PGPR: Biocontrol and Biofertilization

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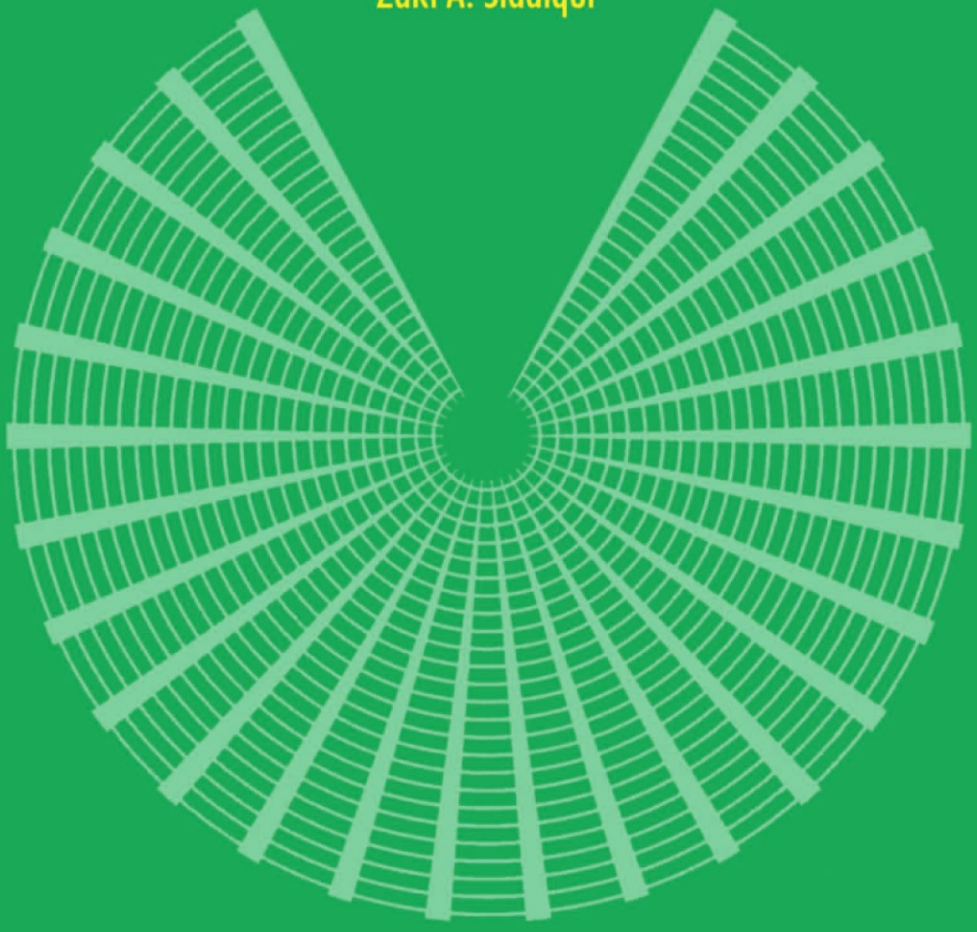
Zaki A. Siddiqui



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Springer

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-4002-4 (HB)
ISBN-13 978-1-4020-4002-3 (HB)
ISBN-10 1-4020-4152-7 (e-book)
ISBN-13 978-1-4020-4152-5 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springeronline.com

Printed on acid-free paper

Printed in the Netherlands.

Dedicated
to
(Late) Prof. Abrar Mustafa Khan
(Professor Emeritus)
and
(Late) Prof. Syed Israr Husain
(My Research Supervisor)

Contents

Preface

Plant growth promoting rhizobacteria (PGPR) are indigenous to soil and plant rhizosphere. These microorganisms are the potential tools for sustainable agriculture. They enhance the growth of a root system and even of an entire plant and often control certain plant pathogens. It is a fascinating subject, multidisciplinary in nature, and concerns scientists involved in plant heath and plant protection. There have been marked advances in this field during the last few decades. This area has been the subject of several reviews, but there is no exclusive text on the subject. This book stresses the need to document the information, developing a unifying theme which treated PGPR in a holistic manner. It deals with biocontrol of plant diseases by PGPR and their role in plant growth promotion, biofertilization and phytohormone production. Since PGPR are the centre of the theme, the book limits itself to the use of PGPR in biocontrol, biofertilization, phytohormone production and their formulations.

The book has eleven chapters and attempts to present balanced information on various aspects of PGPR. Chapter 1 describes the mechanisms of action of different PGPR groups. Physical, chemical and biological factors which affect colonization and the interactions of PGPR with other soil microorganisms and their ecology are dealt in detail. Other chapters deal with PGPR mediated induced resistance, and the biosynthesis of antibiotics by PGPR and role of PGPR in biocontrol of plant pathogens and biofertilization. PGPR action is also considered in phytohormone production and as a potential alternative of plant productivity. Chapter on visualization of interactions of pathogens and biocontrol agents on plant roots using autofluorescent protein markers has provided better understanding of biocontrol process. Proteomics perspective on biocontrol and plant defence mechanism has a separate chapter. An independent chapter has been devoted to formulations of PGPR. Current and future prospects of biocontrol of plant diseases by genetically modified microorganisms are discussed in the last chapter.

The book is not an encyclopedic review. However, an international emphasis has been placed on trends and probable future developments. The chapters incorporate both theoretical and practical aspects, and may serve as base line information for future research through which significant developments can be expected. This book will be useful to students, teachers and researchers, both in universities and research institutes, especially working in areas of agricultural microbiology, plant pathology, and agronomy.

With great pleasure, I extend my sincere thanks to all the contributors for their timely response, excellent and up to date contribution

and consistent support and cooperation. My gratitude to late Prof. Abrar Mustafa Khan, well known Plant Pathologist of India is immense. He along with his students established the section on Plant Pathology in the Department of Botany at Aligarh Muslim University, Aligarh, India. I also express my deep sense of gratitude to late Professor Syed Israr Husain. Prof. Husain was my research supervisor and a student of Prof. Abrar M. Khan. He initiated me into this discipline and was a great source of inspiration to me. I am also thankful to Dr. W. G. Dilantha Fernando, Department of Plant Science, University of Manitoba, Canada, for his encouragement and help during this project. I acknowledge with thanks the valuable assistance from my teachers, friends, well wishers and students. Special thanks are extended to Professors Ainul Haq Khan, Aqil Ahmad, R. P. Singh, Department of Botany, A.M.U. Aligarh, John Robert Pichtell, Ball State University, USA, and also to Drs. Mashiat Ullah Siddiqui, Department of Biochemistry, J.N.M.C, Syed Mashhood Ali, Department of Chemistry, Shamsul Hayat and Lamabam Peter Singh, Department of Botany, A.M.U. Aligarh, India for their encouragement, courtesy and help as this book progressed.

I am also thankful to Department of Science and Technology, Government of India, New Delhi for granting me a research project on PGPR to extend research in the field.

I am extremely thankful to Springer, Dordrecht, The Netherlands for completing the review process expeditiously to grant acceptance for publication. Subsequent cooperation and understanding of its staff, especially of Maryse Walsh and Deignan Margaret is also thankfully acknowledged.

I express sincere thanks to family members, particularly my wife Talat, daughter Zara, son Arsalan, brothers Rafi, Wasi , Atif and Tauqeer, as also to father and uncle for all the support they provided, and regret the neglect and loss they suffered during the preparation of this book.

Finally, I must be gracious to Almighty God who helped me develop and complete a book on **PGPR: Biocontrol and Biofertilization**.

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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

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 1–38.

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 (37-42^{0}C). *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™ 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 X 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.

ACKNOWLEGEMENTS: Many thanks to all the persons who contributed to the improvement of this chapter, for their valuable comments, suggestions and discussions. H. Antoun would like to thank the Natural Sciences and Engineering Research Council of Canada for financial support.

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Chapter 2

INDUCED SYSTEMIC RESISTANCE AS A MECHANISM OF DISEASE SUPPRESSION BY RHIZOBACTERIA

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Abstract: Plant growth-promoting rhizobacteria can suppress diseases through antagonism between the bacteria and soil-borne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens. The generally non-specific character of induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens may be present. Specific *Pseudomonas* strains induce systemic resistance in e.g. carnation, cucumber, radish, tobacco and *Arabidopsis*, as evidenced by an enhanced defensive capacity upon challenge inoculation. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity, indicating specific recognition between bacteria and plants at the root surface. In carnation, radish and *Arabidopsis*, the O-antigenic side chain of the bacterial outer membrane lipopolysaccharide acts as an inducing determinant, but other bacterial traits are also involved. Pseudobactin siderophores have been implicated in the induction of resistance in tobacco and *Arabidopsis*, and another siderophore, pseudomonine, may explain induction of resistance associated with salicylic acid (SA) in radish. Although SA induces phenotypically similar systemic acquired resistance (SAR), it is not necessary for the systemic resistance induced by most rhizobacterial strains. Instead, rhizobacteria-mediated induced systemic resistance (ISR) is dependent on jasmonic acid (JA) and ethylene signaling in the plant. Upon challenge inoculation of induced *Arabidopsis* plants with a pathogen, leaves expressing SAR exhibit a primed expression of SA-, but not JA/ethylene-responsive defense-related genes, whereas leaves expressing ISR are primed to express JA/ethylene-, but not SA-responsive genes. Combination of ISR and SAR can increase protection against pathogens that are resisted through both pathways, as well as extend protection to a broader spectrum of pathogens than ISR or SAR alone.

Key words: disease suppression; ISR; PGPR; SAR.

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 39–66.

1 INTRODUCTION

Plant roots release substantial amounts of carbon- and nitrogen-containing compounds into the surrounding soil. Microorganisms are attracted to this nutritious environment and use the plant exudates and lysates for growth and multiplication on the root surface and in the adjacent rhizosphere soil (Lynch and Whipps, 1991). Because of the rapid consumption of the nutrients, bacterial growth in the rhizosphere remains nutrient-limited, and roots are seldom colonized for more than about 15% of their surface area. Nevertheless, the rhizosphere microflora plays a very important role in plant development and acclimation to environmental stresses (Van Loon and Glick, 2004). Harmful microorganisms, i.e. pathogenic fungi and bacteria, are damaging to the plant, whereas beneficial microorganisms, such as mycorrhizal fungi and many bacterial species, can protect the plant against adverse abiotic and biotic stresses. Since the rhizosphere microflora is extremely diverse, a dynamic interplay between the members of the microbial community occurs, mediated by synergistic and antagonistic interactions, within the limits of the nutrients available (Garbeva *et al.,* 2004). In addition, signals are being exchanged between fungi and bacteria and plant roots, effectively forming a highly dynamic belowground communication network (Van Loon and Bakker, 2003; Somers *et al*., 2004). Although dependent on plant age and soil conditions, the microbial-plant network is maintained for the lifetime of the root and exerts a buffering action on the rhizosphere environment.

Growing roots penetrate new soil layers in which the network is not yet established. This makes growing roots vulnerable to attack by pathogens and insects. For instance, the vascular wilt pathogen *Fusarium oxysporum* tends to penetrate growing plant roots preferentially through the fully undifferentiated tip, after which it establishes itself in the xylem vessels without interference from antagonistic microorganisms (Turlier *et al.,* 1994). However, when growing saprophytically towards the elongating root, *Fusarium* is sensitive to antagonistic actions by other soil microorganisms. Depending on the strategy of a given soil-borne fungal pathogen, competing microorganisms may be more or less effective in counteracting pathogen survival, spore germination, hyphal growth or tissue penetration and colonization (Whipps, 1997; Weller *et al*., 2002). Plants treated with specific rhizosphere microorganisms, notably of certain genera of non-pathogenic bacteria, show improved growth. Hence, these rhizobacteria have been denoted as plant growth-promoting rhizobacteria (PGPR) (Kloepper *et al.,* 1980). PGPR may promote plant growth directly through improving uptake of minerals and water or the production of growth-stimulating compounds, but in many cases improved growth can be attributed to the suppression of deleterious microorganisms that are harmful to the plant (Schippers *et al.,*

1987; Glick *et al.,* 1999). PGPR can, thus, promote plant growth by suppressing diseases caused by various soil-borne pathogens (Van Loon and Glick, 2004).

There is a vast literature describing positive effects of specific strains of rhizobacteria on growth of many plant species in soils in which more or less defined pathogens cause substantial losses (Kloepper *et al.,* 1991). For a number of such strains, mechanisms of disease suppression have been defined. The use of bacterial genetics has allowed mutant analysis to prove that specific traits are responsible. For instance, Fusarium wilt in radish, caused by *Fusarium oxysporum* f. sp. *raphani*, is suppressed by the rhizobacterial strain *Pseudomonas putida* WCS358. A bacterial mutant impaired in the production of the fluorescent siderophore pseudobactin, no longer suppressed disease (Raaijmakers *et al.,* 1995). Using similar approaches, it has been established that effective antagonistic mechanisms can comprise competition for iron through the production of siderophores, production of antibiotics, and secretion of lytic enzymes (Handelsman and Stabb, 1996; Whipps, 2001; Van Loon and Bakker, 2003). In addition, release of non-specific volatile inhibitors, such as HCN, may hamper the activity of pathogenic microorganisms, but can also restrict plant growth (Schippers *et al.,* 1991).

When testing for antagonistic activity of *Pseudomonas fluorescens* strain WCS417 against *Fusarium oxysporum* f. sp. *dianthi* on carnation, it was found that bacteria, when remaining confined to the plant root system, were still protective when the pathogen was slash-inoculated into the stem (Van Peer *et al.,* 1991). Since in this case the rhizobacteria and the pathogenic fungus were never found to contact each other on the plant, the protective effect had to be plant-mediated. Similar observations were made when several strains of PGPR were applied to roots of cucumber, the leaves of which were subsequently challenge inoculated with the anthracnose fungus *Colletotrichum orbiculare* (Wei *et al.,* 1991). The phenomenon was named induced systemic resistance (ISR). Apparently, the inducing rhizobacteria triggered a reaction in the plant roots that gave rise to a signal that spread systemically throughout the plant and enhanced the defensive capacity of distant tissues to subsequent infection by the pathogens. ISR thus extended the protective action of PGPR from their antagonistic activity against soil-borne pathogens in the rhizosphere to a defense-stimulating effect in aboveground tissues against foliar pathogens. As it appeared, the enhanced defensive capacity was expressed in roots as well as in leaves, adding the mechanism of ISR to the list of traits that are effective against soil-borne pathogens (Leeman *et al.,* 1995b). However, ISR, in addition, can reduce damage from pathogens that are active exclusively on foliage, flowers or fruits (Wei *et al.,* 1991; Hoffland *et al.,* 1996). In the past decade, ISR has been established as a mechanism that is effective in bean, carnation,

cucumber, radish, tobacco, tomato, as well as in the model plant *Arabidopsis thaliana*, against different types of pathogens (Van Loon *et al.,* 1998; Ramamoorthy *et al.,* 2001; Kloepper *et al.,* 2004). In addition, ISR has been implicated in several other plant species in which plants, after being treated with specific rhizobacterial strains, showed improved growth in the presence of one or more pathogens.

2 THE MECHANISM OF RHIZOBACTERIA-INDUCED SYSTEMIC RESISTANCE

The generally non-specific character of induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens may be present (Van Loon, 2000). ISR appears phenotypically similar to systemic acquired resistance (SAR), which is the phenomenon that once a plant has been infected by a pathogen and been able to effectively resist it, it has become more resistant to subsequent challenge inoculation by the same and other pathogens and, in some instances, even insects (Sticher *et al.,* 1997; Van Loon *et al.,* 1998). SAR has been studied in detail since the 1960s as to its induction by pathogens, signal-transduction in the plant, and expression in response to challenge inoculation, with tobacco and *Arabidopsis* as model plant species (Ryals *et al*., 1996; Van Loon, 2000). Most effective induction is achieved when the plant reacts to primary infection by a hypersensitive reaction, but necrosis is not a prerequisite for SAR induction. Rather, pathogen elicitors may give rise to hypersensitive necrosis on the one hand, and to the generation of a signal for enhanced resistance on the other hand. Nevertheless, necrotization does contribute to the induction of SAR, and the more systemic signal is generated as tissue necrosis proceeds at a pace that the tissue has sufficient time to react before succumbing. Thus, infliction of rapid necrosis, e.g. by cutting or burning, does not generate sufficient signal to give rise to SAR, whereas damage leading to slowly developing necrotic specks, such as occurs as a result of ozone or heavy metal toxicity, does provoke the state of SAR. To understand the phenomenon of rhizobacteria-mediated ISR it is important to gain insight into the bacterial and plant mechanisms involved and to unravel the requirements for ISR induction, signaling, and expression.

2.1 Induction of ISR

Beneficial rhizobacteria do not obviously damage their host or cause localized necrosis. Therefore, the eliciting factor(s) produced by ISR-

triggering rhizobacteria must be different from elicitors of pathogens, which are defined as compounds that induce defense reactions in the host plant (Ebel and Mithöfer, 1998). There is comparatively little information on the bacterial determinants that trigger ISR (Bakker *et al.,* 2003). However, elicitation shows some similarities to the generation of certain non-specific defense reactions in plant cells that occur in response to general pathogen-associated molecular patterns (PAMPs): common components that are present in microorganisms and appear to be recognized by eukaryotic cells (Gómez-Gómez, 2004; Nürnberger *et al.,* 2004). Crude microbial cell wall preparations, i.e. dead cells, can act as general, non-specific elicitors and induce local or systemic resistance. Cell surface components, such as the outer membrane lipopolysaccharide (LPS) and flagella, can activate the innate immune response in animals and act as triggers of defense-associated reactions in suspension-cultured plant cells and leaves (Gómez-Gómez and Boller, 2002; Erbs and Newman, 2003). Indeed, both these factors of the rhizobacterial strain WCS358 can elicit ISR when applied as purified components to root systems of *Arabidopsis* plants (Bakker *et al.,* 2003; Meziane *et al.,* 2005). Upon challenge inoculation of treated plants with the causal agent of bacterial speck disease, the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* (Pst), the resulting chlorotic and necrotic symptoms on the plants were reduced to an extent comparable to that on plants grown in soil containing wild-type WCS358. Mutants of WCS358 that were defective in the O-antigenic side-chain (OA^-) of the LPS or lacked flagella (fla^-) could still induce systemic resistance, as expected because flagella, or intact LPS, respectively, were still present in these mutants.

Perception of the main constituent protein of bacterial flagella, flagellin, has been studied extensively in suspension-cultured cells of tomato and *Arabidopsis* (Felix *et al.,* 1999; Gómez-Gómez and Boller, 2000). In *Arabidopsis* flagellin is perceived through recognition of a conserved domain within the protein by a leucine-rich repeat – nucleotide binding site – containing receptor-like kinase with a structure similar to that of several major resistance (*R*) genes in plants. Signalling through a mitogen-activated protein (MAP) kinase cascade leads to WRKY transcription factor-mediated activation of defense-related genes and enhanced resistance against Pst and the fungus *Botrytis cinerea* (Asai *et al*., 2002; Zipfel *et al*., 2004). The conserved nature of the flagellin domain being recognized by the receptor would be expected to endow ISR-eliciting activity in *Arabidopsis* on most, if not all, non-pathogenic rhizobacteria. However, this does not appear to be the case. In contrast to WCS358, *Pseudomonas fluorescens* strain WCS374 does not elicit ISR in *Arabidopsis* (Van Wees *et al.,* 1997). Preliminary evidence suggests that flagella of both WCS358 and WCS374 are being recognized by plant suspension cells (L.C. van Loon, unpublished observation). Thus, elicitation of ISR must differ between strains and also

involve other factors. Indeed, induction by purified LPS is highly unlikely to occur through the same receptor kinase as recognizes flagellin.

A non-specific induction of ISR by rhizobacteria is also incompatible with an observed differential induction of systemic resistance in different plant species and, in some cases, even ecotypes (Van Wees *et al*., 1997; Ton *et al.,* 1999). Although some rhizobacterial strains appear to be equally effective in inducing systemic resistance in different plant species, others show narrow specificity, indicative of a plant species-specific recognition between bacteria and receptors on the root surface (Van Loon *et al.,* 1998). As shown in Table 1, of the three WCS strains mentioned earlier, WCS358 elicits ISR in *Arabidopsis* (Van Wees *et al.,* 1997), bean and tomato (Meziane *et al.,* 2005), but not in carnation (Duijff *et al.,* 1993) or radish (Leeman *et al.,* 1995a), WCS374 in radish (Leeman *et al.,* 1995a) but not in *Arabidopsis* (Van Wees *et al.,* 1997), and WCS417 in all five species (Van Peer *et al.,* 1991; Leeman *et al.,* 1995a; Van Wees *et al.,* 1997; Duijff *et al.,* 1998; Bigirimana and Höfte, 2002). Moreover, of ten *Arabidopsis* accessions tested, eight were responsive to WCS417; accessions RLD and Ws-0 were not (Ton *et al.,* 1999).

For a limited number of ISR-eliciting rhizobacterial strains the inducing determinant(s) have been identified through mutant analysis and application of isolated components (Table 2). WCS358 can elicit ISR in *Arabidopsis* not only through its flagella or LPS, but also through its pseudobactin siderophore (Bakker *et al.,* 2003; Meziane *et al.,* 2005). In bean and tomato ISR elicitation by WCS358 depends on both LPS and pseudobactin (Meziane *et al.,* 2005). Siderophores are also acting as an inducing determinant in *Pseudomonas aeruginosa* 7NSK2 (Audenaert *et al.,* 2002), *Pseudomonas fluorescens* CHA0 (Maurhofer *et al.,* 1994), and WCS374 (Leeman *et al.,* 1996), and have likewise been implicated in the elicitation of ISR by *Serratia marcescens* 90-166 on tobacco against the wildfire disease, caused by *Pseudomonas syringae* pv. *tabaci* (Press *et al.,* 1997).

From Table 2, it seems that 7NSK2 elicits ISR in tomato and in bean or tobacco through different determinants. However, this is not necessarily the case. In tomato, as in bean (De Meyer and Höfte, 1997) and tobacco (De Meyer *et al.,* 1999a), bacterially produced salicylic acid (SA) was at first considered to be the inducing factor. Further studies in tomato demonstrated that it is the combination of the siderophore pyochelin and the active oxygen species-generating antibiotic pyocyanin that is responsible (Audenaert *et al*., 2002). Pyochelin contains a SA moiety and, thus, the bacteria need to produce SA for incorporation into pyochelin, making SA a contributing determinant. So far, it has not been clarified whether the involvement of SA in the elicitation of ISR in bean and tobacco is likewise linked to production of the pyochelin siderophore.

Table 1. Differential induction of systemic resistance by *Pseudomonas* spp. in different plant species.

Plant species	*P. putida* WCS358	*P. fluorescens* WCS374	*P. fluorescens* WCS417	References
Arabidopsis	+	-	+	Van Wees *et al*., 1997
Bean	+	nd	+	Bigirimana and Höfte, 2002; Meziane *et al*., 2005
Carnation	-	nd	+	Van Peer *et al*., 1991; Duijff *et al*., 1993
Radish	-	+	+	Leeman *et al*., 1995a
Tomato	+	nd	+	Duijff *et al*., 1998; Meziane *et al*., 2005

-: no induction; +: induction; nd: not determined.

The production of siderophores occurs only under iron-limited conditions. Such conditions are likely to prevail in the rhizosphere, and competition for iron through the production of siderophores is one of the mechanisms of bacterial antagonism against soil-borne pathogens. Thus, siderophore production by specific ISR-eliciting rhizobacteria can play a dual role in disease suppression by depriving resident pathogens from iron locally and by inducing resistance in the plant systemically. Whereas all bacterial siderophores are functional in sequestering iron, not all siderophores elicit ISR. This can be explained by the fact that siderophores produced by different bacteria have very different chemical structures (Höfte, 1993). How siderophores are perceived by plants is presently fully unknown.

Strains WCS374 and WCS417 appear to elicit ISR in radish through more than a single determinant, including an iron-regulated compound with properties resembling a siderophore different from pseudobactin (Leeman *et al.,* 1996). For WCS417, the compound is not known. For WCS374, it was established that this bacterium produces the additional siderophore pseudomonine under iron-limiting conditions (Mercado-Blanco *et al.,* 2001), but it is not clear yet in how far pseudomonine is involved in the elicitation of ISR by this strain in radish.

Antibiotics have also been implicated in ISR. Whereas in tomato, the pyocyanin-producing strain 7NSK2 elicits ISR through this antibiotic in conjunction with the pyochelin siderophore, 2,4-diacetylphloroglucinol (DAPG) has recently been shown to elicit ISR in *Arabidopsis*, establishing

this antibiotic as an inducing determinant of *P. fluorescens* strains CHA0 (Iavicoli *et al.,* 2003) and Q2-87 (Weller *et al.,* 2004). DAPG has likewise been shown to act as the inducing agent in CHA0-mediated ISR in tomato against the root-knot nematode *Meloidogyne javanica* (Siddiqui and Shaukat, 2003). These findings, that rhizobacterially produced DAPG can elicit ISR, suggest that more antibiotics may be capable of eliciting ISR in plants. As in the case of siderophores, antibiotics may thus be taken to play a role not only in microbial antagonism in the rhizosphere, but also in stimulating plant defensive capacity.

The LPS of the three WCS strains is recognized by all plants in which each of these strains was demonstrated to elicit ISR (Van Loon *et al*., 1998). Likewise, purified LPS from *Burkholderia cepacia* strain ASP B 2D has been shown to protect tobacco systemically against black shank disease, caused by *Phytophthora nicotianae* (Coventry and Dubery, 2001), whereas LPS of *Rhizobium etli* strain G12 is a determinant in the induction of systemic resistance in potato roots towards the cyst nematode *Globodera pallida* (Reitz *et al.,* 2002). LPS of different bacterial strains differs in the repeating oligosaccharide moieties of the O-antigenic side-chain, providing a plausible explanation why these LPSs show differential specificity in different plant species. Apparently, the requirements for perception by the plant differ between species. That only a few rhizobacterial strains have been demonstrated to elicit ISR through their LPS may be due to the situation that its involvement for other ISR-eliciting strains has not been investigated. Alternatively, in other strains the structure of the LPS may differ substantially from those of the inducing strains.

Very recently, it was found that certain bacilli, i.e. *Bacillus amyloliquefaciens* IN 937a and *Bacillus subtilis* GB03, can trigger ISR in *Arabidopsis* through a volatile compound, 2,3-butanediol (Ryu *et al.,* 2004). Probably, related compounds are also active. These results show that there are many compounds present on, or released by, rhizobacteria that can elicit ISR in various plant species. Since bacteria abound on the root surface, one might expect all plants in nature to become induced readily at a young stage and remain so as long as the rhizobacteria remain active. However, this does not seem to be the case. Non-bacterized plants growing in raw soil commonly develop more severe symptoms than bacterized plants upon challenge inoculation with a pathogen. For radish, it was established that a minimum of 10^5 colony-forming units per gram of root is required for ISR to be induced by WCS374 (Raaijmakers *et al*., 1995). This value appears to be typical and is seldom reached by any single strain amidst the diverse microbial population in the rhizosphere. Only through inundative applications densities can be increased to the level required for ISR to become established.

Table 2. Bacterial determinants of induced systemic resistance in different plant species.

