

Chapter 7

**BIODIVERSITY AND THE POTENTIAL OF PGPR: PLANT-
MICROORGANISM INTERACTIONS**

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ABSTRACT

During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. This is due to the emerging demand for a reduced dependence on synthetic chemical products, the growing necessity for sustainable agriculture within a holistic vision of development and the need for increased environmental protections. PGPR have yet to fulfill their promise and potential as commercial inoculants. Recent progress in our understanding of their diversity, colonization ability, action mechanisms, formulation, and application should facilitate their development as reliable components in the management of sustainable agricultural systems. Naturally, several efforts have been carried out in order to develop commercial inoculants using these organisms. However, the effect of inoculants on native bacterial populations in the rhizosphere is decisive for

maximizing plant nutrient availability. The vast number of papers dealing with multiple inoculations involving some combination of PGPR and mycorrhiza or rhizobial leads to mixed results, with strong indications that there is some kind of specificity involved. This agrees with the recognized effects of specific plant and bacterial genotypes on the plant-PGPR interactions, and makes the development of practical multiple inoculants a major undertaking. The biodiversity and potential of PGPR for different groups and a visualization of the interactions are shown in the present chapter.

Key words: Plant growth promoting rhizobacteria (PGPR); inoculants; phytohormones; diazotrophic; effectiveness; crop performance; tripartite interaction.

1- INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for their agricultural benefits. Significant increases in the growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper et al., 1980; Seldin et al., 1984; Chen et al., 1994; Zhang et al., 1996; Amara and Dahdoh, 1997; Chanway, 1998; Pan et al., 1999; Catellan, 1999, Bin et al., 2000; Gupta et al., 2000, Biswas et al., 2000; Mariano and Kloepper, 2000; Asghar et al., 2002; Vessey, 2003; Gray and Smith, 2005; Silva et al., 2006; Figueiredo et al., 2008 a,b; Araújo, 2008). Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to a certain plant species, cultivar and genotype (Bashan, 1998; Gupta et al., 2000; Lucy et al., 2004).

PGPR can affect plant growth by different direct and indirect mechanisms (Glick, 1995; Gupta et al., 2000). Some examples of these mechanisms, which can probably be active simultaneously or sequentially at different stages of plant growth, include the following: (1) an increase in mineral nutrient solubilization and nitrogen fixation, increasing nutrient availability for the plant, (2) repression of soilborne pathogens (by the production of hydrogen cyanide, siderophores, and antibiotics and/or the competition for nutrients), (iii) improvements to plant stress tolerance with respect to drought, salinity and metal toxicity, and (iv) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al., 2000). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that hydrolyses ACC, the immediate precursor of ethylene in plants (Glick et al., 1999). By lowering ethylene concentrations in seedlings, and thus its inhibitory effect, these

PGPR stimulate seedling root length (Glick et al., 1999). Bacteria presenting one or more of these characteristics are known as plant growth promoting rhizobacteria – PGPR (Kloepper and Schroth, 1978; Noel et al., 1996).

Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol-PGPB (plant growth promoting bacteria) and PGPB. This classification may include beneficial bacteria that are not rhizosphere bacteria but it is not widely accepted. According to Vessey (2003), numerous species of soil bacteria which flourish in the rhizosphere of plants, but may which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms are collectively known as PGPR. Gray and Smith (2005) have recently shown that PGPR associations depend on the degree of bacterial proximity to the root and the intimacy of association. In general, these can be separated into two categories: (1) extracellular (ePGPR), which exist in the rhizosphere on the rhizoplane or in the spaces between cells of the root cortex, and (2) intracellular (iPGPR), which exist inside root cells in specialized nodular structures.

When diazotrophic organisms showed that they were also able to produce phytohormonal substances, they had their importance emphasized (Rao et al., 1998). Most *Rhizobium* species can produce IAA, and many studies indicate that changes in endogenous auxin concentrations are a prerequisite for nodule organogenesis (Lambrecht et al., 2000). Cytokinin-like compounds were produced and metabolized by *Paenibacillus polymyxa* cultures from the stationary to logarithmic growth phases (Timmusk et al., 1999). An effective *Bacillus* PGPR with ACC deaminase activity has been reported by Ghosh et al., (2003), but legume nodulation was not evaluated. However, Ferguson & Mathesius (2003) found that the auxin/cytokinin ratio may be important for regulating nodule numbers.

2- PGPR – DIAZOTROPHIC: DIVERSITY OF HABITAT AND EFFECTIVENESS

A number of diazotrophic plant growth-promoting rhizobacteria participate in interactions with C₃ and C₄ crop plants (e.g., rice, wheat, maize, sugarcane and cotton), significantly increasing their vegetative growth and grain yields. The mechanisms involved have a significant plant growth-promoting potential, retaining more soil organic-N and other nutrients in the plant–soil system and reducing the need for fertilizer N and P. The data in Table 1 suggest that the diversity of habitat and effectiveness might logically require more than one bacterial strain to obtain the maximum biological effects on plant growth, and

summarize the proposed mechanisms of PGP (plant growth promoting) effects (Kennedy et al., 2004).

Table 1. Biology, and potential role of some diazotrophs promoting crop production (adapted from Kennedy et al., 2004).

