Chapter 1

ECOLOGY OF PLANT GROWTH PROMOTING RHIZOBACTERIA

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Abstract: Chapter presents a discussion on the term PGPR which underlines the need to have a uniform definition to be used by all authors. The actual biodiversity of PGPR will be illustrated by examples of genera and species chosen from the literature and their mechanisms of action for the following different groups: diazotrophs, bacilli, pseudomonads, and rhizobia. As PGPR are introduced in an ecosystem where intense interactions are taking place, we describe how plants, mycorrhiza, and soil fauna can influence the microbial diversity in the rhizosphere. Finally, the beneficial interactions between PGPR and symbiotic microorganisms in the *Rhizobium*-legume symbiosis, and in mycorrhizal plants are discussed. Interactions of PGPR with protozoa and nematodes are also examined.

Key words: arbuscular mycorrhizae; bacteria; fauna; mycorrhizosphere; PGPR; rhizosphere.

1   INTRODUCTION

The rhizosphere is the volume of soil surrounding and under the influence of plant roots, and the rhizoplane is the plant root surfaces and strongly adhering soil particles (Kennedy, 2005). Often, studies of the microbial ecology of the rhizosphere also include the rhizoplane. In this chapter unless specified otherwise, the term rhizosphere will be used to refer to both zones. In the rhizosphere, very important and intensive interactions are taking place between the plant, soil, microorganisms and soil microfauna. In fact, biochemical interactions and exchanges of signal molecules between plants and soil microorganisms have been described and

reviewed (Pinton *et al.*, 2001; Werner, 2001; 2004). These interactions can significantly influence plant growth and crop yields. In the rhizosphere, bacteria are the most abundant microorganisms. Rhizobacteria are rhizosphere competent bacteria that aggressively colonize plant roots; they are able to multiply and colonize all the ecological niches found on the roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper, 2001). The presence of rhizobacteria in the rhizosphere can have a neutral, detrimental or beneficial effect on plant growth. The presence of neutral rhizobacteria in the rhizosphere probably has no effect on plant growth. Deleterious rhizobacteria are presumed to adversely affect plant growth and development through the production of metabolites like phytotoxins or phytohormones but also through competition for nutrients or inhibition of the beneficial effects of mycorrhizae (Nehl *et al.,* 1996; Sturz and Christie, 2003). Kloepper (2003) discussed the problems associated with early research work on deleterious rhizobacteria, resulting from the use of soil-less systems lacking competition from native soil and rhizosphere bacteria, and from the use of a very high number of bacteria to inoculate plants, that can reach log 11.8 per seedling. These experimental conditions would not be encountered in nature, and the concept and nature of deleterious rhizobacteria can be questioned.

1.1   What are plant growth promoting rhizobacteria?

About 2 to 5% of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microflora, exert a beneficial effect on plant growth and are termed plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR are free-living bacteria (Kloepper *et al.*, 1989), and some of them invade the tissues of living plants and cause unapparent and asymptomatic infections (Sturz and Nowak, 2000). These rhizobacteria are referred to as endophytes, and in order to invade roots they must first be rhizosphere competent. It is important to note that the term endorhizosphere, previously used in studies of the root zone microflora, is semantically incorrect and should not be used (Kloepper *et al.,* 1992). The original definition of rhizobacteria was restricted to free-living bacteria to differentiate them from nitrogen-fixing rhizobia and *Frankia*. Overtime, some authors have used a less restrictive definition of rhizobacteria as any root-colonizing bacteria. With the original definition, rhizobia and *Frankia* would not be considered as PGPR, while they would be PGPR with broader definition of rhizobacteria. Hence, it is important for authors to define their terms. It is generally accepted now that growth stimulation resulting from the biological dinitrogen fixation by rhizobia in legume nodules or by *Frankia* in nodules of *Alnus* spp., is not considered as a PGPR mechanism of action (Kloepper, 1993; Kapulnik, 1996; Lazarovits

and Nowak, 1997; Bashan *et al.*, 2004), but rather as the result of the establishment of these well-known symbioses producing nodules. Rhizobia and *Frankia* in that case are designated as the microbial symbiotic partners (microsymbionts) of their homologous plant hosts. Thus, designating rhizobia and *Frankia* species involved in symbiotic associations with higher plants as intracellular PGPR or symbiotic PGPR (Vessey, 2003; Gray and Smith, 2005), is not in agreement with the essence of the original definition of PGPR, and it complicates the study of PGPR since the field of the legume-rhizobia symbioses is so vast and well studied (Vessey, 2003). Several strains of *Burkholderia caribensis* and *Ralstonia taiwanensis* belonging to the β-subclass of proteobacteria are legume-nodulating, they carry *nod* genes very similar to those of rhizobia and they have been designated as β-rhizobia (Chen *et al.*, 2003). Associative dinitrogen fixing bacteria when they do not exhibit morphological modification of the host plant are considered as PGPR. However, rhizobia can also behave like PGPR with non-legume plants and some rhizobia are endophytes (Sessitsch *et al.,* 2002).

PGPR may induce plant growth promotion by direct or indirect modes of action (Beauchamp, 1993; Kloepper, 1993; Kapulnik, 1996; Lazarovits and Nowak, 1997). Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation) and stimulation of disease-resistance mechanisms (induced systemic resistance). Indirect effects originate for example when PGPR act like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Jacobsen, 1997). Based on their activities Somers *et al.* (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth-promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants) and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites). 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 does not seem to have been widely accepted. When studying beneficial rhizobacteria, the original definition of PGPR is generally used: it refers to the subset of soil and rhizosphere bacteria colonizing roots in a competitive environment, e.g. in non-pasteurized or non-autoclaved field soils (Kloepper, 2003). Furthermore, in most studied cases, a single PGPR will often reveal multiple modes of action including biological control (Kloepper, 2003; Vessey, 2003).

2   GENERA OF PGPR

Early studies on PGPR focused more on biological control of plant diseases than on growth promotion, and involved bacteria like fluorescent pseudomonas and *Bacillus subtilis* that are antagonistic to soil-borne plant pathogens (Kloepper *et al.,* 1989). The number of bacterial species identified as PGPR increased recently as a result of the numerous studies covering a wider range of plant species (wild, economically important and tree) and because of the advances made in bacterial taxonomy and the progress in our understanding of the different mechanisms of action of PGPR. Presently, PGPR include representatives from very diverse bacterial taxa (Vessey, 2003; Lucy *et al.,* 2004) and in the following sections we are not giving a thorough description of all the genera and species of PGPR, but rather a few examples to illustrate the biodiversity of these beneficial bacteria.

2.1   Diazotrophic PGPR

Azospirillum known for many years as PGPR was isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates (Steenhoudt and Vanderleyden, 2000) This bacterium was originally selected for its ability to fix atmospheric nitrogen (N_2), and since the mid-1970s, it has consistently proven to be a very promising PGPR, and recently the physiological, molecular, agricultural and environmental advances made with this bacterium were thoroughly reviewed by Bashan *et al.* (2004). Presently PGPR for which evidence exists that their plant stimulation effect is related to their ability to fix N_2 include the endophytes *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter diazotrophicus* and *Herbaspirillum* sp. and, the rhizospheric bacteria *Azotobacter* sp. and *Paenibacillus* (*Bacillus*) *polymyxa*, (Vessey, 2003).

Several plant isolates, previously included in the genus *Azoarcus,* have now separate genera: *Azovibrio restrictus*, *Azospira oryza* and *Azonexus fungiphilus* (Reinhold-Hurek and Hurek, 2000). *Azoarcus* spp. are strictly respiratory bacteria belonging to the β-subclass of the Proteobacteria, and most species have been isolated from roots or stems of Kallar grass, *Leptochloa fusca*; (Hurek *et al.,* 1997). All the plant associated isolates of these genera are unable to use carbohydrates for growth but they use organic acids or ethanol and their optimal growth temperatures are high ($37\text{-}42^0C$). *Azoarcus* sp. strain BH72, which is capable of colonizing the interior of rice (*Oryza sativa* L.) root, has been described as a model for nitrogen fixing grass endophytes (Hurek and Reinhold-Hurek, 2003). *Gluconacetobacter diazotrophicus*, previously known, as *Acetobacter diazotrophicus*, is a Gram-negative bacterium, strict aerobe originally isolated from sugarcane

(*Saccharum officinarum*) roots and stems (Pan and Vessey, 2001). *G. diazotrophicus* has also been isolated from the inner tissues of sweet potato (*Ipomoea batatas*), grass elephant (*Pennisetum purpureum* var. Cameroon), coffee (*Coffea arabica*), finger millet (*Eleusine coracana*) and pineapple (*Ananas comosus*) plants (Muñoz-Rojas and Caballero-Mellado, 2003). *Herbaspirillum* is an endophyte, which colonizes rice, maize (*Zea mays*), sorghum (*Sorghum bicolor*), and other cereals and sugarcane (James *et al.,* 2002). The genus *Burkholderia* contains over 30 species, and the ability to fix atmospheric nitrogen has been established in several plant isolates including *B. vietnamiensis* and *B. Kururiensis* (De Los Santos *et al.,* 2001; Coenye and Vandamme, 2003).