Bacterial strain	Plant species	Determinant	Reference
B. amyloliquefaciens IN937a	*Arabidopsis*	2,3-butanediol	Ryu *et al.,* 2004
B. subtilis GB03	*Arabidopsis*	2,3-butanediol	Ryu *et al*., 2004
P. aeruginosa	Bean	Salicylic acid	De Meyer *et al*., 1999b
7NSK2	Tobacco	Salicylic acid	De Meyer *et al.,* 1999a
	Tomato	Pyochelin + Pyocyanin	Audenaert *et al.,* 2002
P. fluorescens	*Arabidopsis*	2,4-diacetylphloroglucinol	Iavicoli *et al.,* 2003
CHA0	Tobacco	Siderophore	Maurhofer *et al.,* 1994
	Tomato	2,4-diacetylphloroglucinol	Siddiqui and Shaukat, 2003
P. fluorescens Q2-87	*Arabidopsis*	2,4-diacetylphloroglucinol	Weller *et al*., 2004
P. fluorescens	Radish	Lipopolysaccharide,	Leeman *et al.,* 1995b
WCS374		siderophore,	Leeman *et al.,* 1996
		iron-regulated compound	Leeman *et al*., 1996
P. fluorescens	*Arabidopsis*	Lipopolysaccharide	Van Wees *et al*., 1997
WCS417	Carnation	Lipopolysaccharide	Van Peer and Schippers, 1992
	Radish	Lipopolysaccharide	Leeman *et al.,* 1995b
		iron-regulated compound	Leeman *et al*., 1996
P. putida WCS358	*Arabidopsis*	Lipopolysaccharide	Meziane *et al*., 2005
		siderophore,	Meziane *et al*., 2005
		flagella	Meziane *et al.,* 2005
	Bean	Lipopolysaccharide,	Meziane *et al*., 2005
		siderophore	Meziane *et al*., 2005
	Tomato	Lipopolysaccharide,	Meziane *et al*., 2005
		siderophore	Meziane *et al*., 2005
Rhizobium etli G12	Potato	Lipopolysaccharide	Reitz *et al.,* 2002
S. marcescens 90-166	Tobacco	Iron-regulated compound	Press *et al.,* 1997

2.2 Signalling in pathogen-induced systemic acquired resistance

Perception at the root surface is followed by signal transduction leading to the induced state. Whereas SAR is apparent as soon as disease symptoms are visible (Ross, 1961), the time needed for ISR to become established has not been the subject of extensive investigations. However, it appears that it can be reached in as short as one day (Leeman *et al.,* 1995a), but usually it is taken to require several days. Differences may reside in a

differing effectiveness of elicitation or speed with which the original perception is transduced, as well as in the nature and properties of the mobile signal that is needed to propagate the induced state systemically throughout the plant. Often, a period of one week between induction treatment and challenge inoculation is used, because over this period the extent of induced resistance tends to increase.

SAR is commonly taken to remain active for the lifetime of the plant, even though there is detailed evidence that the induced state becomes "diluted" in newly developing foliage (Bozarth and Ross, 1964). ISR can likewise be maintained for weeks, but the level of induced resistance decreases with time (Liu *et al.,* 1995). These observations indicate that elicitor perception and signal transduction confer on the plant an enhanced defensive capacity that is maintained for a considerable length of time. With a single exception (Roberts, 1983), induced resistance has never been shown to be transmissible through seed. Hence, the phenomenon is reminiscent of an epigenetic alteration that is more or less stably maintained. The "memory" effect is conceptually similar to vernalization of seeds or induction of flowering, which are likewise maintained after the initial inducing stimulus has disappeared.

In the case of SAR, mutant and transgenic plants have been instrumental in the identification of critical steps in the signal transduction pathway. The original hypothesis that a phenolic compound structurally resembling SA was required for the establishment of SAR (Van Loon and Antoniw, 1982) was borne out when SA was determined to be an endogenous compound in plants, increasing in amount upon elicitation in tobacco (Malamy *et al.,* 1990) and being transported through the phloem in induced cucumber plants (Métraux *et al.,* 1990). Its role as a key regulator was demonstrated by the use of transformed tobacco and *Arabidopsis* plants carrying the bacterial *NahG* gene (Gaffney *et al.,* 1993). The *NahG* gene encodes the enzyme salicylate hydroxylase, which converts SA into the non-inducing product, catechol. Plants expressing the *NahG* gene, when subjected to induction treatments, no longer express SAR, but can be "rescued" by treatment with SA-analogs, such as 2,6-dichloroisonicotinic acid or acibenzolar-S-methyl, that are not substrates for the SA-hydroxylase (Ryals *et al.,* 1996; Sticher *et al.,* 1997).

The presence of SA in phloem vessels suggested that SA is not only required for the establishment of induced resistance, but also responsible for the systemic nature of the induced resistance by acting as the mobile signal. When SA is applied as a soil drench, it is absorbed by plant roots and transported to other plant parts, where it induces resistance (Van Loon and Antoniw, 1982). However, this transport is likely to take place in the xylem and, thus, to differ from the behaviour of the endogenous SA produced in response to an eliciting pathogen or similar condition. Local application of

SA to leaves induces resistance locally but not necessarily systemically (Van Loon and Antoniw, 1982), even though some SA seems to be transported out of the induced leaf (Shulaev *et al.,* 1995). Indeed, the mobile signal was shown to pass through the petiole of an inducer-treated leaf before an increase in SA in the phloem could be detected (Rasmussen *et al.,* 1991). Moreover, grafting experiments demonstrated that an induction-treated NahG rootstock gives rise to full induced resistance in a wild-type scion, in spite of the fact that SA in the rootstock never accumulated (Vernooij *et al.,* 1994). These results cannot be explained by SA acting as the mobile signal for the systemic induction of SAR. This conclusion is strengthened by recent findings that as a result of a virus-induced hypersensitive reaction, transgenic ethylene-insensitive tobacco plants are fully capable of elevating SA levels and expressing induced resistance locally but, when used as rootstock, fail to transmit the mobile signal to a wild-type scion (Verberne *et al.,* 2003). Nowadays the favoured hypothesis is that upon induction, local SA levels are increased, associated with the generation of a mobile signal that is transported throughout the plant and, in turn, initiates further local SA production in distant leaves. This SA is necessary and sufficient to confer the systemically induced state (Durrant and Dong, 2004).

The trigger for increased SA production in the plant is not known, nor has it been established how SA exerts its resistance-inducing action. However, SA action requires the presence of the protein NPR1, an ankyrin-repeat family protein structurally resembling the inhibitor IF- B, which plays a role in animal innate immunity (Cao *et al.,* 1997). Under the influence of SA, a redox change causes oligomers of NPR1 in the cytoplasm to be reduced to monomers. The monomers are transported into the nucleus, where they interact with specific TGA transcription factors to allow the expression of genes encoding pathogenesis-related proteins (PRs) (Dong, 2004). Several PRs have been shown to have more or lesser anti-pathogen activities (Van Loon, 1997). These conclusions led to the hypothesis that the state of SAR relies on the presence of PRs. However, SA-induced PRs are not active against many pathogens that have been shown to be resisted through SAR, and additional mechanisms must be of major importance in restricting pathogen growth and disease development in SAR-induced plants. Nevertheless, the specific association of PRs with SAR makes PRs convenient molecular markers for this type of induced resistance (Kessmann *et al.,* 1994).

2.3 Signalling in rhizobacteria-induced systemic resistance

SAR has been taken as a paradigm for the systemic resistance induced by non-pathogenic rhizobacteria. However, signalling in ISR appears considerably more complex. Some rhizobacteria are capable of

producing SA and do so in vitro on minimal media in the absence of iron (Van Loon *et al.,* 1998). If these bacteria encounter similar conditions in the rhizosphere of plants, they would be expected to produce SA likewise and induce SAR, mimicking a soil drench with SA solution. However, the SA produced by the bacterium may be incorporated into a SA-containing siderophore, rather than being secreted into the rhizosphere. Under such conditions, induction of systemic resistance might occur depending on whether that siderophore could act as an inducing determinant or not, and on whether the bacterium triggers the SAR pathway or activates a different signalling route.

Several ISR-eliciting rhizobacterial strains have been described to be capable of producing SA, whereas others are not. To determine whether such strains trigger ISR through activation of the SA-dependent SAR pathway, two criteria can be used: the induced systemic resistance should be associated with the induction of PRs, and both ISR and the induction of PRs should be abolished in NahG plants. Induction of systemic resistance by various rhizobacterial strains in the available *NahG*-transformed tobacco, *Arabidopsis* and tomato demonstrated that ISR against tobacco mosaic virus (TMV) and *Botrytis cinerea* is abolished in tobacco and tomato plants, respectively, upon application of 7NSK2 (De Meyer *et al*., 1999a, Audenaert *et al.,* 2002) and in *Arabidopsis* against *P. syringae* pv. *maculicola* after elicitation by *B. pumilus* SE34 (Ryu *et al.,* 2003), whereas it is maintained in all other combinations tested (Table 3). Moreover, mutants of *S. marcescens* 90-166 that had lost the ability to produce SA still induced resistance in tobacco against *Pseudomonas syringae* pv. *tomato* and in cucumber against *Colletotrichum orbiculare* (Press *et al*., 1997).

The requirement of SA production for resistance induction by 7NSK2 was corroborated by the loss of ISR elicitation by bacterial mutant derivatives that were no longer capable of producing SA (De Meyer and Höfte, 1997; De Meyer *et al.,* 1999b; Audenaert *et al.,* 2002). However, upon colonization of tomato roots, SA is required for the production of the SA-containing siderophore pyochelin that elicits ISR in conjunction with the antibiotic pyocyanin. In tobacco, SA-dependent induction of systemic resistance by 7NSK2 was not accompanied by expression of the marker PR-protein, PR-1 (De Meyer *et al.,* 1999a). One explanation could be that tiny amounts of SA that are insufficient for inducing PRs, already suffice to elicit SA-dependent ISR. It is equally possible, however, that upon application of 7NSK2 to tobacco SA might be needed for the synthesis of pyochelin, and elicitation of ISR might involve pyochelin and occur by a SA-independent signalling route. The evidence that WCS358, which does not produce SA, elicits ISR in *Arabidopsis* (Van Wees *et al.,* 1997), and other rhizobacterial strains that can produce SA in vitro either do not elicit ISR [e.g. WCS374 on *Arabidopsis* (Van Wees *et al.,* 1997)], or elicit ISR in a SA-independent way

Table 3. Results of assays for induction of systemic resistance on NahG plants.

Bacterial strain	Plant species/Pathogen	ISR	Reference
B. amyloliquefaciens IN937a	*Arabidopsis*/*Erwinia carotovora*	+	Ryu *et al.*, 2004
B. pumilus SE34	*Arabidopsis*/*P. syringae* pv. *maculicola*	+	Ryu *et al*., 2003
	Arabidopsis/*P. syringae* pv. *tomato*	-	Ryu *et al.*, 2003
	Tobacco/*Peronospora tabacina*	+	Zhang *et al.*, 2002
B. pumilus T4	*Arabidopsis*/*P. syringae* pv. *maculicola*	+	Ryu *et al.*, 2003
	Arabidopsis/*P. syringae* pv. *tomato*	+	Ryu *et al*., 2003
B. subtilis GB03	Arabidopsis/*Erwinia carotovora*	+	Ryu *et al*., 2004
P. aeruginosa	Tobacco/tobacco mosaic virus	-	De Meyer *et al.*,1999a
7NSK2	Tomato/*Botrytis cinerea*	-	Audenaert *et al*., 2002
	Tomato/ *Meloidogyne javanica*	+	Siddiqui and Shaukat, 2004
P. chlororaphis 06	Tobacco/*P. syringae pv. tabaci*	+	Spencer *et al.*, 2003
	Tobacco/*Erwinia carotovora*	+	Spencer *et al.*, 2003
P. fluorescens	*Arabidopsis*/*Peronospora parasitica*	+	Iavicoli *et al.*, 2003
CHA0	Tomato/*Meloidogyne javanica*	+	Siddiqui and Shaukat, 2004
P. fluorescens WCS417	*Arabidopsis*/*P. syringae* pv. *tomato*	+	Pieterse *et al.*, 1996
P. fluorescens 89B61	*Arabidopsis*/*P. syringae* pv. *maculicola*	+	Ryu *et al*., 2003
	Arabidopsis/*P. syringae* pv. *tomato*	-	Ryu *et al*., 2003
	Tomato/*Phytophthora infestans*	+	Yan *et al*., 2002
Serratia marcescens	*Arabidopsis*/*P. syringae* pv. *maculicola*	+	Ryu *et al.*, 2003
90-166	*Arabidopsis*/*P. syringae* pv. *tomato*	-	Ryu *et al*., 2003
	Tobacco/*P. syringae* pv. *tabaci*	+	Press *et al*., 1997

[e.g. *Serratia marcescens* on tobacco (Press et al., 1997) or CHA0 on *Arabidopsis* (Iavicoli *et al*., 2003)] indicates that rhizobacterial production of SA is not generally required for induction of systemic resistance.

Systemically induced resistance in NahG plants rules out an involvement of plant-produced SA also. So far, *NahG* transformants are available in only a few plant species. In other species, an involvement of SA can only be assessed on the basis of systemic accumulation of SA-inducible PRs. Several ISR-eliciting strains were shown to activate the *PR-1a* promoter in a transgenic GUS reporter line of tobacco (Park and Kloepper, 2000), including *Serratia marcescens* 90-166, that was subsequently shown to induce resistance in tobacco in a SA-independent way (Zhang *et al.*, 2002). However, when grown in vitro on nutrient agar medium under gnotobiotic conditions the bacteria do not remain confined to the roots but colonize the entire seedling as well as the surrounding agar, reaching

extremely high densities, which may cause substantial stress to the small seedlings. Induction of the *PR-1a* promoter in greenhouse-grown plants was also observed, but variable, as was a rise in SA in bacterized seedlings (Zhang *et al.,* 2002). These findings contrast with those obtained in *Arabidopsis* after elicitation of ISR by WCS417, in which neither an increase in SA nor PRs were detectable (Pieterse *et al.,* 1996, 2000). Hence, there is very little conclusive evidence for a role of SA in rhizobacteria-mediated ISR similar to its involvement in SAR.

This conclusion seems to be contradicted by a number of observations where SA-inducible PR-proteins were observed in soil-grown plants upon treatment with ISR-eliciting rhizobacteria. However, when applied at high doses to roots of sensitive plant species, specific rhizobacterial strains can be toxic to plant roots, even though no obvious symptoms are apparent on the leaves. Accumulation of proteins with molecular weights corresponding to PR-proteins was observed in tobacco plants after spraying two leaves with purified LPS of *Burkholderia cepacia*, associated with development of ISR against *Phytophthora nicotianae* (Coventry and Dubery, 2001). At the concentrations of LPS used, cell permeability was slightly increased and viability decreased, whereas the proteins induced were not characterized. The characteristic, SA-inducible PRs were expressed in tobacco plants upon triggering of ISR against tobacco necrosis virus (TNV) by CHA0 (Maurhofer *et al.,* 1994). Transformation of a non-SA-producing strain, P3, with a gene cassette for SA biosynthesis, made the transgenic derivative an inducer of ISR against TNV, confirming that SA induces SAR against viruses (Maurhofer *et al.,* 1998). Unfortunately, neither CHA0, nor the SA-producing derivative of P3 seems to have been tested on NahG tobacco. However, on NahG *Arabidopsis* plants CHA0 was still able to elicit ISR against the downy mildew oomycete *Peronospora parasitica* (Iavicoli *et al.,* 2003), suggesting that CHA0 at least has the ability to elicit ISR independently of SA, similar to WCS358 and WCS417 (Pieterse *et al.,* 1996).

Downstream of SA in the SAR signalling pathway, the protein NPR1 plays an essential role. Although SA is not necessarily involved in ISR, NPR1 has been demonstrated to be necessary also for ISR in *Arabidopsis* (Pieterse *et al.,* 1998). Mutant *npr1* plants did not express ISR after treatment with WCS417. Thus, NPR1 seems to play a central role in reaching the induced state, whether triggered by avirulent pathogens or by non-pathogenic rhizobacteria. However, downstream of NPR1, the signalling pathways must diverge again, because SAR is associated with the accumulation of PRs, whereas in ISR-induced plants such accumulation does not commonly occur (Fig. 1). How NPR1 acts in rhizobacteria-mediated ISR is presently unknown.

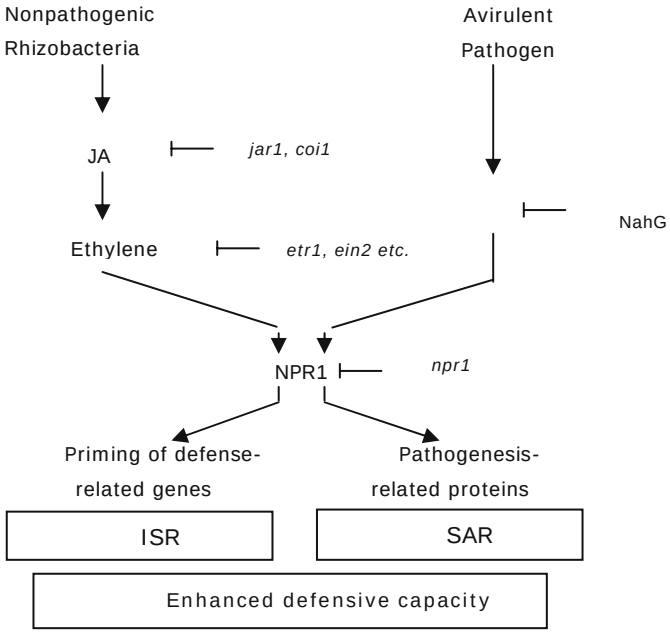

Fig. 1. Current model of signal-transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-induced systemic resistance (ISR). Some non-pathogenic rhizobacteria may trigger a SA-dependent signalling pathway that leads to a state of induced resistance resembling SAR (After Pieterse *et al.,* 1998).

A possible requirement for other regulatory factors implicated in plant defense was tested in bioassays with *Arabidopsis* mutants. In the *jar1* mutant, which has reduced sensitivity to jasmonic acid (JA), WCS417 was no longer able to elicit ISR against Pst, implicating JA in the signalling pathway of ISR (Pieterse *et al*., 1998). *Jar1* encodes a presumed JA-amino acid synthetase that is required to activate JA for signalling (Staswick and Tiryaki, 2004), but the nature and role of the conjugate in the JA signal-transduction pathway is not clear. Spraying *Arabidopsis* plants with the methyl ester of JA (MeJA) induced a systemic resistance identical to that elicited by the rhizobacteria (Pieterse *et al.,* 1998). However, treatment with these rhizobacteria did not increase endogenous JA levels in *Arabidopsis* roots or leaves (Pieterse *et al.,* 2000). Therefore, ISR signalling appears to require responsiveness to JA rather than increased levels of this regulator. It is possible that the sensitivity to JA is increased as a result of elicitation of ISR, and gene expression studies accordingly indicate an enhanced capacity for expression of JA-regulated genes in induced leaves (see below).

Sensitivity to ethylene proved to be likewise required for ISR, as ethylene-insensitive *Arabidopsis* mutants *etr1* and *ein2* were unable to express ISR upon elicitation by WCS417 (Pieterse *et al*., 1998; Knoester *et al.,* 1999). ETR1 encodes an ethylene receptor and the dominant *etr1* mutation causes reduced sensitivity to the hormone (Guo and Ecker, 2004). EIN2 is a membrane protein showing similarity to the Nramp family of metal-ion transporters (Alonso *et al.,* 1999). It plays a central role in the ethylene response, but its biochemical function is unknown. Because many components of the ethylene signalling pathway have been identified, additional ethylene-insensitive mutants (*ein3* – *ein7* and *axr1-12*) were tested, as well as the ethylene-overproducing mutant *eto1-1*. All ethylene-insensitive mutants were impaired in ISR (Knoester *et al.,* 1999), indicating that the expression of ISR requires the complete signal-transduction pathway of this hormone known so far. Surprisingly, also in the *eto1-1* mutant, ISR was abolished. Upon infection with Pst, non-induced *eto1-1* plants developed symptoms to the same extent as non-induced wild-type plants. This observation is difficult to reconcile with the result that exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene that is rapidly converted once taken up by plants, did elicit a systemic resistance to Pst comparable to ISR (Pieterse *et al.,* 1998). Treatment of wild-type Arabidopsis plants with WCS417 did not lead to an increase in ethylene production (Pieterse *et al.,* 2000), but did increase the capacity for ACC conversion in the leaves (Hase *et al.,* 2003). Hence, it appears that, as for JA, it is the sensitivity to the hormone that is required, while the capacity for its synthesis is increased.

The *Arabidopsis* mutant *eir1* is insensitive to ethylene in the roots, but not in the shoots (Roman *et al.,* 1995). In accordance with the requirement for ethylene sensitivity, in bioassays the *eir1* mutant did not express ISR upon treatment with WCS417 to the roots, while it did exhibit ISR when the inducing bacteria were infiltrated into the leaves (Knoester *et al.,* 1999). This result demonstrated that for the induction of ISR in *Arabidopsis* by WCS417, ethylene responsiveness is required at the site of application of the inducing rhizobacteria. The *eir1* mutant is allelic to *pin2*, and lacks a functional auxin efflux carrier protein in roots (Luschnig *et al.,* 1998). A role of auxin in ISR has not been tested and it is presently unclear whether auxin could also be involved. However, ethylene insensitivity of the *eir1* mutant can fully explain its lack of ISR inducibility.

In wild-type *Arabidopsis* plants, ISR can be induced chemically by exogenous application of either MeJA or ACC. In the *jar1* mutant, ACC was still capable of inducing ISR, indicating that responsiveness to ethylene is required after the JA-dependent signalling step. Conversely, MeJA did not induce ISR in the *etr1* mutant (Pieterse *et al.,* 1998). Thus, responsiveness to JA and ethylene are required in this order. Neither MeJA, nor ACC could

elicit ISR in the *npr1* mutant, placing the requirements for both JA and ethylene upstream of NPR1 in the signalling pathway. These results defined a novel signalling pathway for the type of induced systemic resistance elicited by rhizobacteria (Fig. 1).

ISR against *Peronospora parasitica* in *Arabidopsis* in response to root inoculation with CHA0 has likewise been shown to require JAR1, EIR1 and NPR1, and not SA. However, mutants *etr1-1* and *ein2-1* still expressed ISR against *Peronospora parasitica* (Iavicoli *et al.,* 2003), suggesting that the requirements for ISR against this pathogen overlap only partly with those defined for ISR against Pst, as induced by WCS417. On the other hand, ISR activated by two *Bacillus* species through the volatile 2,3-butanediol (cf. Table 1) was found to be independent of SA and dependent on ethylene, yet did apparently not require JA (Ryu *et al*., 2004). ISR elicited in *Arabidopsis* against Pst or *Pseudomonas syringae* pv. *maculicola* by four other PGPR strains was reported to be variably dependent on ethylene and JA (Ryu *et al.,* 2003). These results confirm that, in general, ISR is not dependent on SA, but indicate that instead ISR has a variable requirement for JA and ethylene signalling. The latter does not need to be problematic, because hormone sensitivity is still poorly understood and may vary depending on experimental conditions. In a given situation, either sensitivity to JA or to ethylene, or both, might be limiting.

2.4 Expression of ISR

Expression of ISR upon challenge inoculation with a pathogen is similar to expression of SAR in that disease severity is reduced or the number of diseased plants diminished. This reduction is typically associated with decreased growth of the pathogen and reduced colonization of induced tissues, testifying to the fact that the plant is better able to resist the pathogen (Van Loon, 2000). Neither ISR, nor SAR protects plants completely, in contrast to *R* gene-mediated resistance. However, a decrease in, or slowing down of disease development may save a crop or at least increase yield.

The spectrum of diseases against which ISR and SAR are effective, overlaps only partly (Ton *et al.,* 2002), as could be expected because of the differences in defense signalling described above. It has been demonstrated, mainly in *Arabidopsis*, that pathogens are resisted by either SA-dependent, or by JA- and/or ethylene-dependent defenses, or both (Thomma *et al.,* 2001; Ton *et al.,* 2002). This conclusion was reached because pathogens that are resisted by SA-dependent defenses, cause more severe disease on transgenic NahG than on wild-type plants. Conversely, pathogens that are resisted by JA- and ethylene-dependent defenses cause enhanced disease susceptibility in plant mutants that are defective in JA or ethylene synthesis or signalling. Pst causes increased disease severity in both NahG and *jar1* or

ein2 plants, indicating that this pathogen is resisted by SA-dependent, as well as JA- and ethylene-dependent defenses (Ton *et al.,* 2002). Accordingly, both SAR and ISR are effective against Pst. Moreover, combination of SAR and ISR by induction of SAR through inoculation with an avirulent derivative of Pst on the leaves of plants growing in soil containing ISR-inducing WCS417 bacteria, led to additive protection (Van Wees *et al.,* 2000). This observation demonstrates that SAR and ISR are distinct and complementary mechanisms by which the defensive capacity of plants is enhanced through biotic stimulation. Thus, expression of ISR, while phenotypically similar to SAR, relies not only on a different type of biological inducer, but occurs also through different defense-related activities. Phytoalexins can also contribute to plant resistance. However, *Arabidopsis* mutants that are impaired in the synthesis of the phytoalexin camalexin (*pad1 – pad4*) express normal ISR against Pst (C.M.J. Pieterse, unpublished results), indicating that ISR does not operate through stimulation of phytoalexin production against this pathogen.

In *Arabidopsis*, SAR is most effective against biotrophic pathogens - downy and powdery mildews, as well as viruses -, that are sensitive to SA-dependent defenses. Indeed, PRs, such as PR-1 and PR-5, have been shown to possess antifungal activity against oomycetes (Van Loon 1997), while SA action in resistance to viruses is likely to rely on a different mechanism (Singh *et al.,* 2004). In contrast, ISR is more active against necrotrophic pathogens (Ton *et al.,* 2002) through mechanisms that are yet to be elucidated. It was observed earlier that SAR was not effective against typical necrotrophic fungi, such as *Botrytis cinerea* and *Alternaria brassicicola* (Thomma *et al*., 2000) or bacteria, such as *Erwinia carotovora* (Vidal *et al.,* 1998). Against these pathogens, ISR is effective, be it that the strategy of *Botrytis* to kill its host in advance of tissue colonization hampers the reaction of the plant.

In tobacco, the effectiveness of SAR and ISR against different types of pathogens is largely similar to their differential activities in *Arabidopsis*. However, in tomato the powdery mildew fungus *Oidium neolycopersici* was reported to not be resisted by SA-dependent defenses, while SA was involved in defense against *Botrytis* (Achuo *et al.,* 2004). Thus, the conclusion must be that SA- and JA- or ethylene-dependent defense mechanisms can be effective against different pathogens in different plant species. Therefore, findings from a single pathosystem cannot be generalized, and rigorous experimentation is required to define the potential of SAR or ISR to contribute to enhanced resistance in a particular plant species.

A search for newly induced proteins upon induction of ISR that can be used reliably as markers for the induced state, similar to the PRs associated with SAR, proved negative (Van Wees *et al*., 1999). As must be

concluded from several investigations, the state of ISR is not consistently associated with significant changes in the proteome of the induced plant. Other defense-related activities have been sought that could serve to indicate that ISR was induced and would preferably have a defined role in plant resistance. There are several publications reporting increases in the activities of e.g. chitinase, glucanase, phenylalanine ammonia-lyase (PAL) or peroxidase, as well as in the content of phenolic compounds, in plants treated with ISR-eliciting PGPR. Although specific PR-2 and PR-3, -4, -8 and –11 proteins have glucanase and chitinase activities (Van Loon and Van Strien, 1999), respectively, many glucanases and chitinases in plants are developmentally regulated and induced by various abiotic and biotic stresses through signalling pathways that may, or may not, overlap with those regulating SAR and ISR. While PAL, the key enzyme in phenolic biosynthesis, and oxidative enzymes, such as peroxidase and polyphenoloxidase, can play a role in increased tissue lignification (Barcelo, 1997), as well as the generation of toxic quinones, a causal relationship between these increases and enhanced resistance against specific pathogens has not been well established. Moreover, effective WCS417-triggered ISR in radish was not associated with such changes (E. Hoffland and H. Steijl, unpublished observations), making these parameters unsuitable as markers for the state of ISR. Also, the activities of PAL and peroxidase, and phenolic content, are strongly developmentally regulated and respond sensitively to changes in the physical and chemical environment, as well as to different stresses. That these parameters often change in response to treatment with rhizobacteria indicates that the plants react to the presence of the bacteria, but in how far this reaction is coupled to establishment of ISR is fully unclear. Directly, through stimulation of plant growth, or indirectly, through suppression of deleterious microorganisms, bacterial treatments also promote growth and this, in turn, could lead to increases in e.g. chitinase, glucanase, PAL, peroxidase and phenolics in association with the improved development of the plants. It would be most interesting to determine the effects of ISR-eliciting rhizobacteria on transgenic plants with impaired enzyme activities, but so far such experiments have not been reported.