Diazotrophs	Condition for BNF	Habitat	Energy source	Mechanism of effect	References
<i>Azotobacter chroococcum</i>	Aerobic	Rhizosphere	Organics in soil	BNF	Kennedy and Tchan (1992)
<i>Clostridium</i> spp.	Anaerobic	Soil saprophyte	Organics in soil	BNF	Kennedy and Tchan (1992)
<i>Azospirillum</i> spp.	Microaerobic	Rhizosphere, mildly endophytic in roots, stems and leaves	Organics in soil, root exudates and plant tissue	BNF, PGP	Reinhold and Hurek (1998) Mirza et al. (2000) Okon and Kapulnik (1986)
<i>Herbaspirillum seropedicae</i>	Microaerobic	Endophytic, Rhizosphere	Root exudates	BNF, PGP	Baldani et al. (1986, 2000)
<i>Azoarcus</i> sp.	Microaerobic	Endophytic	Root exudates	BNF	Hurek et al. (1994) Reinhold-Hurek et al. (1993)
<i>Burkholderia vietnamiensis</i>	-	Rhizosphere, Endophytic,	Organics in soil and root exudates	BNF, PGP	Baldani et al. (1997, 2000)
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	-	Endophytic in roots	Root exudates	PGP	Yanni et al. (1997) Biswas et al. (2000)
<i>Rhizobium etli</i> bv. <i>phaseoli</i>	-	Endophytic in roots	Root exudates	PGP	Gutiérrez-Zamora (2001) Martínez-Romero (2001)
<i>Azorhizobium caulinodans</i>	Microaerobic	Endophytic in roots	Root exudates	PGP	Matthews et al. (2001)

<i>Gluconacetobacter diazotrophicus</i>	Microaerobic	Endophytic in roots, stems and leaves	Root exudates and plant tissue	BNF	Baldani et al. (1997) Boddey et al. (1991)
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* BFN, Biological nitrogen fixation; PGP, plant growth promoting.

According to Kennedy et al. (2004), this diversity will need to be carefully considered in the future design of the most efficient inoculant biofertilizers. For example, an important question is whether inoculants should be restricted to a single strain of bacterium, such as *Azospirillum*, or not. If all of the PGP mechanisms can be well expressed in a single strain of bacterium this would simplify the design of inoculant products. However, it would be unlikely that a single strain of bacterium would be capable of optimal activity of all or even most of the growth-promoting mechanisms.

According to Yanni et al., (1997), the diazotroph *Rhizobium leguminosarum* bv. *trifolii* can colonize rice roots endophytically in fields where rice is grown in rotation with Egyptian berseem clover (*Trifolium alexandrinum*), thereby replacing 25–33% of the recommended rate of N fertilizer for rice in field conditions as a result of PGPR effects. Field experiments demonstrated that the inoculation of this bacterium increased mean rice yield by 3.8 t ha⁻¹. This bacterium is also able to colonize the interior of rice roots grown under gnotobiotic conditions. It can significantly increase shoot and root growth, grain yield and agronomic N-fertilizer efficiency, although it is present in rice tissues in low numbers, on the order of 10⁵ c.f.u .g⁻¹ dry weight, which are too low for significant BNF (Yanni et al., 2001).

Many PGPR have the ability to fix N₂, yet rarely is their mode of action aimed for the stimulation of plant growth credited to BNF. PGPR that have the ability to fix N₂, but for which there is little evidence or even counter evidence that their growth stimulation of a specific host plant is due to nitrogenase activity, include *Azoarcus* sp. (Hurek et al., 1994), *Beijerinckia* sp. (Baldani et al., 1997), *Klebsiella pneumoniae* (Riggs et al., 2001), *Pantoea agglomerans* (Riggs et al., 2001), and *Rhizobium* sp. (Antoun et al., 1998; Yanni et al., 2001). It is interesting that so many PGPR are diazotrophs, yet the mechanism underlying their growth promoting effects is not the supply of fixed N to the host. A list of PGPR, based on an ability to fix N₂ *in situ*, are provided in Table 2.

Table 2. Plant growth promoting rhizobacteria (PGPR) based on an ability to fix N₂

PGPR	Relationship to host	Host crops	References
<i>Azospirillum</i> sp.	Rhizospheric	Wheat	Boddey et al., 1986
<i>Azoarcus</i> sp.	Endophytic	Rice	Egener et al., 1999
<i>Azotobacter</i> sp.	Rhizospheric	Maize	Pandey et al., 1998
<i>Bacillus polymyxa</i>	Rhizospheric	Wheat	Omar et al., 1996
<i>Bacillus polymyxa</i>	Rhizospheric	Hybrid spruce	Chanway et al., 2000
<i>Bacillus</i> sp.	Endophytic	<i>Atriplex nummularia</i>	Miranda et al., 2001
<i>Bacillus azotofixans</i>	Rhizospheric	Wheat	Seldin et al., 1984
<i>Paenibacillus durus</i>	Rhizospheric	Wheat	Rosado et al., 1997
<i>Paenibaacillus brasilensis</i>	Rhizospheric	Maize	Van der Weid et al., 2002
<i>Burkholderia</i> sp.	Endophytic	Rice	Baldani et al., 2001
<i>Gluconacetobacter diazotrophicus</i>	Endophytic	Sugarcane	Boddey et al., 2001
<i>Herbaspirillum</i> sp.	Endophytic	Sugarcane	Pimentel et al., 1991

3. PGPR AND RHIZOBIA- POSITIVE EFFECTS ON NODULATION AND ROOT GROWTH

Co-inoculation studies with PGPR and Rhizobia have shown increased plant nodulation and N₂ fixation (Li and Alexander, 1988; Araújo and Hungria, 1999; Vessey and Buss, 2002; Silva et al., 2006; Figueiredo et al., 2008b; Saravana-Kumar et al., 2007; Yadegari et al., 2008; Shehzad et al., 2009). Co-inoculation studies of some *Bacillus* strains with effective Rhizobia resulted in enhanced nodulation and plant growth for green gram (*Vigna radiata* L.) (Sindhu et al., 2002). A variety of rhizosphere microorganisms, including *Bacillus* and

