Modifying the Rhizosphere of Agricultural Crops to Improve Yield and Sustainability: *Azospirillum* as a Model Rhizotroph

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Abstract

The chemicals used in agriculture to increase yields, and to kill pathogens, pests, and weeds, may have a harmful impact on the ecosystem. Current public concerns about the side effects of agrochemicals imposed scientists a new challenge in improving the understanding of cooperative activities among plants and rhizosphere microbial populations. The future goal is the gradual reduction in the use of chemicals without affecting yield or quality of the crops. A new generation of technologies must be developed focusing on the favorably partitioning of the biomolecules produced during the interaction between plants and microbes. The objective of this chapter is to review the current knowledge about the effects of plant growth-promoting rhizobacteria and their potential use as innovative tools for the sustainability of agroecosystems, with emphasis on the *Azospirillum*, and their use in Argentina.

2.1 Introduction

Agricultural intensification has greatly increased the productive capacity of agroecosystems, though it also has unintended environmental consequences including degradation of soil and water resources and alteration of biogeochemical cycles (Drinkwater and Snapp 2007; Lehman et al. 2015). In modern cultivation processes, indiscriminate use of fertilizers, particularly the nitrogenous and phosphorus ones, has led to substantial pollution of soil, air, and water (Gupta et al. 2015). The application of fertilizers on a long-term basis often leads to reduction in soil pH and in

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exchangeable bases, making nutrients unavailable to crops and declining their productivity (Gudzic et al. 2015). To obviate this problem and obtain higher plant yields, farmers have become increasingly dependent on chemical sources of nitrogen (N) and phosphorus (P). Besides being costly, the production of chemical fertilizers depletes nonrenewable resources and poses human and environmental hazards (Joshi et al. 2006). It has also been reported that the excessive use of chemicals not only affects the fertility status of soil and pollutes the environment but also might exert deleterious effects on soil microorganisms (Youssef and Eissa 2014).

By the other side, pathogenic microorganisms that affect plant health are also a major and chronic threat to sustainable agriculture and ecosystem stability, worldwide. Pesticides still represent one of the main pressures from agriculture on human health and ecosystems, though chemical substances placed on the market tend to be less harmful in response to the requirements of the directives and regulations in force, as they undergo more risk assessment (Eurostat. Statistics Explained 2012).

All these setbacks were the consequence of centering agricultural production and soil conservation to cover human needs, without considering either soil stability or soil health (Welbaum et al. 2004). The importance of soil health and quality in relation to sustainable land management is an actual concern (Doran 2002; Karlen 2012).

2.2 The Concept of Soil Health

Soil is a dynamic living matrix, and it is a critical resource in agricultural and food security. Soil health is defined as the sustained capacity of soil to function as a vital living system. This concept is based on that it contains biological elements that are key to ecosystem function within land-use boundaries (Doran and Zeiss 2000; Karlen et al. 2001). These functions are able to sustain biological productivity of soil, maintain the quality of surrounding air and water environments, as well as promote plant, animal, and human health (Doran et al. 1996). Keeping this definition in mind, the quality of a specific kind of soil has been defined as "the capacity to function, within natural or managed ecosystem boundaries, to sustain biological productivity, promote environmental quality, and maintain plant and animal health" (Blanco and Lal 2012). Soil erosion, atmospheric pollution, extensive soil cultivation and grazing, high irrigation, salinization, and desertification not only decrease the productivity of an agricultural land but also perturb or degrade its health. A balance of chemical, physical, and biological components contributes toward maintaining soil quality (Das and Varma 2010). Agroecosystem functioning is governed largely by soil microbial dynamics (Kennedy and Smith 1995). Sustainable and productive agriculture depends on a healthy community of soil microbes. These decompose organic matter and contribute to the biological recycling of chemical nutrients that affect soil fertility. The functioning of agricultural ecosystems, including the health of soil, mostly depends on the interaction between the diversity of primary producers (plants) and decomposers (microbes), which are the two key functional groups that form the basis of all soil ecosystems (Elmqvist et al. 2010).

Microbes balance soil ecology being an integral part of every soil ecosystem. Only 1 g of soil may contain billions of microbes with thousands of different species. Metabolic activities of the microbes, such as plant growth-promoting rhizobacteria (PGPR), cyanobacteria, and fungal organisms (mycorrhiza), and of soil fauna (nematodes, worms, protozoans, etc.) promote soil health and crop productivity. Several biotic or abiotic factors lead to the alteration of the microbial community structure and composition, which may influence directly or indirectly the soil ecosystem, nutrient cycling activity, and crop production (Chaudhry et al. 2012).

In addition, anthropogenic intervention for the management and treatment of soil, involving fertilizers, pesticides, manure, or genetically modified microorganisms and plants, also influences microbial diversity (Nautiyal 2012). Different studies have shown that the application of chemical fertilizers (NPK) enhanced crop yield but affected the diversity in microbial population and their enzymatic activities (Zhang et al. 2015). By the other side, it has been shown that chemical fertilizers could increase the soil microbial biomass, exerting no significant changes in the microbial characteristics of the soil (Nautiyal 2012). Evidences linking direct impact of chemical fertilizers on microbial diversity, function, and phylogeny are still not well documented (Nautiyal 2012).