Recently, transcriptome analyses by cDNA microarrays, RNA differential display, or subtractive hybridization of cDNA libraries have confirmed the notion that rhizobacteria influence plant gene expression to only limited extents. Analysis of the expression of over 8000 genes of *Arabidopsis* plants with ISR elicited by WCS417 revealed changes in the expression of 102 genes in the roots on which the bacteria were present. In contrast, systemically in the leaves, none of the genes examined showed a consistent change, in spite of the fact that, when challenge inoculated, these leaves showed a significant ISR response (Verhagen *et al.,* 2004). Clearly, the roots reacted locally to colonization by the bacteria. Within the first

week transient changes were observed in the expression of hundreds of genes, but these were not associated with the persistent state of ISR. Of the 102 genes whose expression was changed over a longer period, 39 were up-regulated and 63 were down-regulated. Unless a pre-existing factor was released as a result of colonization of the roots by the ISR-eliciting bacteria, the mobile signal that is required for systemic induction is likely to be generated through the action of those genes whose expression was altered. For instance, one or more of the up-regulated genes might encode enzymes required for the synthesis of such a mobile signal. However, it is equally possible that down-regulation of a specific gene might lift the inhibition on a pre-existing or newly induced mechanism. In view of the ethylene dependency of ISR, an increase in a putative ACC oxidase and down-regulation of ethylene response factor 1 (ERF1) and ethylene-responsive element binding factors 1 (EREBP1) and 2 (EREBP2) are particularly interesting.

These results appear to contrast with an analysis of *Arabidopsis* plants treated with the rhizobacterium *Pseudomonas thivervalensis*, which likewise induced systemic resistance against Pst (Cartieaux *et al.,* 2003). Those experiments were conducted in *Arabidopsis* accession Ws-0, which is known to be incapable of expressing ISR (Ton *et al.,* 1999). Hence, the resistance induced by *P. thivervalensis* must be ascribed to a different type of ISR. This type was not specified, but it was reported that the bacterium reduced plant growth by 41% and, at least initially, decreased net photosynthesis. These observations suggest that *P. thivervalensis* behaved as a pathogen on *Arabidopsis* and may have induced the SAR pathway, which is also effective against Pst. Under these conditions, cDNA microarray analysis of approximately 14300 *Arabidopsis* genes revealed that the transcript levels in colonized roots were hardly changed relative to axenic control plants, and none were elevated. In contrast, in shoots the levels of 63 transcripts were modified, including 42 genes that were upregulated. Except for a putative chitinase, no indication of increased *PR* gene expression was evident, however. Induction of resistance against *Erwinia carotovora* in *Arabidopsis* by *Paenibacillus polymyxa* was associated with increased tolerance to drought and changes in the abundance of mRNAs encoding drought stress- and biotic stress- responsive proteins, consistent with a mild pathogenic effect of *P. polymyxa* on *Arabidopsis* (Timmusk and Wagner, 1999). These alterations do not seem typical of ISR.

It was observed earlier that upon challenge inoculation of *Arabidopsis* plants with Pst, SAR-induced plants showed an augmented expression of SA-dependent PR-1 mRNA, whereas plants with ISR accumulated mRNA of the JA-inducible gene *Vsp* to higher levels than non-induced plants (Van Wees *et al.,* 1999). This “priming” effect indicated that induced plants activate defense-related gene expression earlier and to a

greater extent than non-induced plants (Conrath *et al.,* 2002). Indeed, cDNA microarray analysis of WCS417-induced plants revealed 81 genes showing an augmented expression pattern in ISR-expressing leaves after challenge inoculation with Pst (Verhagen *et al.,* 2004). Of these, 51 genes were expressed at at least 1.5 times higher levels, including *Vsp*, the JA- and ethylene-responsive gene *Pdf1.2*, a thaumatin-like gene, a chitinase gene, and a gene encoding EREBP2. The other 30 genes showed a Pst-induced change in WCS417-treated plants only, and appear to be ISR-specific. These included genes that are presumably involved in regulating gene transcription and signal transduction. The majority of the genes were predicted to be regulated by JA or ethylene. Thus, the requirement for JA and ethylene sensitivity in ISR seems to be related to the priming action of defense-related gene expression after challenge inoculation of induced plants.

ISR triggered by *Pseudomonas chlororaphis* O6 upon root colonization of cucumber against target leaf spot, caused by *Corynespora cassiicola*, was likewise associated with a faster and stronger accumulation of transcripts of six distinct genes upon challenge inoculation, as revealed through subtractive hybridization (Kim *et al.,* 2004). Expression of these genes was not induced by O6 colonization alone, and became apparent only after challenge with the pathogen. These results corroborate earlier findings of augmented defense responses upon challenge inoculation of induced plants, such as an increased production of dianthramide phytoalexins after infection by *Fusarium oxysporum* f.sp. *dianthi* of carnation plants induced by WCS417 (Van Peer et al., 1991) and increased cell wall strengthening upon pathogen attack of cucumber and tomato plants pretreated with ISR-eliciting rhizobacteria (Benhamou and Nicole, 1999).

3 SYSTEMICALLY INDUCED RESISTANCE AND PLANT GROWTH

Systemically induced resistance, whether SA-dependent SAR or JA- and ethylene-dependent ISR, both seem to be expressed through an enhanced activation of defense responses upon challenge inoculation. However, whereas SAR is associated with the accumulation of PRs and negatively affects plant growth (Heil, 2002), most of the ISR-triggering rhizobacteria have been selected primarily because of their plant growth-promoting properties. It is quite unclear in how far plant protection through ISR and growth promotion are connected. Besides inducing ISR, PGPR can exert a protective action against those soil-borne pathogens that are particularly prone to attack emerging seedlings. Stimulation of plant growth will lead to increased plant vigour and a shorter period of vulnerability

before adult plant resistance may have become sufficient to limit damage by the pathogen. ISR-eliciting rhizobacteria can be applied on seeds and then will readily colonize emerging plant roots. Thus, seedlings can be better protected already at an early stage (Kloepper *et al.,* 1989; Leeman *et al.,* 1995c).

These properties make ISR-inducing PGPR a useful tool to reduce diseases caused by pathogens that are sensitive to JA- and ethylene-dependent defenses. Moreover, combination of ISR and SAR can increase protection against pathogens that are resisted through both mechanisms, as well as extend protection to a broader spectrum of pathogens than ISR or SAR alone. This provides an attractive strategy when other means of crop protection are limited or absent. However, both ISR and SAR only reduce disease and are usually less effective than physical methods, such as steaming of the soil, or chemical treatments. Notably, the costs of chemical crop protectants are often lower than those of a biological product that requires fermentation on a nutrient medium, extensive formulation, has only limited shelf life, and is less effective under field conditions. Thus, for economic reasons biological crop protectants can only seldom compete with highly effective chemicals. However, ISR is only one of the mechanisms that may be mobilized to counteract plant pathogens in an environmentally friendly and durable way. Integrating ISR-triggering PGPR into disease management programs in conjunction with other strategies will be a worthwhile approach to explore.

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Chapter 3

BIOSYNTHESIS OF ANTIBIOTICS BY PGPR AND ITS RELATION IN BIOCONTROL OF PLANT DISEASES

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Abstract: Plant growth promoting rhizobacteria (PGPR) play a vital role in crop protection, growth promotion and in the improvement of soil health. Some well known PGPR strains are *Pseudomonas*, *Bacillus*, *Azospirillum*, *Rhizobium*, and *Serratia* species. The primary mechanism of biocontrol by PGPR involves the production of antibiotics such as phenazine-1-carboxyclic acid, 2,4-diacetyl phloroglucinol, oomycin, pyoluteorin, pyrrolnitrin, kanosamine, zwittermycin-A, and pantocin. A cascade of endogenous signals such as sensor kinases, N-acyl homoserine lactones and sigma factors regulates the synthesis of antibiotics. The genes responsible for the synthesis of antibiotics are highly conserved. The antibiotics pertain to polyketides, heterocyclic nitrogenous compounds and lipopeptides have broad-spectrum action against several plant pathogens, affecting crop plants. In addition to direct antipathogenic action, they also serve as determinants in triggering induced systemic resistance (ISR) in the plant system. Though antibiotics play a vital role in disease management, their role in biocontrol is questioned due to constraints of antibiotic production under natural environmental conditions. Environmental and other factors that suppress the antimicrobial action of antibiotics have to be studied to exploit the potential of antibiotics of PGPR in crop protection.

Key words: antibiotics; biocontrol; PGPR.

1 INTRODUCTION

Plant pathologists are facing major challenges for the management of soil-borne plant pathogens. Management of plant pathogens with pesticides has resulted in environmental pollution and resistance among pathogens. Subsequently, identification of suppressive soils to various soil borne plant pathogens such as *Gaeumanomyces graminis* var. *tritici*,

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 67–109.

Fusarium oxysporum, *F. solani*, *Phytophthora cinnamomi*, *Rhizoctonia solani* and *Sclerotium cepivorum* limited the disease development in spite of the favorable environment (Cook and Baker, 1983). The suppressiveness was due to the presence of antagonistic microbes. Among various microbes, prokaryotes are omnipresent and have been widely explored for plant disease management. The prokaryotic cells in earth are 2.6×10^{29} (Whitman *et al.*, 1998). Among the wide genetic biodiversity of prokaryotes, plant growth promoting rhizobacteria (PGPR) plays a vital role in the management of plant diseases to increase crop productivity via various mechanisms.

Considerable progress has been made over the past two decades to elucidate the mechanisms by which fluorescent pseudomonads suppress diseases. The primary mechanism of biocontrol by fluorescent pseudomonads involves production of antibiotics such as 2,4-diacetylphloroglucinol (PHL), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazine-1-carboxyclic acid (PCA), 2- hydroxy phenazines and phenazine-1-carboxamide (PCN). In addition to direct antipathogenic action, antibiotics also serve as determinants in triggering induced systemic resistance (ISR) in the plant system and contribute to disease suppression by conferring a competitive advantage to biocontrol agents. Synergism between antibiotics and ISR may further increase host resistance to plant pathogens. Though several modes of action are responsible for the suppression of plant pathogens, this chapter will focus on new insights and concepts in biocontrol of plant pathogens by PGPR through antibiotics.

2 ANTIBIOTICS OF PGPR

Utilization of microbial antagonists against plant pathogens in agricultural crops has been proposed as an alternate to chemical pesticides. Fluorescent pseudomonads and *Bacillus* species play an active role in suppression of pathogenic microorganisms. These bacterial antagonists enforce suppression of plant pathogens by the secretion of extracellular metabolites that are inhibitory at low concentration.

Antibiotics produced by PGPR include 2,4 Diacetyl phloroglucinol, phenazine-1-carboxyclic acid, phenazine-1-carboxamide, pyoluteorin, pyrrolnitrin, oomycinA, viscosinamide, butyrolactones, kanosamine, zwittermycin-A, aerugine, rhamnolipids, cepaciamide A, ecomycins, pseudomonic acid, azomycin, antitumor antibiotics FR901463, cepafungins and antiviral antibiotic karalicin (Table-1). These antibiotics are known to possess antiviral, antimicrobial, insect and mammalian antifeedant, antihelminthic, phytotoxic, antioxidant, cytotoxic, antitumour and plant growth promoting activities.

Table 1. Antibiotics produced by rhizobacteria.

PGPR	Antibiotics	Reference
Pseudomonas sp.	**Antifugal antibiotics**	
	Phenazines	Burkhead *et al.* (1994)
	Phenazine-1-carboxylic acid	Pierson and Pierson (1996)
	Phenazine-1-carboxamide	Chin-A-Woeng *et al.* (1998)
	Pyrrolnitrin	Thomashow and Weller (1988)
	Pyoluteorin	Howel and Stipanovic (1980)
	2,4diacetylphloroglucinol	Shanahan *et al.*(1992b)
	Rhamnolipids	
	Oomycin A	Kim *et al.*(2000)
	Cepaciamide A	Howie and Suslow (1991)
	Ecomycins	Jiao *et al.*(1996)
	DDR	Miller *et al.*(1998)
	Viscosinamide	Hokeberg *et al.*(1998) Nielsen *et al.* (1999)
	Butyrolactones	Thrane *et al.* (2000)
	N-butylbenzene	Gamard *et al.*(1997)
	sulphonamide	Kim *et al.*(2000)
	Pyocyanin	Baron and Rowe (1981)
	Antibacterial antibiotics	
	Pseudomonic acid	Fuller *et al.*(1971)
	Azomycin	Shoji *et al.*(1989)
	Antitumour antibiotics	
	FR901463	Nakajima *et al.*(1996)
	Cepafungins	Shoji *et al.*(1990)
	Antiviral antibiotic	
	Karalicin	Lampis *et al.*(1996)
Bacillus sp.	Kanosamine	Milner *et al.*(1996)
	Zwittermycin A	Silo - Suh *et al.*(1994)
	Iturin A (Cyclopeptide)	Constantinescu (2001)
	Bacillomycin	Volpon *et al.*(1999)
	Plipastatins A and B	Volpon *et al.*(2000)

The major antibiotics that play a vital role in the suppression of plant pathogens are grouped into non-volatile and volatile antibiotics.

- Non-Volatile antibiotics
    - Polyketides (2,4 Diacetyl phloroglucinol; Pyoluteorin; Mupirocin)
    - Heterocyclic nitrogenous compounds (Phenazine derivatives)
    - Phenylpyrrole (Pyrrolnitrin)
    - Cyclic lipopeptides
    - Lipopeptides (Iturin, Bacillomycin, Plipstatin, Surfactin)
    - Aminopolyols (Zwittermycin –A)

- Volatile antibiotics
    - Hydrogen cyanide
    - Aldehydes, alcohols, ketones and sulfides

2.1 Polyketides

Among the various groups of antibiotics produced by the PGPR, the polyketides such as 2,4 Diacetyl phloroglucinol, Pyoluteorin and Mupirocin are highly effective in suppression of plant pathogens.

2.1.1 Diacetyl phloroglucinol (DAPG)

The ubiquitous distribution of fluorescent pseudomonads in the rhizosphere of crop plants has broad spectrum of action in the suppression of fungi, bacteria and nematodes (Keel *et al.*, 1992; Haas and Keel, 2003). Though several mechanisms are in operation to suppress plant pathogens, the antibiotics produced by fluorescent pseudomonads remain as a crucial factor in checking disease development and pathogens. Among the various extracellular metabolites produced, DAPG is of prime importance in plant protection. Three evidences substantiate the involvement of DAPG in crop protection.

- Mutations in the biosynthetic gene cluster of DAPG reduced biocontrol activity of antagonistic bacteria (Keel *et al.*, 1992; Nowak-Thompson *et al.*, 1994).
- Population density of DAPG producers and the antibiotic production was responsible for disease suppression in different soils (Raaijmakers *et al.*, 1999).
- Association of different DAPG producers in the rhizosphere of crop plants was responsible for disease suppression (Raaijmakers *et al.*, 1999).

2.1.1.1 Biosynthesis of DAPG

The polyketide antibiotic DAPG is a phenolic molecule synthesized by the condensation of three molecules of acetyl coenzymeA with one molecule of malonyl coenzymeA to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate PHL utilizing a CHS-type enzyme (Shanahan *et al.*, 1992a). Biosynthetic locus of DAPG is highly conserved. It comprises the biosynthetic genes *phlACBD* (Keel *et al.*, 2000).

2.1.1.2 Phenotypes of DAPG producers

The DAPG producers are grouped into different phenotypes based on the extracellular production of different metabolites including antibiotics and HCN. The major phenotypic groups of DAPG producers include

- 2,4 DAPG and hydrogen cyanide producers
- 2,4 DAPG, hydrogen cyanide and pyoluteorin producers (Keel *et al.*, 1996)
- 2,4 DAPG, pyoluteorin and pyrrolnitrin producers (Nowak-Thompson, 1999; Sharifi-Tehrani *et al.*, 1998).

2.1.1.3 Genetic diversity of phlD among DAPG producers

phlD is an essential gene involved in the synthesis of DAPG. Its diversity was evaluated between the isolates of pseudomonads distributed worldwide. Potential pseudomonads for disease management may be identified functionally, based on their ability to produce 2,4-DAPG. But, all DAPG producers could be taxonomically distinguished as different strains based on the amplified ribosomal DNA restriction analysis (ARDRA) fingerprints. Three to four groups of DAPG producers were distinguished through ARDRA fingerprints. However, it does not explain the complete diversity (Keel *et al.*, 1996; Sharifi-Tehrani *et al.*, 1998; McSpadden Gardener *et al.*, 2000). Hence some other molecular tool has to be deviced for the detection of variation among the different DAPG producers.

As a consequence, utilization of molecular tools such as BOX-PCR and enterobacterial repetitive intergeneric consensus (ERIC-PCR) helped in identification of thirteen to 15 different genotypes among *phl-D* containing strains (McSpadden Gardener *et al.*, 2000). Sixty-four different RAPD genotypes were identified among 150 strains of ARDRA group of *phlD* isolates from maize rhizosphere (Picard *et al.*, 2000). Genotypes identified through RFLP analysis of *phlD* gene was conserved between the isolates. But RAPD analysis of genomic DNA showed a high degree of

polymorphism between DAPG producers (Mavrodi *et al.*, 2001). Hence, there exists a greater genetic diversity among the DAPG producers. Knowledge on diversity of *phlD* gene among DAPG producers are important for assessing the antagonistic potentiality and frequency of horizontal gene transfer between the microbial communities seen in the rhizosphere. It provides a fundamental knowledge for developing a rapid genetic screening system to identify a potential biocontrol strains.

2.1.1.4 Cross talk between DAPG producers

The mechanism of communication between antagonistic *Pseudomonas* and between rhizosphere bacterial communities is gaining importance. Interactions between bacterial communities could lead to either positive or negative effect. N-Acyl-homoserine lactones (AHL) are the signal molecules involved in communication between different bacteria. AHL signals are used for communication between several plant bacterial communities to control the antibiotic gene expression and cell-to-cell communication in a cell density dependent manner termed as quorum sensing (Pierson *et al.*, 1998).

2.1.1.5 Positive cross talk

DAPG induces its own biosynthesis and acts as a diffusible signal at intra and inter population levels. DAPG produced by the genetically distinct pseudomonads (CHAO and Q2-87) in a mixed bacterial population of wheat rhizosphere could be perceived as a positive signal for increasing the synthesis of DAPG by increasing the expression of DAPG biosynthetic genes (Maurhofer *et al.*, 2004). Thus DAPG acts as a signaling compound inducing the expression of its own DAPG biosynthetic genes (Fig 1).

2.1.1.6 Negative cross talk

The negative cross talk also exists between the PGPR, plant pathogens and the abiotic environment. Extracellular metabolites of plant pathogens suppress the expression of biosynthetic genes responsible for antibiotic production. Antipathogenic activity of *P. fluorescens* CHAO against *F. oxysporum* f. sp. *radicis lycopersici* was repressed by fusaric acid produced by pathogen. It repressed the expression of DAPG genes of CHAO strains and was unable to control tomato root and crown rot (Duffy and Defago, 1997; Schnider - Keel *et al.*, 2000). In addition, non-pathogenic isolates of *Fusarium* producing fusaric acid also suppress the expression of DAPG gene in the wheat rhizosphere (Notz *et al.*, 2002). Recent evidence suggests that besides DAPG and fusaric acid a number of other phenolic

metabolites like pyoluteorin and salicylate of microbial and plant origin also affect the production of antimicrobial metabolites in fluorescent pseudomonads (Pierson *et al.*, 1998**;** Schnider - Keel *et al.*, 2000; Fig 1). Apart from fungal metabolites and phenolic compounds, DAPG by itself suppress pyoluteorin produced by other pseudomonads (Haas and Keel, 2003).

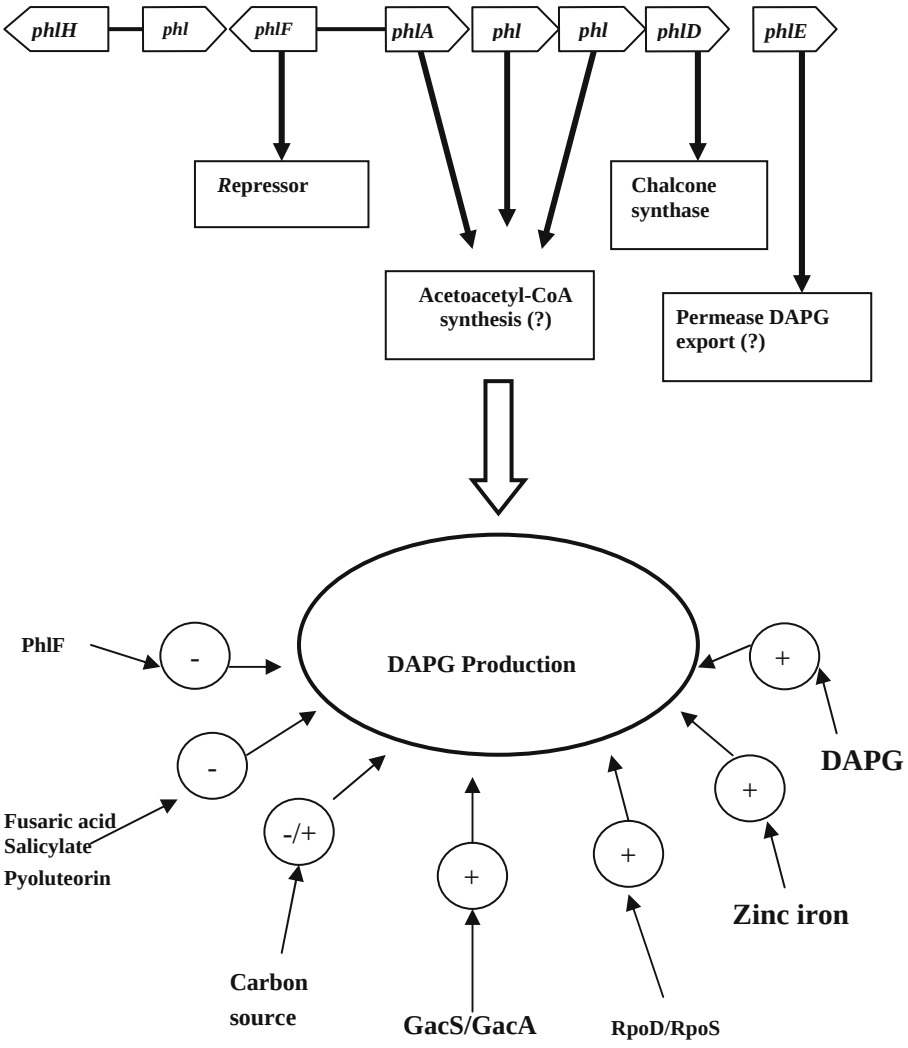

Fig. 1. Biosynthetic genes of DAPG and factors influencing its expression.

2.1.1.7 Factors affecting DAPG production

Biotic and abiotic factors associated with the crop and environment affect the performance of fluorescent pseudomonads (Thomashow and Weller, 1995; Duffy and Defago, 1997; Notz *et al.*, 2002). Biotic factors such as plant species, plant age, cultivar and pathogens alter the expression of the gene *phlA* (Notz *et al.*, 2001). DAPG production is influenced by abiotic factors such as carbon sources and various minerals. Fe3+ and sucrose increased DAPG production in *P. fluorescens* F113, while glucose increased DAPG production in *P. fluorescens* Pf-5 and CHA0 (Nowak-Thompson *et al.*, 1994; Duffy and Defago, 1999). In *P. fluorescens* strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon. Micronutrients Zn2+, Cu2+ and Mo2+ stimulated DAPG production in *P. fluorescens* CHA0 (Notz *et al.*, 2001).

2.1.2 Pyoluteorin

Pyoluteorin (Plt) is a phenolic polyketide with resorcinol ring. The ring is linked to a bichlorinated pyrrole moiety. Biosynthesis of pyrrole moiety is unknown (Kitten *et al.*, 1998; Nowak-Thompsan *et al.*, 1999). It was first isolated from *P. aeruginosa* (Takeda, 1958) followed by *P. fluoresens* Pf-5 and CHAO (Bencini *et al.*, 1983; Bender *et al.*, 1999). Plt has bactericidal, herbicidal and fungicidal properties. Application of Plt to cotton seeds suppressed cotton damping-off (Howell and Stipanovic, 1980).

2.1.2.1 Gene locus for the biosynthesis of Plt

Plt is initiated from proline or a related molecule, which serve as the precursor for dichloropyrrole moiety of Plt. It condenses with three acetate equivalents coupled to chlorination and oxidation. The formation and cyclization of the C-skeleton proceed by the action of a single multienzyme complex (Cuppels *et al.*, 1986; Nowak-Thompsan *et al.*, 1999). Ten genes, *pltLABCDEFG* are involved in the biosynthesis of Plt. Among these ten genes, *pltB* and *pltC* encode type 1 polyketide synthetase. *plt*G encodes a thio esterase, three halogenases are coded by *pltA*, *pltD* and *pltM*. Among the *plt* gene products, PltR is similar to LysR family of the transcriptional activators (Pierson *et al.*, 1998; Nowak-Thompsan *et al.*, 1999). Furthermore, PltR acts as a positive transcriptional activator linked to *phzI* loci of the Phz biosynthetic locus (Pierson *et al.*, 1998; Chin A-Woeng *et al.*, 2003).

2.1.3 Mupirocin

P. fluorescens produces several inhibitory substances with antimicrobial activities. Among the major metabolites pseudomonic acid known as mupirocin is also responsible for its bactericidal activity (Fuller *et al.*, 1971). Mupirocin inhibits isoleucyl-tRNA synthetase and prevents incorporation of isoleucine into newly synthesized proteins (Hughes and Mellows, 1980). Mupirocin producing strains of *P. fluorescens* overcomes the inhibitory effects of antibiotic by altering the target sites, isoleucyl-tRNA synthetase. Mupirocin exhibits a high level of antibacterial activity against *Staphylococci, Streptococci, Haemophilus influenzae* and *Neisseria gonorrheae*. But it is less sensitive against gram positive *Bacilli* and anaerobes (Sutherland *et al.*, 1985). Derivatives of monic acid A, the nucleus of mupirocin was active against a range of mycoplasma species (Banks *et al.*, 1998).

Mupirocin has a unique chemical structure and contains C9 saturated fatty acid (9-hydroxynonanoic acid) linked to monic acid A by an ester linkage. Mupirocin is derived from acetate. The acetate units are incorporated in to monic acid A and 9 - hydroxy nonanoic acid *via* polyketide synthesis. Transposon mutagenesis was used to identify a 60 kb region required for mupirocin biosynthesis in *P. fluorescens* NCIB10586 (Whatling *et al.*, 1995).

2.2 Heterocyclic nitrogenous compounds

Several heterocyclic nitrogenous compounds with antimicrobial action are produced as an extracellular secretion by rhizobacteria. Among those compounds phenazine is a powerful green-pigmented antimicrobial compound (Chin-A-Woeng *et al.*, 1998).

2.2.1 Phenazine

Phenazine is a low molecular weight secondary metabolite, nitrogen containing heterocyclic antimicrobial compound consisting of brightly coloured pigment produced by the bacterial genera pertaining to *Pseudomonas, Burkholderia, Brevibacterium* and *Streptomyces* (Turner and Messenger, 1986; Becker *et al.*, 1990; Thomashow *et al.*, 1990; Gealy *et al.*, 1996; Anjaiah *et al.*, 1998; Tambong and Hofte, 2001). More than 50 naturally occurring phenazine compounds have been described. Few strains of PGPR produce 10 different phenazine derivatives at a same time (Turner and Messenger, 1986; Smirnov and Kiprianova, 1990). Commonly identified derivatives of phenazine produced by *Pseudomonas* spp. are pyocyanin, PCA, PCN and hydroxy phenazines (Turner and Messenger, 1986). Both

PCA and PCN are produced by *P. fluorescens* 2-79 (Thomashow and Weller, 1988), *P. aureofaciens* 30-84 (Pierson *et al.*, 1995) and *P. chlororaphis* (PCL1391) (Chin A- Woeng *et al.*, 1998). Phenazine derivatives aid in long-term survival and ecological competence of these strains in rhizosphere (Mazzola *et al.*, 1992). *Pseudomonas chlororaphis* strain PA-23 was effective in controlling Sclerotinia stem rot of canola in greenhouse and field. *In vitro* assays indicated involvement of antibiotics in the inhibition. PA-23 yielded a 1400 bp fragment characteristic of PCA biosynthetic genes. Sequence analysis of PCR products showed high homology to PCA genes of several *Pseudomonas* strains deposited in the GenBank (Zhang and Fernando 2004a).