Pseudomonas species, are commonly found in the rhizosphere of leguminous and nonleguminous crops (Li and Alexander, 1988; Sindhu et al., 2002). Because of their rapid colonization of the rhizosphere and stimulation of plant growth there is currently considerable interest in exploiting these rhizosphere bacteria to improve crop production. The application of *Bacillus* and/or *Paenibacillus* species to seeds or roots has been shown to cause alterations in the composition of the rhizosphere, leading to increases in the growth and yields of different crops (Li and Alexander, 1988; Vessey and Buss, 2002). Disease suppression in alfalfa by *B. cereus* and enhanced nodulation and seedling emergence in the common bean (Srinivasan et al., 1996; Camacho et al., 2001; Figueiredo et al., 2008b), soybean (Zhang et al., 1996; Araújo and Hungria, 1999; Lambrecht et al., 2000; Vessey and Buss, 2002), cowpea (Silva, et al., 2006, 2007) and pea (Cooper and Long, 1994) have been demonstrated to be beneficial effects on plants. Bacilli are also very attractive as potential inoculants in agriculture, as they produce very hardy spores that can survive for prolonged periods in soil and in storage containers (Bashan, 1998; Nelson, 2004).

Araújo and Hungria (1999) demonstrated the viability of co-inoculating soybean seeds with crude or formulated metabolites, or with cells of *Bacillus subtilis*, to increase the contribution of the biological nitrogen fixation process. Figueiredo et al. (2008b) and Lima (2009) also found nodulation stimulation and increased root dry matter in *Vigna unguiculata* and *Phaseolus vulgaris* that were co-inoculated with *Rhizobia* and *Paenibacillus* (Fig. 1).



Figure 1- Root of cowpea (*Vigna unguiculata* [L.] Walp.) cv “IPA 205” inoculated with *Bradyrhizobium* sp. (BR 3267) (Brady) and co-inoculated with *Bradyrhizobium* sp. + *Paenibacillus graminis* (P-MC 22.13). (Experiment developed in the Leonard jars under greenhouse conditions). (Courtesy of Figueiredo, MVB)

Bacteria mediated increases in root weight are commonly reported responses to PGPR inoculations (Bashan and Dubrovsky, 1996; Bertrand et al., 2001, Vessey and Buss, 2002; Figueiredo et al., 2008a). More importantly, increases in root length and root surface area are sometimes reported (Holguin and Glick, 2001; Silva et al., 2007). These increases in root length and root surface area are important due to the resulting increase in these parameters are more reflective of an increase the volume of soil explored, than that which would be indicated by just increases in root weight. For example, treatment of clipped soybean roots with *Azospirillum brasilense* Sp7 caused a 63% increase in root dry weight, but more than a 6-fold increase in specific root length (root length per unit root dry weight) and more than a 10-fold increase in total root length (Molla et al., 2001).

Table 3 lists papers in which the production of phytohormones has been implicated in growth promotion by biofertilizing-PGPR. Most commonly, IAA producing PGPR are believed to increase root growth and root lengths, resulting in greater root surface areas, which enables the plant to access more nutrients from the soil. Production of other phytohormones by biofertilizing-PGPR has been identified, but not nearly to the same extent as for bacteria that produce IAA. Researchers have begun to identify cytokinin production by PGPR and Gutierrez-Manero et al., (2001) provide evidence that four different forms of GA are produced by *Bacillus pumilus* and *B. licheniformis*.

Table 3. Plant growth promoting rhizobacteria (PGPR) for which evidence exists that their promotion of plant growth is due to influences on or by phytohormones (Modified from Vessey, 2003)

Factor produced	PGPR	Host	References
IAA	<i>Bacillus subtilis</i>	Soybean	Araujo et al., 2005
	<i>Bradyrhizobium</i> sp.	Radish	Antoun et al., 1998
	<i>Enterobacter</i> sp.	Sugarcane	Mirza et al., 2001
	<i>Rhizobium leguminosarum</i>	Radish	Antoun et al., 1998
Cytokinin	<i>Paenibacillus polymyxa</i>	Wheat	Timmusk et al., 1999
	<i>Pseudomonas fluorescens</i>	Soybean	de Salamone et al., 2001
	<i>Rhizobium leguminosarum</i>	Rape and lettuce	Noel et al., 1996
Gibberellin	<i>Bacillus</i> sp.	Alder	Gutierrez-Manero et al., 2001

ACC deaminase	<i>Bacillus pumilus</i>	Rape	Belimov et al., 2001
	<i>Pseudomonas cepacia</i>	Soybean	Cattelan et al., 1999
	<i>Pseudomonas putida</i>	Mung bean	Mayak et al., 1999

Phytohormones, especially auxin, cytokinin, and ethylene, have long been implicated in nodule development. Several reports have suggested that the hormone balance, particularly that between auxin and cytokinin, is part of the nodulation stimulus, but a priori it is not obvious in which direction the balance is shifted (Remans, 2008).

As cited by Hirsch (1992), Thimann in 1936 was the first to propose that a hormone, auxin, could play a role in pea nodulation. Since then, other classical plant hormones such as ethylene (e.g., Zaat et al., 1989) and cytokinin (e.g., Fang and Hirsch, 1998), as well as other signal molecules such as Nod factors (Lerouge et al., 1990), flavonoids (Mathesius et al., 1998), and uridine (Smith et al., 1995), have been shown to participate actively in nodule formation.

