep within the soils, requiring the bacterium to be tolerant of conditions where oxygen and available atmospheric nitrogen concentrations are limited (Upadhyay et al. 2000).

Applications must carefully be assessed before administering any foreign bacterium to a population of native bacteria. If rejection is encountered, survival of the native rhizobacteria as well as the supplemented microorganisms will be unlikely. Native dwelling rhizobacteria can initiate defense mechanisms to combat potential invading microbes by inducing the production of antibiotics or by releasing flavonoids and acting as phytoalexins, which may tax the plant and hinder the plant growth (Parmar and Dufresne 2011). Selecting microorganisms as potential applicants must be carefully tested through rigorous field studies to fully understand interactive traits. Bioinoculants essentially provide the potential of combating both biotic and abiotic factors as well as eliminating the need for harsh insecticides and chemicals, all the while promoting sustainable growth of the microflora within the rhizosphere.

## 8.2 Factors Affecting Sustainable Growth

Overcropping and mass production have become nationwide concerns for many agriculturists for reasons beyond systemic productivity. Traditional methods of chemical and insecticidal applications not only have reduced annual harvests; rather, their use has resulted in altered soil chemistry, disrupting the balance between plant–microbe interactions and chemical/ion/nutrient exchange. More so, using these strategies to increase rate of production has resulted in microbial sterility and decreased diversity of many beneficial microorganisms including rhizobial inhabitants (Wang et al. 2005). For such factors, examining and understanding how old practices, once believed to be the dawn of the agrochemical boom, have become the essence of failed intervention and hindered successive generational growth. Here, we examine some key factors that have hampered soil sustainability, thereby truncating successive growth yields.

### *8.2.1 Overgrazing, Excessive Cropping, and Improper Agricultural Practices*

Maximizing crop yield and fertility has been at the forefront for agribusinesses across the globe, pushing towards high production, quicker harvests, and lower costs. Excessive cropping and the expenditure of resources towards erratic practices such as overgrazing have left soils in dire shape (Kakde et al. 2005). Soil erosion, through less mitigating factors such as water or wind, is one direct consequence of such unsanctioned practices. Soil infertility and degradation in India alone has resulted in uncultivable top soil, reaching approximately 18.5 %, which is a statistical reading recorded as the maximum global loss (Sharma 2005). Also, 175 million ha of land from a total of 329 million ha geographical area is considered to be partially degraded in one form or another caused by excessive cropping and improper agricultural practices (Bhadauria et al. 2010). As such, natural vegetation, essential nutrients, stored organic matter, and microbial entities cannot perform/supply effective quantities to sustain sufficient growth nor provide fertility within the soil flora. Through continuous tilling and agitation, the soil becomes taxed and arid; as a result, organic/biological control methods become less responsive, as essential precursor elements become less available (Krebs 2000). Subsidizing organic controls, such as bioinoculants, to regions devastated by overcropping and overgrazing is a tedious and time-consuming process. Selecting appropriate microorganisms such as PGPR, understanding interactive characteristics of the indigenous microflora, and then mediating symbiosis may provide soil fertility in the long run. Unfortunately, organic turnover is slow and needs time to adapt to environmental conditions before even attempting to correct the damage left by such practices (Upadhyay et al. 2000). As is often the case, farmers and agriculturists turn towards aggressive chemical treatments to essentially save a harvest season,

potentially risking further tillage and mediating negative biological response. Reality is, without aggressive intervention in remediating such soils, natural replenishment of supplement nutrients and organic matter cannot provide an environment needed for supplying sustainable growth conditions.

### *8.2.2 Soil Salinity*

Sustained practices and soil quality are two key characteristics widely recognized as mitigating factors that determine successive growth. Through some abiotic factors and unconventional practices such as overgrazing and application of chemical fertilizers, insecticides, and pesticides, soils have become vulnerable to withering and anoxic conditions. Regions marked by such practices often turn saline as a result (Qadir et al. 2008). Without the necessary remediation tools, salinity can extend over fertility dynamics such as determining viability of microbial entities and consequently altering the rhizoplane structure itself. Salinity and nutrient stresses account for over 100 million ha of damaged farmland across the globe (Ashraf et al. 2009). In India, where pulse crop and leguminous harvests are major economic contributors to a high-population market, 8.5 million ha is considered degraded and highly saline, with 1.3 million ha reflecting in state of Uttar Pradesh (Bhadauria et al. 2010). Characteristics of saline soils are categorized by two parameters: one being soluble salts available in soil and the other, soil reaction. Soluble salts accumulate in soil through waterlogging and secondary salinization mechanisms, whereby these immobilized salts seep into the rhizoplane and adversely prompt changes in physicochemical properties. Such salts can also accumulate through the application of fertilizers, from atmospheric salt depositions, as seen near coastlines and weathering of soil minerals (Wang et al. 2009).

The significance behind "salting of the soils" describes the chemical shift or deviation in soil, which consequently effects rhizospheric competency. As more salt-tolerant bacteria capable of adapting in such environments proliferate, nutrients and minerals sequestered from soil become limited and specific. As a result, the affected region encounters what is described as a shift in microbial hierarchy or dominancy to counteract the chemical shift (FAO and IAEA 2010). This may further be accompanied by a shift in soil composition and plant demographics to reflect such environmental parameters. As the salt concentrations rise, osmotic stresses, which may also be initiated by arid or semiarid conditions, activate the plants to initiate defense mechanisms (Cordovilla et al. 1995). In this process, bacteria may go through a physiological change to try to adapt to the saline condition. Intracellular accumulation of low molecular weight organic solutes, called osmolytes, tries to counteract dehydration parameters in the plant by increases in potassium ($K^+$) concentrations. Increasing $K^+$ level acts to control magnesium ($Mg^{2+}$) flux's during osmotic shock as magnesium ions combat inhibitory response (Zahran 1999). Such a response eliminates any possibility of interacting with the newly colonizing microbes. Microbial activity can be

suppressed by transformation, through physiological changes, accompanied by the release of phytohormones and flavonoids, which may suppress microbial activity. More concerning, however, is the plant itself which may become chemically deprived by not receiving adequate amounts of essential nutrients needed to replenish itself and gear for survival (Singh et al. 2011).

### *8.2.3 Abiotic Factors*

Abiotic stresses have been at the forefront of many failed agribusinesses. Limiting factors, such as soil pH, aridity, aeration, and irrigation, occur naturally due to land topography, limited resources (e.g., water scarcity), climate, and landscape.

#### 8.2.3.1 pH Factors Affecting Sustainable Growth

pH gradients in an ecosystem vary region by region depending on many factors. Of those, salinity, soil composition, and localized plantation are considered influencing factors to pH profiles. Acidic soils require microorganisms that are capable of adapting in pH gradients less than 7. Acidic conditions promote growth factors that stimulate physical dismemberment of soil parameters and plant protective mechanisms, such as cysts and spores, in response to environmental stresses (Sethi et al. 1994). Acidic regions marked by coniferous plantations and aridity often contain soils with limited productivity and marginal diversity. Limited nitrate concentrations are characteristics of acidic soils which reflect truncated nitrogen fixation levels and often are counteracted by high pH, which inhibit growth regulators (Sharma 2005; Torimitsu et al. 1985). In addition, acidic soils are associated with high levels of manganese (Mn), iron (Fe), and aluminum ions ($Al^{3+}$) (Busse and Bottomley 1989). These toxic elements act as inhibitors by disrupting cell differentiation and morphology, suppress nutrient uptake, and undermine plant growth. Aluminum specifically inhibits root growth and phosphorous uptake, while Mn initiates physiological changes, such as black necrotic spots on leaves and chlorosis on leaf margins and cuppings (Busse and Bottomley 1989; Government of Alberta 2002). Essential nutrients such as phosphorus are needed to regulate metabolism and be utilized as energy. Phosphate is returned to soil in organic forms as organic phosphate is readily used by *Rhizobia*. In acidic conditions, phosphorus becomes difficult to attain as the organic element transforms into inorganic phosphate by anionic bonding to cations, such as Al or Fe, becomes fixed to the soil, and essentially is no longer available for nitrogen fixation, thus hindering growth (Wiederholt and Johnson 2005).