The antimicrobial activity of phenazine depends on the rate of oxidative reductive, transformation of the compound coupled with the accumulation of toxic superoxide radicals in the target cells (Hassett *et al.*, 1992 and 1993). Priming the seeds with *P. chlororaphis* effectively controlled seed borne diseases of barley and oats. It is commercially marketed as Cedomon (BioAgri AB, Uppsala, Sweden). Though phenazine plays a vital role in the management of soil-borne pathogens, the chemo taxis and motility of the bacteria decides the antifungal action of the antibiotic producers. The strain that lacks motility fails to exert antifungal action even if it produces antibiotics, due to the lack of rhizosphere colonization. Non-motile Tn5 mutants of *P. chlororaphis* (PCL1391), producer of PCN (chlororaphin) was 1000 fold impaired in competitive tomato root tip colonization compared with the wild type, which was antagonistic to *F. oxysporum* f. sp. *radicis lycopersici* (Chin-A-Woeng *et al.*, 2003). Ecological competence and persistence of *P. fluorescens* 2-79 and *P.aureofaciens* strain 30-84 was attributed to phenazine. But Tn5 mutants of the same were unable to compete with resident microflora (Mazzola *et al.*, 1992).

2.2.2 Biosynthesis of phenazine-1-carboxylic acid (PCA)

The biosynthetic loci of phenazine are highly conserved. Synthesis of phenazine compounds and shikimic acid pathway are closely related in several microorganisms (Turner and Messenger, 1986). Shikimic acid is the basic precursor for synthesis of phenazine and its derivatives (Ingledew and Campbell, 1969). Shikimic acid is converted to chorismic acid, which in turn branches out with amino-2-deoxyisochorismic acid (ADIC) (Callhoun *et al.*, 1972). ADIC serves as the branch point compound of PCA formation (McDonald *et al.*, 2001). Later ADIC is converted to trans-2, 3-dihydro-3-hydroxy anthranilic acid (DHHA). Ring assembly by dimerization of two DHHA moieties resulted in the formation of first phenazine derivative PCA. Dimerization involves oxidation of two molecules of DHHA to the C-3

ketone. The molecules react with each other by nucleophilic addition, dehydration and tautomerization to give 5,10-dihydroanthranillic acid, which is oxidized to PCA (McDonald *et al.*, 2001, Fig.2).

The biosynthetic genes for production of phenazine derivatives have been identified and characterized in several pseudomonads. The production of PCA in *P. aureofaciens* strain 30-84 involves cluster of 5 genes, *phz*FABCD (Pierson *et al.*, 1995). The phenazine biosynthetic operon of *P. fluorescens* 2-79 (Mavrodi *et al.*, 1998; 2004) and *P. chlororaphis* PCL1391

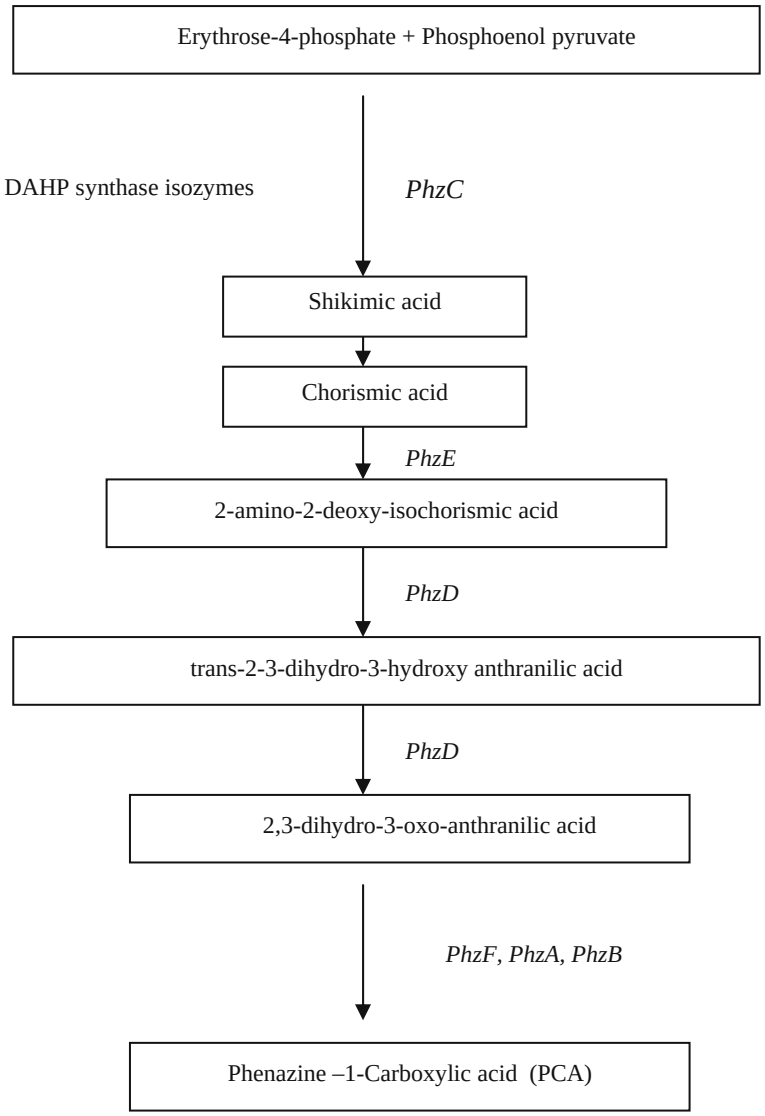

Fig. 2. Biosynthetic pathway of phenazine-1-carboxylic acid (PCA).

(Stover *et al.*, 2000) contain *phzABCDEFG* genes. The gene *phzH* located downstream of the phenazine operon in *P. chlororaphis* PCL1391 is an aminotransferase gene responsible for the conversion of PCA to phenazine-1-carboxamide (chlororaphin), the green phenazine compound characteristic of *P. chlororaphis* (Chin-A-Woeng *et al.*, 1998). Nucleotide sequences in phenazine producers are homologous and have 70-95% identity. The polypeptides encoded by *phzA* and *phzB* are common in all phenazine producers (Chin-A-Woeng *et al.*, 2001). But these genes are not essential for phenazine production instead they code for 163 amino acid, proteins, that help in stabilizing *PhzF* protein. The biosynthetic gene *phzG* located downstream in *P. chlororaphis* PCL1391 is required for PCN synthesis (Chin-A-Woeng *et al.*, 2001).

P. aureofaciens 30-84 contains a novel gene *phzO* located downstream from the core phenazine operon which encode a 55-kDa aromatic monooxygenase. Hydroxylation of PCA by monooxygenase led to the synthesis of 2-OH-PCA a broad-spectrum antibiotic effective against fungal pathogens (Delaney *et al.*, 2001). Two other genes *phzM* and *phzS* were characterized in *P. aeruginosa* PAO1. It code for enzymes that modify phenazine into its related derivativres. The gene *phzM* is located upstream of *phZ A1B1C1D1E1F1G1* operon and it is involved in the production of pyocyanin. The *phzS* gene located downstream from *phzG1* produce a 402-residue protein similar to monooxygenases of bacterial origin responsible for the production of pyocyanin and 1-hydroxy phenazine in *P.aeruginosa* PAO1 (Mavrodi *et al.*, 2001).

P. fluorescens 2-79 has a seven-gene locus *phzABCDEFG* of 6.8-kb. The products of *phzC, phzD* and *phz*E genes are similar to shikimic acid and chorismic acid metabolism. All these genes coupled with *phzF* are required for the production of PCA. *phzG* is similar to pyridoxamine-5'-phosphate oxidases and serves as a source of co-factor for the enzymes required for synthesizing PCA. The genes *phzA* and *phzB* are homologous to each other. It stabilizes multienzyme complex synthesizing PCA. The two new genes *phzX* and *phzY* from *P. aureofaciens* 30-84 produce 2-hydroxy phenazine-1-carboxyclic acid and 2-hydroxy phenazine (Mavrodi *et al.*, 2004).

2.3 Phenylpyrrole antibiotic

The antibiotic of PGPR that belongs to phenylpyrrole group receives much attention due to its broad-spectrum action. The antibiotic pyrrolnitrin belongs to phenylpyrrole group.

2.3.1 Pyrrolnitrin

Pyrrolnitrin (PRN) is a chlorinated phenylpyrrole antibiotic produced by several fluorescent and non-fluorescent pseudomonads. It was first isolated from *Burkholderia pyrrocinia* (Arima *et al.*, 1964). Pseudomonads species such as *P. fluorescens, P. chlororaphis, P. aureofaciens, B. cepacia, Enterobacter agglomerans, Myxococcus fulvus* and *Serratia* sp also produce PRN antibiotics (Hammer *et al.*, 1999). PRN was primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungus *Trichophyton.* Subsequently PRN was developed as an agricultural fungicide (Elander *et al.*, 1968). PRN persists actively in the soil for one month and it does not readily diffuse. But it is released after lysis of host bacterial cell, resulting in the slow release. PRN is effective against the post harvest diseases of apple, pear and cut flowers caused by *Botrytis cinerea* (Janisiewicz and Roitman, 1988; Hammer and Evensen, 1993). It also has strong antifungal action against *R. solani* (El-Banna and Winkelmann, 1988). *P. fluorescens* strains producing PRN reduced take all decline of wheat (Tazawa *et al.*, 2000). *P. chlororaphis* strain PA-23 was effective in controlling Sclerotinia stem rot disease of canola in the greenhouse and field. *In vitro* assays indicated involvement of antibiotics in the inhibition. PA-23 yielded three fragments characteristic of PCA and pyrrolnitrin biosynthetic genes, using primers PrnAF/PrnAR. Sequence analysis of PCR products showed high homology to pyrrolnitrin genes of several *P. fluorescens* and *Burkholderia* sp. strains deposited in the GenBank (Zhang and Fernanado, 2004a).

2.3.2 Genetic organization of pyrrolnitrin

The biocontrol agent, *P. fluorescens* BL915 contains four gene clusters involved in the biosynthesis of antifungal molecule PRN from the precursor tryptophan (Hamill *et al.*, 1970; Chang, 1981). The *prn* operon of 5.8 kb DNA (*prnABCD*) has been completely sequenced. It comprises four ORFs, *prnA, prnB, prnC* and *prnD.* All four ORFs are located on a single transcriptional unit. The four genes encode proteins of identical size. Organization of *prn* genes is identical to the order in which the reactions are catalysed in the biosynthetic pathway. Product of *prnA* gene catalyses chlorination of L-trp to 7 chloro-L-trp to form amino pyrrolnitrin (Hammer *et al.*, 1997). *prnD* gene catalyses oxidation of aminopyrrolnitrin to pyrrolnitrin (Nakatsu *et al.*, 1995). The regulation of *prn* operon occurs through the global regulatory gene, *gacA*. de Souza and Raaijmakers (2003) developed primers from the conserved sequences of pyrrolnitrin, which amplified *prnD* from 18 *Pseudomonas* and 4 *Burkholderia* spp. RFLP

analysis revealed polymorphism within 786bp of *prnD* fragment among *Pseudomonas* and *Burkholderia* spp.

2.3.3 Biosynthetic pathway of pyrrolnitrin

p*rnA* gene encodes a tryptophan halogenase that chlorinate tryptophan to 7-chlorotryptophan (7 CT). *prnB* catalyzes 7CT to phenylpyrrole and decarboxylate to monodechloroamino pyrrolnitrin (MDA). *prnC* produce MDA halogenase and catalyzes a second chlorination in the 3 position of pyrrole ring to form amino-pyrrolnitrin. Enyme coded by prnD oxidizes amino group to a nitro group to form pyrrolnitrin (van Pee *et al.*,1980; Fig 3).

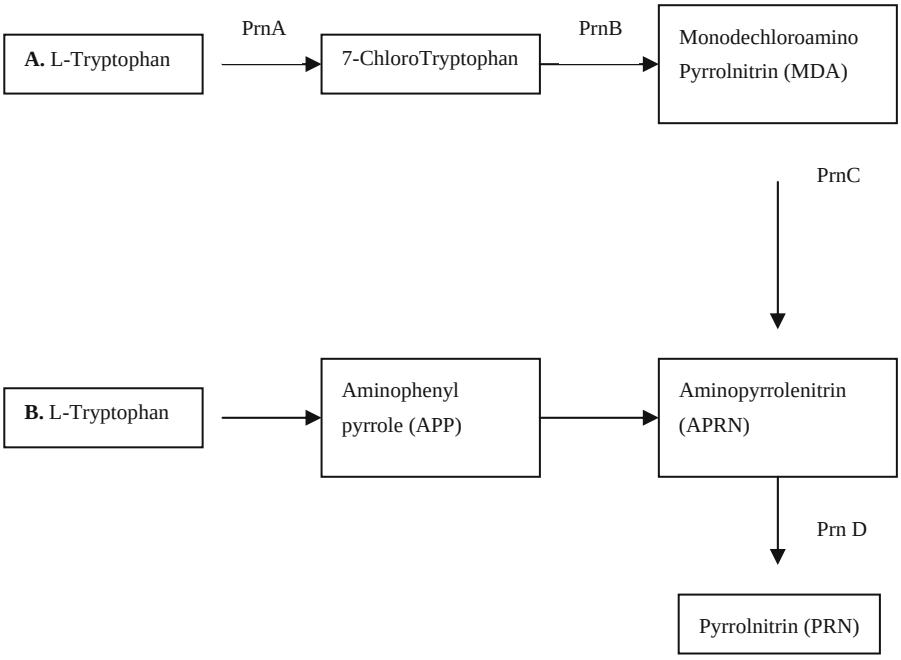

Fig. 3. Pathway for the synthesis of pyrrolnitrin.

2.4 Cyclic lipopeptides

Cyclic lipopeptides (CLPs) are produced by both gram-positive and gram-negative bacteria (Katz and Demain, 1977). Production of different kinds of CLP is common among fluorescent *Pseudomonas* spp. (Nielsen *et al.*, 2002). All CLPs have either 9 or 11 amino acids in the peptide ring with a C_{10} fatty acid at one of the amino acids (Nielsen *et al.*, 2002). Its synthesis is nonribosomal and catalyzed by large peptide synthetase complexes

(Marahiel *et al.*, 1997). CLP is involved in the promotion of bacterial swarming (Givskov *et al.*, 1998; Lindum *et al.*, 1998), with antimicrobial (Takesako *et al.*, 1993; Gerard *et al.*, 1997; Vollenbroich *et al.*, 1997) and biosurfactant properties (Rosenberg and Ron, 1999).

Strains of *P. fluorescens* DR54, 96.578 and DSS73 produce three different CLPs, viscosinamide (Nielsen *et al.*, 2002), tensin (Henriksen *et al.*, 2000), and amphisin (Sorensen *et al.*, 2001) which were antagonistic to *Pythium ultimum* (Nielsen *et al.*, 1998; Nielsen *et al.*, 1999; Thrane *et al.*, 2000) and *R. solani* (Nielsen *et al.*, 2000 and Nielsen *et al.*, 2002). Apart from the antifungal action of viscosinamide it is also involved in the primary metabolism, cell proliferation and strongly binds to the producing cells of the strain DR54 (Nielsen *et al.*, 1999). Tensin and amphisin produced by the strains 96.578 and DSS73 are released into the surrounding medium and suppress the ingress of the pathogen (Nielsen *et al.*, 2000).

Amphisin is a new member of a group of dual-functioning compounds like tensin, viscosin and viscosinamide that have both biosurfactant and antifungal properties. Amphisin is produced at stationary phase. *amsY* gene codes for the synthesis of amphisin synthetase, controlled by two-component regulatory system GacA/GacS (Koch *et al.*, 2002). The ability of *P. fluorescens* strain DSS73 to control *P. ultimum* and *R. solani* arise from amphisin-dependent surface translocation and growth by which the bacterium inhibit *P. ultimum* and *R. solani* (Andersen *et al.*, 2003). *S*ynergistic effect of surface motility and the synthesis of antifungal compounds could efficiently check and terminate growth of pathogen and could prevent the plants from infection by the pathogens.

2.4.1 Durability of CLP in soil

Purified CLPs namely viscosinamide, tensin, and amphisin are highly stable. It was extracted up to 90% (5 µg g^{-1}) when applied to sterile soil. Instead all three compounds degraded within 1 to 3 weeks in nonsterile soil. Concentration of viscosinamide decreased within a week in nonsterile soils augmented with P. *fluorescens* strain DR54 with viscosinamide bound to its cell wall. Addition of strains 96.578 and DSS73 without tensin or amphisin bound to its cell wall did not yield any detectable tensin or amphisin in non-sterile soil. In contrast, germination of sugar beet seeds in nonsterile soil coated with strain DR54 maintained a high and constant viscosinamide level in beet rhizosphere for 2 days. The strains 96.578 and DSS73 exhibited significant production of tensin or amphisin till two days after germination of sugar beet seeds. All three CLPs were found detectable for several days in the rhizosphere. The results thus provide evidence that production of CLPs is habitat specific (produced specifically in rhizosphere)

rather than in the bulk soil, where the rate of degradation is faster (Nielsen *et al.*, 2002).

2.5 Antifungal lipopeptide antibiotics

Bacillus strains produce a broad spectrum of bioactive peptides. A well-known class of such compounds includes the lipopeptides surfactins, fengycin and the iturins compounds (iturins, mycosubtilins and bacillomycins), which are amphiphilic membrane active biosurfactants and peptide antibiotics with potent antimicrobial activities. All these agents occur as families of closely related isoforms which differ in length and branching of the fatty acid side chains and in the amino acid substitutions in the peptide rings (Kowall *et al.*, 1998). The surfactin and iturin compounds are cyclic lipoheptapeptides, contain a beta hydroxy fatty acid and a beta amino fatty acid respectively as lipophilic components.

2.5.1 Iturins

Several strains of *B. subtilis* produce cyclic lipopeptides, which belong to the family Iturin. Iturin A and other antibiotics of their family bacillomycin L, bacillomycin D, bacillomycin F and mycosubtilins are powerful antifungal agents. Iturin A is a cyclolipopeptide containing seven residues of alpha and one residue of beta amino acid. Iturin A has strong antimicrobial action in suppressing *P. ultimum, R. solani, F. oxysporum, S. sclerotiorum and M. phaseolina* (Constantinescu, 2001). Some strains also produce bacilysin and bacillomycin L in addition to Iturin.

Chitarra *et al.* (2003) reported that *B. subtilis* YM10 – 20 produced Iturin like compound that permeabilizes fungal spores and prevents spore germination of *Penicillium roqueforti*. *Bacillus amyloliquefaciens* strain RC-2 produced seven antifungal compounds and inhibited the development of mulberry anthracnose caused by *Colletotrichum dematium* (Hiradate *et al.*, 2002). The antibiotic (Iturin A_2) inhibited other phytopathogenic fungi (*Rosellina necatrix*, *Pyricularia oryzae*), and bacteria (*Agrobacterium tumefaciens* and *Xanthomonas campestris* pv *campestris*) besides *C. dematium in vitro* suggesting that the antibiotics produced by RC-2 has broad spectrum of action against various plant diseases (Yoshida *et al.*, 2001; Yoshida *et al.*, 2002).

Iturin D produced by *B. subtilis* suppressed *C. trifolii*. Crude culture filtrates reduced germination of *C. trifolii* conidia and induced lysis of conidia and formation of inflated germ tubes on germinating conidia (Duville and Boland, 1992). Besson and Michel (1987) isolated antibiotics, iturin D & E from *B. subtilis* producing iturin A. Tsuge *et al.* (2001) reported that *B. subtilis* RB 14 produced an antifungal lipopeptide iturin A. The iturin

A operon is more than 38 kb long and consist four open reading frames, itu D, itu A, itu B and itu C. The itu D gene encodes a putative malonyl coenzyme A transacylase. The second gene itu A, codes a 449-kDa protein similar to fatty acid synthetase, aminoacid transferase, and peptide synthetase. The third and fourth gene, itu B and itu C encode 609 and 297 kDa peptide synthetases. Yu *et al.* (2002) purified three major antifungal compounds from *B. amyloliquefaciens* strains B 94 which has aminoacids Asn, Gln, Ser, Pro and Tyr in a ratio of 3:1:1:1:1. Thus different iturin antibiotics also serve as a major determinant in the management of phytopathogens due to its broad spectrum of action.

2.5.2 Bacillomycin

The antifungal lipopeptide bacillomycin of *B. subtilis* belongs to iturin family and acts with a strict sterol – phospholipid dependence on biomembranes (Volpon *et al.,* 1999). Bacillomycin Lc, being a new antifungal antibiotic of the iturin class differs from Bacillomycin L by sequence changes from aspartate-1 to asparagine – 1 and from glutamine – 5 to glutamate – 5 (Eshita *et al.,* 1995).

B.subtilis produced an antifungal lipopetide bacillomycin D (Besson and Michel, 1992). Similarly Moyne *et al.* (2001) isolated two peptide analogs of bacillomycin D with high antifungal activity against *Aspergillus flavus* from culture filtrate of *B. subtilis* strain Au 195. Peypoux *et al.* (1985) isolated a new antibiotic of the iturin group bacillomycin F which is a mixture of homologous petidolipids. Bacillopeptins, a new iturin group of antifungal antibiotic was isolated from *B. subtilis* FR-2 (Kajimura *et al.,* 1995). Thus different group of antifungal bacillomycin such as bacillomycin Lc, bacillomycin L, bacillomycin D, bacillomycin F and bacillopeptins were identified from different strains of *B. subtilis* were effective against fungal pathogens.

2.5.3 Plipastatin

Plipastatins A and B are antifungal antibiotics belonging to a family of lipopeptides capable of inhibiting phospholipase (A_2) (PLA_2) an enzyme involved in a various cellular processes such as inflammation, acute hypersensitivity and blood platelet aggregation (Volpon *et al.,* 2000). The role of plipstatin in plant disease management has to be explored.

2.5.4 Surfactin

Bacillus subtilis produces another cyclic lipopeptide surfactin with surfactant activity. Surfactin has weak antibiotic activity. *B. subtilis* RB14

produced iturin and surfactin, which had antagonistic activity against *R. solani* (Asaka and Shoda, 1996). *Bacillus* sp. CY22 produced both iturin like antifungal compound and surfactin like biosurfactant (SooJeong *et al.*, 2002).

2.6 Aminopolyols (Zwittermicin A)

Zwittermicin A is a novel bioactive molecule produced by *Bacillus* sp. It is an aminopolyol antibiotic having structural similarities to polyketide antibiotics with broad spectrum of action against various microbes (Silo-Suh *et al.*, 1998; Elizabeth *et al.*, 1999). The diverse biological activity of this novel antibiotics include the suppression of oomycetes diseases of plants and also responsible for the insecticidal activity of *B. thuringiensis* (Emmert *et al.*, 2004). Every gram of soil contains a minimum of 10^4 cfu of Zwittermicin A producers world wide (Raffel *et al.*, 1996). Zwittermicin A is produced by *B. cereus* and *B. thuringiensis* (Raffel *et al.*, 1996) and effective against oomycetes and other pathogenic fungi (Silo-Suh *et al.*, 1998).

2.6.1 Biosynthesis

The gene responsible for the synthesis of Zwittermicin A production and resistance was identified in *B. cereus* UW85 (Silo-Suh *et al.*, 1994). The DNA sequence analysis resulted in the identification of three open reading frames. Two open reading frames had sequence similarity to acyl-CoA dehydrogenases and the acyltransferase domain of polyketide synthases respectively. *orf2* is necessary for antibiotic production. *ZmaR* being the part of the gene cluster, it is essential for the bacterial producer to resist its own Zwittermicin A, but does not have any role in the production of zwittermicin A (Stohl *et al.*, 1999). Synthesis of zwittermicin A has similarities to polyketide synthases (Katz and Donadio, 1993). Genes that encode zwittermicin A biosynthetic enzymes, are involved in the formation of e aminomalonyl-and hydroxymalonyl-acyl carrier protein intermediates (Emert *et al.*, 2004). In addition presence of homologs of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) suggest that zwittermicin A is synthesized by a mixed NRPS/PKS pathway. It enlight that the biostynthetic cluster of zwittermicin A consists 9 open reading frame for the synthesis of zwittermicin A in *B. cereus* UW85 (Table 2), the broad spectrum antibiotic (Emmert *et al.*, 2004).

Table 2. Biosynthetic gene cluster of zwittermicin A and its functions.

Gene	Nucleotide position	No.of amino acids	Function
orf^{3}	78-341	87	Acyl carrier protein
orf^{1}	338-1486	382	Acyl-CoA dehydrogenase
zma^{R}	1483-2610	375	Acetyl transferase (acetylation of zwittermicin A)
orf^{2}	2630-3847	405	Malonyl-CoA-ACP transacylase
orf^{4}	3888-4736	282	3-hydroxybutyryl-CoA dehydrogenase
orf^{5}	4767-5012	81	Acyl carrier protein
orf^{6}	5012-6205	397	Acyl-CoA dehydrogenase
orf^{7}	6202-7779	525	Mycosubtilin synthetase subunit C
orf^{8}	7754-15442	2562	NRPSs/PKSs
orf^{9}*(partial)*	15461-15879	139	Alkanesulfonate monooxygenase

2.7 Volatile antibiotics

2.7.1 Hydrogen cyanide (HCN)

Cyanide is a secondary metabolite produced by gram-negative *P. fluorescens*, *P. aeruginosa*, and *Chromobacterium violaceum* (Askeland and Morrison, 1983). Hydrogen cyanide (HCN) and CO_2 are formed from glycine (Castric, 1977) catalyzed by HCN synthase (Castric, 1994). HCN synthase of *Pseudomonas* sp. oxidize glycine in the presence of electron acceptors, e.g., phenazine methosulfate (Wissing, 1974). *P. fluorescens* CHA0 is an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soil borne fungi (Voisard *et al.*, 1994). HCN production by strain CHA0 suppresses black root rot of tobacco, caused by *Thielaviopsis basicola* (Sacherer *et al.*, 1994). GacA-negative mutants of strain CHA0, defective in synthesis of HCN, antibiotics, and exoenzymes, lost the ability to protect tobacco from black root rot (Voisard *et al.*, 1989).

2.7.2 Aldehydes, alcohols, ketones and sulfides

P. chlororaphis (PA23) isolated from soybean roots produced antifungal volatiles belonging to aldehydes, alcohols, ketones and sulfides. It was inhibitory to all the stages of *S. sclerotiorum* (Fernando *et al.* 2004). Effective antifungal volatiles were benzothiazole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, and nonanal. These substances completely inhibited the growth of mycelium, germination of ascospores and the survival of sclerotia. These volatiles would come in direct contact with the overwintering structures and destruct the sclerotial bodies leading to the reduction in inoculum potential and thereby prevents the disease occurrence (Fernando *et al.* 2004). Bacterial volatiles also promote growth of plants (Ryu *et al.*, 2003a). 2,3-butadienol, enhanced the growth of *Arabidopsis thalliana* (Ryu *et al.*, 2003a), and inhibited the pathogen *Erwinia carotovora* (Ryu et al, 2003b). Production of inhibitory volatiles may increase the survival rate of bacteria in soil, by eliminating potential competitors for nutrients (Mackie and Wheatley, 1999).

3 REGULATION OF BIOSYNTHESIS OF ANTIBIOTICS

Regulation of secondary metabolites production involves:

1. Environment dependent primary sensing
2. A secondary or intermediate level responsible for regulation of antibiotic biosynthesis with other metabolic processes through global regulation and cellular homeostasis
3. A highly specific tertiary level which requires an involvement of regulatory loci that are linked and divergently transcribed from structural genes for antibiotic biosynthetic genes (Elander *et al.*, 1968; You *et al.*, 1998; Duffy and Defago, 1999; Haas *et al.*, 2000; Abbas *et al.*, 2002).