Multiple inoculation experiments during recent decades failed to show a substantial contribution of biological nitrogen fixation to plant growth in most cases. For example, inoculation with different strains of diazotrophs did not relieve the N-deficiency symptoms of unfertilized maize in either field or greenhouse assays (Riggs *et al.,* 2001). It is now clear that associative diazotrophs, like other PGPR, exert mainly their positive effects on plant growth through different direct or indirect mechanisms (Dobbelaere *et al.,* 2003). Kennedy *et al.* (2004) discussed the possibility of improving the plant growth promoting potential of diazotrophs, through the production of high quality inoculant biofertilizers.

2.2   Bacilli

By using the PCR-denaturing gradient gel electrophoresis (DGGE) technique developed to study the diversity of *Bacillus* (including the groups separated as *Paenibacillus*, *Alicyclobacillus*, *Aneurinibacillus*, *Virgibacillus*, *Salibacillus*, and *Gracilibacillus*), Garbeva *et al.* (2003) showed that the majority (95%) of Gram-positive bacteria in soils under different types of management regimes (permanent grassland, grassland turned into arable land, and arable land), were putative *Bacillus* species; *B. mycoides*, *B. pumilus*, *B.megaterium*, *B. thuringiensis*, and *B. firmus*, as well as related taxa such as *Paenibacillus*, were frequently identified by sequencing the DNA bands obtained on DGGE gels. Other Gram-positive bacteria including *Arthrobacte*r spp. and *Frankia* spp. were a minority (less than 6% of the clones obtained). The ubiquity and the importance of *B. benzoevorans* in soils throughout the world were proved by using molecular methodology developed to identify non-culturable bacteria (Tzeneva *et al.,* 2004).

Bacillus spp. are able to form endospores that allow them to survive for extended periods under adverse environmental conditions. Some members of the group are diazotrophs and *B. subtilis* was isolated from the rhizosphere of a range of plant species at concentration as high as 10^7 per gram of rhizosphere soil (Wipat and Harwood, 1999). *P. polymyxa* is a

cytokinin producer (Timmusk *et al.,* 1999) identified as an endophyte of lodgepole pine seedlings (Shishido *et al.,* 1999). However this bacterium is probably not an endophyte, and this misidentification results from the resistance of endospores to the different plant surface disinfection protocols (Bent and Chanway, 2002). *Bacillus* species have been reported to promote the growth of a wide range of plants (De Freitas *et al.,* 1997; Kokalis-Burelle *et al.,* 2002); however, they are very effective in the biological control of many plant microbial diseases.

Under field conditions in Thailand, Jetiyanon *et al.* (2003) observed that a PGPR mixture containing *B. amyloliquefaciens* strain IN937a and *B. pumilus* strain IN937b, induced systemic resistance against southern blight of tomato (*Lycopersicon esculentum*) caused by *Sclerotium rolfsii*, anthracnose of long cayenne pepper (*Capsicum annuum* var. *acuminatum*) caused by *Colletotrichum gloeosporioides*, and mosaic disease of cucumber (*Cucumis sativus*) caused by cucumber mosaic virus (CMV). *Bacillus megaterium* KL39, a biocontrol agent of red-pepper Phytophthora blight disease, produces an antifungal antibiotic active against a broad range of plant pathogenic fungi (Jung and Kim, 2003). *B. subtilis* also synthesizes an antifungal antibiotic inhibiting *Fusarium oxysporum* f. sp. *ciceris,* the agent of fusarial wilt in chickpea (Kumar, 1999) and strain RB14 produces the cyclic lipopeptides antibiotics iturin A and surfactin active against several phytopathogens. This strain has a very good potential to be used for the biological control of damping-off of tomato caused by *Rhizoctonia solani* (Asaka and Shoda, 1996). The best isolates to inhibit *Fusarium roseum* var. *sambucinum*, the causal agent of dry rot of potato tubers, obtained from Tunisian salty salts belonged to the species *B. cereus*, *B. lentimorbus* and *B. licheniformis* (Sadfi *et al.,* 2001). The antifungal activity of the selected isolates was associated with their ability to produce inhibitory volatile substances and diverse and complex lytic chitinases.

2.3   Pseudomonads

Early observations on the beneficial effect of seeds or seed pieces bacterization were first made with *Pseudomonas* spp. isolates, on root crops. By treating potato (*Solanum tuberosum* L.) seed pieces with suspensions of strains of *Pseudomonas fluorescens* and *P. putida*, Burr *et al.* (1978) obtained statistically significant increases in yield ranging from 14 to 33% in five of nine field plots established in California and Idaho. Substantial increase in the fresh matter yield of radish (*Raphanus sativus* L.) was obtained by seed inoculation with fluorescent pseudomonads (Kloepper and Schroth, 1978). Significant growth increases in seedling and mature root weights, and in total sucrose yield were attained in field trials in California and Idaho, by inoculating sugar beet (*Beta vulgaris* L.) with selected strains

of fluorescent *Pseudomonas* spp. (Suslow and Schroth, 1982). Under greenhouse conditions when tested in three different soils, an isolate of *Pseudomonas* sp. consistently caused a significant increase of the maize shoot dry matter yield (Lalande *et al.,* 1989). Several *Pseudomonas* isolates are able to solubilize sparingly soluble inorganic and organic phosphates (Chabot *et al.,* 1993; Rodriguez and Fraga, 1999). Less than 0.5% of the 200 randomly selected isolates obtained from Australian soils were able to use inositol hexaphosphate as sole source of C and P (Richardson and Hadobas, 1997). Further study of 238 isolates obtained from enrichment culture allowed the identification of four unique isolates showing the ability to specifically utilize inositol hexaphosphate, two of them were putative fluorescent (*P. putida*) and two were non-fluorescent pseudomonads (*P. mendocina*). The fluorescent *Pseudomonas* strains exhibited marked phytase activity and liberated up to 81% of P from inositol hexaphosphate. In field trials performed in Quebec (Canada), inoculation with tricalcium phosphate solubilizing *Pseudomonas* sp. 24 caused a significant increase in maize plant height after 60 days of growth and an 18% increase in lettuce shoot fresh matter yield (Chabot *et al.,* 1993). The effects of plant inoculation with *Pseudomonas* and their possible growth promoting mechanisms of action have been reviewed (Lemanceau, 1992; Digat, 1994). The beneficial effects of these bacteria have been attributed to their ability to promote plant growth and to protect the plant against pathogenic microorganisms. Production of indole acetic acid (IAA) by *Pseudomonas putida* GR12-2 plays a major role in the root development of canola (*Brassica rapa*) root system as evidenced by the production of roots 35 to 50% shorter by an IAA-deficient mutant (Patten and Glick, 2002). IAA may promote directly root growth by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. ACC is the direct precursor of ethylene an inhibitor of root growth, and strain GR12-2 like several other bacteria produces ACC-deaminase (Jacobson *et al.,* 1994), which degrades ACC, thus preventing plant production of inhibitory levels of ethylene. Strain G20-18 of *Pseudomonas fluorescens* produced higher amounts of three cytokinins, isopentenyl adenosine, trans-zeatin ribose and dihydrozeatin riboside (Garcia de Salamone *et al.,* 2001). The use of mutants with reduced capacity to synthesize cytokinins, revealed the importance of cytokinin production in the plant growth promoting ability of strain G20-18 (Garcia de Salamone, 2000).