To limit toxicological effects of Mn, $Al^{3+}$, and Mn, remediation is selected towards application of buffering solutions, primarily carbonates and bicarbonates, to counteract acidic stresses (Powell 1994). Alkaline soils between pH 6.5 and 8.5 are preferred conditions where many plants are capable of adapting growth

parameters due to buffering capacities coordinated by host plant and rhizobia. In a study conducted by Bhadauria et al. (2010), using biological intervention for remediating alkaline wastelands, it was observed that appropriate selection of microbes and stimulation of ecological parameters could be maintained. Within a 3-year time frame, soil in such regions marked by alkaline pHs could be reclaimed at pH 8.5, accounting for a growth of 681 diverse tree species and 21 different tree types (Bhadauria et al. 2010). Microbes present in these alkaline soils promote the breakdown of calcium carbonates and calcium hydroxides which act as buffering compounds reducing hydrogen ions from suppressing growth. In such an environment, rhizobial colonies flourish by reducing acidic ions, leading to more available phosphorus, thereby leaving the surrounding rhizosphere as a neutral environment for interactions to occur without inhibitory stresses (Jakasaniya and Trivedi 2004). Selecting biological methods to naturally shift acidic soils towards more alkaline soils is encouraged. This process is quite slow and much work is needed before implementation; however, engineered bioinoculant controls, such as acid- or alkaline-tolerant bacterium which can promote growth in the rhizosphere, are gaining recognition and may be at the frontier of engineered soil inoculants in the near future.

#### 8.2.3.2 The Arid Soil Effect on the Rhizosphere: Limited Aeration and Irrigation

The significance and intensity of the arid soil effect on the rhizosphere and plant growth parameters are largely influenced by land topography, climate, and drainage. The moisture content of the soil without adequate irrigation through evapotranspiration may be lost at higher rates before soil can be replenished (Bhadauria et al. 2010). As a response to such environmental stresses, soil properties have manifested mechanisms in which water and nutrients can be retained, all in lower quantities, permitting potential correspondence with host plants (Vevrek and Campbell 2002). Arid and semiarid soils are fairly porous and aerated, allowing water and nutrients to penetrate into deep layers of soil, serving as a reservoir to existing soil microbes. To access this "reservoir" of nutrients and water, plants must go through physiological changes to accommodate their need for survival (Radwan 2009). In arid and semiarid regions, plant roots must stretch beyond surface layers into deep layers, often ranging between 7 and 10 f. (Rao 2002; FAO and IAEA 2010). This physiological change can be regarded as an adaptive response to aid in survival and, however, often may take several cultivation years before naturally adapting. Plants of tropical regions may not be able to survive in such environmental conditions. These plants are catered towards lateral surface and subsurface elongation in means of attaining water and nutrients. Without intervention in the form of bioinoculant controls, PGPR/AM fungi or a genetically engineered organism, these plants will struggle to survive, as evapotranspiration rates will eventually dehydrate the plant, resulting in shutdown and formation of cysts (Singh et al. 2009).

It is estimated that currently over 70 million ha of farming land is affected by drought/arid-like conditions, with numbers projected to rise. By 2025, the Food and Agriculture Organization (FAO) estimates 1.8 billion people will be affected by water scarcity with length of cultivable growing seasons ranging between 120 days in drylands and 74, or less, days in arid regions (FAO and IAEA 2010; Kassas 2008). Intensive irrigation, tilling, and soil composition are directly affected by precipitation and drainage efficiency in arid regions; however, often such soils are subject to higher saline concentrations, erosion, and soil degradation. As a result, a common mechanism associated with such factors is an increase in the water table due to waterlogging (Kassas 2008). Waterlogging exists through irony as water is the contributing factor leading to aridity. Contrary to how arid soils were described earlier, Sharma (2005) positions waterlogged regions by describing how the actual porous capability of soil in such a region has been truncated due to excessive tilling. The air pockets associated with naturally developed arid and semiarid lands due to environmental parameters are subsequently reduced. As a result, soil is less aerated and more compact with limited capabilities for water penetration deep into soil (FAO 1989). Anoxia is often the mitigating factor which mediates plant and rhizobial death in such practices (Jackson 2004). Sustained respiration and synthesis of metabolites and the rapid exchange of $O_2$ and $CO_2$ become limited and difficult to attain with the influx of water (Setter and Belford 1990). The plant essentially fixates from its own redox reactions as facultative anaerobes eliminate nitrate utilization by converting nitrate into nitrogen gas through a process of denitrification. More concerning is chemical oxides such as Mn and Fe reduced into highly soluble forms $Mn^{2+}$ and $Fe^{2+}$, leading to chemical toxicity that enters roots and disrupts cell morphology and differentiation (Arshad and Frankenberger 1990; Laanbroek 1990).

Further, microbial activity and viability become suppressed as soluble salts accumulate in subsurface layers, resulting in altered chemistry around the roots. With inadequate irrigation or drainage, the waterlogged regions remain stagnated with limited permeability, and as a result evaporation rates increase, leaving behind a highly concentrated saline layer (Sharma 2005). Waterlogging has been estimated to occur approximately in 10 % of all irrigated farmland, resulting in a 20 % decrease in crop productivity (Jackson 2004). Without supplementing biotechnology or corrective irrigational means, soil in such regions will continue to become more saline. As harvest seasons prolong, limited yields, fertility, and allocated growing periods will decline as soil chemistry, microflora, and vegetation will all shift to cope with arid pressures (Sharma 2005).

## 8.3 Effects of PGPR Bioinoculants

The microbial flora within the rhizosphere exists as a continuous complex of interactions and sustained mechanisms. Diverse microbial communities interact among one another in the aim of attaining sustainability, be it through synergism,

neutralism, associated or antagonistic interactions. This is known as developing rhizospheric competency (Nautiyal 2000). These interactions determine soil health and plant viability as a means of characterizing which species will dominate within the rhizosphere (de Selincourt 1996). Through chemical breakdown and uptake of essential nutrients, a dominant species, such as PGPR, will encourage growth and proliferation of plant parameters as well as reduce invasion from competitors by inducing mechanisms that readily fix nitrogen, secrete siderophores for iron utilization, and promote the synthesis of phytohormones (Glick 1995). Either of these mechanisms used by PGPR bioinoculants can provide conditions that stimulate secretion of root exudates from host plants, thereby encouraging colonial growth of the novel species complimentary to the PGPR bioinoculant (Lynch 1990).