3.1 Two-component regulatory system

3.1.1 GacS/GacA system

It is a trans membrane protein that functions as a sensory kinase GacS and the cytoplasmic cognate response regulator GacA protein. It mediates changes in gene expression in response to sensor signals. Phosphorylation of GacS sensor with the interaction of unknown signals activates GacA response regulator. GacA regulates transcription of the target genes. GacS/GacA system exerts a positive impact on cell density-dependent

gene regulation mediated by signal molecule *N*-acylhomoserine lactone (AHL) in *P. aeruginosa*, *P. syringae* and *P. aureofaciens*. Similar system also operates in *P. fluorescens* CHAO, which do not produce AHL. GacS/GacA modulates the expression of exo enzymes, antibiotics and HCN when cells are in transition from exponential to stationary phase (Fuqua *et al.*, 1994; Sacherer *et al.*, 1994; Blumer *et al.*, 1999; Chancey *et al.*, 1999; Elasri *et al.*, 2001; Heeb and Haas 2001).

Bacterial populations in natural ecosystem communicate with each other through chemical signals, released in a cell density-dependent manner, which means a minimum cell number is needed to communicate with each other known as quorum sensing. It operates through amino acids, short peptide hormones and fatty acid-derivatives such as AHLs. The bacteria reach a high population density on the rhizosphere and form a biofilm. It results in the accumulation of fatty acid-derivative, AHL and regulates various physiological processes (Chin A-Woeng *et al*., 2003).

3.1.2 LuxI and LuxR proteins based regulation

Another large family of regulatory systems in biosynthesis of antibiotics has similarity to LuxI and LuxR proteins of *V. fischeri*. This system relies cell concentration dependent communication. LuxI-type proteins synthesize auto inducer molecule AHLs. It diffuses from producer bacteria either passively or by active efflux. AHLs accumulate at high population densities, bind and activate LuxR-type receptor proteins that function as cytoplasmic transcriptional factors or as repressors (Whitehead *et al.*, 2001).

3.2 Sigma factors based regulation

Another level of antibiotic regulation involves sigma factors, which are an integral component of regulation of antibiotics like Phl and Plt as in *P. fluorescens* Pf-5. *rpoD* gene activates the synthesis of antibiotics. Over expression of activator gene *rpoD* or mutation or deletion of suppressor gene *rpoS* increases Phl or Plt production. The genes *rpoD* and *rpoS* encode sigma-factor *s*32 and stationary-phase *s*38 respectively. s factors are required during transcription. Any imbalance of s factors either due to excess of *s*32 or lack of *s*38 might enhance the expression of genes coding for the synthesis of antibiotics (Bangera and Thomashaw, 1996; Howell and Stipanovic, 1979). In addition, pathway-specific regulators have been reported in the regulation of Phl biosynthesis. Phl biosynthetic gene cluster is negatively regulated by the repressor Phl F and positively regulated by PhlH (Delany *et al.*, 2000; Abbas *et al.*, 2002). RNA binding protein RsmA and RsmB regulate Phl production at post-transcriptional level. RsmA is a

translational repressor protein. Both, GacA and RsmA depend on the same specific 'RBS regions' (Ribosome Binding Site), which enhances RsmA-mediated translational repression. Another factor, RsmB exerts a relief to repression. Thus, these molecules of RNA bind and sequester the repressor proteins. Over expression of a regulatory RNA encoded by *prrB* homologue of RsmB restores Phl production in *gacA* and *gacS* mutants. It leads to overproduction of Phl in wild-type *P. fluorescens* (Liu and Romeo, 1997; Romeo, 1998; Blumer *et al.*, 1999; Ma *et al.*, 2001; Abbas *et al.*, 2002).

3.3 Microbial metabolites in antibiotic regulation

Extracellular secretion of metabolites also regulates the synthesis of antibiotics. Synthesis of DAPG is auto induced and repressed by other bacterial extracellular metabolites of strain CHAO. Salicylate, fusaric acid and pyoluteorin have negative effect on DAPG production**.** Salicylate interacts with repressor PhlF and stabilizes its interaction with *phl*A promoter (Abbas *et al.*, 2002).

4 MOLECULAR DETECTION OF ANTIBIOTICS

Identification of antibiotic producers by the isolation of extracellular metabolites and characterization with the standard antibiotic is time consuming and laborious. The availability of sequenced biosynthetic and regulatory genes aid in the development of primers specific to the desired antibiotics of interest. The biosynthetic genes responsible for the production of antibiotics such as zwittermycin A produced by *B. cereus*, 2,4-DAPG, phenazine (PHZs), pyrrolnitrin (PRN) and pyoluteorin (PLT) produced by different *Pseudomonas* sp. has been cloned and either partially or fully sequenced. It helps to enumerate microorganisms capable to produce antibiotics or to evaluate and exploit the diversity among the population without cultivating them. These molecular techniques target conserved DNA sequences with well-defined biosynthetic gene clusters. The sensitivity and specificity of detection depend on the selection or design of appropriate targets, probes, or primers, and on control of the stringency of PCR amplification or DNA hybridization.

Target selection requires amplification of full-length genes (Seow *et al.*, 1997), or the amplification of a well-conserved internal fragment. Amplified fragments commonly range in size from about 600 to 1,000 bp or more and can be analyzed for DNA sequence or restriction fragment length polymorphisms to confirm identity or evaluate genetic diversity within target populations.

phlD gene is an important gene in the biosynthetic pathway of DAPG. Hence the limited distribution of *phlD* gene among bacterial community has made it as a marker gene to fish out DAPG producers. McSpadden Gardener and his coworkers during 2001 cloned and sequenced the major portion of the *phlD* open reading frame from five genotypically different strains. The sequence was screened for the conserved region of the gene specific amplification. Eight different primers were designed and screened. The primers B2PF and BPR4 were highly précised to amplify the target gene. These primers were highly sensitive to even detect as few as log 2.4 cells per sample. This method was used for detecting both inoculants and indigenous DAPG producing pseudomonads (McSpadden Gardener *et al.*, 2001).

Strains that produce Zwittermicin A have a gene responsible for the self-resistance against the action of its own antibiotic. The resistance gene was *zma*R. Usage of *zma*R primers as molecular markers was précised in the detection of zwittermicin A producers (Raffel *et al.*, 1996). It was a more reliable method for identification of zwittermycin A-producers than FAME (fatty acid methyl ester) analysis. Giacomodonato *et al.* (2001) developed primers for the conserved sequences in genes involved in biosynthesis of peptide antibiotics for screening *Bacillus* isolates. Among *Bacillus* isolates that gave a positive signal in PCR, three had an inhibitory effect to *Sclerotinia sclerotiorum*. The strains that failed to amplify did not inhibit fungal growth. Ramarathnam and Fernando (2004) found the presence of zwittermycin A self-resistant gene in the endophytes *Bacillus cereus* strains E4, E8 and E13 isolated from canola with the product size of 1000 bp using the primers 677 and 678. Similarly its presence was also detected in *B. cereus* strain BS8, *B. cereus* strain L and *B. mycoides* strain S (Zhang and Fernando, 2004b). Also Ramarathnam and Fernando (unpublished) have developed two novel primers from Zwittermycin A biosynthetic gene. The primers used for the detection of various antibiotics from rhizobacteria are listed in table 3.

5 BROAD SPECTRUM ACTION OF ANTIBIOTICS BY PGPR

Antibiotics encompass a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms at low concentrations that are deleterious to the growth or metabolic activities of other microorganisms (Fravel, 1988; Thomashow *et al.*, 1997). Antibiotics produced by different PGPR have a broad-spectrum activity.

The broad-spectrum activity of pyrrolnitrin, produced by *Pseudomonas* and *Burkholderia* species, was noticed in 1960s by Japanese scientists (Nishida *et al.*, 1965) who tested and further developed this antibiotic for therapeutic purposes against human pathogenic bacteria and

Table 3. Antibiotics and their primers for the detection of antibiotic producers (Zhang, 2004).

Primer	Sequence	Antibiotics related	Reference
PHZ1	GGC GAC ATG GTC AAC GG	PCA	Delaney *et al.* (2001)
PHZ2	CGG CTG GCG GCG TAT AT	PCA	Delaney *et al.* (2001)
PHZX	TTT TTT CAT ATG CCT GCT TCG CTT TC	PCA	Delaney *et al.* (2001)
PHZY	TTT GGA TCC TTA AGT TGG AAT GCC TCC	PCA	Delaney *et al.* (2001)
PCA2a	TTG CCA AGC CTC GCT CCA AC	PCA	Raaijmakers *et al.* (1997)
PCA3b	CCG CGT TGT TCC TCG TTC AT	PCA	Raaijmakers *et al.* (1997)
Phl2a	GAG GAC GTC GAA GAC CAC CA	2,4-DAPG	Raaijmakers *et al.* (1997)
Phl2b	ACC GCA GCA TCG TGT ATG AG	2,4-DAPG	Raaijmakers *et al.* (1997)
BPF2	ACA TCG TGC ACC GGT TTC ATG ATG	2,4-DAPG	McSpadden Gardener *et al.* (2001)
B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	2,4-DAPG	McSpadden Gardener *et al.* (2001)
BPF3	ACT TGA TCA ATG ACC TGG GCC TGC	2,4-DAPG	McSpadden Gardener *et al.* (2001)
BPR2	GAG CGC AAT GTT GAT TGA AGG TCT C	2,4-DAPG	McSpadden Gardener *et al.* (2001)
BPR3	GGT GCG ACA TCT TTA ATG GAG TTC	2,4-DAPG	McSpadden Gardener *et al.* (2001)

Continued table 3.

BPR4	CCG CCG GTA TGG AAG ATG AAA AAG TC	2,4-DAPG	McSpadden Gardener *et al.* (2001)
PrnAF	GTG TTC TTC GAC TTC CTC GG	Pyrrolnitrin	Carolyn Press, personal communication
PrnAR	TGC CGG TTC GCG AGC CAG A	Pyrrolnitrin	Carolyn Press, personal communication
PRND1	GGG GCG GGC CGT GGT GAT GGA	Pyrrolnitrin	de Souza and Raaijmakers, (2003)
PRND2	YCC CGC SGC CTG YCT GGT CTG	Pyrrolnitrin	de Souza and Raaijmakers, (2003)
PrnCf	CCA CAA GCC CGG CCA GGA GC	Pyrrolnitrin	Mavrodi *et al.* (2001)
PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	Pyrrolnitrin	Mavrodi *et al.* (2001)
PltCreg1F	AGG CAA TCA CTA CCA TCC GTG CGC	Pyoluteorin	de Souza and Raaijmakers, (2003)
PltCreg2r	ATG AGG AGC AGG AGG TGT CGA GCA C	Pyoluteorin	de Souza and Raaijmakers,(2003)
PLTC1	AAC AGA TCG CCC CGG TAC AGA ACG	Pyoluteorin	de Souza and Raaijmakers,(2003)
PLTC2	AGG CCC GGA CAC TCA AGA AAC TCG	Pyoluteorin	de Souza and Raaijmakers,(2003)
PltBf	CGG AGC ATG GAC CCC CAG C	Pyoluteorin	Mavrodi *et al.* (2001)
PltBr	GTG CCC GAT ATT GGT CTT GAC C	Pyoluteorin	Mavrodi *et al.* (2001)
Plt1	ACT AAA CAC CCA GTC GAA GG	Pyoluteorin	Mavrodi *et al.* 2001
Plt2	AGG TAA TCC ATG CCC AGC	Pyoluteorin	Mavrodi *et al.* (2001)
678	ATG TGC ACT TGT ATG GGC AG	Zwittermicin A	Milner *et al.* (1996)
667	TAA AGC TCG TCC CTC TTC AG	Zwittermicin A	Milner *et al.* (1996)

fungi. With respect to plant pathogenic fungi, pyrrolnitrin has antifungal activity against a wide range of Basidiomycetes, Deuteromycetes, Ascomycetes and Oomycetes, including several economically important pathogens like *R. solani, Verticillium dahliae, Pyricularia oryzae, Alternaria* sp., *Botrytis cinerea, P. aphanidermatum, P. ultimum, Rhizopus sp. Aspergillus niger, Fusarium oxysporum, Penicillium expansum, Sclerotinia sclerotiorum* and *Sclerotium rolfsi* (Howell and Stipanovic 1979; Homma *et al.,* 1989; Chernin *et al.,* 1996; Ligon *et al.,* 2000). Furthermore, pyrrolnitrin was also reported to be active against several bacteria, such as *Agrobacterium tumefaciens, Corynebacterium insidiousum, Pseudomonas syringae* pv. *syringae, Xanthomonas campestris, Clavibacterium michiganense, Serratia marcescens* (Chernin *et al.* 1996) and in particular *Streptomyces* species (El-Banna and Winkelmann 1998).

Similarly, DAPG, produced by several strains of *P. fluorescens*, not only have activity against a wide range of plant pathogenic fungi but also have antibacterial, antihelminthic and phytotoxic properties (Keel *et al.* 1992; Thomashow and Weller 1996). Cronin *et al.* (1997) showed that purified DAPG decreased hatching of cysts of the nematode *Globodera rostochiensis* and reduced juvenile mobility. Also zwittermycin A, an antibiotic produced by *B. cereus* and *B. thuringiensis* adversely affects the growth and activity of a wide range of microorganisms, including several plant pathogens.

Zwittermicin A inhibited a wide spectrum of protists, oomycetes, some other fungi and bacteria. The activity was more at alkaline pH. It has synergistic action with kanosamine against *E.coli* and *Phytophthora* (Silo-Suh *et al.,* 1998). UW85 suppressed alfalfa damping off (Silo-Suh *et al.,* 1994), fruit rot of cucumber (Smith *et al.,* 1993) and *Phytophthora parasitica* var. *nicotianae* infection in tobacco (He *et al.,* 1994). Suppression was mainly due to the production of zwittermicin A (Silo-Suh *et al.,* 1994).

6 ANTIBIOTICS OF PGPR IN THE MANAGEMENT OF SOIL-BORNE DISEASES

The significance of antibiotics in biocontrol, and in microbial antagonism has been questioned because of the constraints to antibiotic production in natural environments (Williams & Vickers 1986). Recovery and detection may be hampered by biotic and abiotic complexity, chemical instability of the compound, irreversible binding to soil colloids or organic matter, or microbial decomposition (Thomashow *et al.,* 1997). The first line of evidence of broad-spectrum activity of antibiotics by PGPR was derived from culture filtrates or purified antibiotics (Howell and Stipanovic 1979;

Kang *et al.* 1998; Nakayama *et al.*, 1999). Suppression of Pythium root rot of cucumber was improved by enhancing the production of DAPG and pyoluteorin in *P. fluorescens* strain CHA0 (Maurhofer *et al.,* 1992; Fenton *et al.*, 1992) (Table 4).

Seed bacterization of tomato and chilli with a talc based consortia comprising of *P. fluorescens* and *P. chlororaphis* performed better in reducing the incidence of damping-off (Kavitha *et al.*, 2003). It also increased the biomatter production. *In vitro* assay explained the role of phenazine in suppressing *P. aphanidermatum* the causal agent of damping-off. Aerugine [4-hydroxymethyl-2-(2-hydroxyphenyl)-2-thiazoline] was

Table 4. Antibiotics of PGPR in the management of soil-borne diseases.

Antibiotics	**PGPR**	**Pathogen**	**Crop**	**Reference**
DAPG	*Pseudomonas* sp.	*P. ultimum*	Sugar beet	Shanahan *et al.* (1992b).
DAPG	*P. fluorescens* (CHAO)	*Theilaviopsis basicola*	Tobacco	Keel *et al.* (1992).
Aerugine	*P. fluorescens*	*Phytophthora*	Pepper	Lee *et al.*(2003)
		C. orbiculare	Cucumber	Lee *et al.*(2003)
Phenazine	*Pseudomonas sp.*	*F. oxysporum*	Tomato	Chin-A-Woeng *et al.* (1998)
PCA	*P. fluorescens*	*G. g.* Var. *tritici*	Wheat	Thomashow and Weller (1988)
Pyrrolnitrin	*Burkholderia cepacia*	*F. sambucinum*	Potato	Burkhead *et al.*(1994)
Pyrrolnitrin	*P. fluorescens*	*R. solani*	Cotton & Cucumber	Hammer *et al.* (1997)
Pyrrolnitrin	*P. fluorescens*	*V. dahliae*	Cotton	Howell and
		T. basicola	Cotton	Stipanovic (1979)
Pyrrolnitrin	*P. cepacia*	*F. sambucinum*	Potato	Burkhead *et al.* (1994)
Pyrrolnitrin	*P. cepacia*	*Sclerotinia sclerotiorum*	Sunflower	McLoughlin *et al.* (1992)
Viscosinamide	*P. fluorescens*	*R. solani*	Sugar beet	Nielsen *et al.*(1998)
		P. ultimum		
Pantocin A,B	*P. agglomerans*	*Erwinia herbicola*	Apple	Wright *et al.* (2001)
Pyoluteorin	*P. fluorescens*	*Pythium spp.*	Cotton	Howell and
		Pythium spp	Sugarbeet	Stipanovic (1980)

effective against *C. orbiculare*, *P. capsici*, and *P. ultimum* (MICs - 10 $\mu g\,ml^{-1}$). Treatment with aerugine suppressed development of Phytophthora disease on pepper and anthracnose on cucumber (Lee *et al.*, 2003). Spray of PA23 (*P. chlororaphis*) to canola during 50 per cent blooming controlled the infection of *S. sclerotiorum* (Savchuk and Fernando, 2004). Application of PA23 through rhizome and soil suppressed the incidence of rhizome rot caused by *P. aphanidermatum* (Nakkeeran *et al.*, 2004).

7 ISR MEDIATED BY BACTERIAL ANTIBIOTICS

In several PGPR, more than one determinant is operative in triggering systemic resistance (ISR) in plant. The involvement of antibiotics production in ISR has not been investigated in detail. Pyoluteorin and DAPG reduced growth of sweet corn, cress and cucumber and in turn the stress caused by these antibiotics may also trigger resistance (Maurhofer *et al.*, 1992). Pyocyanin induced ISR in radish against Fusarium wilt of tomato (Leeman *et al.*, 1995 and Audenaert *et al.*, 2001; 2002). But a salicylic acid or pyocyanine mutant of wild type *P. aeruginosa* 7NSK2 was unable to induce resistance against *B. cinerea* (Audenaert *et al.*, 2001). It was hypothesized that the pyochelin precursor salicylic acid was produced in nanograms on roots. It was converted to siderophore pyochelin. The pyochelin and pyocyanine act synergistically to produce active oxygen species that cause cell damage and it subsequently leads to induced resistance (Audenaert *et al.*, 2001). Rhizosphere colonization of *P. fluorescens* protected tomato from wilt disease by accumulating the pool of DAPG around tomato roots (Aino *et al.*, 1997). Hence the accumulation of DAPG in the roots might be as a signal to trigger ISR (Haas and Keel, 2003).

8 FACTORS MODULATING ANTIBIOTIC PRODUCTION AND ITS EFFICACY

The inconsistencies of antibiotics are attributed to the involvement of biotic and abiotic factors. The factors include:

- Physical factors
- Acetylation
- Variation in sensitivity
- Cell concentration
- Growth phase

8.1 Physical factors

8.1.1 pH, temperature and soil moisture

The physical factors that affect antibiotic production are temperature (Shanahan *et al.* 1992b), soil moisture (Georgakopoulos *et al.* 1994), and pH (Ownley *et al.* 1992). Chin-A-Woeng *et al.* (1998) observed that at pH 5.7, the *in vitro* antifungal activity of phenazine-1-carboxamide was 10 times higher than phenazine-1-carboxylic acid (PCA). PCA activity was completely abolished under less acidic conditions. The activity of DAPG is more active against *Pythium* species at acidic than at neutral to alkaline pH *in vitro* (de Souza *et al.*, 2003).

In general secondary metabolites accumulate until the beginning of stationary phase. In *P. fluorescens* CHA0, 2,4-DAPG and MAPG accumulate until the beginning of stationary phase. Subsequently, there after the concentrations of the two metabolites decreased. At 18°C accumulation and degradation rates of 2,4-DAPG were slowed down and the concentrations was doubled than the concentration at 30°C.

Microbes in the rhizosphere depend on substrates liberated from the root or shoot for their growth and for the antibiotic production. Incorporation of alfalfa seedling exudates to the culture medium enhanced the production kanosamine in *B. cereus* by 300% (Milner *et al.*, 1996).

8.1.2 Nutrients

Nutrient sources like carbon, inorganic phosphate and minerals influence the production of antibiotics by *P. fluorescens* (Duffy and Defago, 1999). DAPG production by all the strains was stimulated in glucose-amended medium. But stimulation of DAPG production by zinc occurred in a strain-specific manner. Phosphate repressed DAPG production in *B. cereus* (Millner *et al.*, 1996) and phenazine production in *P. fluorescens* (Slininger & Jackson 1992). Zn2+, NH4Mo2+, and glucose stimulated production of PHL. Production of PLT was stimulated by Zn^{2+}, Co^{2+}, and glycerol but was repressed by glucose. Fructose, mannitol, and a mixture of Zn2+ and NH4Mo2+ increased pyrrolnitrin production. Co2+, fructose, mannitol, and glucose increased pyochelin production. Interestingly, production of its precursor salicylic acid was increased by different factors, i.e., NH_4Mo^{2+}, glycerol, and glucose. The mixture of Zn^{2+} and NH_4Mo^{2+} with fructose, mannitol, or glycerol further enhanced the production of PHL and PLT compared with either the minerals or the carbon sources used alone.

8.2 Level of acetylation

The phloroglucinol derivative 2,4-DAPG was more antifungal than MAPG and PG. The mycelial growth of *P. ultimum* var. *sporangiiferum* was completely inhibited at a concentration of 32 µg/ml. But for MAPG and PG at least a 10 fold higher concentration was necessary to exert antimicrobial action. Hence the level of acetylation decides the antimicrobial action of DAPG (deSouza *et al.*, 2003).

8.3 Variation in sensitivity

The antimicrobial action of the antibiotics found to differ between the different stages of life cycle of the pathogen and between the species. This is an important factor in the biological efficiency of the antibiotics. Various propagules of *P. ultimum* that are part of the asexual stage of the life cycle differed considerably in their sensitivity to DAPG (deSouza *et al.*, 2003).

8.4 Cell concentration / Growth stage

Synthesis of antibiotics by fluorescent pseudomonads responds to cell density, showing higher expression in stationary phase. For *Pseudomonas aureofaciens* 30-84, it has been demonstrated that the cell-density-dependent regulation response known as quorum sensing interacts with this regulatory response (Pierson *et al.*, 1995). A genomic Tn*5* insertion mutant of *P. putida* showed 90% decrease in *rpoS* promoter activity, resulting in less RpoS in a cell at stationary phase (Lange *et al.*, 1995; Kojic and Venturi, 2001)**.**

9 CONCLUSIONS

Nature is bestowed with an enriched biodiversity of PGPR. The dominant bacterial microfloras in the PGPR community include *Pseudomonas* spp., and *Bacillus* spp. The research over the last decade has resulted in the introduction of several well-characterized *Pseudomonas* spp. that helps in understanding regulation and organization of the biosynthetic gene clusters involved in the production of antibiotics. The knowledge on the regulation of antibiotics will lead to the development of PGPR with improved reliability and efficacy. Molecular communication between different genera and species of PGPR might help in the selection of compatible strains to be released under field conditions. The antibiotic

DAPG acts as a signal molecule to trigger the gene expression in the related species of *Pseudomonas*. But at the same time the presence of antibiotic producers like pyoluteorin suppress the expression and production of DAPG by fluorescent pseudomonads. Though DAPG and pyoluteorin pertains to the same class namely polyketides the expression of one type suppress the other. Apart from it the communication and interaction of soil-borne pathogens with PGPR also suppress the expression of the gene in fluorescent pseudomonads for the production of DAPG.

The research on the communication between different types of antibiotic producers, its interaction with abiotic environment, plant pathogens and the plant is only in its stage of infancy. Intensification of research in this field will help in understanding the interaction of PGPR, pathogen, plant and abiotic environment around the rhizosphere. This will facilitate the researchers to fish out better biocontrol agents that overcome the negative cross talk in the environment around the rhizosphere.

Knowledge on the distribution of antibiotic genes and the ecology of the organisms in the natural environment could facilitate the introduction of non-indigenous strains and would also favour in the selection of better biocontrol strains that are suited to different ecological conditions and for different crops. The increasing understanding of the role of AHL signal molecule in the production of antifungal metabolites through quorum sensing and the identification of promoters that can be induced or boosted in the rhizosphere opens new areas for the development of novel biocontrol agents.

Though antibiotics play a vital role in the management of plant diseases, chemotaxis and motility of the bacteria decides the antifungal action of the antibiotic producers. Antibiotic producers are highly effective in suppression of plant pathogens *in vitro*. However, the quantity of antibiotics produced under field conditions in the rhizosphere are below the minimal inhibitory concentration required for the suppression of plant pathogens. Availability of antibiotics below the minimum level might be due to the biotic and abiotic complexity of the soil and due to the irreversible binding to soil colloids or organic matter or microbial degradation. Even under these circumstances if the antibiotic producers are able to control plant diseases it may be due to the involvement of systemic resistance mediated by the antibiotics at very low concentration or due to the interaction of antibiotics with other extra cellular metabolites that may trigger ISR. The interaction effect of antibiotics, hydrolytic enzymes, lipopolysacchrides, hydrogen cyanide and active oxygen species involved in induction of systemic resistance has to be explored. Though antibiotics of PGPR play a key role in plant disease management, the research gaps in suppressing the antimicrobial action has to be intensified to exploit the usage of antibiotics in disease management.

Since the quantum of antibiotic produced in the rhizosphere is less than the inhibitory level, understanding of the synergistic action of antifungal proteins produced by the rhizobacteria coupled with ISR mechanisms will be a promising strategy to overcome the inconsistent biocontrol activity against pest and diseases. Development of consortial formulation of PGPR with different modes of action and compatible signaling interaction between the bacterial strains should be developed so that the sensitive receptors in the plant rhizosphere can perceive the signals and trigger resistance in the plant to overcome the attack of the pests and pathogens.

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Chapter 4

PGPR: PROSPECTIVE BIOCONTROL AGENTS OF PLANT PATHOGENS

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Abstract: Plant growth promoting rhizobacteria (PGPR) are indigenous to soil and the plant rhizosphere and play a major role in the biocontrol of plant pathogens. PGPR can profoundly improve seed germination, root development and water utilization by plants. These rhizobacteria can stimulate plant growth directly by producing growth hormones and improving nutrient uptake or indirectly by changing microbial balance in the rhizosphere in favour of beneficial microorganisms. They can suppress a broad spectrum of bacterial, fungal and nematode diseases. PGPR can also provide protection against viral diseases. The use of PGPR has become a common practice in many regions of the world. Although significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies, results in the field have been inconsistent. Recent progress in our understanding of their diversity, colonizing ability, mechanisms of action, formulation and application should facilitate their development as reliable biocontrol agents against plant pathogens. Some of these rhizobacteria may also be used in integrated pest management programmes. Greater application of PGPR is possible in agriculture for biocontrol of plant pathogens and biofertilization.

K**ey words**: biocontrol; PGPR; plant diseases; plant pathogens; rhizosphere.

1 INTRODUCTION

Biocontrol broadly refers the use of one living organism to curtail the growth and proliferation of another, undesirable one. Biocontrol can be defined as “any condition under which a practice whereby survival or activity of a pathogen is reduced through the agency of another living organisms (except by man himself) with the result there is a reduction in incidence of disease caused by pathogens” (Garrette,1965). Rhizosphere microorganisms may provide a front line defense against pathogen attack

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and are ideal for use as biocontrol agents (Weller, 1988). Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health (Handelsman and Stabb, 1996). Disease suppression by biocontrol agents is the manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. Biocontrol of plant pathogens is now an established sub discipline of plant pathology and more than 80 biocontrol products have been marketed worldwide for the control of plant diseases (Paulitz and Belanger, 2001).