Pseudomonads are well known for their involvement in the biological control of several plant pathogens. Alabouvette *et al.* (1993) showed that in addition to non-pathogenic *Fusarium oxysporum*, *P. fluorescens* and *P. putida* are the main candidates for the biological control of fusarium wilts. The fluorescent pseudomonads are involved in the natural suppressiveness of some soils to fusarium wilts, and they have been applied

successfully to suppress fusarium wilts of various plant species (Lemanceau and Alabouvette, 1993). For many pseudomonads, production of metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) is the primary mechanism of biocontrol (Weller and Thomashow, 1993). By using a bacterial mutant unable to produce HCN, Gallagher and Manoil (2001) were able to show that *P. aeruginosa* PAO1 kills the nematode *Caenorhabditis elegans* by cyanide poisoning. *P. aeruginosa* 78 produce a polar substance, heat labile, sensitive to extreme pH values causing *in vitro* juvenile mortality of *Meloidogyne javanica*, the root -knot nematode (Ali *et al.,* 2002). Several evidence indicate that siderophore production when iron is limited is responsible for the antagonism of some strains of *P. aeruginosa* against *Pythium* spp. the causal agents of damping-off and root rot of many crops (Buyens *et al.,* 1996; Charest *et al.,* 2005). The antibiotics produced by bacterial biocontrol agents and their role in microbial interaction, were reviewed by Raaijmakers *et al.,* (2002). *P. fluorescens* CHAO isolated and intensively studied by the group of G. Défago in Switzerland produces several bioactive compounds (antibiotics, siderophores, HCN, indole acetic acid) giving it one of the broadest spectra of potential biocontrol and growth-promoting mechanisms of known PGPR (Weller and Tomashow, 1993). Production of 2,4-diacetylphloroglucinol by CHAO is an important mechanism of suppression of take-all of wheat and black root rot of tobacco (Keel *et al.,* 1992). The production of a novel lipopeptide antibiotic (AFC-BC11) is largely responsible for the ability of *Burkholderia cepacia* to effectively control damping-off of cotton caused by *Rhizoctonia solani* in a gnotobiotic system (Kang *et al.,* 1998). Many strains of pseudomonads can indirectly protect the plants by inducing systemic resistance against various pests and diseases (Van Loon *et al.,* 1998; Ramamoorthy *et al.,* 2001; Zehnder *et al.,* 2001). In Canada, *Pseudomonas* spp. were developed for the biological control of *Pythium* diseases in hydroponics systems for greenhouses (Paulitz and Bélanger, 2001). In a spring cucumber crop, *P. corrugata* strain 13 and *P. fluorescens* strain 15 produced 88% more marketable fruit, while in a fall crop with severe disease pressure due to higher slab temperatures, both strains significantly increased by 600% the marketable fruit. Strain 15 also increased fruit production in treatments not inoculated with pathogen (Paulitz and Bélanger, 2001). Several reports show the critical role-played by fluorescent *Pseudomonas* spp. in naturally occurring soils that are suppressive to fusarium wilt (Mazzola, 2002), and take-all caused by the fungus *Gaeumanomyces graminis* var. *tritici* (Weller *et al.,* 2002). Finally, *P. putida* isolated in the province of Quebec, from a soil selected for its important suppressive effect against the causal agent of potato silver scurf (*Helminthosporium solani)*, reduced the disease severity by 70% after 30 days at 15^0C and by 22% after 18 days at 24^0C (Martinez *et al.,* 2002).

2.4   Rhizobia

Rhizobia and bradyrhizobia are well known as the microbial symbiotic partners of legumes, forming N_2-fixing nodules. However these bacteria also share many characteristics with other PGPR. In fact rhizobia can produce phytohormones, siderophores, HCN; they can solubilize sparingly soluble organic and inorganic phosphates, and they can colonize the roots of many non-legume plants (Antoun *et al.,* 1998). Under greenhouse condition, radish dry matter yield was increased by inoculation with strains of *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae* and *Sinorhizobium meliloti*. The highest stimulatory effect (60% increases as compared to the uninoculated control) was observed with strain Soy213 of *B. japonicum* (Antoun *et al.,* 1998). In a series of field experiments performed between 1985 and 1993, Höflich *et al.* (1994) observed that inoculation with strain R39 of *R. leguminosarum* bv. *trifolii*, significantly (P<0.05) stimulated the shoot dry matter yield of maize, spring wheat (*Triticum aestivum* L.) and spring barley (*Hordeum vulgare* L.). In pot experiments, inoculation of wheat with some strains of *R. leguminosarum* bv. *trifolii* isolated from Morocco increased shoots dry matter yield by 16 to 19% and grain yield by 23 to 25%, as compared to the uninoculated control (Hilali *et al.,* 2001). Chabot *et al.* (1996) obtained under field conditions the stimulation of growth of maize and lettuce (*Lactuca sativa* L.) by inoculation with dicalcium phosphate solubilizing strains of *R. leguminosarum* bv. *phaseoli*. Similar stimulations were observed when mycorrhizal lettuce was inoculated with strains of *S. meliloti* under gnotobiotic conditions (Galleguillos *et al.,* 2000). Inoculation of barley in pots with the tricalcium phosphate solubilizing strain *Mesorhizobium mediterraneum* PECA21 significantly increased the plant dry matter yield, and its content in N, K, Ca and Mg (Peix *et al.,* 2001). *Azorhizobium caulinodans* is nitrogen fixing bacterium forming stem and root nodules on their legume host *Sesbania rostrata* (Ndoye *et al.,* 1994). In the presence of the flavonoid naringenin strain ORS571 of *A. caulinodans* is able to colonize the roots of *Brassica napus* (O'Callaghan *et al.,* 2000). Several reports indicate that rhizobia are endophytes of non-legume plants. McInroy and Kloepper (1995) isolated *B. japonicum* from the roots of cotton (*Gossypium hirsutum* L.) and sweet corn. *Rhizobium giardinii* (Reiter *et al.,* 2002) and *S. meliloti* (Sturz *et al.,* 1999) were also identified as endophytes of potato. Photosynthetic bradyrhizobia were also found as natural endophytes of the African wild rice *Oryza breviligulata*, the ancestor of the African cultivated rice, *O. glaberrima* (Chaintreuil *et al.,* 2000). In regions where legumes are cultivated in rotation with non-legumes, rhizobia are frequently found as endophytes of the non-legume plant involved in the rotation. In Egypt, for over 7 centuries,

production of rice has benefited from the rotation with Egyptian berseem clover (*Trifolium alexandrinum*); and 3-4 strains of *R. leguminosarum* bv. *trifolii* were true rhizobial endophytes of rice, and were able to promote rice growth and productivity under laboratory and field conditions (Yanni *et al.*, 1997). *Rhizobium etli* is a natural endophyte of maize traditionally cultivated for thousands of years in Mesoamerica, in association with beans (*Phaseolus vulgaris*) (Guttiérrez-Zamora and Martinez-Romero, 2001). Lupwayi *et al.* (2004) observed that in the bulk soil, rhizosphere or rhizoplane of barley, wheat and canola the populations of rhizobia were greater when these crops were grown in rotation after pea as compared to monoculture, and *R. leguminosarum* bv. *viciae* colonized the root interiors of the three plants.

Rhizobia have a good potential to be used as biological control agents against some plant pathogens. Strains of *S. meliloti* are antagonistic to *Fusarium oxysporum* (Antoun *et al.*, 1978), and rhizobia antagonistic to *F. solani* f. sp. *phaseoli* isolated from commercial snap bean, appeared to have a good potential for controlling fusarium rot (Buonassisi *et al.*, 1986). Ehteshamul-Haque and Ghaffar (1993) observed under field conditions that *S. meliloti*, *R. leguminosarum* bv. *viciae*, and *B. japonicum* used either as seed dressing or as soil drench reduced infection of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* spp., in both leguminous (soybean; *Glycine max* and mungbean; *Vigna radiata*) and non-leguminous (sunflower; *Helianthus annuus* and Okra; *Abelmoschus esculentus*) plants. In a field naturally infested with *Pythium* spp. inoculation of pea (*Pisum sativum* L.) and sugar beet with strain R12 of *R. leguminosarum* bv. *viciae*, isolated from lentil (*Lens culinaris*) in Alberta Canada, significantly increased seedling emergence four weeks after planting (Bardin *et al.*, 2004). This strain was as effective as *Pseudomonas fluorescens* 708 a biological control agent of *Pythium* sp. (Bardin *et al.*, 2003). In one field experiment performed with sugar beet in august 2001, rhizobia R12 was as effective as the fungicide Thiram[TM] used as seed treatment to control *Pythium* diseases. Two other strains R20 and R21 isolated from pea showed comparable results and are potentially good biocontrol agents against *Pythium* diseases in pea and sugar beet. Reitz *et al.* (2000) showed that the lipopolysaccharides of *R. etli* G12 induce the systemic resistance to infection by the cyst nematode *Globodera pallida* in potato roots.