Interaction and uptake are of essence the mitigating factors for successive colonization and proliferation of PGPR with an existing indigenous population. The concentration of bacteria surrounding the rhizosphere as per gram of soil compared to that of the bacteria found existing in aggregates dispersed throughout the soil is generally found at much higher folds (Lynch 1990). This accounts for the high levels of metabolic activity occurring within root regions. Nutrients such as atmospheric nitrogen, phosphorus, and carbon are readily available in agro-rich regions. Rokhzadi et al. (2008) demonstrated nutrient acquisition capabilities by studying the interactions of symbiotic bacterium *Mesorhizobium ciceri* and nonsymbiotic rhizobacteria from the *Azospirillum*, *Azotobacter*, and *Pseudomonas* genra on growth and yield of *Cicer arietinum* (Rokhzadi et al. 2008). Combined inoculation with mutually inclusive traits promote symbiotic activities that often result in increased nutrient acquisition by activating host characteristics that allow recognition and release of root exudates into soil (Sindhu et al. 2002). Rhizobacteria mutually respond by uptaking soil nutrients and fixating them so they can be used for plant synthesis. Stimulation inevitably results from the sustained nutrient supply and exchange within the root, promoting cellular respiration and differentiation in plant tissues (Rokhzadi et al. 2008; Zhang et al. 2011). This mechanism is similar in both bacteria and fungi, displaying characteristics of growth fixation, uptake, and release of nutrients by host plant and surrounding microbes (Zhang et al. 2011).

### *8.3.1 PGPR's Mechanism of Biocontrol Within the Rhizosphere*

Under environmental norms, mechanisms implemented to support PGPR's inhabitation, growth, and proliferation in the rhizosphere lie towards secondary methods of protection to complement the symbiotic invasion: the production of phytohormones and the release of flavonoids and phytoalexins (Parmar and Dufresne 2011). These are secreted as a by-product of an endosymbiotic interaction to provide the plant with support and protection against invasive or damaging stresses (Glick 1995). We will focus on the role of phytohormones on plant growth

promotion and then try to correlate these findings with mechanisms employed by PGPR treated in *Cicer* dwelling rhizospheres.

#### 8.3.1.1 Phytohormones Supplemented with PGPR Inoculants

Phytohormones are described as plant growth-promoting hormones active in regulating response to biotic and abiotic stresses through synergistic or antagonistic actions. This is referred to as signaling cross talk (Schmelz et al. 2003). These hormones induce cell elongation, aid in cell division and differentiation, and promote lateral root development to allow nutrients and minerals to be sequestered from distant and localized regions, essentially aiding in plant versatility under conditions of limited surface nutrient availability (Hong et al. 1991). We will focus primarily on auxins as hormones responsible for plant growth promotion due to the interactive traits displayed against PGPR. Auxins are defined for their characteristic as a plant hormone containing indole-3-acetic acid (IAA), which, through its synthesis, stimulates rapid cell growth and differentiation (Cleland 1990). PGPR accelerate cell differentiation through its capacity to synthesize auxin, a role typically reserved to plants which now is able to transmit dual synthesis, accelerating cell growth and proliferation (Gaudin et al. 1994). However, Gaudin et al. (1994) suggested, to positively benefit from maximum auxin synthesis, the primary objective is to distinguish between the degree of auxin synthesis in plants, void of PGPR, and compare that to the level of auxin synthesis when a bioinoculant, such as PGPR, is supplemented in the rhizosphere (Gaudin et al. 1994). To understand such characteristics, a wheat plant was supplemented with a mutant of *Azospirillum brasilense* strain, and trace quantities of IAA synthesis were found (Glick 1995). Compared to the wild-type strain, production of IAA was much limited, and as a result, limited IAA cannot effectively promote formation of laterals roots, thereby eliminating such physiological characteristics associated with PGPR stimulation (Glick 1995).

A study done by Khalid et al. (2004) demonstrated how effective PGPR is as an auxin synthesizer and to which degree PGPR's characteristics can promote survival in foreign rhizospheres. The team used tryptophan (L-TRP) as their method of control. The significance behind L-TRP is that it is an amino acid readily secreted in root exudates, which holds as a precursor for the biosynthesis of auxins in plants and microbes (Frankenberger and Arshad 1995). PGPR supplemented in L-TRP-deficient soils were found to synthesize plant auxins in varied amounts; however, when comparing this number to TRP-positive PGPR-deficient inoculums, PGPR prompted synthesis at much higher levels even in the absence of TRP. In the presence of TRP, auxin synthesis was heightened several folds, with Glick (1995) suggesting that PGPR symbiotically coordinates uptake and chemical breakdown with the plant's vascular system in an effort to enhance auxin synthesis (Glick 1995). Khalid et al. (2004) also tested the versatility of PGPR strains inoculated in sterile and non-sterile soils, a test to understand compatibility of PGPR with the indigenous microflora. Monitored through auxin synthesis analysis, it was found

that PGPR effectively prompted auxin synthesis at higher levels in both sterilized and non-sterilized soils as compared to the preexisting uninoculated strains. However, uninoculated strains in non-sterilized soil provided higher degrees of synthesis compared to single strain PGPR inoculants in sterilized soils. Furthermore, PGPR inoculated in non-sterilized soils, in tandem with the preexisting microflora, substantially accelerated auxin synthesis, which was achieved through the secretion of plant growth-promoting substances which encourage other PGPR bioinoculants to take forth in the mutually inclusive interaction (Okon and Vanderleyden 1997). Once established, the symbiotic partners fix nitrogen, promote metabolic activities, and indirectly stimulate the plant to release much needed exudates to enrich the soil (Parmar and Dadarwal 1997). As a result, PGPR-inoculated plants showed early germination, early development and flowering, and increases in dry weight of root and shoot parameters, all of which correlated to higher yields and increased biomass (Khalid et al. 2004).

### 8.3.2 *Synergistic Relations of PGPR with Native* Cicer Rhizobia

*Cicer arietinum*, also known as chickpea, is among the high-demand pulse crop selection geared towards serving Middle Eastern and south Asian diets. With such demands, productivity and marketing of *Cicer* and other pulse crops is essential in establishing successive harvests and generational fertility to serve a largely vegetarian population (Reddy et al. 2000). Traditionally, production was often heightened through the use of chemical fertilizers and insecticidal sprays to combat invasive species and promote soil fertility; however, after subsequent application, physiological side effects and growth yield began to shrink. Rajasthan's state environmental policy 2010 reported truncated growing periods and necrosis of cellular components in plant, and indigenous populations began to propagate, suppressing biological activity and shifting the plant towards defense/survival mechanisms. Through such a response, production inevitability falters as host symbiosis no longer can be sustained, bringing about a microbial shift and consequently altering the interactions within the rhizosphere (Department of Rajasthan 2010).

Coupled with the interactions of applied bioinoculants, PGPR can exist in any form that promotes growth and fixation between host and the native microflora population (Glick 1995). PGPR regulates successive growth and coordination through two mechanisms in *Cicer* sp. and other leguminous crops: endosymbiosis interactions with host *Rhizobia* and biocontrol stimulatory response mechanisms and differentiation in cellular components (Zhang et al. 2011; Glick 1995). PGPR and *Rhizobium* interactions are marked by selective integration and release of intermediary metabolites that induce uptake and growth. Such metabolites include flavonoids; phytohormones, such as auxins as mentioned earlier; iron-chelating

siderophores; and antibiotics (Glick and Pasternak 2003). It is well known that *Rhizobia* are equipped with specialized *Nod* genes; these *Nod* genes are inactive in the absence of host legumes, such as *Cicer*. Together with *Nod* factors, signal transduction between symbionts is expressed through an affinity for receptor and signaling molecules adjacent to *Nod* (Tilak et al. 2010). *Nod* genes induce response in the epidermis near the distal part of the nodule infection zone where infection threads and bacteria are released (Mirabella et al. 2005). These infection sites harvest regions that provide additional occupancy for *Rhizobia* to colonize, enhance the solubilization of inorganic phosphates, and provide protection for the plant from phytopathogens (Hayat et al. 2010). This ultimately promotes growth through enhanced nodule and root hair formation along root structures and phosphorus utilization, heightening physiological function of plant respiration and indirectly prompting soil fertility (Tilak et al. 2010).