Some bacteria are associated with roots of crop plants and exert beneficial effects on their hosts and are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR inhabit the rhizosphere, the volume of soil under the immediate influence of the plant root system, and favors the establishment of a large amount of active microbial population. Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere microorganisms (Handelsman and Stabb, 1996). The rhizosphere is subject to dramatic changes and the dynamic nature of the rhizosphere creates interactions that lead to biocontrol of diseases (Rovira, 1965, 1969, 1991; Hawes, 1991; Waisel *et al*., 1991). PGPR are free-living bacteria that may impart beneficial effects on plants. PGPR enhance emergence, colonize roots and stimulate overall plant growth. PGPR also improve seed germination, root development, mineral nutrition and water utilization. They can also suppress diseases of plants. The manipulation of the crop rhizosphere by inoculation with PGPR for biocontrol of plant pathogens has shown considerable promise (Handelsman and Stabb, 1996; Siddiqui and Mahmood, 1999; Nelson, 2004). Biocontrol of plant diseases is particularly complex because these diseases mostly occur in the dynamic environment at the interface of the plant root as well as in the aerial parts of plants. Numerous recent reviews present comprehensively the variety of microbial biocontrol agents (Weller, 1988; Handelsman and Stabb, 1996; Siddiqui and Mahmood, 1995a, 1996, 1999; Whipps, 2001; Weller *et al*., 2002; Bakker *et al.*, 2003). This chapter presents recent advances in our understanding of the biocontrol of plant diseases. This understanding will facilitate the application of PGPR for the biocontrol of plant diseases under field conditions.

2 MECHANISMS OF DISEASE SUPPRESSION

PGPR can directly stimulate plant growth in several different ways. They can:

- Fix atmospheric nitrogen
- Synthesize several plant hormones
- Solubilize minerals
- Synthesize enzymes that can modulate plant hormone levels

A particular plant growth promoting bacterium may possess one or more of these mechanisms. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one more phytopathogenic organism by:

- Producing siderophores that limit the available iron to the pathogen
- Producing antibiotics that kill the pathogen
- Inducing systemic resistance in plant

PGPR also cause cell wall structural modifications and biochemical/ physiological changes leading to the synthesis of proteins and chemicals involved in plant defense mechanisms. PGPR has been successfully used for the biocontrol of fungal, nematode, bacterial and viral diseases of plants in different parts of the world (Tables 1-4). Some of the biocontrol mechanisms that have been dealt with in detail and will be discussed are as follows:

- Interactions of PGPR with pathogens
- Interactions of PGPR with plants
- Interactions of PGPR in the rhizosphere

Table 1. Effects of PGPR on fungal diseases of plants.

PGPR	**Fungus**	**Effect**	**Reference**
P. fluorescens	*Gaeumannomyces graminis* var. *tritici*	Strains of *P. fluorescens* may be involved in the suppression of *G. graminis var. tritici.*	Cook and Rovira , 1976
P. fluorescens	*Pythium sp.*	In *Pythium* contaminated sites, significant increases were observed in plant height, number of heads and grain yield of winter wheat.	Weller and Cook, 1986
Pseudomonas spp (fluorescent strains)	*Gaeumannomyces graminis*	27% yield increase due to biocontrol of bacteria in winter wheat under field conditions.	De Freitas and Germida, 1990
Pseudomonas fluorescens	*Fusarium* sp.	Observed induced resistance and phytoalexin accumulation in carnation.	Van Peer *et al*., 1991
P. cepacia R55, R85 *P. putida* R104	*Rhizoctonia solani*	Increase of 62-78% of dry weight of winter wheat grown in *R. solani* infected soil.	De Freitas and Germida, 1991
B. licheniformis, A. faecalis	*Macrophomina phaseolina*	Reduced root-rot disease of chickpea.	Siddiqui and Mahmood, 1992

Continued table 1.

P. aureofaciens Q2-87	*G. graminis* var. *tritici*	Inhibition of fungus was demonstrated both *in vitro* and *in vivo*.	Harrison *et al.*, 1993
P. putida *P. fluorescens* *P. alcaligenes*	*Sclerotium rolfsii*, *Fusarium*	Reduced the incidence of disease caused by *S. rolfsii* in bean, and fusarium wilt of cotton and tomato.	Gamliel and Katan, 1993
B. subtilis	*M. phaseolina*	*B. subtilis* was superior to *P. lilacinus* for the management of *M. phaseolina* on chickpea.	Siddiqui and Mahmood, 1993
B. subtilis	*Fusarium udum*	Increased shoot dry weight and reduced wilt of pigeonpea.	Siddiqui and Mahmood, 1995b
B. subtilis	*M. phaseolina*	*B. subtilis* resulted in greater shoot dry weight of chickpea than with any fungal filtrate.	Siddiqui and Mahmood, 1995c
P. fluorescens	*F. oxysporum* f. sp. *raphani* *A. brassicicola*, *F. oxysporum*	Protected radish plants through induction of systemic resistance against these pathogens.	Hoffland *et al*., 1996
P. chlororaphis 2E3,O6	*Fusarium culmorum*	Strong inhibition of the fungus on spring wheat in the field.	Kropp *et al*., 1996
P. putida, *S. marcescens*, *Flavomonas oryzihabitans*, *B. pumilus*	*Colletotrichum orbiculare*	PGPR mediated ISR was operative under field conditions against naturally occurring anthracnose of cucumber.	Wei *et al*., 1996
B. pumilis *B. subtilis*, *Curtobacterium flaccumfaciens*	*Colletotrichum orbiculare*	Mixture of these PGPR strains as seed treatment caused disease reduction on cucumber.	Raupach and Kloepper, 1998
Pseudomonas PsJN	*Verticillium dahliae*	Reduced disease incidence in tomato.	Sharma and Nowak, 1998
P. fluorescens	*Fusarium udum*	Wilt incidence was reduced in pigeonpea.	Siddiqui *et al*., 1998
P. corrugate, *P. aureofaciens*	*Pythium aphanidermatum*	Induced systemic resistance in cucumber roots.	Chen *et al*., 1999.
P. putida, *B. subtilis*, *E. aerogenes*, *E.agglomerans*, *B. cereus*	*Pythium* sp	Most strains increased root length of cucumber in *Pythium*-infected plants *in vitro.*	Uthede *et al*., 1999
B. subtilis *P. putida*	*Pythium aphanidermatum*, *F. o.* f. sp. *cucurbitacearum*	Growth and yield of lettuce and cucumber were increased and disease severity reduced.	Amer and Utkhede, 2000

Continued table 1.			
B. pumilus SE34 *S. marsescens* 90-166	*Cronartium quercuum* f. sp. *fusiforme*	Two bacterial isolates out of 8, significantly reduced number of galls and induced systemic resistance against fusiform rust on Loblolly pine.	Enebak and Carey, 2000
B. subtilis AF1	*Aspergillus niger, Fusarium udum*	AF1 supplemented with chitin or chitin material showed better control of crown rot and wilt diseases of ground nut and pigeonpea.	Manjula and Podile, 2001
Pseudomonas fluorescens	*Rhizoctonia solani*	Mixture of 3 strains reduced disease and promoted growth of rice.	Nandakumar *et al*., 2001.
S. marcescens 90-166	*Colletotrichum orbiculare*	Seed treatment suppressed anthracnose of cucumber.	Press *et al.*, 2001
Pseudomonas fluorescens	*Colletotrichum capsici*	Increased accumulation of enzymes involved in phenyl propanoid pathway and PR-proteins in hot pepper.	Ramamoorthy and Samiyappan, 2001
P. fluorescens 4-92	*M. phaseolina*	*P. fluorescens* increased disease resistance by 33% in chickpea.	Srivastava *et al.*, 2001
Pseudomonas PsJn	*Botrytis cineria*	PsJn inhibits growth of *B. cineria* by disrupting cellular membrane and cell death.	Barka *et al.*, 2002
Bacillus species BC121	*Curvularia lunata*	Showed high antagonistic activity against *C. lunata*.	Basha and Ulaganathan, 2002
Pseudomonas aeruginosa, Pseudomonas fluorescens	*Colletotrichum lindemuthianum*	*P. aeruginosa* induced resistance only in resistant interactions while *P. fluorescens* induced resistance in susceptible and moderately resistant interactions on bean.	Bigirimana and Hofte, 2002
P. fluorescens	*R. solani, F. oxysporum*	Out of 40 strains, 18 strains showed strong antifungal activity.	Kumar *et al.*, 2002
P. fluorescens 89B61 *B. pumilus*SE34	*Phytophthora infestans*	Elicited systemic protection against late blight of tomato and reduced disease severity.	Yan *et al*., 2002
P. fluorescens strains Pf1, FP7	*Cnaphalocrocis medinalis*	Mixture of two strains performed better than the individual strains in reducing sheath blight of rice.	Radja Commare *et al*., 2002
P. fluorescens Pf1	*F. oxysporum* f. sp. *lycopersici*	Pf1 protected tomato plants from wilt disease.	Ramamoorthy *et al*., 2002a
P. fluorescens, P. putida	*Pythium aphanidermatum*	*P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper.	Ramamoorthy *et al*., 2002b

Continued table 1.			
P. fluorescens	*Colletotrichum falactum*	Induced systemic resistance against red rot of sugarcane.	Viswanathan and Samiyappan, 2002
P. aeruginosa PNA 1	*Fusarium udum, F. oxysporum* f. sp. *ciceris*	*P. aeruginosa* protected pigeonpea and chickpea from Fusarium wilt.	Anjaiah *et al*., 2003
Bacillus pumilus	*Sclerospora graminicola*	Out of 7 PGPR strains, maximum vigor index resulted from treatment with strain INR7 followed by IN937b.	Niranjan Raj *et al*., 2003
Pseudomonas fluorescens	*Sclerospora graminicola*	The isolates offered protection ranging from 20 to 75% against downy mildew to pearl millet.	Niranjan Raj *et al*., 2004
P. putida	*F. oxysporum* f. sp. *melonis*	Control on muskmelon achieved by seed treatment of *P. putida* strain 30 was 63% and 46-50% for strain 180.	Bora *et al.*, 2004
Bacillus subtilis Burkholderia cepacia	*R. solani*	Combination of *B. subtilis* RB14-C with *B. cepacia* BY can lead to greater damping-off suppression than by these strains separately.	Szczech and Shoda, 2004
P. fluorescens FP7	*Colletotrichum gloeosporioides*	Suppressed the anthracnose pathogen on mango leading to improved yield attributes.	Vivekananthan *et al.,* 2004
P. fluorescens A. chroococcum	*Alternaria triticina*	*P. fluorescens* caused greater reduction in *A. triticina* infected leaf area than *A. chroococcum.*	Siddiqui and Singh, 2005a
Bacillus and fluorescent pseudomonads isolates	*Fusarium udum*	Four isolates, namely Pa116, P324, B18 and B160, have shown antifungal activity.	Siddiqui *et al*., 2005

Table 2. Effects of PGPR on plant parasitic nematodes.

PGPR	**Nematode**	**Effect**	**Reference**
Bacillus thuringiensis	*Meloidogyne* sp.	Prevented *M. incognita* from forming galls on tomato.	Ignoffo and Dropkin, 1977
244 isolates	*M. incognita*	Only 125 bacterial isolates imparted positive effect on tomato and cucumber, rarely on both and negative effect on nematodes.	Zavaleta- Mejia and VanGundy, 1982
Serratia marcescens	*M. incognita*	Bacterium produced a volatile metabolite and was nematoxic.	Zavaleta-Mejia, 1985
Number of isolates	*Globodera pallida*	Seed treatment reduced nematode penetration of potato roots.	Racke and Sikora, 1985

Continued table 2.			
354 isolates	*M. incognita*	*P. fluorescens* (strains JOB204, JOB 209) and *Bacillus*(JOB203) were most effective and clover plants treated with these bacteria had fewer galls and large root.	Becker *et al*., 1988
Bacillus subtilis, B. cereus, B. pumilus, Pseudomonas	*M. incognita, Heterodera cajani, H. zeae, H. avenae*	Most effective isolates against all tested species were *B. subtilis* and *B. pumilus*. The non-cellular extract exhibited high larvicidal properties.	Gokte and Swarup, 1988
Bacillus subtilis	*Meloidogyne sp. Rotylenchulus reniformis*	Reduced nematode reproduction and galling on cotton, tomato, peanut and sugar beet.	Sikora, 1988
Agrobacterium radiobacter	*Globodera pallida*	Reduced nematode infection by 40% when sprayed on seed pieces of potato.	Sikora *et al.*, 1989
290 isolates	*Heterodera schachtii*	Eight isolates were antagonistic to *H. schachtii*, 3 isolates were identified as *P. fluorescens.*	Oostendorp and Sikora ,1989a
8 isolates	*H. schachtii*	Nematode penetration was reduced by 6 of 8 isolates tested	Oostendorp and Sikora, 1989 b
Bacillus licheniformis, P. mendocina	*M. incognita*	*B. licheniformis* caused greater reduction in nematode multiplication than *P. mendocina* on tomato.	Siddiqui and Husain, 1991
Bacillus licheniformis, Alcaligenes faecalis	*M. incognita*	*B. licheniformis* caused greater reduction in nematode multiplication than *A. faecalis* on chickpea.	Siddiqui and Mahmood ,1992
Pseudomonas aureofaciens	*Criconemella xenoplax*	One strain inhibited nematode multiplication in greenhouse test.	Westcott and Kluepfel, 1992
Pseudomonas aureofaciens	*Criconemella xenoplax*	Bacteria suppressed population of ring nematode.	Kluepfel *et al.*, 1993
B. cereus	*M. javanica*	Inhibited penetration of nematodes on tomato roots.	Oka *et al.*, 1993
B. subtilis	*M. incognita* race3	*B. subtilis* reduced nematode multiplication and improved growth of chickpea.	Siddiqui and Mahmood, 1993
P. fluorescens	*Panagrellus* sp.	Bacteria cultivated on plate count broth reduced nematodes up to 57.4%.	Weidenborner and Kunz ,1993
Bacillus thuringiensis	*C. elegans, R. reniformis, P. penetrans*	Isolate 371 of bacterium reduced nematode populations on tomato and strawberry.	Zuckerman *et al*., 1993
Pseudomonas solanacearum	*R. reniformis*	Slight inhibition of nematode activity on aubergine roots.	Kermarrec *et al*., 1994
B. subtilis	*M. incognita* race3	Seed treatment with bacteria reduced nematode multiplication on chickpea.	Siddiqui and Mahmood, 1995 c

Continued table 2.

B. subtilis	*H. cajani*	Bacteria reduced nematode multiplication on pigeonpea.	Siddiqui and Mahmood, 1995b
Endophytic bacterial stains	*M. incognita*	Reduced galling of cotton roots by root-knot nematode.	Hallmann *et al*., 1997
P. fluorescens	*M. javanica*	Reduced nematode multiplication and morphometrics of *M. javanica* females on tomato in different soil.	Siddiqui and Mahmood ,1998
P. putida, *P. fluorescens*	*R. similis*, *Meloidogyne* spp.	Inhibited invasion of *R. similis* and *Meloidogyne* spp. in banana, maize and tomato.	Aalten *et al*., 1998
B. sphaericus, *Agrobacterium radiobacter*	*Globodera pallida*	Rhizobacteria systemically induced resistance against potato cyst nematode.	Hasky-Gunther *et al*., 1998
P. fluorescens	*H. cajani*	Reduced multiplication of *H. cajani* on pigeonpea.	Siddiqui *et al.*, 1998
B. subtilis	*M. javanica*	Greatest growth of tomato and high reduction in nematode multiplication occurred when ammonium sulphate was used with *B. subtilis* and *G. mosseae*.	Siddiqui and Mahmood , 2000
P. fluorescens, *Azotobacter chroococcum*, *Azospirillum brasilense*	*M. javanica*	Use of *P. fluorescens* with *Glomus mosseae* was better at improving chickpea growth and reducing galling and nematode multi-plication than other treatments.	Siddiqui and Mahmood , 2001
P.fluorescens (strains GRP3 and PRS9)	*M. incognita*	GRP3 strain was better in reducing galling and nematode multiplication than PRS9.	Siddiqui *et al.*, 2001
P.fluorescens, *Azospirillum brasilense*, *Azotobacter*, *chroococcum*, Microphos	*M. incognita*	Best management of *M. incognita* was obtained when Microphos culture (mixture of P. *straita*, *B. polymyxa* and *Aspergillus niger*) was used with *A. chroococcum* and *A. brasilense*.	Siddiqui *et al.*, 2002
P. aeruginosa, *P. fluorescens*	*M. javanica*	Bare root dip or soil drench treatment reduced nematode penetration into tomato roots.	Siddiqui and Shaukat , 2002
P. fluorescens, *Azotobacter chroococcum*	*M. incognita*	Greater biocontrol of *M. incognita* was observed when *P. fluorescens* was used with the straw of *Zea mays*.	Siddiqui and Mahmood ,2003
Fluorescent pseudomonads	*Heterodera cruciferae*	Growth and hatching of nematode eggs were inhibited	Aksoy and Mennan, 2004
P. fluorescens, *Azotobacter chroococcum*, *Azospirillum brasilense*	*M. incognita*	*P. fluorescens* was better at improving tomato growth and reducing galling and nematode multiplication than *A. chroococcum* or *A. brasilense*.	Siddiqui, 2004

Continued table 2.			
P. straita	*M. incognita*	Reduced reproduction of *M. incognita* on pea.	Siddiqui and Singh, 2005b
Bacillus and fluorescent pseudomonads isolates	*M. incognita, H. cajani*	Four isolates of *Pseudomonas* and 2 of *Bacllus* (Pa70, Pf18, Pa116, Pa324, B18 and B160) were considered potentially useful for the biocontrol of nematodes.	Siddiqui *et al.,* 2005

Table 3. Effects of PGPR on bacterial diseases of plants.

PGPR	**Pathogenic bacteria**	**Effect**	**References**
P. fluorescens	*Xanthomonas compestris* pv. *citri*	Control of citrus canker by siderophore production.	Unnamalai and Ganamanickam, 1984
Fluorescent pseudomonads spp.	*Gaeumannomyces graminis*	Two new strains suppressed take-all disease in the field.	Weller *et al.,* 1985
P. putida W4P63	*Erwinia carotovora*	Increased yield of Rosset Burbank potato and suppressed soft rot potential of tubers.	Xu and Gross, 1986
P. fluorescens A506	*E. amylovora*	Reduction in the population size of *E. amylovora* in pear flowers with *P. fluorescens* was due to competition.	Wilson and Lindow, 1993
P. fluorescens WCS417	*P. syringae* pv. *tomato*	*P. fluorescens* protected radish through induction of systemic resistance against a virulent bacterial leaf pathogen.	Hoffland *et al.,* 1996
P. fluorescens M29 and M40	*P. solanacearum*	Isolate M40 reduced tomato wilt significantly.	Kim and Misaghi, 1996
P. putida, S. marcescens, Flavomonas oryzihabitans, B. pumilus	*P. syringae* pv. *lachrymans*	PGPR strains caused significant protection against pathogen on cucumber.	Wei *et al.,* 1996
P. fluorescens A506	*E. amylovora*	Strain A506 and antibiotics acted additively in the control of frost and fire blight disease.	Lindow *et al.,* 1996
B. pumilis, B.subtilis, Curtobacterium flaccumfaciens	*P. syringae* pv. *lachrymans, Erwinia tracheiphila*	Seed treatment of strains mixture caused reduction in angular spot and wilt of cucumber.	Raupach and Kloepper, 1998
Fluorescent pseudomonads	*Ralstonia solanacearum*	All three strains suppressed wilt of tomato and increased yield.	Jagadeesh *et al.,* 2001

Continued table 3.			
P. fluorescens	*Xanthomonas oryzae* pv. *oryzae*	Showed resistance to the rice bacterial blight pathogen.	Vidhyasekaran *et al*., 2001
Azospirillium brasilense	*P. syringae* pv. *tomato*	Prevented bacterial speck disease development and improved tomato growth.	Bashan and Bashan, 2002
Serratia J2, *Pseudomonas*, *Bacillus* BB11	*Ralstonia solanacearum*	All the three strains suppress wilt of tomato and increase yield.	Guo *et al*., 2004
B. cereus, *B. lentimorbus*, *B. pumilus*	*Xanthomonas compestris* pv. *compestris*	Incidence and severity of black rot of cabbage were reduced when antagonists were applied.	Massomo *et al.*, 2004

3 INTERACTIONS OF PGPR WITH PATHOGENS

3.1 Siderophore production

Iron is an essential nutrient for all living organisms. In the soil it is unavailable for direct assimilation by microorganisms because ferric iron (FeIII), which predominates in nature, is only sparingly soluble and too low in concentration to support microbial growth. To survive, soil microorganisms synthesize and secrete low-molecular-weight iron-binding compounds (400-1,000 daltons) known as siderophores. Siderophores bind FeIII with a very high affinity. The bacterium that originally synthesized the siderophores takes up the iron siderophore complex by using a receptor that is specific to the complex and is located in the outer cell membrane of the bacterium. Once inside the cell, the iron is released and is then available to support microbial growth. PGPR can prevent the proliferation of fungal and other pathogens by producing siderophores that bind most of the FeIII in the area around the plant root. The resulting lack of iron prevents pathogens from proliferating in this immediate vicinity. The PGPR out-compete the pathogens for available iron, thus causing death of the latter. Plants are not affected by the localized depletion of soil iron as most plants can grow at much lower iron concentrations (about 1000 fold less) than microorganisms.

Microbial siderophores vary widely in overall structure but most contain hydroxamate and catechol groups which are involved in chelating the ferric ion (Neilands, 1995). The involvement of the siderophore in disease suppression is based on:

- Inhibition in the antagonistic activity of PGPR by addition of dissolved ferric ion *in vitro* and *in vivo*.
- Ineffectiveness of siderophore minus PGPR mutants to suppress pathogens.

- Inhibition in the growth of pathogens *in vitro* and increase in plant growth by purified siderophores.

Suppression of soil borne plant pathogens by siderophore producing pseudomonads was observed (Bakker *et al*., 1986, 1987; Becker and Cook, 1988, Loper, 1988) and the wild type strain was more effective in suppressing disease compared to non-siderophore-producing mutants. Siderophore production is an important feature for the suppression of plant pathogens and promotion of plant growth. Fluorescent siderophore production was observed as a mechanism of biocontrol of bacterial wilt disease in the fluorescent pseudomonads RBL 101 and RSI 125 (Jagadeesh *et al.*, 2001). Press *et al.* (2001) reported the catechol siderophore biosynthesis gene in *Serratia marcescens* 90-166 and associated with induced resistance in cucumber against anthracnose.

Table 4. Effects of PGPR on viral diseases of plants.

PGPR	**Viruses**	**Effects**	**References**
Bacillus uniflagellatus	Tobacco mosaic virus	Cultures and extracts from cultures reduced numbers of lesions from TMV.	Mann, 1965
P. fluorescens CHAO	Tobacco necrosis virus	Reduction in TNV leaf necrosis in *P. fluorescens* treated tobacco plants.	Maurhofer *et al*., 1994a
P. fluorescens, Serratia marcescens	Cucumber mosaic virus	Treatment of cucumber or tomato plants with PGPR induced systemic resistance against CMV.	Raupach *et al*., 1996
Bacillus amyloliquefaciens, B. subtilis, B. pumilus	Tomato mottle virus	Disease severity ratings were significantly less in all PGPR powder based treatments.	Murphy and Zehnder, 2000
Bacillus amyloliquefaciens B. subtilis, B. pumilus	Cucumber mosaic cucumo virus(CCMV)	PGPR mediated ISR occurred against CCMV following mechanical inoculation on tomato.	Zehnder *et al.*, 2000
Bacillus amyloliquefaciens	Pepper mild mottle virus (PMMoV)	*Bacillus* induced systemic resistance against PMMoV in tobacco via salicyclic acid and jasmonic acid dependent pathways.	Ahn *et al*., 2002

The capacity to utilize siderophores is important for the growth of bacteria in the rhizosphere (Jurkevitch *et al.*, 1992) and on the plant surface (Loper and Buyer,1991). Specific siderophore producing *Pseudomonas* strains rapidly colonized roots of several crops and colonization of roots

resulted in yield increases (Schroth and Hancock, 1982). Enhanced plant growth caused by pseudomonad strains was often accompanied by the reduction in pathogen populations on the roots. There is convincing evidence to support a direct role of siderophore mediated iron competition in the biocontrol activity exhibited by such isolates (Leong, 1986; Loper and Buyer, 1991). The antagonism depends on the amount of iron available in the medium; siderophores produced by a biocontrol agent and sensitivity of target pathogens (Kloepper *et al*., 1980; Weger *et al*., 1988). Production of ALS 84 and siderophore may contribute to the biocontrol of crown gall by *Agrobacterium rhizogenes* K84 especially under conditions of iron limitation (Penyalver *et al*., 2001).

Iron nutrition of the plant influences the rhizosphere microbial community structure (Yang and Crowley, 2000) and the role of the pyoverdine siderophore produced by many *Pseudomonas* species has been clearly demonstrated in the control of *Pythium* and *Fusarium* species (Loper and Buyer, 1991; Duijff *et al*., 1993). Pseudomonads also produce two other siderophores, pyochelin and its precursor salicylic acid, and pyochelin is thought to contribute to the protection of tomato plants from *Pythium* by *Pseudomonas aeruginosa* 7NSK2 (Buysens *et al*., 1996). Different environmental factors can also influence the quantity of siderophores produced (Duffy and Défago, 1999).

3.2 Antibiotic production

One of the most effective mechanisms that PGPR employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. Evidence for the direct involvement of antibiotic production in PGPR- mediated disease suppression has come from two types of experiments:

- Non-antibiotic producing mutants of several disease-suppressive bacterial strains were unable to prevent phytopathogens to cause damage in plants.
- When an antibiotic-producing strain was genetically manipulated to overproduce antibiotics the resultant strain protected plants against pathogens more effectively than the wild type strain.

There are numerous reports of the production of antifungal metabolites by bacteria *in vitro* that may also have activity *in vivo*. Metabolites include ammonia, butyrolactones, 2,4-diacetylphloroglucinol (Ph1), HCN, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (Pln), viscosinamide, xanthobaccin, and zwittermycin A (Milner *et al*., 1996; Keel and Défago, 1997; Whipps, 1997; Kang *et al*., 1998; Nielsen *et al*., 1998; Kim *et al*., 1999; Nakayama *et al*., 1999; Thrane *et al*., 1999). To demonstrate a role for

antibiotics in biocontrol, mutants lacking production of antibiotics or antibiotics over-producing mutants have been used (Bonsall *et al*., 1997; Chin-A-Woeng *et al*., 1998; Nowak-Thompson *et al*., 1999). Alternatively, the use of reporter genes or probes to demonstrate production of antibiotics in the rhizosphere is becoming more common place (Kraus and Loper, 1995; Raaijmakers *et al*., 1997; Chin-A-Woeng *et al*., 1998). Indeed, isolation and characterization of genes or gene clusters responsible for antibiotic production have now been achieved (Kraus and Loper, 1995; Bangera and Thomashow, 1996; Hammer *et al*., 1997; Kang *et al*., 1998; Nowak-Thompson *et al*., 1999). Significantly, both Phl and PCA have been isolated from the rhizosphere of wheat following introduction of biocontrol strains of *Pseudomonas* (Thomashow *et al*., 1990; Bonsall *et al*., 1997; Raaijmakers *et al*., 1999), confirming that such antibiotics are produced *in vivo*. Further, Ph1 production in the rhizosphere of wheat was strongly related to the density of the bacterial population present and the ability to colonize roots (Raaijmakers *et al*., 1999). PCA from *Pseudomonas aureofaciens* has even been used as a direct field treatment for the control of *Sclerotinia homeocarpa* on creeping bent grass (Powell *et al*., 2000).