3   EFFECTS OF INOCULATION WITH PGPR ON THE PLANT-SOIL-MICROBE ECOSYSTEMS

In order to have a beneficial effect on a target plant, PGPR are introduced in large number by seed or seed piece inoculation, with the aim

of having good root colonization, a prerequisite for the successful use of PGPR. However, this inoculation process might have other non-target effects on plants, microorganisms and other members of the soil fauna like protozoa and nematodes. Winding *et al.* (2004) reviewed recently the non-target effects of the use of bacterial biocontrol agents suppressing root pathogenic fungi. The introduction of antibiotic-producing bacteria into the rhizosphere caused in some cases significant non-target effects; however they were generally small in scale and limited to a growth season, and have not been proven to affect soil health. Regardless of the statistics and techniques used, culture-dependent (BIOLOG, FAME, PLFA) or culture independent (PCR-DGGE), it was frequently observed that the introduction of bacterial biocontrol agents affected microbial community structures, and these temporary effects are probably of minor importance for soil functioning (Winding *et al.,* 2004). Later in this chapter we will be showing how PGPR can influence some beneficial symbiosis, like the *Rhizobium*-legume or the plant-mycorrhizae, but let us first see how some important constituents of the soil-plant-microbe ecosystems affect soil microbial structure which may have an important effect on the outcome of inoculation with PGPR.

3.1   Factors influencing soil microbial structure and activity

3.1.1   Mycorrhizae

More than 80% of all land plant species form symbiotic associations with mycorrhizae (Sylvia, 2005). Following mycorrhizal colonisation, the functions of the root become modified both through the mycorrhizal fungus acting as a sink for the photoassimilate and through hyphal exudation. This may be expected to lead to changes in both qualitative and quantitative release of exudates in the mycorrhizosphere (Hodge, 2000). The rhizosphere concept has therefore been widened to take into consideration the fact that plant root are commonly mycorrhizal resulting in the term "mycorrhizosphere". The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus and it includes the more specific term "hyphosphere" which refers only to the zone surrounding individual hyphae (Johansson *et al.,* 2004). Plant root-colonization with arbuscular mycorrhizal (AM) fungi can affect bacterial communities associated with the roots directly by providing energy-rich carbon compounds derived from host assimilates and transported to the mycorrhizosphere via fungal hyphae, by fungal induction of pH changes, by fungal exudates (inhibitory or stimulatory compounds) or by competition. Indirect effects of AM fungi can result from modification of soil structure or plant root exudates (Johansson *et al.,* 2004). A greater number of the PGPR *Azotobacter chroococcum* and

Pseudomonas fluorescens were attracted towards tomato roots colonized by *Glomus fasciculatum* compared to non-vesicular-arbuscular mycorrhizal tomato roots (Sood, 2003). By using mutants of *A. brasilense* and *R. leguminosarum* altered in the production of extracellular polysaccharides, Bianciotto *et al.* (2001) showed the involvement of these polysaccharides in the attachment of these bacteria to the structures of AM fungi. In soil, an extensive network of AM fungi develops and PGPR are usually associated with fungal surfaces (Bianciotto and Bonfante, 2002). The symbiotic AM fungi *Gigaspora margarita*, *Scutellospora persica* and *Scutellospora castanea*, contain endosymbiotic bacteria closely related to the genus *Burkholderia* (Bianciotto *et al.,* 2000). Minerdi *et al.* (2001) observed the presence of *nif* genes in *Burkholderia* the endosymbiont of *G. margarita*. The ecological importance of the presence of these rare examples of bacteria living in symbiosis with fungi remains to be elucidated. Barea *et al.* (2002) summarized the different interactions taking place in the mycorrhizosphere that improve plant fitness and soil quality. Villegas and Fortin (2001) used a two compartment Petri plate system, and roots of carrot (*Daucus carota* L.) transformed with *Agrobacterium rhizogenes* to study the solubilization of tricalcium phosphate by the AM fungus *Glomus intraradices* and by phosphate solubilizing bacteria. When ammonium was used as sole nitrogen source *Pseudomonas aeruginosa*, and mycorrhizal and non-mycorrhizal roots of carrot and the mycelium of *G. intraradices* exhibited some P solubilization activity. Inoculation of the non-mycorrhizal carrot roots with *P. aeruginosa* showed a slight non-significant increase in the amount of P solubilized. However, when the inoculated roots were infected with *G. intraradices* a substantial significant increase in P solubilization was observed clearly indicating the presence of a synergistic effect caused by the fungus. When nitrate was used as sole nitrogen source, important solubilization activities were only observed as results of the interactions between *G. intraradices* and the two P-solubilizing bacteria *P. aeruginosa* and *P. putida* (Villegas and Fortin, 2002).

3.1.2   **Plant effect**

In comparison to the bulk soil, the number of microorganisms in the rhizosphere is always substantially higher because of the plant influence. There are also changes in the biodiversity of microorganisms caused by this "rhizosphere effect" which was defined by Badalucco and Kuikman (2001) as any physical, chemical or biological change occurring within the root sphere or even indirectly mediated by its excretions and organic debris. Plant genes play an important role in the interaction between plant and beneficial symbiotic (and probably asymbiotic) microorganisms, as indicated by the observed variations in the response of different plant cultivars to the same

introduced organism. Plant genotype affects the response to inoculation with
PGPR because it affects root colonization by the introduced bacteria, as well
as the total population size of microbial communities on plant and it
probably also affect the composition of those communities (Smith and
Goodman, 1999). Lemanceau *et al.* (1995) used biochemical and
physiological tests to compare the diversity of the soilborne populations of
fluorescent pseudomonads in flax (*Linum usitatissinum* L.) and tomato
(*Lycopersicon esculentum* Mill.) grown in the same soil. The populations
isolated from uncultivated soils were different from those isolated from
plants (rhizosphere, rhizoplane or root tissue), and analysis of the bacterial
isolates indicated that plant has a selective influence on fluorescent
pseudomonads and the selection was more strongly expressed with flax than
with tomato plants. Further study with 317 isolates of fluorescent
pseudomonads revealed that in the vicinity of flax and tomato roots,
denitrifiers were more abundant than in the uncultivated soil, and it was
hypothesized that denitrification could be a selective advantage for the
denitrifiers in the root environment and that this process could contribute to
modify the specific composition of the bacterial communities in the
rhizosphere (Clays-Josserand *et al.,* 1995). The genetic variability of the
cultivable *Burkholderia cepacia* populations in the rhizosphere of maize
grown under field conditions in Italy, decreased as plants were getting older
indicating that in the selection of *B. cepacia* strains to be used as inoculants
for maize, plant growth stage is an important factor among others that should
be taken into account (Di Cello *et al.,* 1997). Comparable results suggesting
a marked influence of time on microbial pools were observed with pot
grown maize plants (Baudoin *et al.,* 2002). By using direct DNA isolation
and the PCR-DGGE technique Duineveld *et al.* (1998) observed that the
rhizosphere effect in chrysanthemum plants grown in pots influenced only a
minor fraction of the total bacterial community represented by weak bands
on the DGGE gel. Normander and Prosser (2000) also did not observe any
difference between DGGE patterns of bulk soils and rhizosphere in barley
grown in pots. Different results are obtained from the rhizosphere of plants
grown under field conditions. In fact, under field conditions, the DGGE
fingerprints obtained from the rhizosphere of strawberry (*Fragaria ananassa*
Dutch.), oilseed rape (*Brassica napus* L.) and potato showed plant dependent
shifts in the relative abundance of the rhizosphere populations, which
became more pronounced in the second year of growing the same crop
(Smalla *et al.,* 2001). The perennial strawberry plant had rhizosphere
communities' pattern quite different from those of the two similar patterns
obtained with the annual plants oilseed rape and potato. In studying
microbial diversity in soil, molecular techniques based on PCR have been
used to overcome the limitations of culture-based methods; however these

techniques have their own limitations, which have been reviewed by Kirk *et al.* (2004).