Changes in microbial parameters are correlated with the soil organic carbon content, as all the soil organisms essentially need a carbon source for their survival, and not to the application of P and N. This is the reason why soils poor in organic matter are usually poor in microbial activities. Crop productivity greatly depends upon the amount of available nutrients in the soil, which is governed by transformations of soil microbial biomass. Thus, the growth and activity of microorganisms are functions of soil properties, such as nutrition, texture, pH, temperature, and moisture content, and they are sensitive indicators of changes in these soil properties. The optimal functioning of each organism usually appears as a part of small well-structured communities carrying species which are interdependent on each other. In this context, augmentative approaches study the possibility of isolating organisms from the vast pool of biodiversity, with any special enhanced activity, and introducing them in the ecosystem. These activities can be also enhanced by manipulation such as drainage (aeration) or crop rotation. This approach can involve the use of selected wild-type organisms or genetically modified organisms which have their function introduced or enhanced by the use of recombinant DNA. Nevertheless, such constructed organisms may not fit ecologically as the comparable wild types (Prakash et al. 2011). Future marketing of transgenic bioinoculant products and their release into the environment as eco-friendly alternations to agrochemicals will depend on the generation of the biosafety data required for the registration of PGPR agents.

2.3   The Rhizosphere

The rhizosphere, the interface between growing roots and the mineral world in the soil, provides a particular ecosystem where ecological feedbacks, chemical interactions, and inter-organism communication take place. According to root nearness, soil can be divided in three main zones: (1) the rhizoplane or root surface, (2) the ectorhizosphere that portion of the soil under root influence, and (3) soil that is devoid of plants (Manthey et al. 1994). The ectorhizosphere, the rhizoplane, and the root cortex are together called the rhizosphere.

Rhizosphere affects and even transforms a large soil environment, including all of the so-called bulk soil (Richter et al. 2007). Plants alter the rhizobacterial community by releasing different substrates, which can vary from single sugar components to complex aromatic structures, and therefore selecting for increased numbers of certain taxa and/or functional groups of bacteria (Kravchenko et al. 2003). Microorganisms can also influence the plant by promoting or inhibiting growth (Glick et al. 1998). Many of the interactions between microbes and plants are still unknown.

During the past years, there has been an increased recognition of the role that biological processes play in soil function and in sustainable crop production (Nautiyal 2012). A recent major strategy to counteract the rapid decline in environmental quality is to promote sustainable agriculture. The objective is to sustain high production with the gradual reduction in the use of fertilizers and pesticides and the greater use of the biological and genetic potential of plant and microbial species. In this sense, the strategies that relay on sustainable agricultural techniques do not harm the environment or health, not only for animals and human beings but also for soil.

2.4   Using Microbes to Attain Higher Benefits in Sustainable Agriculture

2.4.1   Beneficial Microbial Modes of Action on Plants

The application of PGPR as biofertilizers and biocontrol agents is being considered as an alternative or supplemental way of reducing the use of chemicals in crop production (Kloepper et al. 1989; Vessey 2003; Maheshwari 2011). In addition to the plants ability to modify their physiology and metabolism, certain rhizosphere microorganisms can help plants to either avoid or partially overcome environmental stresses (Govindasamy et al. 2008). PGPR that were isolated from heavy metal polluted soils are able to enhance plant growth and development under heavy metal stress conditions, such as in the presence of arsenic (Reichman 2014), cadmium (Guo and Chi 2014), or both zinc and cadmium (Pereira et al. 2015). This line of research is oriented to soil bioremediation attaining in addition a boost effect on crop growth.

Sustainable approaches are those that not only aim to improve short-term crop yields but also to assure the maximum long-term performance, protecting the ecology of agricultural systems and the interests of the farmers. The so-called microbial

technologies deal with restructuring the crop rhizosphere by inoculating crops with beneficial microorganisms and using cultural practices that enrich indigenous beneficial ones. The long-term sustainability of agricultural systems is highly dependent on effective handling of the indigenous resources of agroecosystems. Actual methods, used to investigate microbial structure and composition, include culture-dependent and molecular methods. The first ones can detect less than 1 % of the microorganisms present in soil. The second ones include high-throughput DNA-sequencing techniques with the potential to detect, cost-effectively, low abundant uncultivable microbial species (Roesch et al. 2007). Another tool is the phospholipid fatty acids profiles determination that provides a wide-ranging measurement of microbial communities at the phenotypic level. Although these profiles do not give information on species composition, they reveal the fingerprint of community structure. Actually, they are considered as a robust tool that consistently discriminates between different communities (Kaur et al. 2005). During the last 20 years, the new biotechnologies have opened new scenes for the enhancement of sustainable agriculture production. These advances have made possible to take advantage of soil microorganisms for improving crop productivity. They also offer an economically attractive and ecologically viable choice to reduce external inputs.

When applied as inoculants, the so-called PGPR enhances plant growth by a wide variety of mechanisms that have been classified by their direct or indirect modes of action. The first comprises the production of bacterial metabolites, mainly phytohormones, that stimulate plants (Dobbelaere et al. 2003) but also include volatiles (Ping and Boland 2004; Santoro et al. 2015) and signal molecules like nitric oxide (Creus et al. 2005; Molina Favero et al. 2008). Lowering the ethylene level in plants (Glick et al. 2007), improving the plant nutrient status by mobilizing nutrients in soils or fixing atmospheric N (Hayat et al. 2010), and stimulating disease resistance in plants by triggering induced systemic resistance response (Van Wees et al. 1997) are also direct modes of action. Indirect effects are originated on the ability of some PGPR to constraint other soil microbes thus giving pathogenic ones less chance to develop (Lugtenberg and Kamilova 2009).