The first antibiotics clearly implicated in biocontrol by fluorescent pseudomonads were the phenazine derivatives (Handelsman and Stabb,1996) that contribute to disease suppression by *P. fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84, which control take-all of wheat (Weller and Cook, 1983; Brisbane and Rovira, 1988). *P. fluorescens* strain CHAO produces hydrogen cyanide**,** 2,4-diacetylphloroglucinol, and pyoluteorin, which directly interfere with growth of various pathogens and contribute to disease suppression (Voisard *et al.,* 1989; Keel *et al.,* 1990,1992; Maurhofer *et al.,* 1994b). Furthermore, a quantitative relationship between antibiotic production and disease suppression is suggested by the enhanced production of 2,4-diacetylphloroglucinol and pyoluteorin accomplished by adding extra copies of a 22-kb fragment of DNA that improves suppression of *Pythium* on cucumber (Maurhofer *et al.,* 1992). Antibiotic DAPG has been shown to act as the inducing agent in CHAO-mediated ISR in tomato against root-knot nematode *Meloidogyne javanica* (Siddiqui and Shaukat, 2003) and suggest that more antibiotics may be capable of eliciting ISR in plants.

3.3 Enzyme Production

Biocontrol of *Phytophthora cinnamomi* root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily *et al*., 1996) and control of *Phytophthora fragariae* var. *rubi* causing raspberry root rot was suppressed by the application of actinomycete isolates selected for the production of ß-1,3-, ß-1,4- and ß-1,6-

glucanases (Valois *et al*., 1996). Chitinolytic enzymes produced by both *Bacillus cereus* and *Pantoea* (*Enterobacter*) *agglomerans* also appear to be involved in biocontrol of *Rhizoctonia solani* (Chernin *et al*., 1995, 1997; Pleban *et al*., 1997). Tn*5* mutants of *E. agglomerans* deficient in chitinolytic activity were unable to protect cotton and expression of the *chiA* gene for endochitinase in *Escherichia coli* allowed the transformed strain to inhibit *R. solani* on cotton seedlings. Similar techniques involving Tn*5* insertion mutants and subsequent complementation demonstrated that biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* was due to the production of extracellular protease (Dunne *et al*., 1997). The incidence of plant disease caused by the phytopathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* was reduced by using a ß -1,3-glucanse producing strain of *Pseudomonas cepacia* which was able to degrade fungal mycelia. Many of the bacterial enzymes that can lyse fungal cells, including chitinases and ß -1,3- glucanse, are encoded by a single gene.

4 INTERACTIONS OF PGPR WITH PLANTS

4.1 Induced Resistance

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon *et al*., 1998). Use of selected PGPR strains was shown to trigger a plant mediated resistance in above ground plant parts (Van Peer *et al*., 1991; Wei *et al*., 1991). This type of resistance is often referred to as induced systemic resistance (ISR) and has been demonstrated in many plant species including bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis thaliana* (van Loon *et al*.,1998). Rhizobacteria-mediated ISR resembles phenotypically with classic pathogen induced resistance, in which non-infected parts of a previously pathogen infected plant become more resistant to further infection. This form of resistance is referred as systemic acquired resistance (SAR) (Ross, 1961). The difference between ISR and SAR is that ISR is induced by non-pathogenic rhizobacteria while SAR is induced systemically after inoculation with necrotizing pathogens. Moreover, ISR is independent of salicyclic acid but involves jasmonic acid and ethylene signaling while SAR requires salicyclic acid as a signaling molecule in plants. ISR is accompanied by the expression of sets of genes distinct from the PR genes while SAR is accompanied by the induction of pathogenesis related proteins. Both ISR and SAR are effective against a broad spectrum of plant pathogens (Kuc 1982; van Loon *et al*., 1998).

The effectiveness of ISR and SAR to a range of viral, bacterial, fungal and oomycete pathogens was tested on *Arabidopsis*. *Arabidopsis thaliana* L. has many features favoring its use as a model in studies of PGPR (O'Callaghan *et al*., 2000). In this model system, the non-pathogenic rhizobacterial strain *P. fluorescens* WCS417r was used as the inducing agent (Pieterse *et al.,* 1996) to trigger ISR in several plant species (Van Peer *et al*., 1991; Leeman *et al*., 1995; Duijff *et al.,* 1998; Bigirimana and Hofte, 2002). Colonization of *Arabidopsis* roots by *P. fluorescens* WCS417r protected the plants against different plant pathogens (Pieterse *et al.,* 1996; Van wees *et al.,* 1997.,Ton *et al.,* 2002). Protection against different pathogens was expressed both in reduction in disease symptoms and inhibition of pathogen growth. Since rhizobacteria were spatially separated from pathogens, the mode of disease suppression in the plants is through ISR. The ability to develop ISR appears to depend on the host / rhizobacterium combination (Pieterse *et al.,* 2002) and suggests that specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR. Several bacterial components as potential inducers of ISR are involved including outer membrane lipopolysaccharides and iron regulated siderophores (Leeman *et al.,* 1995; van Loon *et al*., 1998).

Changes that have been observed in plant roots exhibiting ISR include: (1) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (Benhamou *et al*., 1996*a, b, c*, 2000; Duijff *et al*., 1997; Jetiyanon *et al*., 1997; M'Piga *et al*., 1997); (2) increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase (M'Piga *et al*., 1997; Chen *et al*., 2000); (3) enhanced phytoalexin production (Van Peer *et al*., 1991; Ongena *et al*., 1999); and (4) enhanced expression of stress-related genes (Timmusk and Wagner, 1999). However, not all of these biochemical changes are found in all bacterial–plant combinations (Steijl *et al*., 1999). Protection from diseases by biocontrol and its consistency in the field are generally not sufficient to compete with conventional methods of disease control. Combine use of antagonistic microorganisms with different mechanisms of action may improve efficacy and consistency of biocontrol agents (De Boer *et al*., 1999). Moreover, combination of ISR and SAR that results in an enhanced level of protection against specific bacterial pathogens (Van Wees *et al.,* 2000) offers great potential to integrate both forms of induced resistance in agricultural practices. Induced resistance appears to be more useful for the management of viral diseases of plants where other management strategies are not generally successful.

4.2 Root colonization

Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg *et al.*, 2001). PGPR generally improves plant growth by colonizing the root system and pre-empting the establishment of, or suppressing deleterious rhizosphere microorganisms (Schroth and Hancock, 1982). PGPR must be able to compete with the indigenous microorganisms and efficiently colonize the rhizosphere of the plants to be protected**.** Colonization is widely believed to be essential for biocontrol (Weller, 1983; Parke, 1991) and a biocontrol agent should grow and colonize the surface of plant. The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots (Benizri *et al*., 2001; Bloemberg and Lungtenberg, 2001). Colonization or even initial population size of the biocontrol agent has been significantly correlated with disease suppression (Parke, 1990; Bull *et al.*, 1991).

Under field conditions percolating water probably plays an essential role in the passive distribution of bacteria on roots (Liddell and Parke, 1989) Osmotolerance is also correlated with colonization ability (Loper *et al*.,1985). Cell surface characteristics influence the attachment of bacteria to roots which may be necessary for colonization (Vesper, 1987; Anderson *et al.*, 1988). Certain mutants that affect accumulation of secondary metabolites also influence colonization of plant roots in the field (Mazzola *et al*., 1992, Carroll *et al.*, 1995). Analysis of mutants indicates that prototrophy for amino acids and vitamin b1, rapid growth rate, utilization of organic acids and lipopolysaccharide properties contribute to colonization (Lugtenberg *et al.*, 1996).

Use of confocal laser scanning microscopy (CLSM) in combination with organisms differentially labeled with auto fluorescent proteins (AFPs) allowed the simultaneous visualization of both the pathogen and the biocontrol agent on the root under disease controlling conditions in the gnotobiotic system. Seedlings grown in a gnotobiotic sand system infected with pathogen and biocontrol agent may be studied via *in vitro* setup. These studies may contribute to our understanding of root colonization and biocontrol processes.

A variety of bacterial traits and specific genes contribute to colonization but only few have been identified (Benizri *et al.*, 2001; Lugtenberg *et al*., 2001). These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion and quorum sensing (Lugtenberg *et al*., 2001). Competition of introduced bacteria with indigenous microorganisms already present in the

soil and rhizosphere of the developing plant is another important aspect for root colonization.

4.3 Genetic variations in the host

Plants vary in their ability to support and respond to beneficial microorganisms (Handelsman and Stabb, 1996). The ability to support certain biocontrol agents varies among plant species and among cultivars. Some plants appear to attract and support biocontrol agents which are antagonistic to pathogens (Neal *et al*., 1973; Azad *et al*., 1985). Legumes vary in their response to the PGPR *B. polymyxa* (Chanway *et al.*, 1988) and *Bacillus* isolates from wheat roots enhanced growth of wheat in a cultivar-specific manner (Chanway *et al.*, 1988). Plant species vary in their ability to induce genes for pyluteorin biosynthesis in *P. fluorescencs* (Kraus and Loper, 1995) probably due to variation in composition of root exudates among species. Moreover, different cultivars vary in terms of survival or disease incidence in the presence of a pathogen and biocontrol agent (Liu *et al.*, 1995, King and Parke, 1996). Strains of *P. fluorescens* that overproduce pyoluteorin and 2, 4-diacetyl-phloroglucinol provide superior disease suppression compared with the parent strain in some host pathogen combinations and not others, and effect correlate with host, and not pathogen, sensitivity to antibiotics (Maurhofer *et al*., 1995).

5 INTERACTIONS OF PGPR IN THE RHIZOSPHERE

5.1 Interactions with the microbial community

Many biocontrol agents suppress disease effectively in the laboratory but fail to do so in the field. These biocontrol agents may be affected by indigenous soil microbial communities and they may also influence the community into which they are introduced. Certain fluorescent pseudomonads displace resident microflora in some cases reducing populations of deleterious microorganisms (Yuen and Schroth, 1986). Manipulation of introduced PGPR populations may lead to enhanced suppression of other soil borne plant pathogens. Limited induced soil suppressiveness can also be achieved through shills in microbial community structure and function by several cultural practices (Kloepper *et al.*, 1999). This may include the application of organic manures and plant straw (Siddiqui and Mahmood, 2003; Siddiqui, 2004), inclusion of antagonistic

plants in cropping systems and other integrated pest management approaches.

5.2 Interactions of PGPR strains

In general, a single biocontrol agent is used for biocontrol of plant disease against a single pathogen (Wilson and Backman, 1999). This may sometimes account for the inconsistent performance by the biocontrol agent, because a single agent is not active in all soil environments or against all pathogens that attack the host plant. On the other hand, mixtures of biocontrol agents with different plant colonization patterns may be useful for the biocontrol of different plant pathogens via different mechanisms of disease suppression. Moreover, mixtures of biocontrol agents with taxonomically different organisms that require different optimum temperature, pH, and moisture conditions may colonize roots more aggressively, improve plant growth and efficacy of biocontrol. Naturally occurring biocontrol results from mixtures of biocontrol agents rather than from high populations of a single organism. The greater suppression and enhanced consistency against multiple cucumber pathogens was observed using strain mixtures of PGPR (Raupach and Kloepper, 1998).

Incompatibility of the co-inoculants may sometimes arise and thus inhibit each other as well as the target pathogens (Leeman *et al*., 1996). Thus an important prerequisite for successful development of strain mixtures appears to be the compatibility of the co-inoculated microorganisms (Baker, 1990; De Boer *et al*., 1997). A biocontrol product composed of a mixture of strains is more costly than a product composed of single strain due to increased costs of production and registration of such a product. Regardless, however, greater emphasis on the development of mixtures of biocontrol agents is needed, because they may better adapt to the environmental changes that occur throughout the growing season and protect against a broader range of pathogens. Mixtures of microorganisms may increase the genetic diversity of biocontrol systems that persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms (Pierson and Weller, 1994). Multiple organisms may enhance the level and consistency of biocontrol by a more stable rhizosphere community and effectiveness over a wide range of environmental conditions. In particular, combination of fungi and bacteria may provide protection at different times, under different conditions, and occupy different or complementary niches.

6 A PRACTICAL CONTROL SYSTEM USING PGPR

Selection of effective strains of bacteria is of prime importance for the biocontrol of plant pathogens. Isolation of bacteria from pathogen suppressive soils may increase the chances of isolating effective strains (Cook and Baker, 1983). The suppressive soil becomes apparent where the severity or incidence of disease is lower than expected as compared to that in surrounding soil (Cook and Baker, 1983). To obtain the effective strains the isolation of bacteria should be conducted from the same environment in which they will be used (Weller *et al.,* 1985). The ability to colonize roots and resistance against antibiotics are other parameters necessary to screen the effective strains (Siddiqui *et al*., 2005). Screening of biocontrol agents by a seedling bioassay chamber is required to determine the compatibility of an antagonist with the microflora of a field soil (Randhawa and Schaad, 1985). Selection of field effective strains can also be facilitated by a greenhouse assay. The important considerations in the development of the assays in the greenhouse are the inoculum potential of the pathogen (Weller *et al*., 1985), and environmental conditions and dose of the bacterium (Xu and Gross, 1986). Many factors such as temperature, soil moisture and soil texture influence the survival and establishment of bacteria. Formulation and application methods are often of paramount importance in effecting biocontrol (Papavizas and Lumsden, 1980).

PGPR have great potential in the biocontrol of plant pathogens but the use of these rhizobacteria by farmers in the field is still lacking. The most obvious reasons for the limited use thus far are the limited numbers of PGPR formulations available and inconsistent performance of these formulations. A separate chapter is devoted on PGPR formulations in this book and aspects related to formulations are not covered in this chapter. Mixtures of different strains are required to overcome inconsistency in their biocontrol performance. These mixtures of rhizobacteria may be used as seed treatment which may be useful in reducing the quantity of bacterial inoculum required. Moreover, this will facilitate systemic spread of the bacterial inoculum along the surface of the developing root system, and their antagonistic activity on the root surface during the early root infection by the pathogens. Rhizobacteria suspensions or formulations can also be mixed with organic manures in large vessels. They can be stored at 30-35^{o}C for 5-10 days, mixing each day with water to keep them moist (Siddiqui and Mahmood, 1999). Within 10 days bacteria will attain high populations and this organic manure can be used at planting or after planting for the biocontrol of plant pathogens and better plant growth in the field.

7 CONCLUSION

Numerous studies have indicated that PGPR have great potential in the biocontrol of plant pathogens but most of the studies have been conducted in sterilized soil and in pots. There is an urgent need to conduct studies under field conditions. Evaluation of PGPR by greenhouse assays for effective strains is required before their application as biocontrol agents (Siddiqui *et al.,* 2005). Root colonization by PGPR is also important to increase their potential as biocontrol agents. Studies on the physical and chemical factors of soil which affect root colonization are needed. Moreover, use of mixture of effective strains of PGPR is advisable compared with use of single strain. The use of organic amendments with effective strains of PGPR is recommended, as organic materials are thought to encourage the growth of organisms that compete with or destroy pathogens (Siddiqui and Mahmood, 1999). PGPR may also be used with fungal biocontrol agents and with arbuscular mycorrhizal fungi for greater beneficial effects. The absence of commercial interest in the biocontrol of plant pathogens by PGPR is also a major obstacle to progress. It is hoped that the future will see greater use of PGPR for the biocontrol of plant pathogens and biofertilization.

Acknowledgement: Author is thankful to Department of Science and Technology, Government of India, New Delhi for granting a research project (SP/SO/A22/2001) on PGPR.

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Chapter 5

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Abstract: Many bacteria and fungi can enhance plant growth. The present review is limited to plant growth promoting rhizobacteria (PGPR). However, it includes endophytic bacteria that show plant growth enhancing activity as well. Also the best studied bacterial mechanisms of plant growth promotion are discussed, with a special emphasis on biological nitrogen fixation and synthesis of phytohormones, including less understood mechanisms like inhibition of plant ethylene synthesis, degradation of organic-P compounds, phenazine-related mineral solubilization, and synthesis of lumichrome. In addition, examples of PGPR genes that show activation in the interaction with plants, and beneficial events resulting from plant-bacterial interactions like stress relief and enhancement of other ecological associations are presented. Plant growth promoting activity and more precisely, crop yield enhancement are the final effects of the different mechanisms that PGPR possess and are the applicative goal of the agricultural microbiology research. Despite the undoubted economic and ecological benefits of utilizing some PGPR species as biofertilizers, the application of such a species must be very carefully assessed because of their importance as opportunistic pathogens in nosocomial infections and in patients with other diseases. On this basis, PGPR species must be selected for producing safe biofertilizers. Strain selection, as also the number of the bacterial cells, and characteristics of the bacterial cultures used in the production of biofertilizers, as well as, results of inoculation of different crops and cultivars with *Azospirillum* under field conditions are also included in the discussion.

Key words: bacterial inoculation; endophytic bacteria; nitrogen fixation; phytohormones; rhizosphere bacteria; plant growth promoting rhizobacteria.

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 143–172.

1 INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) have been studied for long. It has been suggested in the last few years that endophytic N_2-fixing bacteria may be more important than rhizospheric bacteria in promoting plant growth because they escape competition with rhizosphere microorganisms and achieve close contact with the plant tissues (Assmus *et al.*, 1995; Döbereiner, 1992). The well known genera of PGPR are *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*, but some of these genera include endophytic species as well. The best-characterized endophytic bacteria include *Azoarcus* spp, *Gluconacetobacter diazotrophicus*, and *Herbaspirillum seropedicae*. Novel *Burkholderia* species, for instance, *B. unamae* (Caballero-Mellado *et al*., 2004) and *B. tropica* (Reis *et al.*, 2004) have the potential for promoting plant growth (Caballero-Mellado *et al.*, 2003) and are found in rhizospheric and endophytic association with different agricultural crops. Bacterial mechanisms of plant growth promotion include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, synergism with other bacteria-plant interactions, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like phosphorus, iron and minor elements, and growth enhancement by volatile compounds. However, the expression of such bacterial activities under laboratory conditions does not guarantee in association with a host plant. This is especially true of nitrogen-fixation as abundantly expressed in culture media by many bacterial species. The mechanisms of plant growth promotion have been analyzed in different organisms, especially in *Azospirillum* spp. and in few other PGPR (Vande Broek *et al.*, 2000; Lucy *et al.*, 2004). In this review, *Azospirillum* as a model for studying mechanisms of plant growth promotion will only be covered briefly but some other models and new mechanisms will be presented in more depth.

Many definitions and interpretations of the term biofertilizer exist (Vessey, 2003). In this chapter, a biofertilizer is a product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop yield through different mechanisms. The term biofertilizer as used here could include products containing bacteria to control plant pathogens, but these are frequently referred to as biopesticides (Siddiqui and Mahmood, 1999; Burdman *et al.*, 2000; Vessey, 2003).

2 MECHANISMS FOR PLANT GROWTH PROMOTION

2.1 Associative nitrogen fixation

Many PGPR and endophytic bacteria can grow diazotrophically, and either be symbiotic or non-symbiotic. Young (1992) has reviewed the phylogenetic classification of nitrogen-fixing organisms, but in the last few years many novel N_2-fixing species belonging to different genera have been described. For instance, the associative *Burkholderia* species such as *B. vietnamiensis* (Gillis *et al*. 1995), *B. kururiensis* (Estrada-de los Santos *et al*., 2001), *B. unamae*, *B. xenovorans*, and *B. tropica* (Caballero-Mellado *et al*., 2004; Goris, *et al*., 2004; Reis *et al*., 2004), as well as the legume nodulating species like *B. phymatum*, *B. tuberum* (Vandamme, *et al*., 2002) and *Ralstonia taiwanensis* (Chen *et al*., 2001); as also the species of *Azoarcus* that include *A. communis*, *A. indigens* (Reinhold-Hurek *et al*., 1993) and *A. tolulyticus* (Zhou *et al*., 1995). Furthermore, in the Acetobacteraceae family has been described *Gluconacetobacter johannae* and *G. azotocaptans* (Fuentes-Ramírez *et al*., 2001), *Swaminathania salitolerans* (Loganathan and Nair, 2004), and possibly *Asaia bogorensis* (Weber *et al*., 2003a; Yamada *et al*., 2000). A genomic-based survey for nitrogen-fixing genes indicates that approximately 5% of prokaryotes could carry nitrogen fixation-like genes (Raymond *et al*., 2004). Nitrogen-fixation genes are plasmid borne in some species, but most prokaryotes have chromosomal *nif* genes. Hence, it would not be so simple for them to loose *nif* genes. Hitherto, the presence of the novel superoxide-dependent nitrogen fixing system detected in *Streptomyces thermoautotrophicus* (Gadkari, *et al*., 1992) has not been reported in other bacteria, raising the possibility that several known or unknown PGPR could have it.

Many N_2-fixing bacteria have been found in rhizospheric and endophytic association but the transference of biologically fixed nitrogen has been demonstrated only in a few systems. Sugarcane has been a model in which BNF has been observed (Boddey, 1995; Yoneyama *et al*., 1997). Nevertheless, the organism(s) responsible(s) for such activity has not been fully established. Since the description of *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988; Gillis *et al*., 1989), presently *Gluconacetobacter diazotrophicus* (Yamada, *et al*., 1997) has been suspected as one of the contributors of nitrogen to sugarcane. Sugarcane plantlets inoculated with a wild type strain of *G. diazotrophicus*, and incubated in an atmosphere enriched with $^{15}N_2$, incorporated a significantly higher proportion of $^{15}N_2$ than the plantlets inoculated with a *G. diazotrophicus nifD* mutant (Sevilla *et al*., 2001).

As with *G. diazotrophicus* in sugarcane, the inoculation of *Oryza sativa* IR36 with *Azoarcus* sp. BH72 did improve the plant growth, but this was not dependent on nitrogen fixation since a *nifK* mutant showed the same effect as the wild type strain, and besides the presence of NifH was not detected in inoculated plantlets (Hurek *et al*., 1994). Nevertheless, other experiments show participation of nitrogen fixation in that association. Fusion transcripts of *nifH* were detected in rice inoculated with *Azoarcus* sp. BH72 when amended with a small amount of carbon source, or even without it (Egener *et al*., 1999), although, in later plants the detection of the reporter of *nifH* was considerably minor and belated. Similarly, in Kallar grass strain BH72 was found to contribute to nitrogen incorporation and abundant transcripts of *nifH* were detected in both plants grown in greenhouse and in non-inoculated ones grown in the field (Hurek *et al*., 1997; Hurek *et al*., 2002).

Considering carbon supply is one of the limiting factors for associative nitrogen fixation in non-legumes (Chelius and Triplett, 2000; Vande Broek, *et al.*, 1993), it would be desirable to look for cultivars that excrete photosyntates in adequate amounts in order to find effective nitrogen fixation in rhizospheric associations. Gyaneshwar *et al*. (2002) found significant uptake of $^{15}N_2$ by one rice variety that exuded a great quantity of carbon compounds.

For successful plant-bacteria interaction, both genotype of organisms and the environmental conditions play very important role. The influence of the plant genotype has been documented, for example, wild rice species and traditional races of rice support a higher population of *Azoarcus* sp. than modern varieties (Engelhard *et al*., 2000). Even though it has not been tested if those associations exhibit nitrogen fixation activity, the wild rice *Oryza officinalis* gave ARA positive activity and also incorporated $^{15}N_2$ in an inoculation experiment with a homologous *Herbaspirillum* isolate (Elbeltagy *et al.*, 2001). Not only traditional rice varieties, but also some modern ones can exhibit nitrogen fixation: thus the variety IR42 inoculated with *H. seropedicae* Z67 showed low, but significant incorporation of $^{15}N_2$ (James *et al*., 2002), and rice cultivars NIA-6 and BAS370 inoculated with either *Azospirillum lipoferum*, *Azospirillum brasilense*, *Azoarcus* or *Zoogloea*, showed considerable nitrogen fixation activity after six weeks of inoculation in gnotobiotic experiments (Malik *et al.*, 1997). The recently described acetobacterium species *Asaia bogorensis* (Yamada *et al*., 2000) has been demonstrated to enhance growth of pineapple plants (Weber *et al*., 2003a, 2003b), probably, through N_2-fixation activity (Weber *et al.*, 2003a), or, by producing phytohormones (Santoyo-Páez, Jiménez-Salgado and Fuentes-Ramírez, unpublished results). In plant-bacteria interaction the available nitrogen for the roots has great influence. It has been found that the amount of accessible nitrogen has a negative relationship with the *G.*

diazotrophicus populations that colonize sugarcane (Fuentes-Ramírez *et al*., 1999; Muñoz-Rojas and Caballero-Mellado, 2003; Muthukumarasamy *et al*., 1998) and reduce the diversity of diazotrophs associated to wild and modern rice (Tan *et al*., 2003).

Though biologically fixed nitrogen has been found in a small number of non-legumes, this activity could have a great impact on the ecology of wild and cultivated ecosystems. The isolation of spontaneous *A. brasilense* mutants with greater acetylene reduction activity in *vitro* association with wheat (Katupitiya, *et al*., 1995; Pereg Gerk, *et al*., 2000), indicates that populations in nature are greatly flexible and some highly nitrogen-fixing genotypes could be favored in certain conditions.

The contribution of BNF to the growth of sugarcane plants has been observed. It seems to depend on the cultivar (Boddey *et al*., 1991; Lima *et al*., 1987), environmental conditions (Baldani *et al*., 2002), and microorganisms. With a plant-gene expression-approach, an active role of the plant was found in the development of the association with bacteria, and also a bacterial species-specific response of the plant (Nogueira *et al*., 2001). There are fundamentals of BNF that we still do not know i.e. the identity of the organisms that have greater potential to transfer nitrogen, or whether they are located inside the plant or in the rhizosphere. Endophytes have been suggested to be the organisms that have a higher potential to transfer nitrogen to the plant, but data indicate that endophytic BNF as a large nitrogen source has drawbacks also, for example, the number of nitrogen-fixing bacteria that inhabit the inside of plants may vary. For instance, compared to Rhizobia (10^9 cells per nodule and around 10^{11} per plant; James *et al*., 2000) the population of diazotrophs inside the plant is low. There are probably no more than 10^6-10^7 per g of the plant inside the sections most colonized, and even much less in most of the tissues of the plant. Moreover, in sugarcane, the *G. diazotrophicus* numbers decline rapidly as the plant grows (Muñoz-Rojas and Caballero-Mellado, 2003). Additionally, the bacterial numbers that are active, and the amount of their activity, could be restricted in most of the plants, since, endophytes like *G. diazotrophicus* have been observed to preferentially colonize the xylem (Fuentes-Ramírez *et al*., 1999; James *et al*., 1994), where carbon sources are limited (Welbaum *et al*., 1992). The rhizosphere is not as adverse an environment for BNF as it could be though; the most important plant growth promotion activity of a semi-quiescent community of endophytes could be the release of phytohormones that are needed in nanograms for exerting positive effects on the plant.

2.2 Phytohormones

Plant growth regulators participate in the growth and development of cells, tissues, organs, and in fact the entire plant. These compounds are active in plants in very minute amounts and their synthesis is extremely regulated. Plants not only produce phytohormones but also, numerous plant-associated bacteria both beneficial and harmful, produce one or more of these substances (Dobbelaere *et al.,* 2003). Among the PGPR species, *Azospirillum* is well known for its ability to excrete phytohormones such as gibberellins (Bottini *et al.,* 1989; Janzen *et al*., 1992), cytokinins (Tien *et al*., 1979) and auxins (Mascarua-Ezparza *et. al*., 1988; Omay *et al*., 1993; Reynders and Vlassak, 1979). Many studies suggest the involvement of indole-3-acetic acid (IAA), produced by *Azospirillum*, in morphological and physiological changes of the inoculated plant roots (Harari *et al*., 1988; Kapulnik *et al*., 1985; Tien *et al*., 1979). It is noteworthy that bacterial plant-dependent response induces IAA synthesis by *Pantoea agglomerans* (Brandl and Lindow, 1997), and also, greater auxin production by rhizospheric strains of *P. polymyxa* than by non-rhizospheric isolates (Lebuhn *et al*., 1997). Differential behavior of the isolates in relation to the proximity to plant tissues could be linked to a great competitiveness of the more actively phytohormone-synthesizing strains. Inoculation experiments of single, or mixtures of strains, previously isolated from different distances from roots, could help in determining this issue. Also, it would be exciting to determine if the rhizosphere gradient of plant exudates participates in determining a differential response in the bacterial synthesis and release of phytohormones. Particularly for bacterial IAA synthesis, the finding of Vande Broek *et al*. (1999) that this phytohormone induces some bacterial genes could be useful for designing tools to determine the differential response. In rhizospheric colonization of rice by *Pseudomonas stutzeri* A15, Rediers *et al*. (2003) found expression of *miaA* whose product could be involved in the production of the cytokinin *trans*-zeatin in association with the plant. Many inoculation effects in plants have been attributed to bacterial synthesis and release of phytohormones. Expression of an auxin-responsive promoter in *Arabidopsis* indicated that plants really detect the bacterial synthesized IAA released in the rhizosphere (O'Callaghan *et al*., 2001).