#### 8.3.2.1 Growth Promotion by *Pseudomonas* spp.

*Pseudomonas* spp. have been identified as novel forms of PGPR that act synergistically with indigenous populations to promote growth and proliferation of plant parameters (Antoun and Prévost 2005). In vivo and in vitro studies have shown that supplementing *Pseudomonas* spp. as a PGPR-directed bioinoculant causes significant increases in nodule yield, weight, and root and shoot biomass of various legumes and marked increases in soil fertility (Parmar and Dufresne 2011). The secondary function observed with *Pseudomonas* spp. while promoting nitrogen fixation and symbiosis with the native bacteria population is to reduce infection from phytopathogens by acting as an antagonist towards soilborne plant pathogens (Khare et al. 2011).

Production of indoleacetic acid (IAA) stimulates cell elongation and cell division by activating aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Jacobson et al. 1994). The use of *Pseudomonas fluorescens* as a biological control method for chickpea wilt was demonstrated by supplementing *P. fluorescens* within the rhizosphere. This not only promoted growth and interactions of the native rhizobacteria but acted as an antagonist towards *Fusarium oxysporum* f. sp. *ciceri* (Vidhyasekaran and Muthamilan 1995). *Fusarium oxysporum* f. sp. *ciceri* is one of the most devastating known soilborne fungal pathogens keen on disrupting cellular processes and translocation of water and nutrients through development of spores, causing vascular wilt, chlorosis, flaccidity, and discoloration in chickpea plants (Cho and Muehlbauer 2004; Buddenhagen and Workneh 1988). The stages of infection caused by *Fusarium oxysporum* f. sp. *ciceri* in chickpea xylem vessels were captured by Gupta et al. using scanning electron microscopy (Gupta et al. 2010). Symptoms of the pathogenic infection were analyzed over a subset of 4 days postinoculation (DPI) in a susceptible breed of chickpea plant, JG62, to measure degree of wilt and internal vascular disintegration. At 4 DPI, the onset of beginning stages of tissue damage in the xylem vessels was seen with microspores beginning to propagate within the xylem tissue interior. At 8 DPI, larger numbers of spores

were found causing pronounced vascular tissue damage in the xylem. At 12 DPI, the appearance of microconidia was apparent, translating into complete demolishment of the original structure of the xylem tissue. In comparison, using the wilt-resistant WR315 chickpea plant, it was found that, at 15 DPI, no damage or sporadic activity within the xylem was noticed. Only after 22–24 DPI, fungal spores were detected and after 28 DPI colonization and slight tissue damage were seen; however, no fungal division was present (Gupta et al. 2010).

Using synergistic biocontrol methods, Nautiyal supplemented *P. fluorescens* NBRI1303 as an active antagonist to pathogenic *Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia bataticola*, and *Pythium* sp., three of the most devastating pathogens affecting chickpea (Nautiyal 1997b). When inoculated among chickpea seed cultivars with *P. fluorescens* NBRI1303, seed germination increased by 25 %, the number of diseased plants reduced by 45 %, and seedling dry weight and shoot as well as root length increased between 16 and 18 %. These results suggested that the PGPR bacterium (*P. fluorescens*) actively and aggressively interacted with chickpea *Rhizobia* to colonize and mediate mechanisms to readily promote, sequester, and regulate nutrient–soil homeostasis, all the while maintaining suppressive behavior of phytopathogenic species (Nautiyal 1997a, b). Recently, Maheshwari et al. (2011) reported co-inoculation of urea- and DAP-tolerant *Sinorhizobium meliloti* and *Pseudomonas aeruginosa* as an integrated approach of growth enhancement of *Brassica juncea*. Gupta et al. (2010) further elaborated on this notion of wilt resistance using *Pseudomonas* spp. in chickpea seedlings. Phenotypic changes in chickpea plants over a subset of three 4 DPI intervals with infected JG62 and resistant WR315 were studied. Despite slight yellowing of the roots, WR315 plants after 12 DPI remained unaffected as compared to JG62, which by 8 DPI began showing major signs of wilt, chlorosis, browning of roots, and retardation of branching and growth. By 12 DPI, the plant suffered major loss with chlorosis, accompanied by root blackening due to increased phenolic deposition (Gupta et al. 2010). Similar antagonistic interactions were also observed by Parmar and Dadarwal (1997), studying sustainability and effectiveness through co-inoculation of rhizosphere bacteria such as *Bacillus* spp. (*Pseudomonas* sp.) with chickpea *Rhizobium*. Results indicated significant increases in nodule weight, nitrogen uptake, and root and shoot biomass. *Pseudomonas* spp. "CRP55b" strain acted symbiotically to induce increased production of flavonoids like compounds in roots on seed bacterization accounting for enhancement in growth and percent yield (Parmar and Dadarwal 1997). More recently Rokhzadi et al. (2008) used a combination of bioinoculant strains *Azospirillum* spp., *A. chroococcum* 5, *Mesorhizobium ciceri* SWR17, and *P. fluorescens* P21 to mimic similar responses against chickpea cultivars. The combined effects of the bioinoculants and PGPR strain enhanced nitrogen and phosphorus consumption and availability, increased supply of nutrients, and enriched production of growth-promoting substances. Secondary effects markedly reduced phytopathogen populations and competitively inhibited antagonistic populations. These factors accounted for improved nodulation, increased dry matter content in roots and shoots, and promotion of grain, biomass, and protein yields (Rokhzadi et al. 2008). Inoculation with PGPR increased growth

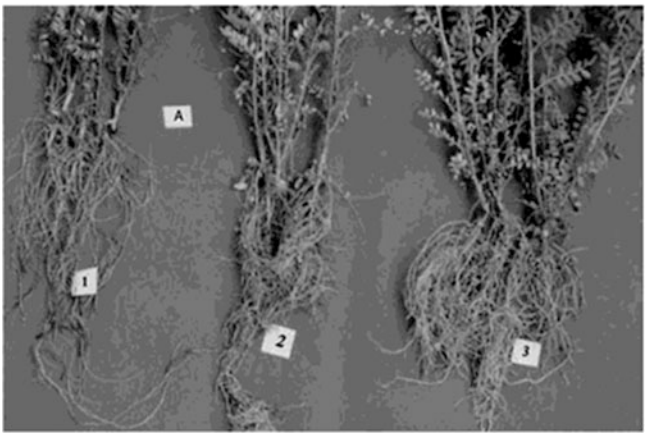


**Fig. 8.1** Comparing *Cicer arietinum* (chickpea) growth inoculated with modified rhizobial symbionts (Parmar and Dadarwal 1999)

parameters in chickpea plants with increased biomass and lateral root formation in our studies (Fig. 8.1).

#### 8.3.2.2 Growth Promotion by *Bacillus* spp.

*Bacillus* spp. is another type of soil-dwelling rhizobacteria often used for its growth-promoting capabilities. *Bacillus* spp. mode of action orients itself towards providing biological control methods through suppression of plant pathogenic organisms, production of iron-chelating siderophores, and release of antibiotics (Timmusk et al. 1999; Pal et al. 2000; Chakraborty et al. 2006). The capacity of *Bacillus* spp. to sequester iron and other heavy metal compounds from soil prevents redox reactions from converting heavy metal compounds into radical forms, which are known toxicological elements geared towards suppressing plant metabolism (Miethke and Marahiel 2007; Tian et al. 2009). Furthermore, the siderophore activity prevents pathogenic organisms from uptaking iron; starving the pathogens thus creates an environment unsuitable for growth and infection (Saharan and Nehra 2011).