It is well established that ethylene inhibits nodulation in several legumes such as *Pisum sativum*, *Melilotus alba* (Lee and LaRue, 1992), *Medicago truncatula* (Penmetsa and Cook, 1997), *Medicago sativa*, *Lotus japonicus*, and *Macroptilium atropurpureum* (Nukui et al., 2000). However, it does not seem to affect nodulation in *Glycine max* (Lee and LaRue, 1992, Schmidt et al., 1999, Nukui et al., 2000). In pea, nodule organogenesis is blocked quite early, when the infection thread is either in the epidermis or in the outer cortex (Guinel and LaRue, 1991).

Phytohormone production (according to Araujo et al. (2005), particularly that of IAA, has been repeatedly observed in rhizobacteria and in symbiotic bacteria such as those in the genus *Bradyrhizobium* (Boddey and Hungria, 1994) while associative nitrogen-fixing bacteria produce auxins, gibberellins and cytokinins (Tien et al., 1979).

Changes in auxin and cytokinin levels have also been implicated in the normal infection and nodulation processes of various legumes (Ferguson and Mathesius, 2003). Both direct measurements of auxin levels (Boot et al., 1999) and the use of transformants with auxin-sensitive reporters such as GH3:gusA (Mathesius et al., 1998) suggest that shortly after exposure of legume roots to rhizobia or the appropriate Nod factor there is a decline followed by an increase in auxin levels in the infection zone for rhizobia. Likewise, changes in cytokinin levels have been associated with the normal nodulation process (Badenoch-Jones et

al., 1987), including the cytokine-induced expression of several early nodulin genes, e.g., Enod2, Enod12A and Enod40 (Mathesius et al., 2000).

Reports of the inhibitory effect of ethylene on the nodulation process have been acquired for several different symbiont partners. Discoveries have indicated that ethylene levels in plant roots can be reduced by some strains of rhizobia. At least one strain, *Bradyrhizobium elkanii*, produces rhizobitoxine, an inhibitor of 1-aminocyclopropane-1-carboxylate (ACC) synthase, which is one of the key enzymes in the ethylene biosynthetic pathway. The synthesis of rhizobitoxine can enhance nodulation by *B. elkanii* in the host plant, *Macroptilium atropurpureum*, probably by suppressing its ethylene biosynthesis (Yuhashi et al., 2000). The existence of the enzyme ACC deaminase, which can degrade the immediate precursor of ethylene in plants, has also been found in some strains of rhizobia (Ma et al., 2002).

4- PGPR ACTIVITY OF RHIZOBIA WITH NONLEGUME CROPS

The beneficial effect of the symbiotic association between rhizobia and legumes is well known and has been intensively investigated. However, many studies show that rhizobia can form associations with other economically important grain crops (maize, rice, and wheat) and with vegetable crops (lettuce and radishes). These new associations can be beneficial for non-legume plants, but it can also have deleterious effects. Therefore, it is important, when crop rotations or intercrop systems are used, to select strains of rhizobia that will have PGPR effects on both of the involved plants (Antoun et al., 1998) (Table 4).

Table 4. Some direct evidence of PGPR activity of rhizobia with nonleguminous plants (adapted from Antoun et al., 1998)

Bacteria	Plant stimulated by inoculation		Type of assay	References
	Preferred symbiotic	Non-legumes		
<i>Bradyrhizobium japonicum</i>	Soybean	Radishes	Greenhouse	Antoun et al., 1998
<i>Rhizobium</i>	Clover	Maize	Field	Höflich et al., 1994

<i>leguminosarum</i>		Wheat	Field	Höflich et al., 1994
<i>bv. trifolii</i>		Rice	Field	Yanni et al., 1997
		Radishes	Greenhouse	Antoun et al., 1998
<i>Rhizobium</i>	Bean	Lettuce	Field	Chabot et al., 1996
<i>leguminosarum</i>		Maize	Field	Chabot et al., 1996
<i>bv. phaseoli</i>		Radishes	Greenhouse	Antoun et al., 1998
<i>Rhizobium</i>	Pea	Canola	Gnotobiotic	Noel et al., 1996
<i>leguminosarum</i>		Letucce	conditions	
<i>bv. viciae</i>				
<i>Sinorhizobium</i>	Alfalfa	Radishes	Greenhouse	Antoun et al., 1998
<i>meliloti</i>				

Until recently, the rhizobial life cycle was considered to consist of two niches: the legume nodule (as a symbiont) and the soil (as a heterotroph) (Yanni et al., 2001). Thus, in the rotation years, during which a legume crop is not grown, it was believed to persist saprophytically in soil until another legume crop was grown. Now, it is known that the rhizobial life cycle can include the (endo) colonization of roots from non-legume crops. Endophytic rhizobia and other bacteria have been found to increase the yields of non-legume crops (Yanni et al., 1997; Biswas et al., 2000; Riggs et al., 2001), but there is no conclusive evidence that the benefits involve symbiotic nitrogen fixation (James, 2000; Yanni et al., 2001). These bacteria increase yields by stimulating plant growth, increasing disease resistance, or improving the plant's ability to withstand environmental stresses like drought (Sturz and Nowak, 2000; Dobbelaere et al., 2003).