Both symbiotic and nonsymbiotic associations between organisms in the rhizosphere rely on interacting factors and chemical signals that operate on time and space scales. Among these, compounds of hormonal nature play major roles. To make the picture more complex, all these factors vary with water content, temperature, nutrients and soil structure, and others (Molina Favero et al. 2007).

2.4.2 Microbial Inoculants as Components in Current Agronomical Practices

The progress made in the last three decades in the understanding of the diversity of PGPR, along with their colonization abilities, and modes of action has facilitated their application as a new component in the management of sustainable agricultural systems. The practical application of living bacteria as inoculants was quite controversial from the beginning, because the response of crops is not completely

predictable and depends on many ecological and agro-technological factors. Nevertheless, much progress has been made in this field, leading to an ever-growing and successful application of rhizobacteria in several regions of the world, especially in South and Central America. Diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and nonsymbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*) rhizobacteria are now being used worldwide as inoculants to promote plant growth and/or to protect crops and attain higher yields.

There are a number of studies revealing the benefits of bacterial inoculation even though chemical application is accomplished as a current agronomical practice. Beneficial bacteria showed certain and variable grade of compatibility with herbicides (Ahemad and Khan 2010, 2011a), insecticides (Ahemad and Khan 2011b, c), and fungicides (Pereyra et al. 2009; Ahemad and Khan 2011d, 2012) even under stress conditions like soil heavy metal contamination (Wani and Khan 2010; Ma et al. 2011), salinity (Mayak et al. 2004), or water stress (Creus et al. 2004; Pereyra et al. 2009).

The leading countries in field applications of PGPR are Mexico with estimated 300,000 ha inoculated fields in 2007 and Argentina where over 220,000 ha of wheat and corn were commercially inoculated with *Azospirillum* in 2008 (Bashan and Hartmann 2009). Particularly, *Azospirillum* sp. has been commercially used on a relative large scale in Argentina, Mexico, Europe, South Africa, and India, mainly on cereals but also on other crops (Fuentes-Ramirez and Caballero-Mellado 2005; Bashan and Hartmann 2009; Díaz-Zorita and Fernández-Canigia 2009). Many of these studies showed promising results as "Microbial Technologies."

Regarding on the benefits they induce on crops, PGPR can be classified as phytostimulators, biofertilizers, and biocontrol agents, depending on the proposed use of the commercial product. Nevertheless, certain groups of bacteria show overlapping applications. Here, we describe proved cases of the application of PGPR on crops that can be ascribed to one or more of these groups.

2.4.2.1 Phytostimulators

A major goal to improve agricultural performance and increase food production is to attain high yields, even at low soil fertility or without intensive fertilization. To achieve this goal, root development and physiology appear to be central. Roots are dynamic anchorages of plants. They not only support the whole plant growth but also its physiological activity. Treatments to achieve greater adventitious rooting, increased number of lateral roots, higher length, and density of root hairs are targets of many research projects in plant biology (Molina Favero et al. 2007). It is generally accepted that root activity modifies the habitat of microorganisms and these, in turn, could trigger changes in the overall plant behavior. Some PGPR stimulate plant growth directly by the production of substances that mimic plant hormones. However, to produce these stimulatory effects on plants, the first step is the proper root colonization by bacteria that attach to root surface forming clumps or biofilms on it (Salcedo et al. 2015). As a primary target, root is the organ that shows the first stimulating bacterial effects. This is particularly remarkable in plants inoculated with *Azospirillum* spp. (Okon 1985).

The production of plant growth regulators, mainly auxins, cytokinins, and gibberellins, is the most commonly invoked mechanism for plant growth promotion exerted by PGPR (Bashan and de Bashan 2010). Auxin, cytokinin, gibberellin, abscisic acid, and jasmonate production has been reported in several associative and endophytic diazotrophic species of many genera such as *Azospirillum*, *Klebsiella*, *Gluconacetobacter*, *Azoarcus*, *Herbaspirillum*, *Enterobacter*, *Bacillus*, *Achromobacter*, *Acetobacter*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Xanthomonas*, and *Azotobacter* (Ping and Boland 2004; Tsavkelova et al. 2006; Baca and Elmerich 2007). Auxins and cytokinins are important regulators of plant development, regulating processes involved in the determination of the root architecture (Overvoorde et al. 2010). Nevertheless, auxins are thought to play the major role in stimulating root growth by rhizobacteria (Dobbelaere et al. 2003). Cytokinins play important roles for plant developmental processes from seed germination to senescence, including maintenance of stem cell systems in shoots and roots, organogenesis, leaf senescence, and interacting with auxins both participate in root vascular development and the control of shoot branching (Castillo et al. 2015). Early work from Barea et al. (1976) found that at least 90 % of the bacteria isolated from the rhizosphere of important crops were able to produce cytokinin-type compounds in chemically defined medium.

Gibberellins play an important role in the early stages of plant development by enhancing shoot and root growth and increasing root hair density, though they also regulate many aspects of reproductive growth in plants. Bottini et al. (1989) were the first to confirm the ability of *Azospirillum* sp. to produce gibberellins in chemically defined culture medium. Gibberellic acid production and their conjugates metabolism by *Azospirillum* sp. were summarized by Bottini et al. (2004).