3.1.3   Soil fauna

Soil fauna has an important function in regulating rhizosphere microbial processes and therefore affect plant growth (Bonkowski *et al.,* 2000). Protozoa are essential components of the soil ecosystem and they consume in general more than 50% of the bacterial productivity, enhancing nutrient cycles and energy flows to the benefit of microorganisms, plants and animals (Foissner, 1999). There is about 1600 known protozoan species living in terrestrial environment, however as indicated by studies with ciliates, these represent about 20 to 30% of the species actually present, most of which are still not described. Grazing by a mixed assemblage of soil protozoa (seven flagellates and one amoeba) had significant effects on the bacterial community structure in a soil microcosm, as revealed by the PCR-DGGE as well as the community level physiological profiling determined with the Biolog plates (Ronn *et al.,* 2002). Grazing favoured Gram-positive bacteria closely related to *Arthrobacter* spp. The effects of rhizobacteria on root architecture seem to be mediated by protozoan grazing, particularly by naked amoeba, which are the most important bacterial grazers in soil (Bonkowski, 2004). The presence of the amoebae *Acanthamoeba* sp. induced changes in root morphology of watercress (*Lepidium sativum* L.) seedlings resembling hormonal effects and increased the proportion of IAA producing rhizosphere community (Bonkowski and Brandt, 2002). By changing the physical structure of soil and the distribution of resources, the activities of earthworms alter the habitat for many different types of organisms (Amador and Görres, 2005). Hendriksen and Hansen (2002) observed that the vegetative cells of the insecticide bacterial strain *Bacillus thuringiensis* var. *Kurstaki* DMU67R, were present in the gut of the non-target earthworm species *Lumbricus rubellus, L. terrestris* and *Apporrectodea caliginosa*. In *A. caliginosa* DMU67R, spore germination seemed to be restricted to the gut and sporulation occurred after defecation. These results suggest that survival in the soil of *B. thuringiensis* is a dynamic process involving germination, cell divisions and sporulation in specific microhabitats. Knox *et al.* (2003) tested in sand based microcosms, the effect of three species of nematodes (*Caenorhabditis elegans*, *Acrobeloides thornei* and *Cruznema* sp.) on wheat rhizosphere colonization by three Gram-negative PGPR (*Pseudomonas corrugata* and two strains of *P. fluorescens*) and a Gram-positive PGPR (*B. subtilis*). Irrespective of the bacterial or nematode species, rhizosphere colonization by the tested PGPR was substantially increased by the presence of nematodes. In developing new plant inoculants containing PGPR, the effect of soil fauna is an important

factor that should not be overlooked, and the possibility of developing a mixed inoculant containing for example beneficial protozoa should be considered and further investigated.

3.1.4   Abiotic factors

Soil physical and chemical properties (pH, humidity and water availability, temperature, redox, salinity, texture, stability of aggregates, fertility, organic matter content), the presence or absence of pesticides and other xenobiotic substances are examples of well known abiotic factors that can directly or indirectly affect plant growth and their interaction with soil microflora and fauna. Abiotic factors can also directly influence PGPR activity and probably their effect on plant growth and the dynamics of root microbial communities. Duffy and Défago (1999) studied the environmental factors that modulate the biosynthesis of antibiotic and siderophore by the disease-suppressive strain *P. fluorescens* CHAO. The production of the antibiotic 2,4-diacetylphloroglucinol was stimulated by Zn^{2+}, NH_4Mo^{2+} and glucose, and production of pyoluteorin was stimulated by Zn^{2+}, Co^{2+} and glycerol and was repressed by glucose. The production of the siderophore pyochelin was increased by Co^{2+}, fructose, mannitol and glucose. Comparison of strain CHAO with a genetically diverse collection of 41 *P. fluorescens* biocontrol strains indicated that the effect of some factors like the stimulation of 2,4-diacetylphloroglucinol by Zn^{2+} and glucose was strain dependent (Duffy and Défago, 1999).

3.2   Root colonization by introduced PGPR

Failure of PGPR to produce a desired effect after seeds inoculation is frequently associated with their inability to colonize plant roots. In fact, root colonization is a very complex phenomenon involving several steps and influenced by many biotic and abiotic parameters, and has been reviewed by Benizri *et al.* (2001). Mechanisms involved in the establishment of a successful interaction between PGPR and plant roots have been reviewed and discussed (Somers *et al.,* 2004). Latour *et al.* (2003) described a strategy used during the last decade to study traits involved in the rhizosphere competence of fluorescent pseudomonads. First, the diversity of indigenous populations associated with plant roots was compared with that of the uncultivated soils in order to identify traits discriminating between the two populations. Comparing a wild-type strain to mutants affected in the corresponding phenotypes, allowed the determination of the involvement of the identified traits in rhizosphere competence. Finally, traits shared by populations adapted to the rhizosphere were identified by comparing the metabolism and the competitiveness in the rhizosphere of a collection of

bacterial strains. The results obtained indicated that rhizosphere competent pseudomonads are particularly efficient in using pyoverdine-mediated iron uptake system and in reducing nitrogen oxides (Latour *et al.,* 2003).

Quorum sensing (also called autoinduction) is a well-understood mechanism of bacterial cell-to-cell communication and it conveys the concept that certain traits are only expressed when bacteria are crowded together. In plant pathogenic bacteria, traits regulated by quorum sensing include the production of extracellular polysaccharides, degradative enzymes, antibiotics, siderophores, and pigments, as well as motility and biofilm formation (von Bodman *et al.,* 2003). *N*-acyl-homoserine lactones (AHLs), are the most commonly reported type of quorum sensing signals, and interestingly production of this molecule is more common among plant-associated *Pseudomonas* spp. than among soil borne species, confirming the importance of quorum sensing in plant associated bacterial communities (Elasri *et al.,* 2001).

4   INTERACTIONS BETWEEN PGPR AND OTHER MICRO-ORGANISMS

Research on the interactions between PGPR and other soil microbes has been mainly focused on their benefits for increasing yield of different plant crops. Soil is a complex environmental system, and the beneficial effects of PGPR interactions are often strain and plant dependant. However, the importance of these interactions is clearly seen by the increasing number of studies looking for synergism between PGPR with symbiotic organisms (rhizobia, mycorrhiza), and with other soil microorganisms and some constituents of the fauna.

4.1   PGPR and symbiotic organisms

4.1.1   PGPR and rhizobia

Symbiotic nitrogen fixation in legumes is accomplished by rhizobia inside root nodules. This process is dependant on the efficiency of the *Rhizobium* strain involved and on its competitiveness for nodulation against indigenous soil rhizobia, and is influenced by environmental factors. Increasing symbiotic nitrogen fixation is rational since legume crops are an important source of protein and are environmentally safe, avoiding the use of nitrogen fertilizers. Rhizobial strain selection and legume breeding are conventional approaches to improve this process and, more recently; molecular approaches have demonstrated their potential. The exploitation of

PGPR in combination with *Rhizobium* also constitutes an interesting alternative to improve nitrogen fixation.

Free-living diazotrophs, *Azotobacter* and *Azospirillum* increase nodulation and yield of several legume species such as soybean, winged bean, pea, chickpea, sulla clover, vetch, clover, alfalfa and *Macroptilium atropurpureum* after co-inoculation with their respective rhizobial symbionts (Singh and Subba Rao, 1979; Burns *et al.,* 1981; Iruthayathas *et al.,* 1983; Sarig *et al.,* 1986; Yahalom *et al.,* 1987). The mechanisms involved in the beneficial interaction *Azospirillum-Rhizobium* with clovers have received considerable world-wide attention. However, negative effects of *Azospirillum* on nodulation of clover have also been reported under artificial experimental conditions (agar plate assay) in the presence of some strains of *R. leguminosarum* bv. *trifolii* (Plazinski and Rolfe, 1985a). This inhibition occurs when the cell ratio of *Rhizobium*:*Azospirillum* is about 1:2000 or when *Azospirillum* is inoculated 24 h before or after the *Rhizobium*. From a series of subsequent experiments (Plazinski and Rolfe, 1985a; 1985b), it was concluded that *Azospirillum* could block the capacity of some rhizobial strains to produce root hairs curling (the first step of nodulation). In the case of increased nodulation, the significant increase in root hairs number and length in the presence of the *Rhizobium-Azospirillum* mixture suggested that *Azospirillum* can create additional infection sites, which can be occupied later by rhizobia. This hypothesis is strengthened by a further study using a Gus-reporter gene (Tchebotar *et al.,* 1998), in which an equal mixture of *Azospirillum lipoferum-R. leguminosarum* bv. *trifolii* increased nodulation in clovers, and *Azospirillum* was observed colonizing tap root, root hairs and sites near or on the nodules.