*Bacillus* spp. mode of interaction and growth compared to *Pseudomonas* spp. is much similar. Both PGPR are capable of effectively solubilizing insoluble phosphate, hence commonly described as phosphate-solubilizing microorganisms (PSM). Both genera have potential to increase yield and biomass content, and both mediate symbiosis with plant and validate expression on biocontrol mechanisms (Saharan and Nehra 2011). In a study oriented towards understanding inoculated characteristics of PGPR and *Rhizobium* in chickpeas, Verma et al. (2010) found significant increases in nodule formation, nodule dry weight and nutrient concentration, root and shoot biomass production, and grain and straw

yield. Tests were conducted in field for a period of 2 years using inoculated seeds and control (no bacteria), *Rhizobium* spp., *Rhizobium* spp. + *A. chroococcum*, *Rhizobium* spp. + *P. fluorescens*, and *Rhizobium* spp. + *B. megaterium*. The only considerable differences captured between coinoculated *Rhizobia* + *Pseudomonas* and *rhizobia* + *Bacillus* were the following: earlier combinations produced higher levels of indoleacetic acid (IAA), siderophore production, HCN utilization, and the inhibition of *Fusarium oxysporum* as compared to rhizobia +*Bacillus megaterium*. This difference in IAA levels account for *Pseudomonas* spp. ability to synthesize tryptophan, which, as described earlier, is one of the most recognized auxins involved in promoting cell and stem elongation (Verma et al. 2010). *Bacillus* spp. marked advantage as a bioinoculant lies towards its capacity to survive within extremes and irregular environments. *Bacillus* spp. has been recognized as moderate halophiles or halotolerant bacteria. In the rhizosphere, *Bacillus cereus* 80 is capable of adapting to various concentrations of salt, ranging in soil from 0 to 5 % NaCl (Welsh 2000). In cultivating chickpea, Indian farmlands are often characterized by soil salinity or alkalinity, requiring corrective remediation and inoculation with successive strains to counteract such adverse conditions (Bhadauria et al. 2010). Through seed inoculation of *B. subtilis*, growth factors in chickpea were maintained at various conditions and often showed enhanced biomass and nodule formation (Siddiqui and Mahmood 1995). Furthermore, *B. subtilis*, a well-recognized antagonistic bacterium against phytopathogens, can survive high soil and arid conditions ranging from 30 to 60 °C (Brock 1978; Edwards 1990). These physiological traits marked *Bacillus* spp. as one of the most recognized and versatile PGPR species. In association, as a single bioinoculant or selected part of a co-inoculant, *Bacillus* spp. and *Pseudomonas* spp. exhibit traits that induced proliferation geared towards suppressing soilborne pathogens and promoting plant growth parameters.

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

When considering fungi as a source of soil inoculums, often negative connotations propelled by the intensive degradation by fungal species (e.g., *Fusarium oxysporum*) are contributing factors to agricultural condemnation. However, recent advances towards biotechnology have identified fungal species capable of promoting successive growth and increasing soil fertility (Sharif and Moawad 2006). The major groups of fungi that establish mutualistic symbiosis are categorized for their ability to interact with the roots of various plant species, referred to as mycorrhizal symbionts (Ahmad et al. 2008a). Arbuscular mycorrhizal fungi (AMF) have been identified as existing entities in most agroecosystems, colonizing the root cortex biotrophically and establishing a mycelium bridge (hyphal network), connecting root to surrounding microhabitats (Egamberdiyeva et al. 2004). AM fungi are

considered as obligate microbial symbionts, dependent on colonization of host plants to maintain viability in the system. This mutually exclusive relationship benefits the host through correspondence with the mycorrhizal hyphal network, providing a larger surface area for absorption of essential immobile ions such as phosphate, copper, and zinc needed by the plant for sustaining growth (Paraskevopoulou Paroussi et al. 1997; Masoumeh et al. 2009). Mycorrhizal symbiosis also provides the plant with versatility against various biotic and abiotic stresses through formation of stable soil aggregates, selective proliferation of synergistic microbial colonies, and formation of macropore structures in soil to facilitate aeration and water penetration to deep surface layers (Piotrowski et al. 2004). These compositional structure modifications and branching complexes allow nutrients to be sequestered from various deep soil reserves, mandating a push towards plant fitness and tolerance, increasing the probability of survival when subsurface nutrient concentrations are limited or faced with harsh environmental conditions (Ahmad et al. 2008b).

*Macrophomina phaseolina* (tassi) is a common root rot fungus, infecting about 500 plant species, one of which being *Cicer arietinum* (Srivastva et al. 2001). *Rhizobia* provide an initial barrier to fungal pathogens; however, through the use of AM fungi species, potential for remediating pathogenesis while promoting growth is possible (Siddiqui and Akhtar 2009; Ozgonen and Erkilic 2007; Akkopru and Demir 2005). Akhtar and Siddiqui (2010) studied the influence of four AM fungi species, *Glomus intraradices*, *G. aggregatum*, *G. claroideum*, *and Glomus* sp., for biocontrol of *M. phaseolina* on *Cicer arietinum* pod growth, nodulation, chlorophyll, nitrogen, phosphorus, potassium concentrations, and effectiveness of controlling root rot. The experimental design consisted of five randomized blocks, each with different treatments: (1) *G. intraradices*, (2) *G. aggregatum*, (3) *G. claroideum*, (4) *Glomus* sp., and (5) control in the presence and absence of *M. phaseolina* (Akhtar and Siddiqui 2010). The plants were harvested 90 days after inoculation and grown in sandy loam soil mixed with washed river sand and farm yard manure at 3:2:1. The inoculation of all four AM fungi species without treatment of *M. phaseolina* exercised all growth parameters as compared to the uninoculated control. Increases in shoot dry weight, number of pods per plant, the number of nodules per root system, nitrogen, potassium, phosphorus, chlorophyll, and degree of root colonization by AM fungi were all exhibited after the 90-day harvest period, with *G. intraradices* optimizing greatest yields. Under the influence of *M. phaseolina*, interestingly enough shoot dry weight also increased, recording higher percentages, and then control and non-pathogen treatment. This gain corresponded to the increased shoot dry weight of pathogenic fungus manifested through AM fungi colonization, however; this also resulted in considerable decreases to the number of pods per plant as compared to non-*M. phaseolina* treatment (Akhtar and Siddiqui 2010). The number of nodules per root system stayed relatively the same, while root colonization of AM fungi was found to be considerably lower, suggesting formulation of spores and/or the activation of plant defense mechanisms, inhibiting growth and colonization (Demir and Akkopru 2005). Through the influence of AM fungi on *M. phaseolina*-treated plants, a

reduction in root rot index was seen, suggesting that the uninoculated control (index of 4) was less effective in secreting enzymes and biocontrol compounds necessary to maintain viability after infection (Pozo et al. 1999).