Experiments with IAA-attenuated mutant bacteria inoculated on wheat (Barbieri and Galli 1993; Dobbelaere et al. 1999) or those carried with dwarf rice or maize deficient in the production of physiologically active gibberellins (Castillo et al. 2015) are strong evidence that the production of phytohormones by associated bacteria accounts for the phytostimulatory effects. In addition, the study of the expression profiles of inoculated plants would help to understand the complex metabolic changes produced upon inoculation. The transcript profile of in vitro grown sugarcane inoculated with *G. diazotrophicus* and *H. rubrisubalbicans* revealed differentially expressed genes related to auxins, gibberellins, and ethylene (Nogueira et al. 2001). The transcriptional profile of rice plants inoculated with *H. seropedicae* identified expressed sequence tags (ESTs) involved in auxins and ethylene pathways that are regulated during the association (Brusamarello-Santos et al. 2012).

Apart from the production of plant growth regulators, the decrease in the levels of ethylene in inoculated plants is another proposed phytostimulatory effect (Glick 2004). Some rhizosphere and endophytic bacteria produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase). The activity of ACC deaminase can reduce ACC content from the ethylene biosynthesis pathway in plants (Desbrosses et al. 2009). ACC deaminase-containing rhizobacteria bound to a plant act as a sink for ACC, thereby lowering ethylene levels in plant tissues. The result of the functioning of this enzyme is an increase in the growth of plant roots and shoots and a reduction of the inhibitory effects of ethylene synthesis especially during stressful conditions (Glick 2004).

The phytostimulatory effects of PGPR on plants were also studied in greenhouse and field conditions. Experiments involving *Azospirillum* inoculation during the 1990s were carried out in many countries including Israel, France, Belgium, Argentina, Uruguay, Mexico, and South Africa. These experiments' results were summarized in two interesting reviews by Okon and Labandera-González (1994) and Dobbelaere et al. (2001). They concluded that inoculation with *Azospirillum* resulted in significant yield increases in the magnitude of 5–30 % in about 60–70 % of the experiments. The beneficial effects were mainly observed in lighter soils under intermediate levels of fertilizer (N, P, and K) and water regimes. These pioneer reviews on the application of bacteria at the field lead to establish the first basis for designing larger experiments to assess the conditions and management practices for attaining positive and reproducible results.

Veresoglou and Menexes (2010) concluded in an excellent report on a meta-analysis conducted on 59 available articles to evaluate the extent, to which *Azospirillum* might contribute to wheat growth properties, that a mean increase of 8.9 % in seed yield and of 17.8 % in aboveground dry weight resulted from inoculation of wheat with *Azospirillum*. Other crops like corn, sorghum, rice, and legume showed yield increases in ranges from 5 % to 30 % over non-inoculated controls (Dobbelaere et al. 2001; Díaz-Zorita and Fernández-Canigia 2009; Hungria et al. 2010; Helman et al. 2011).

2.4.2.2 Biofertilizers

Apart from the described increasing effects on growth and yield, PGPR application might also enhance nutrient uptake from soils, thus reducing the need for fertilizers and preventing water contamination with nitrate and phosphate accumulation in agricultural soils (Bashan and de Bashan 2010). The expanded root system can improve the efficiency of the uptake of soil nutrients and fertilizers. This can be accomplished either by increasing the volume of explored soil or by enhancing nutrients uptake rates per root surface unit. The first possibility is the best established for PGPR as the better exploration of soil allows a major accessibility to micro-sites where low mobile nutrients could be enriched. A reduction in fertilizer application would lessen the effects of water contamination from fertilizers and lead to economical savings for farmers. These savings would increase the cost/benefit ratio, a crucial aspect for sustainable agriculture in many developing countries. Symbiotic processes leading to enhanced N fixed by *Rhizobium* and *Bradyrhizobium* species or P availability by mycorrhizal fungi have been the most studied up today. These two genera of microorganisms are well known, and many researchers have shown the contribution in N and P, respectively.

Though in less magnitude, associative nonsymbiotic bacteria can contribute for fixing N (Welbaum et al. 2004) or remobilizing of nonmobile P sources by acid production (Ahemad and Kibret 2014). When applied from outside as inoculants, PGPR facilitate resource acquisition (N, P, and essential minerals). In this sense, the theoretical needs of chemical inputs could be decreased. Nevertheless, the balance of P and other elements is a long-term variable that must be analyzed to determine if the system is sustainable. Although the cumuli of knowledge in PGPR effects and

their modes of action are very large, actually the information on practical biofertilization techniques to lesser chemical inputs is still scarce.

A two-season field study was performed in the south of Vietnam to assign the effects of a product containing a pseudomonad, two bacilli and soil yeast, on rice. Results indicated that application improved significantly the N use efficiency by rice, saving 43 kg N ha^{-1} with an additional yield of 270 kg ha^{-1} in the two consecutive seasons (Cong et al. 2009). The extra efficiency was shown by the fact that both treatments, biofertilizer with the application of about 40 and 60 kg less N-fertilizer and urea alone full dose, reached the same maximum yields in two successive harvests on the same plots (Cong et al. 2009).

Results obtained from a 3-year field research conducted to test whether microbial inoculants could be used to increase maize yield and to enhance nutrient uptake were published by Adesemoye et al. (2008). They showed that inoculated plots removed higher amounts of N, P, or K from the soil, potentially reducing nutrient losses to the environment.