The ability of other PGPR species to improve nodulation is documented for many legume species. In general, enhanced nodulation allows higher nitrogenase activity resulting in superior dry matter yield. However, the results vary depending on the experimental system used. Under field conditions, nine PGPR strains of *Serratia proteamaculans*, *S. fonticola*, *P. fluorescens* and *P. putida*, tested individually or in combination with *R. leguminosarum*, increased emergence, vigor, nodulation, nitrogenase activity and root weight of lentil, but had no effect on pea. Laboratory studies showed that the two best strains in field studies gave similar results with lentil grown in pot and sand column systems, but not in Leonard jar or growth pouch systems (Chanway *et al.,* 1989). The potential for using fluorescent *Pseudomonas* and *Rhizobium* in pea production has been shown in field studies where there was a reduction in the number of *Fusarium oxysporum* infected peas grown in infested soils, and an improvement of plant growth in term of shoot height and dry weight. The strains used exhibited antifungal activity and produced siderophores (Kumar *et al.,* 2001). However, strains of *Pseudomonas putida* identified as plant

deleterious, produced extracellular metabolites regulated by iron that inhibit the growth of *R. leguminosarum* and have a negative impact on its chemotaxis, indicating that the initial pea root infection process could be disrupted (Berggren *et al.,* 2001). Other studies showed that antifungal rhizobacterial isolates of *Rahnella aquatilis* and *S. proteamaculans* increased the yield of pea and lentils in field soils, and they were selected for possible development of commercial inoculants (Leung *et al.,* 2003; 2004). Growth promotion mechanism on pea was investigated using 2,4-diacetylphloroglucinol (DAPG) producing *P. fluorescens* and its negative mutant (De Leij *et al.,* 2002). High concentrations of DAPG were found in pea rhizosphere, suggesting that DAPG can act as a plant hormone-like substance, inducing morphological changes in the plant that can lead to enhanced infection and nodulation by *Rhizobium*. A novel interaction between *Streptomyces lydius* WYEC108, known as a biocontrol agent and a siderophore producer, and the *Rhizobium*-pea symbiosis was shown to enhance overall growth of the plant (Tokala *et al.,* 2002). Root and nodule colonization by this streptomycete is probably one of the mechanisms that promote nodule number and growth, and improve bacteroids vigor by favoring iron assimilation.

The presence of PGPR can influence the ability of rhizobia to compete with indigenous populations for nodulation. This was demonstrated with green gram (*Vigna radiata*) grown in a non-sterile soil, in which two strains of *Enterobacter* co-inoculated with two strains of *Bradyrhizobium* sp. (*Vigna*) did increase nodule occupancy of the two rhizobial strains. *Bradyrhizobium* sp. strain S24 occupied 60% of nodules in single inoculation and this value was increased to 81% in the presence of *Enterobacter* strain EG-ER-1. The other *Enterobacter* isolate (KG-ER-1) increased nodule occupancy of *Bradyrhizobium* strain Cog15 from 77 to 88% (Gupta *et al.,* 1998). However, it seems that PGPR strains have no effect on the *in vitro* growth of *Bradyrhizobium*, as demonstrated by the same authors using 10 *Bradyrhizobium* strains co-inoculated with 14 PGPR strains, including the same *Enterobacte*r strains (Gupta *et al.,* 2003). Five *Bacillus* spp. strains and two *Enterobacter* strains increased yield of green gram, while nodulation and nitrogen fixation (acetylene reduction activity, ARA) were increased only in combination with *Bradyrhizobium* strain cog15. In a field study, a consortium of three PGPR inoculated to cowpea resulted in a better nodulation and nitrogen fixation than what was observed using *Bradyrhizobium* sp. (*Vigna*) alone. However, dual inoculation with *Bradyrhizobium* sp. and the PGPR consortium improved all growth parameters (Gulati *et al.,* 2001).

In a study with *B. japonicum*, 18 root colonizing bacteria belonging to the genera *Pseudomonas* and *Aeromonas* spp. did not interfere with the nodulation capacity of soybean, but three of these strains increased nodule

numbers and others enhanced plant growth (Polonenko *et al.,* 1987). Similar strain dependent effects have also been reported in a study where co-inoculation with *P. fluorescens* 2137 increased the colonization of *B. japonicum* on soybean roots, nodule numbers and ARA while coinoculation with *P. fluorescens* WCS365 had the opposite effects (Chebotar *et al.,* 2001). The same study also suggests that the high root colonization of *P. fluorescens* 2137 could enhance nodulation by the release of growth-promoting substances that stimulate *B. japonicum*. Lian *et al.* (2001) observed that a strain of *Bacillus circulans* produces a chemical compound analog to the nod factor of *B. japonicum*. This compound causes root hair deformation activity on soybean.

PGPR can also overcome the inhibitory effect of low temperature on the *B. japonicum*-soybean symbiosis. It was shown that application of the PGPR strains *S. protamacluans* 1-102 or *S. liquefaciens* 2-68 co-inoculated with *Bradyrhizobium* allowed a better plant growth, higher nitrogen fixation and nodule numbers at root zone temperatures of 15°C and 25°C (Zhang *et al.,* 1996; 1997). This was reflected in field studies where these PGPR accelerated nodulation and nitrogen fixation under short growing seasons (Dashti *et al.,* 1998). The optimal co-inoculation dose is 1×10^8 cells per soya seedling, for both PGPR strains (Bai *et al.,* 2002a). The combination of these PGPR with genistein flavonoid responsible for the induction of nodulation genes, did not cause additional improvement in nodulation and nitrogen fixation in field studies (Pan *et al.,* 2002), although some combined treatments of PGPR plus rhizobia preincubated with genistein stimulated growth under certain low root temperatures (Dashti *et al.,* 2000). However, an inducible activator, possibly an LCO (lipo-chitooligosaccharide) analogue to the rhizobial signal to legumes stimulating nodule formation, could be responsible for the growth-promoting activity of strain 1-102 (Bai *et al.,* 2002b). In another study of co-inoculation with *B. japonicum*, two strains *of Bacillus subtilis* (NEB4 and NEB5) and a strain of *B. thuringiensis* (NEB17), isolated from nodules of field-grown soybean plants, enhanced soybean plant growth in greenhouse and field experiments (Bai *et al.,* 2003). Strain NE-B17 is the most suitable for use in soybean production systems because it provided the highest nodule number and weight, and shoots and roots dry weight.

Stimulation of nodulation and plant growth has also been reported for chickpea (*Cicer arietinum*) using *Pseudomonas* strains that are antagonistic to fungal pathogens (*Aspergillus* sp*., Fusarium oxysporum, Pythium aphanidrematum* and *Rhizoctonia solani*) in co-inoculation with *Mesorhizobium* (Goel *et al.,* 2000). This resulted in the formation of 68 to 115% more nodules, compared to single inoculation with *Mesorhizobium*. The beneficial effect on plant shoot dry mass was more pronounced with HCN-producing *Pseudomonas* strain (Goel *et al.,* 2002).

Synergism between *Rhizobium,* PGPR and phosphate solubilizing bacteria (PSB) is also advantageous for legume crops, as observed earlier with chickpea (Alagawadi and Gaur, 1988). Dual inoculation of *Rhizobium* and *P. striata* or *B. polymyxa* (PSB) increased plant growth parameters, nodulation, nitrogenase activity, and N and P uptake. PSB also increased the available P content of the soil. The possibility of producing a common inoculant containing a mixture of a PGPR (*Pseudomonas* KB-133), a PSB (*B. megatherium*) and a *Rhizobium* sp. strain (COC 10) efficient for blackgram nodulation and yield, has been recently demonstrated (Prasad and Chandra, 2003;Gunasekaran *et al.,* 2004).

Finally, inoculation modes of PGPR and rhizobia may result in variable effects on legume growth, and this may depend on the phase of the process modified by PGPR: infection, nodulation or nitrogen fixation. This was concluded from results showing that PGPR strains (*P. fluorescens*, *Chryseobacterium balusim* and *Serratia fonticola*) and S*inorhizobium fredii* gave the most significant increases on plant growth yield when they were inoculated at different times (PGPR or *S. fredii* first). Co-inoculation had no effect, probably due to competition between the PGPR and *S. fredii* (Lucas Garcia *et al.,* 2004)

4.1.2   PGPR-mycorrhizae interaction

It is widely reported that mycorrhizal symbiosis influences growth, water and nutrient absorption of plants, and protects them from root diseases. The AM fungi are important because they are associated to about 80% of plant species. They reside as spores, hyphae and propagules, and the extraradical hyphae act as a bridge between soil and roots. Plant root colonization proceeds with the growth of intraradical hyphae and with the formation of arbuscules located in cortical cells. It is now clear that development of endo or ectomycorrhizae cause rhizosphere microbial changes which can result in interactions among rhizosphere microbes (Bianciotto and Bonfante, 2002). For example, AM fungal endosymbiotic bacteria have been reported, but their functional significance is not clear, indicating the complexity of the mycorrhizal interactions with bacteria (Bianciotto and Bonfante, 2002).