5- FREE-LIVING -PGPR

Free-living plant growth-promoting rhizobacteria –PGPR can be used in a variety of ways when plant growth enhancements are required. The most intensively researched use of PGPR has been in agriculture (Dobereiner and Baldani, 1993) and horticulture (Gomes et al., 2003). Several PGPR formulations are currently available as commercial products for agricultural production (Okon and Labandera and Gonzalez, 1994; Lazzaretti and Bettioli, 1997; Date, 2001; Schisler et al., 2004; Adesemoye et al., 2008). Developing areas of PGPR usage include forest regeneration (Chanway et al., 2000; Bent et al., 2001) and phytoremediation of contaminated soils (Burd et al., 2000). As the mechanisms of plant

growth promotion by these bacteria are unraveled, the possibility of more efficient plant-bacteria pairings for novel and practical uses will follow (Lucy et al., 2004). Some indirect mechanisms used by PGPR include antibiotic protection against pathogenic bacteria, reduction of iron available to phytopathogens in the rhizosphere, synthesis of fungal cell wall-lysing enzymes, and competition with detrimental microorganisms for sites on plant roots. Direct mechanisms of plant growth by PGPR include the acquisition of bioavailable phosphorus for the plant, nitrogen fixation, the sequestration of iron by siderophores, the production of hormones like auxins, cytokinins and gibberellins, and the reduction of plant ethylene levels (Glick et al., 1999).

6- PGPR WITH AM FUNGI AND RHIZOBIA

PGPR research has been somewhat hampered by the highly specific environment-plant genotype-bacterial genotype interactions that have been frequently found and by the not so infrequent discrepancies between ex-plant and plant experiments (Antoun et al., 1998; Valdenegro et al., 2001; Picard and Bosco, 2005; Timms-Wilson et al., 2005). This characteristic is even more important when dealing with the effect of PGPR on the rhizobial, micorrhizal or tripartite interaction with plants because the number of genotype combinations exponentially increases for each new element under consideration. In a few cases, the authors refer to a strain without even identifying the species while in others the strain is not named. Both of these situations can be troublesome for a subsequent comparison of results (Valdenegro et al., 2001). That said, quite a few papers have demonstrated field (Roesti et al., 2006; Adesemoye et al., 2008; Yadegari et al., 2008) or greenhouse (Gryndler et al., 2008; Jäderlund et al., 2008) advantages in the use of multiple partners, including a point very well argued by Felici et al. (2008), according to whom the use of microbiological mixtures would allow the mixing of different physiological processes without the need for genetic engineering. On the other hand, in the same paper, the authors did not find any synergistic effects for the dual inoculation of *Bacillus subtilis* (a biocontrol agent) and *Azospirillum brasilense* (a growth promoting bacteria) on tomato (*Lycopersicon esculentum*) samples.

It is important to notice that a large number of the papers dealing with the use of a combination of PGPR strains do not include either rhizobial or mycorrhizal partners in the interaction. A few of those researchers working with one of the legume nodulating bacteria do not use it in the traditional way, which is to enhance nitrogen fixation in legumes, but instead

use it as a PGPR in its own right with non-legume crops (Siddiqui et al., 2007; Parveen et al., 2008; Hossain and Mårtensson, 2008). In fact, through the literature found on the subject, it would seem that the most important topic in regards to multiple inoculation is some variant of biological pest control, usually of phytopathogenic bacteria (Nandakumar et al., 2001; Ryu et al., 2006; Behn, 2008; Parveen et al., 2008).

Considering only those papers that deal with mycorrhizal fungi-PGPR mixtures, we observed that one of the main research efforts is concentrated on the possible use of phosphate-solubilizing bacteria to enhance the advantage of mycorrhizal with respect to plant phosphorus nutrition, as exemplified by Roesti et al. (2006). In this paper, the authors obtained 3000 isolates from wheat (*Triticum aestivum*) fields in India, and selected 20 of those considered to be the most promising from both phosphate-solubilization and IAA production standpoints. These strains were selected from areas with different management strategies (low input and yield; low input with average yield and high input and yield), and although the authors found that plant growth stadium, crop management history and PGPR mix had significant effects, altogether they explained less than 60 % of the overall variation, even though the effect of PGPR inoculation was stronger than that observed for mycorrhizal inoculation.

On another line of research regarding the interaction between PGPR inoculation effects on the mycorrhizal symbiosis, the main effect expected from the PGPR is the traditional biological control role of PGPR (Jaizme-Vega et al., 2006; Behn, 2008; Jäderlund et al., 2008). Most often these papers deal with the control of some soil born microbial pathogen, as illustrated by the control of wheat take-out (a fungi - *Gaeumannomyces graminis* - induced *Triticum aestivum* disease) (Behn, 2008), but a few papers also deal with other pathogens such as the *Meloidogyne javanica* nematode on papaya (*Carica papaya*) (Jaizme-Vega et al., 2006). In general, this effect is considered to be indirect, with the eventual advantage of using the double inoculation being due to the decrease in vigor loss of the plant because of the pathogen.

Although these should not be considered PGPR, there is a whole class of bacteria involved with mycorrhizal symbiosis. These are the so-called Mycorrhiza Helper Bacteria (MHB), and they can be defined as bacteria that are endocellular to mycorrhizal fungi, usually vertically inherited, and beneficial to the symbiosis (Bianciotto et al., 2004; Duponnois and

Kisa, 2006; Bending, 2007; Frey-Klett et al., 2007; Ramachandran and Ravindran, 2008; Bonfante and Anca, 2009), such as those presented on Figure 2.