#### 8.3.3.1 PGPR Interactions with AM Fungi as a Potential Bioinoculant

Diversity in the rhizosphere and surrounding microhabitats is marked by various interactive microfloras, stimulating mechanisms to promote or suppress microbial activity. AM fungi establish host specificity by infecting the host cortical cells, forming arbuscules along the plant root architecture. In this, the soil-dwelling *Rhizobium* and PGPR bacteria interact through endosymbiosis, forming an AM fungal endosymbiotic bacteria capable of promoting rhizobial interactions with mycorrhizae and plant (Bianciotto and Bonfante 2002). The typical rhizobacteria–AM fungi interaction describes PGPR as the "mycorrhizae-helper microorganism/bacteria," active in stimulating mycelial growth and/or enhancing mycorrhizal formation (Garbaye 1994). PGPR or soil-dwelling *Rhizobia* interact with the mycorrhizal fungi by adhering to fungal spores and hyphal structures, initiating exposure and spread to other microorganisms capable of symbiosis within the rhizosphere (Bianciotto and Bonfante 2002). As PGPR or *Rhizobia* interact with the host plant, the rate of exudate expulsion increases. When aided by the presence of AM fungi, the secretion of root exudates stimulates mycelial growth in the rhizosphere and initiates root penetration by the fungus (Azcon-Aguilar and Barea 1992).

Furthermore, as Azcon-Aguilar and Barea (1992, 1995) observed, the rhizobial interaction influences presymbiotic stages of AM fungal development such as spore germination and mycelia growth, when coupled by the release of plant hormones, instigate AM establishment within the rhizosphere and root cortex. Such morphological transformations induce physiological changes within the plant and surrounding environment to complement the interaction. Symbiosis alters the chemical composition of root exudates through changes in host's physiology, establishing shifts in mineral nutrient disposition of plant tissues, carbon allocation and utilization, and hormonal balances. However, physical development of AM mycelium in the rhizosphere/rhizoplane induces the synthesis and metabolism of essential plant and microbial parameters by acting as an abundant source of carbon (Barea et al. 2005). Secretion, uptake, and availability of root exudates, phytoalexins and, phenolic compounds become more abundant, prompting soil composition to become systemically modified to accommodate elevated interactions (Duponnois et al. 2005), thereby inducing physiological changes in the rhizobial community, marketing both quantitative and qualitative production of viable active symbionts, such as PGPR (Barea et al. 2005). This well-nourished and rich region of interaction and growth of mycorrhizae and mycelia is referred to as the mycorrhizosphere (Linderman 1988; Gryndler 2000). In the mycorrhizosphere, the principle of interaction is oriented towards promoting phosphorus uptake. Through the extensive branching between AM fungal mycelium and host root

structures, access to phosphate ions in soil can be elevated, extending beyond the phosphate depleting zone and into deeper regions in soil (Smith and Read 1997). Aside from providing the vessel for transport and available carbon, AM fungi contributed to phosphorous capture by linking the biotic and geochemical portions of the soil ecosystem, thereby affecting both phosphorous cycling rates and patterns (Jeffries and Barea 2001).

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

#### 8.3.3.2 Promotion by AM Fungi–PGPR Symbiosis

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

In fact, the postinoculation period between 45 and 90 days was marked by significant levels of growth through collective combinations of PSM on root

infection and spore density (Zaidi et al. 2001). This persistent symbiotic behavior between AM fungi, PGPR, and rhizobia suggested similar results can be obtained in environmentally stressed soils where viable growth is hindered due to source availability. AM fungi species *Glomus intraradices* as a co-inoculant with *P. fluorescens* exhibited varying deficit intensities. Individually, in water-deprived soil, *P. fluorescens* (Pf) had limited grain and biomass production, while co-inoculation with AM fungi increased assimilation of phosphorus and nitrogen concentrations, equivalent to that of chemical phosphorus treatment. However, when inoculated in water-deficient soil, dual inoculation with phosphorus fertilizer and AM + Pf inoculation significantly increased grain phosphorous and nitrogen concentrations as compared to uninoculated well-watered treatments (control). Root colonization was significantly higher in applications with dual inoculants, against control (uninoculated) and phosphorus fertilizer treatment in well-watered soils (Ehteshami et al. 2007). Such increased levels of colonization coincide with increased ACC-deaminase and chitinase activity (Shaharoona et al. 2006). Further, Ehteshami et al. (2007) suggest these gains market proliferation through the aid of plant hormones (phytohormones) and release of regulatory metabolites to counteract and maintain vitality during erratic intensities of water deficit (Ehteshami et al. 2007). Earlier, Subramanian et al. (2006) suggested that the increased absorptive surface area and densely proliferated root growth in the mycorrhizosphere complement increased root colonization and infection. These characteristics support the use of bioinoculants as potential remediation tools to combat water-deficit stresses. However, water uptake through a plant vascular system can be hindered if severe stresses disrupt root architecture and distribution, thereby affecting the rate of water absorption per unit root (Auge 2001). In such case, naturally occurring bioinoculants may not be as effective to counteract such stresses; however, a tool is out there to market biocontrol with higher degree of success and adaptability: the development of anti-pathogens and genetically engineering bioinoculative strains.

## 8.4 Engineering Bioinoculants as Remediation Tools in Agriculture

With an increase of biotic and abiotic stresses plaguing agricultural sustainability, scientists are aggressively pushing towards biological controls as a means of primary remediation. Through the study of soil ecology and interaction, scientists harvest the knowledge of symbiosis existing as a biological phenomenon involving dynamic changes in the genome, metabolism, and signaling network (Kawaguchi and Minamisawa 2010). Dynamic changes in genome are of particular interest, as genetic engineering provides the capacity to manipulate biological growth-promoting strains to correspond with indigenous microflora in order to maximize productivity in harsh soils (Upadhyay et al. 2000). By mimicking indigenous traits, engineered bioinoculants are capable of adapting to various stresses through

production of antimetabolites to inhibit nodule occupancy of native *rhizobia*, enhance regulation of plant–microbe signaling, adapt to environmental stresses, and enhance nutrition and Exudates sequestration and usage (Archana 2010). Others have linked engineering bioinoculants through beneficial relation of the plants to resist soilborne pathogens, become better hosts to symbiotic microbes, remediate toxic waste, and even attract communities of soil microbes to enhance plant growth (O'Connell et al. 1996). These methods have been provided to increase growth, fertility, and viability throughout harvesting seasons so farmers are capable of competing and succeeding against the demanding agriculture market without sacrificing quality and yield.

### *8.4.1 Engineering Bioinoculants as Anti-pathogens*

Scientists are seeking innovative ways to engineer the rhizosphere in the aim to create a biased rhizosphere which essentially engineers the plants to secrete nutrients that specifically enhance the growth of mutualistic microbes (O'Connell et al. 1996). In such an attempt, to maximize efficiency, selecting to control root rot and pathogen invasion was of primary concern. Without adequate pathogen control, invasive species will try to persist as the engineered rhizosphere is now the epicenter of nutrient and chemical exchange. A lag phase between plant–microbe symbioses may hinder development and seed fertilization due to the initial competition in the rhizosphere, depriving both the plant and microbes of essential energy and compounds needed for sustained cellular and respiratory functions (Glick 1995). As mentioned earlier, to control chickpea root rot, *P. fluorescence* NBRI1303 was supplemented in soil to act as a pathogen antagonist towards *R. bataticola*, *F. oxysporum* f. sp. *ciceri*, and *Pythium* sp. (Nautiyal 1997a). Engineering, without genetic manipulation, a chickpea rhizosphere-competent strain involved greenhouse assays to evaluate the root-colonizing capacity of native chickpea rhizosphere. By selecting out and inoculating the spontaneous chromosomal Rifr strains to seeds, without checking for mutation, the isogenic form of the Rifr strain could be compared against survival and competition with that of the isogenic parent and one another to exhibit specific traits. These strains could then be added to a mixture of isolates and observed for stable growth and treatment against soilborne pathogens or pests (Nautiyal 1997a). The NBRI1303 was identified as the first reported single biocontrol bacterium active against the three most devastating pathogenic fungi of chickpea (Nautiyal 1997b). Rifampicin-resistant mutant *P. fluorescens* strain NBRI1303R confirmed NBRI1303 capacity to control pathogen infection by observing the rapid and aggressive root colonization. In particular, strain NBRI1303 reduced the number of diseased plants by 45 %, significantly promoted seed germination, and increased yield, length, and overall biomass of chickpea (Nautiyal 1997b).