Interactions of AM fungi with other soil organisms have been described with regards to their effect on mycorrhizal development and functioning. Some interactions such as grazing of the external mycelium by soil organisms are detrimental, while other including PGPR can promote mycorrhizal functioning (Hodge, 2000). Rhizobacteria showing a beneficial effect on mycorrhizae are often referred to as "mycorrhizae-helper microorganisms". Linderman and Paulitz (1990) reviewed the interactions between mycorrhizae and groups of bacteria such as nitrogen-fixing bacteria,

PGPR including phosphate-solubilizing bacteria and biocontrol agents. Bacteria associated to mycorrhizal fungi adhere to fungal spores and hyphal structures and thus spread to the rhizosphere (Bianciotto and Bonfante, 2002). Recently, Bianciotto *et al.* (2004) observed strong evidence of a vertical transmission of endobacteria through the AM fungus vegetative generation. However, antagonistic effects are often reported in the AM fungi-PGPR interactions. Positive interactions often result in plant growth improvement.

Inoculation with both free living nitrogen fixing bacteria such as *Azospirillum brasilense* or *Azotobacter* and with AM fungi increase plant productivity. The nitrogen-fixing bacteria stimulate root colonization by AM fungi and increase their number of internal vesicles; they also alter rhizosphere rhizobial populations (Linderman and Paulitz, 1990). It is not clear whether the enhancement of plant growth is due to free nitrogen fixation or to the production of plant-growth promoting substances. On the other hand, a study estimated that associative nitrogen fixation by *Bacillus* could contribute in part to the growth promotion effect observed with *Pinus contorta* inoculated with the mycorrhizal fungus *Wilcoxina mikolae* (Chanway and Holl, 1991).

Some studies considered free-nitrogen fixers like other PGPR species, without reference to nitrogen fixation activity. For instance, in a study using the nitrogen-fixer *A. chroococcum* and *P. fluorescens*, the chemotaxis of these two PGPR towards roots of mycorrhizal tomato plants (*Glomus fasciculatum*) was an important step of communication for root colonization (Sood, 2003). It was found that *G. fasciculatum* alters the characteristics of root exudates which are chemoattractants specific for each PGPR, amino acids for *P. fluorescens* and sugars for *A. chroococcum*. In dual inoculation with *Glomus mosseae*, *B. coagulans* was superior to *A. chroococcum* in enhancing plant biomass of *Simarouba glauca* (Sailo and Bagyaraj, 2003). Different combinations between three PGPR species (*A. chroococcum*, *Azospirillum brasilense* and *Burkholderia cepacia*) and two AM fungi (*Glomus clarum* and *G. fasciculatum*) did not show the same trends on root colonization or on the nutritional status of onion and tomato, the highest mycorrhizal colonization was achieved by *Azospirillum brasilense* co-inoculated with each AM species on tomato and by single inoculation with *G. fasciculatum* on onion (Pulido *et al.,*2003). Finally, mycorrhization of wheat and maize was not affected by different *Azospirillum* species or by a genetically modified derivative of *A. brasilense* overproducing indole-3-acetic acid, indicating again variations in PGPR-AM fungi interactions (Russo *et al.,* 2005). On the contrary, a biofertilizer containing a mixture of N-fixer (*A. chroococcum*), P solubilizer (*B. megaterium*) and K solubilizer (*B. mucilaginous*) and AM fungus (*G. mosseae or G. intraradices*) increased growth and nutrient uptake of maize,

enhanced root colonization by the AM fungus and improved soil properties such as organic matter content and total N (Wu *et al.,* 2005)

The effect of PGPR strains (*Pseudomonas cepacia, P. aeruginosa, P. fluorescens* and *P. putida*) on growth and interactions of spring wheat with AM fungi in field studies varied with the PGPR strain used. Wheat harvest index was increased by pseudomonads and root biomass was reduced by one PGPR strain while two others increased root dry weight in the 15 cm zone (Germida and Walley, 1997). More evidence of positive interactions between AM fungi and PGPR on wheat has been shown in field experiments conducted in New Delhi, India. Different combinations from 11 PGPR and five AM fungi affected plant yield and weight and uptake of micro- and macro-nutrient, and these benefits allowed a reduction of fertilizer application by up to 50% (Singh and Adholeya, 2003).

The use of PGPR and AM mycorrhizae has been attempted with the aim of protecting plants against pathogens. The interactions of biocontrol PGPR with AM fungi are often contradictory and probably depend on the tested bacterium, the plant species and the environmental factors. In a study with wheat, some strains of *Pseudomonas* spp. and *Bacillus* spp. showed a better biocontrol effect against *Gaeumannomyces graminis* when applied alone than when used with soil inoculation with AM fungi (Ksiezniak *et al.,* 2001).

The combination PGPR and ectomycorrhizae have been studied for enhancing growth of tree seedlings in nurseries, but the effect of PGPR is either beneficial or detrimental for mycorrhization, depending on the study. For instance, in a study with Douglas-fir, dual inoculation with *P. fluorescens* strain BBc6R8 and the ectomycorrhiza *Laccaria bicolor* increased mycorrhizal colonization from 45 to 77% depending of the dose of bacterial and fungal inocula used (Frey-Klett *et al.,* 1999). Two years after inoculation, *Pseudomonas* cells could not be detected in the soil, but the height of the mycorrhizal Douglas-fir was increased, even by the lowest bacterial dose used. When co-inoculated, *L. bicolor* and *P. fluorescens* strain BBc6 significantly inhibited mycorrhizal development in *Eucalyptus diversicolor* (Dunstan *et al.,* 1998). However, in the same study, a PGPR effect was observed with an unidentified bacterium, allowing 49% more shoot dry weight than the uninoculated control. Studies with *Bacillus* species showing reduction in mycorrhizal colonization of loblolly pine suggest high metabolic costs of mycorrhizal maintenance in the presence of some rhizobacteria (Vonderwell and Enebak, 2000). This is also confirmed in a greenhouse study with pine*,* where both *B. licheniformis* CECT 5106 and *B. pumilus* CECT 5105 promoted growth of *Pinus pinea* without the synergistic effect of mycorrhizal inoculation with *Pisolithus tinctorius* (Probanza *et al.,* 2001). The absence of a synergistic effect of the same two *Bacillus* strains

combined to *P. tinctorius* was also observed with oak (Domenech *et al.,* 2004).

Antagonistic or synergistic interactions reported above may be related to physical and chemical interactions between AM fungi and PGPR. First, the degree of attachment to spores and hyphae of AM fungi depends on the PGPR strain, and it was suggested that extracellular soluble factors (bacterial material) produced around the attached bacteria may mediate bacterial-fungal interactions, and that AM fungi are vehicles for the colonization of plant roots by rhizobacteria (Bianciotto *et al.,* 1996). Secondly, the chemotaxis of PGPR towards AM mycorrhizal roots could be an important step of communication between these microorganisms for root colonization and could depend on mycorrhizal root exudates which are chemoattractants for PGPR (Sood, 2003).

4.1.3 Interactive effects of PGPR with AM fungi and *Rhizobium*-legume symbioses

The possibility of optimizing plant growth by managing interactions between AM fungi, PGPR and the *Rhizobium*-legume symbiosis has been considered as a promising avenue and synergism resulting from these interactions has been demonstrated earlier. For example, dual inoculation of the legume clover with AM fungi and PGPR resulted in higher shoot dry weight and nodulation than inoculation with mycorrhizae or PGPR alone (Meyer and Linderman, 1986). Some studies indicated that extracellular metabolites could be responsible for the synergism. In fact, the addition of PGPR cell-free culture filtrate to the mycorrhizal and nodulated legume *Hedysarum coronarium* resulted in maximum plant growth and nutrient uptake in comparison to PGPR washed cells or the whole bacterial cultures (Azcòn, 1993). However, in other experiments with beans (*Phaseolus vulgaris*), bacterial culture of fluorescent *Pseudomonas* co-inoculated with *Glomus etunicatum* increased root growth, nodulation and N and P uptake (Silveira *et al.,* 1995).