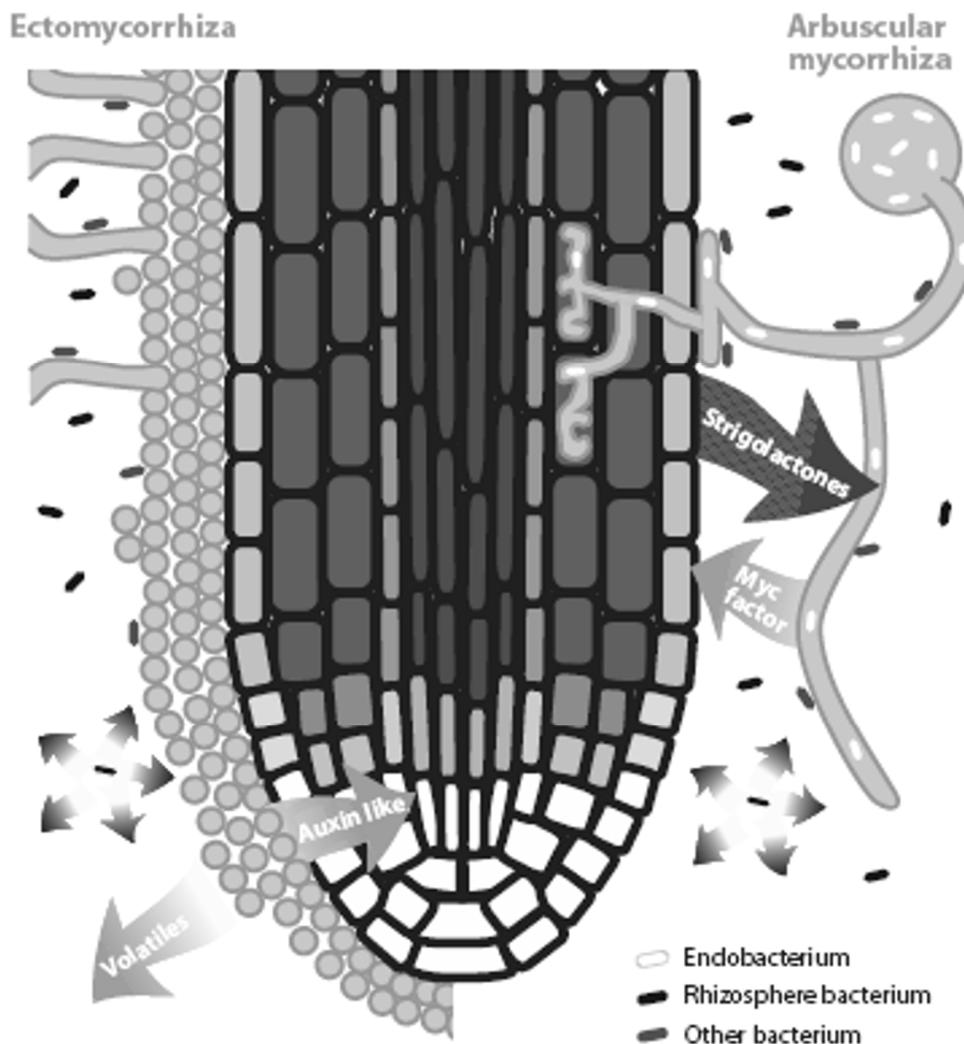


Figure 2. Some interactions among plants, mycorrhizal fungi (ectomycorrhiza on the left, arbuscular mycorrhiza on the right), and bacteria. Endobacteria are confined to AM fungi and grow from the spores toward the intraradical mycelium; rhizosphere bacteria release diffusible factors that may be beneficial detrimental to mycorrhization; other bacteria establish physical contact with the fungus-root surface and may have positive or negative effects. Arrows indicate release of diffusible factors (strigolactones, Myc factors, volatiles, and auxin-like molecules) perceived by the receiving partner (Bonfante, P. and I. A. Anca , 2009).

One point that may be clear from the results of several different papers is that MHB may have effects that are quite similar to the gamut of effects observed in plants by PGPRs, such as nutrient mobilization (Rajeshkumar et al., 2009), production of hormone-like substances

(Duponnois and Kisa, 2006), and suppression of pathogens (Riedlinger et al., 2006). The observed vertical transmission indicates that these bacteria may evolve into some kind of organelle in the future, although at present this transmission is still “leaky,” as evidenced by the strong reduction in MHB population after as few as four generations of spore production by *Gigaspora margarita* isolate BEG34 under in-vitro axenic conditions (Bianciotto et al., 2004). Although these bacteria have been frequently found on arbuscular mycorrhizal, a vast majority of papers dealing with their action mechanisms have dealt with ectomycorrhiza because these can be cultivated *in vitro*, which is sadly not the case for arbuscular mycorrhizal except when some kind of modified root system is used (Bianciotto et al., 2004). Even in this case, it is still necessary to use a three-component system, whereas with ectomycorrhiza a simpler two-component system may be used (Becker et al., 1999; Frey-Klett et al., 1999; Brulé et al., 2001; Poole et al., 2001; Bending et al., 2002; Founoune et al., 2002a; Founoune et al., 2002b; Gamalero et al., 2003; Schrey et al., 2005; Aspray et al., 2006; Riedlinger et al., 2006; Deveau et al., 2007; Schrey et al., 2007; Hryniewicz et al., 2009; Kataoka and Futai, 2009; Kataoka et al., 2009).

While the literature regarding the interaction between bacteria and mycorrhizal fungi has been increasing in recent years, as shown above, the triple interaction between PGPR, mycorrhiza and plants has not been the subject of such a dramatic increase, perhaps because of the cumulative complexity of the system and the many possible combinations of individual elements. Again, when arbuscular mycorrhiza are examined, the situation is even more complex because of its obligatory symbiosis. On the other hand, literature on the interaction between legume, their nodulating bacteria and PGPR is more abundant than that on mycorrhizal multiple inoculation (Chebotar et al., 2001; Valdenegro et al., 2001; Gupta et al., 2003; Tank and Saraf 2003; Lucas García et al., 2005; Remans et al., 2007; Siddiqui et al., 2007; Dutta et al., 2008; Figueiredo et al., 2008b; Yadegari et al., 2008) and seems to concentrate mostly on the same subject areas, with the obvious lack of MHB studies.