In a large study conducted during 2002–2006 growing seasons, the performance of a commercial inoculant based on INTA Az-39 strain of *A. brasilense* was evaluated in 297 experimental field trials in the Pampas region of Argentina (Díaz-Zorita and Fernández-Canigia 2009). At all sites, the sown wheat varieties were regionally adapted and recommended for high yielding environmental and crop management conditions. N and P fertilization were applied when necessary according to recommendations based on chemical soil analysis and suggested protocols for each local site. Wheat grain yield from those 297 experimental sites varied in a range from 850 to 8050 kg ha^{-1} according to the management. The yield average increase was 260 kg ha^{-1}, equivalent to 8.0 % of the mean wheat yield attained under the dry land farming conditions found in the region. Positive responses were determined in about 70 % of the sites, depending mostly on the attainable yield, and independently of fertilization and other crop, and soil management practices. This is in agreement with the reported efficiency estimated from green house and field studies conducted in different parts of the world (Okon and Labandera-González 1994; Dobbelaere et al. 2001). The interaction between inoculation and N and/or P fertilization was also analyzed. As it was expected, fertilized wheat yield was enhanced with respect to that of unfertilized crop. However, regardless of the fertilization practice, inoculation significantly and positively affected yield, with mean yield responses of 259 and 260 kg ha^{-1} for unfertilized and fertilized wheat, respectively.

Sugarcane inoculation with *G. diazotrophicus* also resulted in improved N uptake (Suman et al. 2005). Studies on rice inoculated with ten different associative and endophytic diazotrophs, including *Paenibacillus* sp., *Bacillus* sp., *Burkholderia* sp., *Herbaspirillum* sp., and *Azorhizobium* sp., indicated that bacterial inoculation had a significant positive impact on N uptake and on shoot and root growth (Islam et al. 2009).

Despite of the large reservoir of P on earth, the amount of soil available P-forms to plants is generally low because the majority of soil P is found in insoluble forms, while plants absorb it only as soluble ionic phosphates (Bhattacharyya and Jha 2012). Organisms coupled with phosphate-solubilizing activity often termed as phosphate-solubilizing microorganisms may provide the available forms of P to the plants and

hence comprise a viable substitute to chemical P fertilizers (Khan et al. 2006). Bacteria of the genera *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* are reported as the most significant phosphate-solubilizing bacteria (Bhattacharyya and Jha 2012).

The increment of plant P nutrition by inoculation with PGPR can be explained by different mechanisms. One is the capability these soil bacteria have to solubilize inorganic P as a consequence of the action of low molecular weight organic acids they are able to synthesize and excrete (Zaidi et al. 2009). Another different mechanism is the mineralization of organic P that occurs through the synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters. Nevertheless, both mechanisms can coexist in the same bacterial strain (Ahemad and Kibret 2014).

Many strains of *Pseudomonas* are able to solubilize P in soil and increase its availability to plants (Sundara et al. 2002). The beneficial effects of the inoculation with phosphate-solubilizing bacteria as single or combined inoculants are well documented (Ahemad and Kibret 2014). Positive results of inoculation with phosphate-solubilizing bacteria of soybean (Fernández et al. 2007), sorghum (Vikram 2007), wheat (Afzal and Bano 2008), and many other crops were reported.

Seed inoculation with *H. seropedicae* enhanced the N content in leaf of maize (12 %) under soil acidity conditions without N supply (Inagaki et al. 2015). Inoculation of PGPR in acidic sandy soil (4.5–5.0 pH) resulted in higher P concentration in the leaf tissue of maize, indicating increase of P solubilization promoted by the diazotrophic bacteria (Inagaki et al. 2015).

Microorganisms such as those of the genera *Aspergillus*, *Bacillus*, and *Clostridium* were found to be efficient in potassium solubilizing by excreting organic acids that directly dissolve rock potassium or chelate silicon ions to solubilize and mobilize it in different crops (Mohammadi and Sohrabi 2012; Parmar and Sindhu 2013). Nevertheless, little research has been done on potassium solubilization, which is the third major essential macronutrient for plant growth.

By the other side, iron is an essential compound for most living organisms. However, despite its abundance on earth, and the micromolar concentrations required for cell growth, it is biologically unavailable in most environments. Its availability in nature is limited by the rapid oxidation of the ferrous form to the very insoluble ferric form, which aggregates into insoluble oxy-hydroxide polymers. Also reduced ferrous form might induce Fenton reaction producing free radicals which are deleterious to cellular macromolecules (Halliwell and Gutteridge 1984). To fulfill their iron needs, bacteria have multiple iron acquisition systems. One of relevant importance for rhizobacteria relies on molecules (siderophores and hemophores) synthesized and released by bacteria into the extracellular medium; these molecules scavenge iron or heme from various sources (Wandersman and Delepelaire 2004). Siderophores are low molecular weight compounds produced by microorganisms under limited availability of iron. These compounds are able to bind iron from the environment and to transfer it into the bacterial cell (Stintzi et al. 2000). Studies with PGPR showed that siderophore-mediated iron uptake systems present in these microorganisms exert a strong influence on the whole microbial

community that can be quite beneficial to the plant (Kloepper et al. 1980). Yet in spite of many researches, it remains to be elucidated if the true effect relies on a better plant iron nutrition or if it is a biocontrol on the pathogenic bacteria for the quest of iron in the rhizosphere. The probable implication of siderophores produced by PGPR has been considered as a potential way to improve plant growth, nodulation, and N_2 fixation in iron-deficient conditions (Fernández-Scavino and Pedraza 2013). Plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or a ligand exchange reaction. Pandey et al. (2005) observed that *Pseudomonas aeruginosa* GRC1, a prolific producer of hydroxamate-type siderophores in iron-deficient conditions enhanced the growth of *Brassica campestris* in field trials. More recently, Radzki et al. (2013) showed that siderophores produced by *Chryseobacterium* C138 provided iron to iron-starved tomato plants in hydroponics culture.