Selecting PGPR and AM fungi from polluted soils has been shown to be a valuable ecological approach to promote effective *Rhizobium*-legume symbiosis in these soils. In an experiment with clover growing in soil contaminated with Cd, an indigenous AM fungus plus the indigenous PGPR *Brevibacillus* enhanced shoot biomass from 18% (at 13.6 mg Cd kg^{-1}soil) to 35% (at 85.1 mg Cd kg^{-1}soil) and nutrition (N, P, Zn and Ni content) and reduced Cd transfer from soil to plants by up to 37.5%. There was also a strong positive effect of *Brevibacillus* sp. on nodule formation (Vivas *et al.,* 2003a). The same tendency was observed in Pb contaminated soils, where co-inoculation with an indigenous PGPR strain, identified as *Brevibacillus*, and a mixture of AM fungal indigenous species, could enhance plant growth,

mycorrhizal infection, nitrogen and phosphorus content (Vivas *et al.,* 2003 b). There was also a decrease in the amount of Pb absorbed in clover, probably due to the increased root biomass resulting from the production of IAA by the PGPR strain. Thus, autochthonous microorganisms applied as inocula are important for plant tolerance and growth in polluted soils.

The use of isotopic dilution techniques (^{15}N and ^{32}P) have been found useful to evaluate the interactive effects of microbes (*Rhizobium*, mycorrhizal fungi, phosphate-solubilizing bacteria) and rock phosphate fertilizer on N and P uptake by *Medicago sativa* (Toro *et al.,* 1998). The mixed microbial inoculation treatments used more P from the labile fraction in soils than from rock phosphate, but the total plant P uptake was far higher in AM mycorrhizal plants. *Enterobacter* inoculation seems to improve the use of rock phosphate in the rhizosphere of non-mycorrhizal plants. There was enhanced N fixation rates in plants inoculated with *Rhizobium* and AM fungi compared to rates achieved by *Rhizobium* alone.

4.2 PGPR vs. other microbes: mediated biocontrol and induced systemic resistance

The effect of the introduction of PGPR on rhizosphere community has not been intensively studied, since many experiments have been performed under gnotobiotic or greenhouse conditions. However a recent study strongly indicates that increases in plant growth can be attributed to changes in the rhizosphere microbial community due to the presence of the inoculated PGPR in soils (Ramos *et al.,* 2002). This study showed that the PGPR *B. licheniformis* improved European alder growth and induced different changes in phospholipids profile and culturable bacteria according to the soil used.

Most studies on PGPR interactions with other soil microorganisms and with soil fauna have been focused on biocontrol or induced systemic resistance against fungal, bacterial and viral diseases and against insect and nematode pests. A recent review on the induction of systemic resistance by PGPR in crop plants underlines the potential of *Pseudomonas* species for commercial exploitation and the potential of developing mixed inoculants against various pathogens attacking the same crop (Ramamoorthy *et al.,* 2001). PGPR cause plant cell wall modifications and physiological changes that lead to the synthesis of compounds involved in plant defense mechanisms. Lipopolysaccharides, siderophores and salicylic acid are major determinants of PGPR that mediate induced systemic resistance.

4.3   PGPR vs. soil fauna

The interactions between plant roots, microorganisms and animals play a determinant role in nutrient cycling and in the availability of mineral nutrients to plants. The process of "the microbial loop" in soil is initiated by the release of root exudates that increase microbial biomass. Nutrients sequestered during microbial growth are re-mobilized for plant uptake due to the microbial consumption by soil fauna (Griffiths, 1994). Protozoa and nematodes are very important in this process, representing 70 and 15% respectively of total respiration of soil animals (Sohlenius, 1980; Foissner, 1987). Protozoa and saprozoic nematodes show indirect plant growth promoting effect, mainly due to their important contribution in N mineralization (Griffiths, 1994). It is thus important to increase knowledge of their interactions with rhizobacteria, especially with the PGPR, to fully understand and manage soil living organisms for optimizing plant growth.

4.3.1   PGPR-protozoa interactions

Interactions between protozoa and rhizobacteria in the rhizosphere are well-known to increase plant growth through the mechanism identified as "the microbial loop in soil" (Bonkowski, 2003). The beneficial effect of protozoa on plant growth is not only due to nutrients released from consumed bacterial biomass, but also by their effects on root architecture and the resulting change of the composition of microbial communities in the rhizosphere. This effect is similar to a "plant-growth-promoting" or "hormonal" effect (Bonkowski, 2002). In experiments with watercress in the presence of *Acanthamoeabae* (Protozoa: Amoebida) , the root system was greater and more branched and there was an increase in the proportion of IAA producing rhizosphere bacteria, further indicating hormonal effect on plant growth (Bonkowski and Brandt, 2002). IAA did not originate from amoebal metabolism, but resulted from the changes in the composition and activity of microbes. It is likely that hormone production is stimulated by selective amoebal grazing of rhizosphere bacteria and thereby favoring certain bacteria capable of promoting plant growth by producing hormones.

4.3.2   PGPR-nematodes interactions

The PGPR-nematodes interactions have been extensively studied with the aim to manage plant-parasitic nematodes. These studies involve the selection of bacteria that can be used as biocontrol agents against nematodes. The genera involved include *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pseudomonas*, *Serratia* and *Streptomyces* (Siddiqui and Mahmood, 1999).

In the last few years, other bacterial species have shown biocontrol potential against nematodes. Bacteria isolated from the root of nematicidal plants, and identified as *Stenotrophomonas maltophilia*, *Bacillus mycoides* and *Pseudomonas* sp. reduced *Trichodorid* nematodes density on potato by 56% to 74%. These bacteria were characterized for production of hydrolytic enzymes, HCN, phenol oxidation and antifungal activity (Insunza *et al.,* 2002). *Rhizobium etli* has been reported to have a biocontrol effect against the nematode *Meloidogyne incognita* and showed the capacity to colonize plant roots and nematode galls (Hallmann *et al.,* 2001). *Azotobacter*, *Azospirillum*, *Rhizobium* sp. and the mycorrhiza *Glomus* have been reported to reduce galling and nematode *Meloidogyne javanica* infesting chickpea (Siddiqui and Mahmood, 2001).

Nematodes influence the colonization of roots by pathogenic and beneficial organisms, but little is known on the interactions with their natural antagonists in the rhizosphere (Kerry, 2000). Based on phylogenetic studies, it was proposed that the origin of parasitism in the root-knot nematode *Meloidogyne* spp. may have been facilitated through horizontal gene transfer from soil bacteria. Root-knot nematodes and rhizobacteria occupy similar niches in the soil and roots, suggesting the possibility for genetic exchange (Bird *et al.,* 2003).

Non parasitic nematodes can also play an important role in the colonization of the rhizosphere by PGPR in the absence of percolating water. Three species of nematodes (*Caenorhabditis elegans*, *Acrobeloides thornei* and *Cruznema* sp.) promote rhizosphere colonization of four strains of beneficial bacteria in sand-based microcosm system. Nematodes should be considered as important vectors for bacterial rhizosphere colonization (Knox *et al.,* 2003).

5   CONCLUSION

There is overwhelming evidence in the literature indicating that PGPR can be a true success story in sustainable agriculture. In fact, through their numerous direct or indirect mechanisms of action, PGPR can allow significant reduction in the use of pesticides and chemical fertilizers. These beneficial events producing biological control of diseases and pests, plant growth promotion, increases in crops yield and quality improvement, can take place simultaneously or sequentially. Plant age and the soil chemical, physical and biological properties will greatly influence the outcome of PGPR inoculation. Presently, the absence of a universal magic PGPR bioinoculant formulation for each important field crop, simply reflects the complexity of the interactions and of the molecular signal exchanges taking place in the soil-plant-organisms ecosystems. There are in the literature

several examples of important synergism observed on plant growth when the inoculants used contain a mixture of organisms. To develop future beneficial inoculants for field grown crops, one approach should consider performing inoculation assays with a consortium containing a mixture of soil organisms instead of a single strain. A consortium could contain a mixture of PGPR stimulating plant growth at different growth stages, and showing one or more of the known PGPR mechanisms of action. It could also contain beneficial symbiotic organisms like AM fungi, rhizobia and mycorrhizae helper bacteria. Finally this consortium will probably contain some beneficial protozoa and nematodes as well.

Another valuable approach could be the exploitation of single microbes in which the mechanism of action is well understood and the environmental conditions showing significant beneficial plant growth effects are well defined. Many examples in literature showed that the same strain of PGPR can be effective with different plant species and in different soil types and regions. Inoculants containing one micro-organism could be easier to produce and commercial formulation more stable ensuring better cell viability.

Single or consortium inoculants will have to be developed by taking into account the soil of the region and the general crop management systems used. PGPR inoculants will have to be compatible with the agrochemicals as well as the soil organic amendments used, and their development will also have to take carefully into account the long term crop rotation systems.

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