The first subject area concerns the nutritional effects of PGPR, most often phosphorus solubilization (Remans et al., 2007; Afzal and Bano, 2008). On this line of research, results tend to be positive under greenhouse conditions, but more ambiguous in field research. This may partly be due to the large variability observed for phosphorus, added to the incredibly high biodiversity observed in soils. This is even more important because such a large proportion of the rhizosphere biota shows some level of phosphate solubilization (Johri et al.,

1999; Dhiman and Saraf, 2003; Hara and Oliveira, 2004; Barroso and Nahas, 2005; Chung et al., 2005; Alikhani et al., 2006; Daimon et al., 2006; Ahmad et al., 2008; Chaiharn and Lumyong, 2009). This is considered an important effect, particularly because of the usual P deficiency of most tropical soils, and the energy demands of biological nitrogen fixation, which leads to the general acknowledgement of high P demands on this system (Franco and Faria, 1997; Araújo et al., 2000; Burity et al., 2000; O'Hara, 2001; Lum and Hirsch, 2003; Ogoke et al., 2003; Kochian et al., 2004; Cardoso and Kuyper, 2006).

The second major line of research, also similar to the one observed with mycorrhizas, deals with phytopathogen control (Gupta et al., 2003; Siddiqui et al., 2007; Dutta et al., 2008; Hossain and Mårtensson, 2008; Parveen et al., 2008). Again, most often the control is of microbial pathogens, but there are some works involving nematodes (Siddiqui et al., 2007). As mentioned earlier, there are frequent interactions between the genotypes involved, which in this case constitute at least a four-way relationship between plants, rhizobia, PGPR and the pathogen. Moreover, this still does not take into account the soil and climate variables. Should we really be surprised about the variability of the results?

That said, there is still a large margin for improvement in the technology, which holds the promise of reducing the ways by which chemicals are used to control diseases. This may be very important, and even more so if we take into account the usually high cost of these chemical products.

The last major line of work on the multiple inoculation of plants with rhizobia and PGPR pertains to the effect of phytohormone production on nodulation and nitrogen fixation (Remans et al., 2007; Figueiredo et al., 2008b; Cassán et al., 2009). One area that is unique to this biological system is the use of PGPR in inducing nod factor production by rhizobia (Zhang et al., 1996; Dashti et al., 1997; Dashti et al., 2000; Lian et al., 2002).

7- PGPR INOCULANTS – PROGRESS AND CHALLENGES

A PGPR inoculant is a formulation containing one or more beneficial bacterial strains (or species) in an easy-to-use and economical carrier material, which may be organic, inorganic, or synthesized from defined molecules (Bashan, 1998). Inoculants containing PGPR have three purposes: to suppress plant diseases (bioprotectants), to improve nutrient

uptake by plants (biofertilizers) and to produce phytohormones (biostimulants) (Arshad and Frankenberger, 1993). Bioprotectants have been used the most and have been commercialized to control biological plant diseases (Kloepper, 1993). Bacteria genera like *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia* and *Agrobacterium* are natural biological control agents that have been strongly studied and marketed throughout world (Khalid et al., 2004). The main mechanism for disease suppression involves the induction of systemic resistance and production of antibiotics (Kloepper, 1993). Biofertilizers are also studied and marketed for the increase in nitrogen fixation and uptake from the nitrogen-fixing bacteria associated with roots (Vessey, 2003). These biofertilizers are called inoculants and allow a reduction in chemical nitrogen fertilizer use.

The development of PGPR inoculants involves several steps (Arshad and Frankenberger, 1993): a) isolation of bacteria from roots; b) laboratory screening of putative PGPR against pathogens or for plant growth promotion in soil-less cultures; c) greenhouse screening of putative PGPR to protect plants against pathogens or promote growth in potted soil; d) field screening of the most effective putative PGPR in cropped soil (crop variety and different soil types examined); e) refinement of commercial inoculums; f) eco-toxicological testing and substantiation of PGPR claim prior to registration; g) registration of the product for commercial use.

Stringent quality assurance at various steps during production ensures the production of consistently high quality inoculants (Baudoin et al., 2008; Cassán et al., 2009). Carrier alternatives to peat are being investigated and several experimental formulations based on polymers have been evaluated (Bashan, 1998; Denardin and Freire, 2000; Schuh, 2005; Fernandes Junior, 2006; Tumelero and Denardin, 2008). These polymers, used as potential bacterial carriers, offered substantial practical advantages over peat (Amiet-Charpentier et al., 1999). These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses and release them to the soil gradually as soil microorganisms degrade the polymers. They can be stored dry at ambient temperatures for prolonged periods, offer consistent batch quality and a better-defined environment for the bacteria, and can be manipulated easily according to the needs of specific bacteria or crops (Bashan, 1998). These inoculants can be amended with nutrients to improve the short-term survival of the bacteria upon inoculation, especially those that are associative plant growth-promoting bacteria (Date, 2001; Cassán et al., 2009).

The development of formulation characteristics, to ensure survival and activity in the field and compatibility with chemical seed treatments, has been the focus of PGPR research with respect to agricultural applications. This research, among other things, optimizes growth conditions before the formulation and develops vehicles and other appropriate technology for specific applications (Date, 2001; Bashan et al., 2002; Baudoin et al., 2008; Amutha et al., 2009).