2.4.2.3 Biological Control Agents

Some root-colonizing bacteria are able to both suppress disease in host plants by the production of inhibitory compounds that restrain soil pathogen growth and, at the same time, stimulate growth and defense responses in host plants. Biological control is, thus, considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Gerhardson 2002; Lugtenberg and Kamilova 2009).

The mechanism of pathogen growth inhibition is due to diverse metabolic abilities of biocontrol bacteria that produce inhibitory allelochemicals. These compounds include iron-chelating siderophores, antibiotics, and antifungal metabolites like HCN, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, viscosinamide, and tensin (Compant et al. 2005; Bhattacharyya and Jha 2012). Biocidal volatiles, lytic enzymes, and detoxification enzymes produced by some bacteria are also other metabolic biocontrol compounds (Compant et al. 2005).

As it was stated in the preceding section, iron is an essential element with low bioavailability. Under iron-limited conditions, the strongly producers of siderophores are correlated with an enhanced capacity to niche occupancy required by many rhizobacteria. Although various bacterial siderophores differ in their abilities to bind iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Compant et al. 2005).

Pseudomonas spp. are ubiquitous bacteria in agricultural soils and have many traits that make them well suited as biocontrol agents of soilborne pathogens (Weller 2007). Weller (2007) proposed that pseudomonads are able to improve plant growth by suppressing either "major" pathogens (produce well-known root or vascular diseases with obvious symptoms) or "minor" pathogens (parasites or saprophytes that damage mainly juvenile tissue such as root hairs and tips and cortical cells). In addition to pseudomonads, the genus *Bacillus* is widely recognized as a powerful biocontrol agent. *B. subtilis* and other bacilli are potentially useful as biocontrol agents due to their broad host range and their ability to form endospores and to produce different biologically active compounds with a broad spectrum of activity (Nagorska et al. 2007).

On the other hand, colonization of roots with PGPR can lead to systemic resistance in the plant providing protection against several types of pathogenic diseases.

The protection is typically manifested as both a reduction in disease symptoms and an inhibition of pathogen growth. This process appears to be phenotypically similar to pathogen-induced systemic acquired resistance. This effect of rhizobacteria is referred to as an induced systemic resistance (ISR) and has been demonstrated in different plant species, e.g., bean, carnation, cucumber, radish, tobacco, and tomato, and in the model plant *Arabidopsis thaliana* (Van Wees et al. 1997). Elicitation of ISR by plant-associated bacteria was initially demonstrated using *Pseudomonas* spp. and other Gram-negative bacteria (van Loon and Glick 2004). Various specific strains of *Bacillus* also elicit a significant reduction in the incidence or severity of various diseases on a diversity of hosts (Kloepper et al. 2004). Many individual bacterial components induce ISR, such as lipopolysaccharides, flagella, siderophores, cyclic lipopeptides, DAPG, homoserine lactones, and volatiles like acetoin and 2,3-butanediol (Lugtenberg and Kamilova 2009).

Root inoculation of *A. thaliana* ecotype Columbia with *Pseudomonas fluorescens* CHA0 partially protected leaves from the oomycete *Peronospora parasitica* (Iavicoli et al. 2003). Using mutants derived from strain CHA0 (pyoverdine deficient, exoprotease deficient, HCN deficient, pyoluteorin deficient, or DAPG deficient), it was demonstrated that DAPG production in *Pseudomonas fluorescens* is required for the induction of ISR in *Peronospora parasitica* (Iavicoli et al. 2003). Although DAPG is a known antifungal compound, it was also demonstrated to affect the physiology of plants and other eukaryotes (Keel et al. 1992). Although the mode of action of DAPG is not fully understood, it inhibited primary root growth and stimulated lateral root production in tomato seedlings (Brazelton et al. 2009). In this sense, there seems to be overlapping effects of some secondary metabolites. Apart from exerting direct biocontrol by competing with pathogens for the resources or the niche occupancy, they can also stimulate the host growth or induce ISR.

Whatever the mechanism involved in the control, the ability of biocontrol agents to compete in the rhizosphere is crucial to produce their benefits when commercial products are applied in the field. This competence comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable period, in the presence of the indigenous microflora (Parke 1991; Lugtenberg et al. 2001). Given the importance of rhizosphere competence as a prerequisite for an effective biological control, understanding root-microbe interaction, and the genetic and environmental factors that affect rhizosphere colonization, will significantly contribute to improve the efficacy of products based on biocontrol agents.

2.5   *Azospirillum* as a Model Rhizotroph

Among PGPR, the species of *Azospirillum* have gained the reputation of being the most studied plant-associative bacteria (Bashan et al. 2004). When present in plants in proper amounts, they stimulate the density and length of root hairs, the rate of appearance of lateral roots, and root surface area (Okon and Labandera-González 1994). These effects cause roots to take up more water and mineral nutrients

resulting in faster plant growth. Under appropriate agronomic conditions, these processes would increase crop yield (Creus et al. 2004). In general, they contribute to reduce the burden of soil nutrient loss in arable lands, to counteract part of the negative effects of water and saline stresses on plant growth, and to help plants avoid or minimize contaminant uptake (Barassi et al. 2007). Moreover, in view of the high input of agrochemicals in contemporary crop production and the likelihood that *Azospirillum* inoculation could be regularly used in the near future in regular crop production, studies on the interaction of *Azospirillum* inoculants with common pesticides are essential (Pereyra et al. 2009).