Treatment using PGPR has also provided an alternative against combating viral diseases without the use of abrasive chemical pesticides and sprays through induced

systemic resistance (ISR), which characterizes increased synthesis of defense enzymes (M'piga et al. 1997; Zehnder et al. 2000). ISR was best described by Kirankumar et al. (2008) expressing resistance against the tomato mosaic virus, reporting a reduction of weight up to 59.0 % with a mean disease incidence recording at 55.98 % (Kirankumar et al. 2008; Cherian and Muniyapppa 1998). Several of the PGPR isolates were able to control early blight disease of tomato caused by *Alternaria solani* through induced system resistance (Earnapalli et al. 2005). ISR's mechanism behind establishing resistance lies through PGPR's ability to conform physiological and biochemical reactions of the host, resulting in the synthesis and secretion of defense chemicals against pathogenic organisms (Van Loon et al. 1998). As a result, phenol content, peroxidase and phenylalanine ammonia lyase, (PALase) enzymes witnessed a multiple fold of augmented activity. The major biological properties of phenolic compounds are reflected towards establishing antimicrobial activity, while peroxidase is a key enzyme in the biosynthesis of lignin and oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates, which has been correlated with viral disease resistance (Saini et al. 1988; Bruce and West 1989). PALase is responsible for biosynthesis of various defense chemicals in phenylpropanoid metabolism and promotes plant functions that elicit strength and repair of the cell wall, antimicrobial activity, and signaling (Daayf et al. 1997). In addition, ISR-expressing plants have the capacity to convert, aminocyclopropane-1-carboxylate (ACC), an essential precursor molecule to ethylene biosynthesis, which acts as a suppressant against phytopathogens during initial stages of pathogen attack (Niranjan Raj et al. 2005).

Understanding the signaling pathways and supplementing advantageous microbes to the rhizosphere mediate selective remediation where biological recognition and response are tightly monitored. *P. fluorescens* supplemented in soil has shown remarketed beneficence in growth of various legumes, with secondary characteristics geared towards reflecting sustained pathogen control. *P. fluorescens* produce salicylic acid, which acts as local and systemic signaling molecule, inducing resistance in plants through activation and adherence to secondary plant hormones, jasmonic acid, and ethylene (De Meyer and Hofte 1997). Signaling compounds such as salicylic acid (SA) and ethylene (ET) play roles in regulating and inducing basal resistance. SA is a key regulator of pathogen-induced systemic acquired resistance (SAR), while ET is initiated through rhizobacteria-mediated induced systemic resistance (ISR) (Niranjan Raj et al. 2005). Root colonization of *A. thaliana* by *P. fluorescens* WCS417r has shown to elicit ISR against *P. syringae* pv. tomato (PST) (Knoester et al. 1999). Mutant ethylene-response *P. fluorescens* WCS417r strains revealed ISR function suppression, while SAR function remained unaffected (Knoester et al. 1999). SAR differs with regard to its capacity to be effective against pathogens that non-induced plants are resisted through SA-dependent defenses, while ISR are effective against pathogens in non-induced plants and dependent on ET-producing compounds (Ton et al. 2002). Knoester et al. (1999) found diminished ethylene production in roots/leaves and limited expression of the ethylene biosynthetic enzymes, ACC synthase and ACC oxidase, and suggested that the expression of ISR requires complete submission of the signal

transduction pathway. Thus, the potential to mediate signal transduction on *P. fluorescens* WCS417r strains with nonmutant ET-dependent pathways is possible and can be implemented to similar biochemically inducing ISR pathways in plants.

#### 8.4.1.1 Engineering Resistance Through Rhizospheric Competency

Rhizospheric competency and ecology is a complex correlation between abiotic and biotic factors. Supplementing bioinoculants or PGPR in the rhizosphere and proposing effective microbe and plant symbiosis are a process that in vitro is highly effective. However, in field conditions, factors such as soil chemistry, mineral availability, and diversity of phytopathogen species may be delimiting factors to sustained colonization and effective pathogen control (Glick 1995). The capacity of PGPR to initiate defense mechanisms against phytopathogens requires engineering to characterize specific traits complementing a particular pathogen genome. This process, all be it highly effective, requires tedious interaction monitoring and genetic manipulation to suppress or activate specific molecular markers or sequences that complement, methylate, and destroy pathogen DNA/RNA (Prins et al. 2008). As a result of this complexity, biotechnology ventured into understanding soil characteristics and whether it is possible to use soil chemistry as a novel characteristic to engineer PGPR and utilize rhizosphere components for diverse suppression of various phytopathogens. Understanding PGPR and such soil characteristics, earlier Castignetti and Smarrelli (1986) suggested supplementing the rhizosphere with PGPR that are capable of producing and secreting siderophore molecules with a very high affinity for iron ($Fe^{3+}$).

The theory behind selecting high-affinity iron-binding siderophore molecules lies parallel to the fact that $Fe^{3+}$ is only sparingly available in nature at a sustainable soil pH of 7.4 (Neilands et al. 1987). By engineering the PGPR to secrete siderophore that binds at higher affinities, most of the available $Fe^{3+}$ in the rhizosphere is quickly taken up, leaving the surrounding area barren and essentially starving pathogens through the lack of iron uptake (O'Sullivan and O'Gara 1992). Biotechnology can engineer the bacterium to contain a receptor on the outer cell membrane that specifically compliments the iron–siderophore complex, transports it back to the microbial cell, and encourages utilization for microbial growth and proliferation (O'Sullivan and O'Gara 1992; Neilands and Leong 1986).

In an attempt to justify this mechanism of pathogen resistance, Vandenbergh and Gonzalez (1984) tested pathogen resistance against *F. oxysporum* in tomatoes by using a mutant strain of *P. putida* that overproduced siderophore molecules. The study revealed that overproduction of siderophore molecules in the mutant *P. fluorescens* strain was better suited to provide protection against *F. oxysporum* as compared to the wild-type *P. fluorescens* strain (Vandenbergh and Gonzalez 1984). Similarly, a mutant *P. aeruginosa* strain incapable of producing siderophore molecules was tested for its efficiency to control pathogen, *Pythium* sp., in tomato plants. Results confirmed *Pythium sp*. infection in tomato, as parameters marketing iron consumption were solely induced by *Pythium* sp., rendering the PGPR

siderophore complex inactive (Buysens et al. 1994). *Pseudomonas* sp. WCS417r strain was previously identified for the bacteria's capacity to induce systemic resistance through an ethylene-dependent signaling pathway. This strain has also shown marketability in inducing systemic resistance to Fusarium wilt on carnation caused by *F. oxysporum* f. sp. *dianthi* (Fod). Duijff et al. (1993) demonstrated this by using mutant WCS417r, defective in its capacity for siderophore biosynthesis (sid-), and compared this to *Pseudomonas putida* strain WCS358r. The team inhibited conidial germination by purified pseudobactins, which are siderophore molecules of *Pseudomonas* species, and found that the ferrated pseudobactins inhibited germination significantly less than the unferrated pseudobactins. Furthermore, sid-mutant WCS358 was ineffective in inhibiting Fod, whereas sid-WCS417r was still able to inhibit Fod. Treatment with WCS358r strain on carnation was able to reduce fusarium wilt, suggesting inhibition of Fod was induced solely on siderophore-mediated competition for iron. WCS417r strain significantly reduced wilt incidence, while mutant sid-WCS417r strain showed intermediate effectiveness in reducing wilt, suggesting WCS417 strain mechanism of pathogen control extends beyond siderophore inhibition, involving multiple mechanisms of control (Duijff et al. 1993). Such binding capacities essentially mediate effective biocontrol of disease through competitive bacterium–pathogen interactions where sustainability is dependent on soil parameters. This mechanism can be sustained by plants even at low $Fe^{3+}$ concentrations as plants are independent of the physical uptake process and, however, dependent on PGPR siderophore uptake and release into plant cellular components (Crowley et al. 1988; Wang et al. 1993). Thus, engineering rhizobacteria to compliment soil characteristics and actively suppress pathogens through competitive antagonisms is one method of active pathogen inhibition through rhizosphere competency.