When registering and marketing products with PGPR a large number of constraints are found (Mathre et al., 1999). The U.S. market, based on the information of the committee of biological products from the American Phytopathology Society (APS) in 2005, has registered the following products: ten products based on *Bacillus* (**BioYield**TM, **Companion**TM, **EcoGuard**, **HiStick N/T**TM, **Kodiak**TM, **Meplus**TM, **Serenade**TM, **Sonata**TM, **Subtilex**TM, **YieldShield**TM), two based on *Burkholderia cepacia* (**Deny**TM and **Intercept**TM) and five based on *Pseudomonas* (**AtEze**TM, **Bio-save**TM, **BlightBan**TM, **Frostban**TM, **Spot-Less**TM). Most of these products are in powder soluble formulations. Different genera of bacteria have been studied as PGPR, however, research and development investments have been concentrated on *Pseudomonas* and *Bacillus*. Work on *Pseudomonas* has been focused on alternatives to improve the survival of this species of bacteria in commercial formulations. Furthermore, bacteria from the genus *Bacillus*, which are tolerant to desiccation and heat, have a longer life in commercial formulations; this explains the greater availability of commercial products based on *Bacillus* (Araujo, 2008).

Naturally, several efforts have been carried out in order to develop commercial inoculants using these organisms. However, the effect of inoculants on bacterial native populations in the rhizosphere is decisive for maximizing plant nutrient availability (Nelson, 2004; Figueiredo et al., 2008a; Cassán et al., 2009)

The prospect of manipulating crop rhizosphere microbial populations by inoculating with beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field (Bowen and Rovira, 1999). Recent progress in elucidating the biological interactions that occur in the rhizosphere and the practical requirements for inoculant formulation and delivery has been useful in increasing the production of PGPR inoculants. There are inconsistencies between results from studies in the field and studies in the greenhouse or growth-chamber and these varying results are a dominant barrier to the widespread use of PGPR inoculants (Bashan,

1998). One of the challenges in developing PGPR inoculants is ensuring that an effective selection and screening procedure is in place so that the most promising organisms are identified and brought forward (Arshad and Frankenberger, 1993). Effective strategies for the initial selection and screening of rhizobacterial isolates are required. It may be important to consider host plant specificity or adaptation to a particular soil, climatic condition or pathogen when selecting the isolation conditions and screening assays (Chanway et al., 1989; Bowen and Rovira, 1999). Following this point of view, some recent studies show a promising trend in the field of inoculation technology with *Pseudomonas* (Kumar et al., 2007), *Azospirillum* (Fulchieri and Frioni, 1994), and *Bacillus* (Ryder et al., 1999).

Other approaches for selecting biological control agents involve isolating samples from soils that are suppressive to that pathogen (Weller et al., 2002). Additionally, application of PGPR for the control of fungal pathogens in greenhouse systems shows considerable promise (Paulitz and Belanger, 2001) because of to the consistent environmental conditions and high incidence of fungal disease in greenhouses. Achieving consistent performance in the field, where there is heterogeneity in the abiotic and biotic factors and competition with indigenous organisms, is more difficult. Knowledge of these factors can aid in determining the optimal concentration, timing and placement of an inoculant, and devising soil and crop management strategies that can enhance the survival and proliferation of the inoculant (Gardener and Fravel, 2002). The concept of engineering or managing the rhizosphere to enhance PGPR function by manipulating host plants, substrates for PGPR, or agronomic practices, is gaining increasing attention (Mansouri et al., 2002). Development of better formulations to ensure survival and activity in the field and compatibility with chemical and biological seed treatments is another area of focus. Approaches include the optimization of growth conditions prior to formulation and the development of improved carriers and application technologies (Bashan, 1998; Bowen and Rovira, 1999; Date, 2001; Mathre et al., 1999; Yardin et al., 2000).

Recent initiatives by the pesticide regulatory departments of European and North American governments have renewed interest in biopesticide technologies as alternatives for pest management, following plans to deregister many chemical pesticides (Hynes and Boyetchko, 2006). According to these authors, strains of *Bacillus subtilis* are being used in USA and Canada, while *Paenibacillus polymyxa* is being used in Korea, *Pseudomonas chlororaphis* is being used in Austria, Finland, and Sweden, *Streptomyces griseoviridis* is being used in Canada and *Phlebiopsis gigantea* is used in the UK.

Although PGPR was first used for promoting plant growth and for the biocontrol of plant diseases, much attention has recently been paid to bioremediation with PGPR (Narasimhan et al., 2003). Phytoremediation is a new and promising approach to removing contaminants in the environment. The application of PGPR has been extended to remediate contaminated soils in association with plants. Of all the present contaminants, the profound impact of organic pollutants has attracted worldwide attention (Zhang et al., 2007). Recent examples of the bioremediation of organic contaminants by PGPR involve the use of *Pseudomonas putida* for polychlorinated biphenyl degradation (Narasimhan et al., 2003), *Pseudomonas fluorescens* for trichloroethylene degradation (Villacieros et al., 2005), *Azospirillum lipoferum* for oil degradation (Muratova et al., 2005) and *Enterobacter cloacae* for polycyclic aromatic hydrocarbon degradation (Huang et al., 2005).

CONCLUSION

There are many potential uses for PGPR in agriculture, horticulture and forest regeneration. Knowledge of the complex environment of the rhizosphere, the mechanisms of action of PGPR, and the practical aspects of inoculant formulation is important in the search for new PGPR products. The success of these products will depend on the ability of PGPR to survive and compete in the rhizosphere environment. Genetic enhancement of PGPR strains to enhance colonization and effectiveness may involve the addition of one or more traits associated with plant growth promotion. For this reason, there is an urgent need for research to clearly define what bacterial traits are useful and necessary for different environmental conditions and plants so that optimal bacterial strains can be selected. However, field experiments are needed to provide a better understanding of the biological efficacy of increased yields in crop systems. For the short and medium term future, additional studies need to be conducted on the effectiveness of different and novel inoculant formulations.

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