Azospirillum spp. are included into the alpha subclass of *Proteobacteria* belonging to the IV rRNA superfamily (Xia et al. 1994). After the recent reclassification of *Azospirillum irakense* to *Niveispirillum irakense* and *Azospirillum amazonense* to *Nitrospirillum amazonense* based on their polyphasic taxonomic characteristics, at present this genus encompasses 15 valid species (Young et al. 2015). *A. brasilense* is the most used as biofertilizer. *Azospirillum* is not a plant-specific bacterium but a general root colonizer. Although it has first been isolated from and studied on cereal crops, at present there are more non-cereal species successfully inoculated with *Azospirillum* (Bashan and de Bashan 2010).

Azospirillum congregates several characteristics present in different microorganisms that make it a valuable PGPR. The very first studies on *Azospirillum*-inoculated subtropical grasses (*Z. mays*, *O. sativa*, and forages such as *Digitaria* spp.) attributed the growth promotion effects primarily to the biological N_2 fixation exerted by the bacteria (Döbereiner and Day 1976). Even though this characteristic could be extremely valuable in agriculture, later field studies failed to demonstrate a significant N_2 fixation in *Azospirillum*-inoculated crops (Vande Broek et al. 2000). Further studies ascribed the positive bacterial effects on plants to morphological and physiological changes in the inoculated roots, which would lead to an enhancement of water and mineral uptake (Okon and Kapulnik 1986). *Azospirillum brasilense* produces plant growth regulators mainly IAA, which is associated with the beneficial effects observed after inoculation (Baca and Elmerich 2007). Several mechanisms have been postulated to explain how *Azospirillum* enhances growth and development of plants (Bothe et al. 1992; Bashan and Holguin 1997; Steenhoudt and Vanderleyden 2000; Bashan and de Bashan 2010). Nevertheless, to date no unique mechanism had been established to explain the growth promotion capability of these bacteria. Instead, the most accepted hypothesis postulates that a sum of events accounts for the general plant growth promotion effect (Bashan and Holguin 1997).

It was previously reported that the cell wall is a target for *A. brasilense* growth promotion (Creus et al. 2004; Pereyra et al. 2010). Plant cell growth is constrained by the primary cell wall which consists of cellulosic microfibrils embedded in a matrix of interwoven noncellulosic polysaccharides and proteins. Cucumber seeds inoculated with *Azospirillum* resulted in seedlings presenting larger hypocotyls. Cell wall dynamics of these inoculated plants was affected including greater acid-induced cell wall extension and lower activity of two important enzymes from the cell wall metabolism, NADH oxidase and ferulic acid peroxidase (Pereyra et al. 2010). These lesser activities, coupled with a lesser content of ferulic acid,

responsible of the stiffening of the cell wall, could be another mechanism accounting for the growth promotion induced by *Azospirillum* (Dal Lago et al. 2015).

These and other physiological changes observed in the inoculated plants subjected to abiotic stresses were reported. *Azospirillum*-inoculated wheat (*T. aestivum*) seedlings subjected to mild osmotic stress developed significant higher coleoptiles, with higher fresh weight and better water status than non-inoculated seedlings (Alvarez et al. 1996; Creus et al. 1998). A larger root system was evident in *Azospirillum*-inoculated wheat seedlings growing either under well-irrigated or water stress conditions (Pereyra et al. 2006). It was also proved that part of the negative effects, mild and severe salt stresses would cause on wheat seedlings were significantly reversed in *Azospirillum*-inoculated roots (Creus et al. 1997). Fresh weight, fresh weight/dry weight, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus et al. 1997). Vessel size has an important role in the adaptation to water stress environmental conditions. *A. brasilense*-inoculated wheat seedlings showed wider xylem vessels and less negative water potential in their coleoptiles when grown exposed to osmotic stress (Pereyra et al. 2012). The induction of wider xylem vessels by inoculation might imply an enhanced coleoptile hydraulic conductance which in turn could explain the better water status observed in plants. Indeed, field experiments carried out with different *Azospirillum* strains in *S. bicolor*, *Z. mays*, and *T. aestivum* have shown significantly increased yields, enhanced mineral uptake, and less canopy temperature (Sarig et al. 1988; Okon and Labandera-González 1994; Casanovas et al. 2003; Creus et al. 2004). In this sense, inoculation technology with *Azospirillum* could be extended to arid soils to protect crops against drought. Under drought conditions, inoculated plants responded in a different way to water stress compared to non-inoculated ones. They showed significantly higher water content, relative water content, water potential, apoplastic water fraction, and lower cell wall modulus of elasticity values (Creus et al. 2004).

Although *Azospirillum* is not considered to be a classic biocontrol agent of soilborne plant pathogens, there have been reports on moderate capabilities of *A. brasilense* in biocontroling crown gall-producing *Agrobacterium* (Bakanchikova et al. 1993), bacterial leaf blight of mulberry (Sudhakar et al. 2000), and bacterial leaf and/or vascular tomato diseases (Bashan and de-Bashan 2002a, b). In addition, *A. brasilense* can restrict the proliferation of other nonpathogenic rhizosphere bacteria (Holguin and Bashan 1996).