#### 8.4.1.2 Engineering PGPR-Mediated Antibiotic Resistance

PGPR-mediated antibiotic resistance has provided scientists another avenue of integrated phytopathogenic suppression through direct involvement of antibiotic genes displaying antiviral, antimicrobial, antifeedant, phytotoxic, antioxidant, cytotoxic, and plant growth-promoting activities (Glick 1995; Fernando et al. 2005). Maurhofer et al. (1992) engineered a wild-type *Pseudomonas fluorescens* CHA0 strain to overproduce antibiotics pyoluteorin and 2, 4-diacetylphloroglucinol (DAPG). The strain was tested for its ability to protect cucumber plants against disease caused by *Pythium ultimum* and compare it to levels of wild-type *P. fluorescens* CHA0 inhibition. Together with similar findings of Schnider et al. (1994), Maurhofer and team elucidated strong correlation of increased synthesis of antibiotics by mutant *P. fluorescens* CHA0 strain results in better protection and suppression of *P. ultimum* in cucumber as compared to wild-type *P. fluorescens* CHA0 (Maurhofer et al. 1992; Schnider et al. 1994). DAPG and pyoluteorin are antibiotics classified as nonvolatile polyketides produced by *P. fluorescens* capable of a broad spectrum of actions against pathogenic fungi, bacteria, and nematodes

(Haas and Keel 2003). To actively suppress invasive species, *P. fluorescens* relay signaling molecules such as N-acyl-homoserine lactones (AHL) to mediate communication between different rhizobial dwelling bacteria as a means of antibiotic gene expression (Pierson et al. 1998). DAPG induces its own biosynthesis and acts as a diffusible signal for increasing the synthesis of DAPG by increasing the expression of DAPG biosynthetic genes (Maurhofer et al. 2004). The regulation of secondary metabolite production involves a two-component regulatory system, consisting of cellular homeostasis and transcription of antibiotic biosynthetic genes (Elander et al. 1968; Haas et al. 2000). A complex known as the GacS/GacA system acts to facilitate active response to changes in gene expression and sensory signals once AHL in most *Pseudomonas* sp. is recognized, exerting a positive impact on cell density-dependent gene regulation. Upon activation, GacS/GacA modulates expression of exoenzymes, antibiotics, and HCN during cellular transition from exponential to stationary phase of growth to mandate cell-to-cell communication and establish competency when antimetabolites are released in soil medium (Fuqua et al. 1994; Sacherer et al. 1994; Heeb and Haas 2001). Several other genetic regulators and signaling genes are involved, but for the purpose of explaining systemic antibiotic regulation, AHL and the GacS/GacA system are sufficient. These regulatory genes, coupled with the symbiotic soil bacterium, diversify PGPR's capacity to initiate, selectively suppress, and regulate the rhizosphere from incidence of attack (Fernando et al. 2005).

Antibiotics produced by various PGPR have a broad spectrum of activity. With *P. fluorescens* synthesizing DAPG, Cronin et al. (1997) used purified DAPG against nematode *Globodera rostochiensis* to exemplify suppressive abilities of the PGPR. Cronin et al. (1997) observed a decrease in the emergence of nematode cysts and reduced juvenile mobility. Similarly, *B. cereus* and *B. thuringiensis* exhibited pathogen resistance by producing antibiotic, Zwittermicin A (Fernando et al. 2005). Bacillus strains that produce Zwittermicin A are found at a minimum of $10^4$ cfu/g of soil worldwide and contain a gene responsible for self-resistance against the action of its own antibiotic (Raffel et al. 1996). *Helicoverpa armigera* (pod borer) and a homopteran group of sucking insects, *Aphis craccivora*, represent two of the most potent pests to chickpea growth (Das 2005). Insecticidal Cry proteins derived from *Bacillus thuringiensis* (Bt) are transcribed into *Cicer arietinum* genomes (Cowgill and Lateef 1996). Cry proteins are classified as δ-endotoxins that bind to the midgut epithelial cells, inducing osmotic lysis in the invading pest, causing reduced activity and eventual death (Herrera-Estrella et al. 2005). High expression of Bt lines carrying the Cry2Aa gene has shown to confer near-complete protection, reporting 98 % mortality of *H. armigera* larvae (Sarmah 2006). Such a characteristic enables *B. thuringiensis* to persist as a novel insecticidal strain in suppressing oomycete disease of plants and other pathogenic fungi (Emmert et al. 2004; Silo-Suh et al. 1998). Thus it is recognized that with the utility of PGPR, isolating genes that encode the biosynthesis of antibiotics engineered or naturally found in expressing resistance can provide optimal growth and sustained resistance against a wide range of phytopathogens (Glick 1995; Gill and Warren 1988). Furthermore, by secreting antibiotics in the rhizosphere, the proliferation of

unwanted soil microorganisms indirectly becomes limited, reducing occupancy and competition for nutrients, thus prompting ideal parameters for sustainability (Glick 1995).

## 8.5 Conclusions and Future Perspectives

Biotechnology has revolutionized modern-day agriculture. The use of bioinoculants encourages selective integration of compatible rhizobia and genetic traits which correspond to the host and surrounding environment. PGPR and AM fungi market trait specificity within the rhizosphere through active chemical fixation, nutrient cycling, and induced methods of pathogen resistance. *Cicer arietinum* (chickpea) biomass, yield, nodulation, dry weight, and root and shoot lengths all increased, while incidence of root rot and infection from pathogenic organisms decreased. As co-inoculants, PGPR and AM fungi elicited greater response as compared to chemical alternatives such as insecticidal sprays and fertilizers. By reducing chemical alternatives, soil chemistry is managed through biological fixation and reduction. In doing so, beneficial rhizobacteria adapt and proliferate at higher levels, establishing a colony where continuous feedback is generated and competition is controlled. Hormones and iron-chelating compounds such as phytoalexins and siderophores released by the chickpea plant mediated control around the rhizosphere, establishing an interactive zone favorable to rhizobia expressing particular lines of symbiosis. In harsh or heavily deprived environments, the use of antibiotics and development of transgenic plants and PGPR enable pathogen-derived and pathogen-induced systemic resistance towards combating abiotic and biotic stresses. With acquired/selected genetic traits, plants and microbes are able to perform and enhance growth parameters without sacrificing quality. A continuous effort to establish rhizosphere competency using mutually inclusive rhizobia and enhanced resistance against a broad range of pathogens and viruses is being made; however, many tests and trials must be conducted before marketing for public applications. The push for such developments will take time and patience from both farmers and biotechnologists; however, the possibility to sustain growth in a once infertile piece of land is worth the wait.

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