It is agreed that the benefits *Azospirillum* imposes on plants rely upon root colonization. In this sense, the formation of complex bacterial communities on the roots, known as biofilms, is crucial. Previous studies showed that nitric oxide production by *A. brasilense* Sp245 was responsible, at least in part, of the effects on root growth and proliferation (Creus et al. 2005). Nitric oxide is also a signaling molecule implicated in biofilm formation and was shown to regulate the formation of biofilm in *A. brasilense* Sp245 (Arruebarrena di Palma et al. 2013). Biofilm dynamics is of enormous importance for *Azospirillum* to exert beneficial effects on plants. So, the mechanisms operating in these phenomena are intensively and actively investigated.

Finally, in the last years, biofertilizers composed of mixed species are being used. They showed a better impact in crop yields than single species ones.

Ruiz-Sánchez et al. (2011) reported that rice inoculation with *Glomus intraradices* and *A. brasilense* increased growth under water stress. Combined formulations based on pseudomonads, *Azospirillum*, and many other PGPR microorganisms are also available for agronomic purposes. However, still little information is available about interspecies and multispecies interactions. There are several field experiments of single species seed inoculation with *Azospirillum* or *Pseudomonas*, but there is really very limited agronomical data regarding co-inoculation with both microorganisms (Valverde et al. 2015). A series of field trials with dual inoculation was conducted in Argentina during the seasons 2010–2013. The performance of *A. brasilense* single inoculation was compared to a combined formulation containing also *P. fluorescens* in wheat and corn fields. In all cases, *A. brasilense* alone or in combination with *P. fluorescens* had a positive effect on plant biomass at all three N fertilization levels essayed. The potential yield predicted was higher for dual inoculation, mainly when N-fertilizer was applied.

Thought, the number of spikes per m^2, a good predictor of the potential yield, was higher for dual inoculation, mainly when N-fertilizer was applied. This effect was translated into higher grain yield for dual inoculation than for single *A. brasilense* application (Valverde et al. 2015). The same effect on maize yield was observed in many locations, with or without N fertilization, though always major effect is observed under slight N fertilization. In a study conducted during 2009 growing season, the performance of a commercial combined inoculant based on INTA Az-39 strain of *A. brasilense* and *P. fluorescens* was evaluated in the presence of different levels of N, in an experimental field trial in Balcarce, Argentina. The sown maize variety was regionally adapted and recommended for high yielding environmental and crop management conditions. P fertilization was not necessary, and N (urea) was applied at sown, in three treatments: no N, half the dose (125 kg ha^{-1}), and complete dose (250 kg ha^{-1}), according to recommendation based on chemical soil analysis. Grain yield varied in a range from 9930 to 11,995 kg ha^{-1} according to the treatment (Fig. 2.1). The percentage of yield average increases due to inoculant application were 3.3 %, 16.6 %, and 2.5 % when no N, half dose, and complete dose were applied (Table 2.1). This result is a clear picture of the benefit inoculation exerts, in view of the achievement of greater yields reducing N applications. Co-inoculation is a promising field to be considered in the development of new biofertilizers.

2.6 Outlook and Conclusion

Organic farming differs from conventional agriculture in the production process, and it relies on techniques such as crop rotation, green manure, and biological pest control to maintain the soil productivity instead of chemical fertilizers and pesticides (Zhengfei et al. 2005). Several researchers have demonstrated that organic farming leads to improved soil quality with higher microbiological activity (Nautiyal 2012). Research must be focused in exploring bacterial structure, including PGPR consortium changes under different cropping practices and systems, and get a better
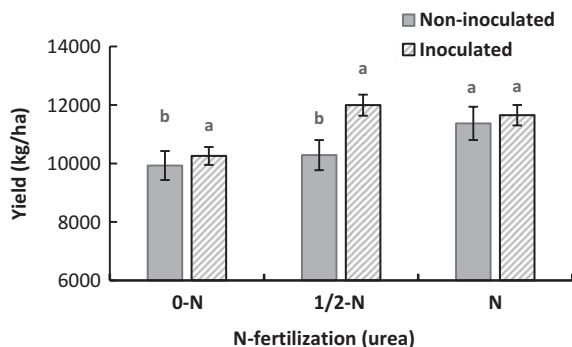
Fig. 2.1 Yield (kg ha^{-1}) of *Zea mays* inoculated with a formulation containing *A. brasilense* and *P. fluorescens* with three different doses of N-fertilizer applied as urea: 0-N, no urea applied; ½-N, half dose; and N, complete dose according to recommendation based on chemical soil analysis. Bars represent averages of four blocks as replicas. Small bars are standard deviation. Different letters within each level of N fertilization represent significant differences ($p < 0.05$) according to Duncan test

Table 2.1 Percentage of yield increase over non-inoculated control for each N-level

	N fertilization urea		
	0-N	½ -N	N
Increase over non-inoculated	3.3 %	16.6 %	2.5 %

understanding on how to build soil holistic ecology to maintain the health and productivity of plants. Long-term experiences have shown that neither the organic manure nor the chemical fertilizers alone can achieve sustained high yields. Integrated use of organic manures, biofertilizers, and chemical fertilizers, therefore, remains the only promising option in improving crop productivity.

Sustainable agriculture strategies should maintain the biodiversity of PGPR in the soil which might be affected by agricultural practices (Mäder et al. 2002; Esperschutz et al. 2007; Sugiyama et al. 2010). Studies aimed to understand and integrate plant responses during association, based on the profiling of plant gene expression, are a great help. New alternatives should be taken in mind for the use of bioinoculants. Some of them might include extending the technology to other valuable crops such as fruits, vegetables, and flowers, developing new formulations, and including multi-strain bacterial consortia. Also the optimization of growth conditions, self-life of PGPR products, and application alternatives should be considered.

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