The general effects on the plant can be direct, that is through plant growth promotion, or indirect, that is through improving plant nutrition via the better development of the roots, and it is difficult to distinguish between them. Additionally, plant associated bacteria could induce phytohormones synthesis. The elevation of root IAA level in lodgepole pine plantlets, inoculated with *Paenibacillus polymyxa*, and, of dihydroxyzeatin riboside root concentration in plants inoculated with *Pseudomonas fluorescens* (Bent *et al*., 2001), might be attributed to the induction of plant hormone synthesis

by the bacteria, however the uptake of bacterial synthesized phytohormones can not be excluded, since both *P. polymyxa* and *Pseudomonas* sp. produce cytokinins and IAA *in vitro* (Akiyoshi *et al.* 1987; Bent *et al.*, 2001; Srinivasan *et al.*, 1996; Timmusk, *et al.*, 1999).

The participation of bacterial IAA in plant growth promotion has been demonstrated in the interaction between canola and *P. putida* GR12-2. The roots inoculated with an *ipdC* mutant, a key gene in the synthesis of IAA, grew significantly less than the roots inoculated with the wild type strain (Patten *et al.*, 2002). Other bacterial synthesized phytohomones that participate in growth promotion are the gibberellins. Exposition of alder seedlings to an inhibitor of gibberellin biosynthesis produces a dwarf phenotype. Inoculating the plantlets with gibberellin-producing *Bacillus pumilus* and *B. licheniformis* strains reversed that phenotype, and the same effect was observed when GA_3 was added (Gutierrez-Manero *et al.*, 2001). *G. diazotrophicus* also produces gibberellin GA_3, indole-3-acetic acid and gibberellin GA_1 (Bastián *et al.*, 1998; Fuentes-Ramírez *et al.*, 1993). Therefore, the induction of sugarcane growth by a *nif*$^-$ mutant of *G. diazotrophicus* under conditions of N fertilization (Sevilla *et al.*, 2001) might be accredited to the release of any of those phytohormones. TLC extracts of supernatant of the acetic bacterium *A. bogorensis*, and isolates of *Asaia* spp. obtained from the nance (*Byrsonima crassifolia*) plant, showed that this bacterium secretes IAA to the medium (Santoyo-Páez, Jiménez-Salgado and Fuentes-Ramírez; unpublished results). This suggests that the growth promoting effect of *Asaia bogorensis* in pineapple, reported by Weber *et al.* (2003a, 2003b) could be related to bacterial synthesis and release of IAA.

Bacteria related to the Rhizobia are nitrogen fixers in legumes and are known to contribute to the growth of non-legumes. Inoculation of *Bradhyrhizobium* in cotton improved nutrient uptake, which was attributed to IAA (Hafeez *et al.*, 2004). Similarly, growth responses in sunflower inoculated with *Rhizobium* sp. (Alami *et al.*, 2000), and in lettuce plants inoculated with *R. leguminosarum* (Noel *et al.*, 1996), were attributed to an increased nitrogen uptake besides bacterial phytohormone synthesis.

In addition to growth alterations, bacterial synthesized phytohormones have effects on the plant metabolism. IAA released by *Pantoea herbicola* seems to induce a much localized nutrient leakage affecting only the plant cells closest to the bacterial cells (Brandl and Lindow, 1998). Finally, IAA and cytokinins have inhibitory effect on the plant hypersensitive response (Robinette and Matthysse, 1990).

Although *in vitro* and *in vivo* activities do not always relate to each other, non-identified strains isolated from wheat rhizosphere showed a relationship between *in vitro* production of auxins and growth promoting capability. The isolates that *in vitro* produced higher quantity of auxins also had the highest promoting capability (Khalid *et al.*, 2004). This suggests that

in vitro screening for promoting mechanisms could be useful for selecting strains for potential application. Studies on plant hormone-synthesizing bacteria have focused on rhizospheric and endophytic bacteria, but it is known that epiphytes also produce such substances (Lindow and Brandl, 2003). The phytohormone-mediated roles of bacterial ephyphitic communities on plants are yet not clear.

The future of biofertilizers based on hormone-producing bacteria seems very promising. Large numbers of experiments have shown that bacterial participation raises the phytohormone levels in plants. This may be via bacterial synthesis or through bacterial induction of plant hormone synthesis but both offer economical and ecological advantages.

2.3 ACC deaminase activity

Ethylene exposition induces different observable changes in plants, including reduction in the growth rate (Abeles *et al.*, 1992). This is especially true in stressed dicot plants, since monocots are less sensitive to ethylene (Holguin and Glick, 2001). It has been proposed that PGPR may enhance plant growth by lowering the plant ethylene levels (Glick *et al*., 1998). In these cases, the immediate precursor of ethylene is 1-aminocyclopropane-1-carboxylate (ACC). This compound is hydrolyzed by bacteria-expressing ACC-deaminase activity. Ammonia and α-ketobutyrate, products of this hydrolysis, are used by the ACC-degrading bacterium as nitrogen and carbon sources (Honma and Shimomura, 1978). Bacteria belonging to phylogenetically distant genera such as *Alcaligenes* sp., *Bacillus pumilus*, *Pseudomonas* sp. and *Variovorax paradoxus* (Belimov *et al*., 2001) as well as, *Azoarcus*, *Azorhizobium caulinodans*, *Azospirillum* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Burkholderia vietnamiensis* and others (Dobbelaere *et al.,* 2003) were identified by their ability to grow on minimal media containing ACC as sole nitrogen source. Recently, expression of ACC deaminase activity was found in many strains of *Burkholderia unamae* and *B. vietnamiensis*, and the ACC deaminase gene (*acd*S) was also detected in these species as well as in *B. phymatum*, *B. xenovorans*, and *B. caribiensis* (Onofre-Lemus and Caballero-Mellado, unpublished results). It is postulated that ACC can be exuded to the rhizosphere and then degraded by plant-associated bacteria resulting in a final growth promoting effect (Glick *et al*, 1998). Mutations in ACC deaminase prevent the promoting effect of *Pseudomonas putida* in canola (Glick *et al*., 1994). The nutrient status of the plant, and the availability of nutrients, seems to be determinant for the plant growth activity of ACC deaminase carrying bacteria (Belimov *et al*., 2002). Plants grown under P-limiting or high N conditions were not enhanced by PGPR (Belimov *et al*., 2002). The role of ACC deaminase in growth promotion of plants is evident

in an inoculation experiment with *A. brasilense* Cd. This is a non-ACC deaminase strain, carrying an ACC deaminase gene from *Enterobacter cloacae* UW4. This strain promoted more growth of tomato seedlings than the wild type strain (Holguin and Glick, 2003). Considering that many phylogenetically distant genera are capable of expressing ACC deaminase activity, and, in order to determine the applicability of ACC utilizing bacteria in extensive cultivation of crops, especially of dicots, field experiments are required to determine their real growth promoting effect.

2.4 Other plant growth promoting mechanisms and expression of genes

Novel plant growth promoting mechanisms include phytase degrading organic phosphate compounds, mineral reduction by phenazines, synthesis of lumichrome, and production of volatile compounds. It is well known that phosphate-solubilizing bacteria can increase the availability of P to plants in deficient soils. These bacteria solubilizate phosphate through the production of acids, and possibly by means of other mechanisms as well (Nautiyal, *et al.*, 2000). Diverse bacteria, including *B. subtilis*, *K. terrigena*, *Pseudomonas* spp., and *Streptomyces griseus* produce phytases (Greiner *et al*., 1997; Kerouvo *et al*., 1998; Moura *et al*., 2001; Richardson and Hadobas, 1997). The role of phytases in raising the accessibility to P in organic compounds was demonstrated in maize-*Bacillus amyloliquefaciens* interaction experiments (Idriss *et al.*, 2002). Plants inoculated with a phytase secreting *Bacillus amyloliquefaciens* strain, or amended with its culture filtrate showed increased growth compared to non-inoculated plants, when grown under P-limitation.

Phenazines produced by bacteria are known for their antifungal activity, and consequently, improve the competitiveness of the producing strains. Recently, it has been shown that phenazines produced by *Pseudomonas chlororaphis* can reduce minerals (Hernández *et al*., 2004). Hence, such compounds could increase the availability of diverse nutrients, including iron, to the associated plants.

Lumichrome, a metabolite of riboflavin, is a molecule that enhances the respiratory rate of roots and increases the plant size as well (Phillips *et al*., 1999). This compound can be the product of photodegradation of riboflavin (Treadwell Jr., and Metzler, 1972). However, it can also be synthesized by *Sinorhizobium meliloti*, *Pseudomonas*, and possibly other plant associated bacteria (Phillips *et al.*, 1999; Yanagita and Foster, 1956). Apparently, lumichrome or even riboflavin producing-rhizospheric bacteria can benefit through organic carbon as also CO_2 release from lumichrome-induced roots (Phillips *et al*., 1999; Yang *et al*., 2002). In addition to other

bacterial metabolites, the volatile compounds 2,3-butanediol, and acetoin produced by *Bacillus* spp., were found to promote growth of *Arabidopsis thaliana* (Ryu *et al*., 2003). The suggested mechanism involves the cytokinin-signaling pathway.

One primary aim in microbial ecology is to determine the activity exhibited by the organisms that inhabit a particular habitat, and in particular, PGPR-plant associations. The interest is to find out what is needed for establishing the interaction, and, what happens when the interaction is established. In *Pseudomonas fluorescens*, a putative recombinase, located in the locus *xerC/sss*, is necessary for colonizing tomato plants (Dekkers *et al.*, 1998), but, the answer as to why that recombinase is at all required has yet not been determined.

In vivo expression technology (IVET) is an efficient tool that has allowed great advances in finding genes that are activated under particular environmental conditions (Mahan *et al.*, 1993). The study of plant colonization by *P. fluorescens* using IVET has found that expressed genes are related to nutrient acquisition, stress responses, biosynthesis of phytohormones, and antibiotics, and also to a type III secretion system, antioxidation, chemotaxis, and detoxification of aromatic compounds (Gal *et al.*, 2003; Preston *et al*., 2001; Rediers *et al*., 2003). Loci that seem to have special importance are the gene for a type III secretion system, the gene coding an acetylated cellulose polymer, and genes for detoxification of aromatic compounds. Similarly, genes particularly important for adhesion of *P. putida* to seeds are involved in pathogenesis, and efflux of toxic substances. Also genes with phenotypes are implicated with motility and chemotaxis (Espinosa-Urgel *et al*., 2000). Seed colonization by *E. cloaceae* seems to be highly related to the metabolic capability for using carbon sources that are exuded, since phosphofructokinase is needed for successful colonization of seeds that exude low amounts of carbohydrates (Roberts *et al.*, 1999). In the interaction of *Azoarcus* sp. with rice seedlings, it has been observed that the *pilAB* locus, encoding type IV pili, is necessary for the adhesion of the bacterium to the plant (Dorr *et al.*, 1998).

2.5 Environmental stress relief

Several associations between plants and beneficial bacteria show a protective response under restrictive environmental conditions. Wheat and faba beans subjected to saline stress showed greater growth when inoculated with *Azospirillum*, compared to non-inoculated plants (Bacilio *et al.*, 2004; Hamaoui *et al*., 2001). This favorable effect may be attributable directly to bacteria or indirectly to the effect on plant physiology. The production of microbial metabolites like polysaccharides modifies the soil structure, and has a positive effect on plants grown in water stress. Growth parameters of

sunflower plants under water stress inoculated with an exopolysaccharide (EPS)-producing *Rhizobium* sp. were greater than in uninoculated plants (Alami *et al*., 2000). Promotion effect in wheat plants was also observed after inoculation with an EPS-producing *Pantoea agglomerans* isolate (Amelal *et al*., 1998). In wheat plants inoculated with *Paenibacillus polymyxa*, the aggregation of rhizospheric soil depended on a bacterial polysaccharide that enlarged the amount of soil adhering to roots (Bezzate *et al*., 2000; Gouzou *et al*., 1993).

Bacteria can also stimulate the plant to turn on particular metabolic activity like increasing its exudates, and consequently, improve rhizospheric soil qualities (Heulin *et al.,* 1987). In the same way, inoculation of *Arabidopsis* with *P. polymyxa* the water-stress gene ERD15 is switched on (Timmusk and Wagner, 1999). Inoculated plants show improved response against pathogenic colonization and drought stress in comparison to control plants. Hence it seems that inoculation induces protection against biotic agents, and also against abiotic ones.

Overall, PGPR can protect a plant, against aggressive environmental and particularly hostile soil conditions through the bacterial release of soil structure-improving substances, and by inducing the plant to activate stress responsive mechanisms. In hostile soils, the use of bacteria that allow plants to thrive are probably the best option to obtain good yields at lesser ecological costs.

2.6 Improvement of other microorganism-plant interactions

PGPR can improve beneficial associations between Rhizobia and leguminous plants, as also between different plants and mycorrhiza. Bean plants co-inoculated with *Rhizobium etli* and *R. tropici* and *Azospirillum brasilense* had more nodules than plants inoculated only with one of the two Rhizobia (Burdman *et al*., 1996). Nodule occupancy was increased by co-inoculation of *Enterobacter* with *Bradyrhizobium* in green gram (Gupta *et al*., 1998). Additionally, the co-inoculation of a *Bradyrhizobium* strain with an *Enterobacter* or with a *Bacillus* isolate increased the dry weight of shoots and the grain yield in comparison with uninoculated plants or with plants inoculated with either of the strains alone (Gupta *et al.*, 1998). Similarly, the co-inoculation of soybean with *Serratia proteamaculans* 1-102 or *S. liquefaciens* and *Bradhyrhizobium japonicum* 532C increased nitrogen fixation, as well as the number of root nodules and the plant biomass, both at 25 and 15°C (Bai *et al*., 2002a; 2002b; Dashti *et al*., 1998). Generally, the benefits for the plant in the rhizobia-plant associations are debilitated at low temperatures; therefore, the enhancement of the rhizobial interaction of soybean at 15°C has special significance for the cultivation of this legume under temperate conditions. The enhancement of the *Bradyrhizobium*-

soybean interaction might be associated with substances secreted by *S. proteamaculans* 1-102, since it was also observed that the plant weight increased by adding the supernatant of the strain 1-102 to the plant (Bai *et al.*, 2002b). The PGPR mechanism for promoting nodulation is not precisely known, the secretion of substances resembling lipo-chitoologosaccharides could enhance the nodulation (Bai *et al*., 2000b; Burdman *et al*., 1996). Additionally, the plant could be induced to produce more flavonoids. For instance, in common bean inoculated with *Azospirillum* the flavonoids synthesized by the induction of *Azospirillum* were able to trigger the nod response of *R. etli* (Burdman *et al*., 1996). Generally, plant-associated bacteria by themselves may synthesize flavonoid-like substances capable of activating nodulation by Rhizobia (Parmar and Dadarwal, 1999). Additionally, a phytohormone effect on nodulation produced a higher density of root hairs, root length, and increased respiration rate in beans inoculated with *Azospirillum* than uninoculated plants (Burdman *et al*., 1996; German *et al*., 2000; Vedder-Weiss *et al*., 1999; Dobbelaere *et al*., 2003).

Different experiments have shown that mycorrhizal associations can be enhanced by the co-inoculation with bacteria. Arbuscular mycorrhization of red clover by *Glomus mosseae* increased when a strain of *Paenibacillus brasilensis* was also inoculated (Artursson *et al*., 2004). The mycorrhizal development and alkaline phosphatase activity of extraradical hyphae in maize was enhanced by inoculation with *P. putida*. The leaf area of maize was enlarged by the co-inoculation of mycorrhizal fungi and bacteria (Vosatka and Gryndler, 1999). Douglas-fir plantlets developed a greater height and a higher index of ectomycorrhization with *Laccaria bicolor* by inoculating the plants with *Pseudomonas fluorescens* (Frey-Klett *et al*., 1999). The capability of mycorrhizal formation by related fungi, *Laccaria fraterna*, in *Eucaliptus diversicolor* was also increased significantly by *P. fluorescens*, *B. subtilis*, *Bacillus* sp. or *Pseudomonas* sp. (Dunstan *et al*., 1998). Generally, bacteria belonging to different taxonomic groups can enhance the mycorrhizal associations or the growth of mycorrhizal plants or both. Even Rhizobia, which contribute to plant growth solely through N_2-fixation in legumes, participate in interactions. *Sinorhizobium meliloti* contributed to lettuce growth in mycorrhizal association, where a combination with *Glomus intraradices* increased the shoot biomass more than 100% comparing with uninoculated plants (Galleguillos *et al*., 2000). A particular characteristic of at least some enhancing-mycorrhiza bacteria is their capability to adhere to the hyphae. It has been observed that bacterial extracellular polysaccharides are not only important for cellular adhesion to plants but also to mycorrhizal fungi (Bianciotto *et al*., 2001). The importance of the bacterial adherence as a prerequisite for possible improvement of mycorrhizal growth or for mycorrhiza-plant interaction is yet not clear.

The PGPR can induce mycorrhizal formation by stimulating hyphal growth out of the roots and also through-provoking sporulation (Dunstan *et al.*, 1998; Tiwari *et al*., 2004). The natural association between the mycorrhizal fungi *Tuber borchii* and unknown nitrogen-fixing bacteria suggested the possibility of transfer of fixed nitrogen to the fungi. In fact, RT-PCR analysis of fruiting bodies of the fungi identified *nifH* transcripts, indicated a possible nitrogen fixation (Barbieri *et al.*, 2004). Although the bacterial mechanisms for enhancing mycorrhization are not entirely known, some of them could be the following: transfer of nutrients to the hyphae; interacting with bacterial synthesized hormones; synthesis of hyphal growth stimulating substances or induction to the plant to produce these substances, (Bécard *et al*., 1992); improved receptiveness of the plant; enhancement of soil structure; intervention in the recognition process; stimulation of germination; and improvement of hyphal growth (Bécard *et al*., 1992; Burdman *et al*., 1996; Nair *et al*., 1991; Parmar and Dadarwal, 1999; Tsai and Phillips, 1991; Xie *et al*., 1995; Garbaje, 1994).

The applicability of co-inoculations has been proved for certain associations, but field experiments with a large number of crops are required to determine the real importance of biotic and abiotic factors in these interactions.

3 BIOFERTILIZERS, APPLICATIONS AND OPINIONS

Many studies in glasshouse and fields have assessed the effect of rhizobacteria and endophytic species on plant growth, grain yield of annual crops, and the cultivars of different crops to save fertilizers, or to diminish pollution caused by agrochemicals, or, both. *Azospirillum* head the list of PGPR assessed worldwide in tens of experiments (Burdman *et al*., 2000; Dobbelaere *et al*., 2001 and 2003; Okon and Labandera-González, 1994; Lucy *et al*., 2004; Vessey, 2003). Diverse studies have been published about the effects of other rhizobacteria on plant growth (Kennedy *et al*., 2004; Lucy *et al*., 2004). In addition to *Pseudomonas* and *Bacillus* species (Alam, *et al*., 2001; Cakmakci *et. al*., 2001; Glick *et al*., 1994; Kokalis-Burelle *et al*., 2002), other PGPR and endophytic bacteria, such as *Enterobacter* (some of them currently *Pantoea*), *Klebsiella pneumoniae*, *Burkholderia* (formerly *Pseudomonas*) *cepacia* and *Stenotrophomonas* (formerly *Pseudomonas* and later *Xanthomonas*) *maltophilia*, have received increasing attention in recent years, because of their association with important crops and potential to enhance plant growth (Dong *et al*., 2003; Chelius and Triplett, 2000; Sturz *et al*., 2001; Verma *et al*., 2001). Despite the undoubted economic and

ecological benefits of some bacterial species as biofertilizers the application of such PGPR must be very carefully assessed because of their importance as opportunistic pathogens in nosocomial infections and in patients with diverse diseases. For instance, *S. maltophilia* is often found in the rhizosphere and in association with cultivated plants such as maize, potato, wheat, and others (Blondeau, 1980; Garbeva *et al*., 2001; Heuer and Smalla, 1999; Juhnke *et al.*, 1987; Lambert *et al*., 1987; Sturz *et al.*, 2001). Moreover, *S. maltophilia* produces high amounts of indole-3-acetic acid (Berg and Ballin, 1994; Berg *et al*., 1996). However, this bacterium has been associated with bacteremia, endocarditis, cystic fibrosis, urinary tract infections (Friedman *et al*., 2002; Khan and Mehta, 2002; Marchac *et al*., 2004; Vartivarian *et al.*, 1996), and many other diseases in humans (Nicholson *et al.*, 2004; Senol, 2004). Similarly, *B. cepacia* and the nitrogen-fixing species *B. vietnamiensis* are often associated with plants (Estrada-de los Santos *et al*., 2001; Dalmastri, *et al*., 1999; Di Cello, *et al*., 1997; Fiore *et al*., 2001; Trân Van *et al*., 1994), and their activity as PGPR has been documented (Bevivino *et al*., 2000; Trân Van *et al*., 1994; Trân Van *et al.*, 2000), but, both species have been isolated from patients with cystic fibrosis (Coenye *et.al;* 1999; Frangolias *et al.*, 1999; Vandamme *et al*., 1997). Biofertilizer formulation using opportunistic pathogens must be not justified because they are found in most soils and are plant-associated. The analysis of phenotypic and genotypic features from clinical and environmental isolates of *S. maltophilia* has revealed that the grouping of strains is independent of source (Berg *et al*., 1999). In fact, it has been suggested that environmental sources could be an important mode of transmission of *S. maltophilia* (Denton *et al.*, 1998). On this basis, biofertilizers containing opportunistic pathogens commonly found in soils and plants will represent significant risk for human health. Bacterial biodiversity is enormous and so it is not necessary to use opportunistic pathogens in the production of new biofertilizers. It would suffice to search among the myriads of bacteria for novel species that promote plant growth and crop yields.

Among the PGPR, *Azospirillum* species heads the list of bacteria used in commercial products (Burdman *et al*., 2000; Lucy *et al*., 2004). Yet except those formulated with *Azospirillum*, all these products are applied to crops as biopesticides or biocontrol agents. *Azospirillum* inoculants are available for maize in Europe and in South Africa (Dobbelaere *et al*., 2001). This incipient commercialization seems to be based in tens of field inoculation experiments with *Azospirillum* carried out during the 1980s and early 1990s. These experiments were carried out on many crops under a variety of soil and environmental conditions, which frequently resulted in significant yield increases ranging from 5 to 30% (Okon and Labandera-Gonzalez, 1994). The extensive commercialization of PGPR biofertilizers has been limited worldwide, yet Latin America has shown increased interest

in the application of *Azospirillum* inoculants during last few years. For instance, in Mexico the Autonomous University of Puebla in collaboration with the Rural Development Ministry (Secretaría de Desarrollo Rural) in Puebla State, in 2002 produced *Azospirillum*-based biofertilizers for 15,000 ha of maize, wheat, barley and sorghum (Mascarúa-Esparza, M. A. and Carcaño-Montiel, M., personal communication). Also in Mexico, a large field-inoculation program (around 600,000 ha) with maize, wheat, sorghum and barley was carried out in 1999 through the Ministry of Agriculture Research Institute (INIFAP) in collaboration with the Nitrogen Fixation Research Center (CIFN-UNAM). Due to positive responses in 1999 (Dobbelaere *et al*., 2001) the demand by farmers reached about 1.5 million ha of crop fields in 2000. Presently, the company ASIA (Asesoría Integral Agropecuaria, S.A.) in Mexico sells a product for maize and sorghum, and another for wheat and barley, containing a mixture of *A. brasilense* strains. Companies in South America are developing new products based on *Azospirillum* species, e.g., Lage & Co. in Uruguay and Nitrasoil and Nitragin in Argentina. Despite the numerous positive results, often it is claimed that the commercialization of PGPR (especially of *Azospirillum* species) biofertilizers on a large scale has been limited due to the variability and inconsistency of field results (Bashan and Holguin, 1997; Lucy *et al*., 2004; Vande Broek *et al*., 2000; Vessey, 2003). Inconsistency and variability in yield responses have been attributed to adverse conditions such as interaction of rhizospheric organisms (Lucy *et al*., 2004; Vande Broek *et al*., 2000; Vessey, 2003), physical and chemical conditions of the soil (e.g., low pH), poor ability of the PGPR strain to colonize the plant roots, environmental factors including high mean temperatures, and, low rainfall during the growing season (Lucy *et al*., 2004; Vande Broek *et al*., 2000), as well as to host cultivars (Vassey, 2003). Undoubtedly, many or all these factors could be involved in the lack of consistent responses and successful application of biofertilizers. However, often the so-called "variability and inconsistency" of field results due to inoculation with *Azospirillum*, is based in the comparison of experiments carried out in different years, and consequently, with different environmental conditions, or, with different cultivars, as well as, in soils with different characteristics, and, not taking into account many others varying factors, such as, strains inoculated, and the number and physiological state of the bacterial cells, etc. On the basis of such comparisons, it is obvious that there exists a huge "inconsistency and variability" in the response of crops to bacterial biofertilizers. This great variability and inconsistency has been observed in the application of mineral fertilizers in diverse crops cultivated under different environmental and soil conditions, and the yield response to mineral fertilizers has been recorded. The experiments were carried out in the same or different sites and on the same crops, and in different years and varying environmental conditions.

The results were reported as "fertilized or control treatments" in studies of inoculation with *Azospirillum* (Albrecht *et al.*, 1981; Zaady *et al.*, 1994; Kapulnik *et al*., 1983; Kapulnik *et al*., 1987). Unfortunately, researchers who emphasize the so-called "variability" of field results when PGPR performance is evaluated do not take into account the variability of yield response of crops to mineral fertilizers. However, it is well known that soil is a highly variable environment and expected results are sometimes hard to reach (Bashan 1998) either with PGPR biofertilizers or with mineral fertilizers. Table 1 shows the response of crops to *Azospirillum* inoculation and to mineral fertilizers.

Table 1. Field inoculation of cereals with *A. brasilense* in different regions of México (summer of 1999).

State/crop/cultivar	N level kg ha^{-1}	Grain yield (kg ha^{-1})		Difference (%)
		Control*	Inoculated	
Campeche				
Maize cv. H-515	110	4,590	5,100	+ 10
Chiapas				
Maize cv. H-515	120	3,862	4,125	+ 6
Michoacán				
Maize cv. H-515	110	6,406	6,887	+ 8
Guanajuato				
Sorghum cv. D-65	90	6,235	6,486	+ 4
Morelos[†]				
Sorghum cv. D-65	90	5,335	6,223	+ 17
Sorghum cv. D-65	90	4,105	4,900	+ 19
Puebla				
Sorghum cv. D-65	80	7,604	8,025	+ 6
Tlaxcala				
Barley cv. Esmeralda	0	1,444	2,387	+ 65
Hidalgo				
Barley cv. Esmeralda	0	1,600	2,590	+ 62

*Non-inoculated control

[†]Different sites in the same state

Data in Table 1 are based on evaluation made by farmers and INIFAP agronomists in areas from 1 to 2 ha in a large field-inoculation program carried out in 1999 in different states of Mexico. Although the same cultivar of maize, sorghum or barley was used, the variability of yield

response of crops to *Azospirillum* inoculation, as well as, to mineral fertilizers is clear. These results strongly suggest that the variation of the response was due to different environmental and soil conditions. However, the consistency of field results due to inoculation with *Azospirillum* is noteworthy.

Although different soil types can influence the effectiveness of PGPR (Kloepper *et al*., 1980), the bacterial strain of a particular species, as well as, the number and physiological state of the cells, play an important role for obtaining the expected beneficial effect on the plant growth promotion. Many inoculation experiments using a particular PGPR strain have been carried out as if such a strain could be universally successful in any soil and with any host plant. This is particularly true with *Azospirillum brasilense* strains Cd or Sp7, which were used worldwide in tens of experiments to inoculate a great variety of plant species (Lucy *et al*, 2004; Okon and Labandera-Gonzalez, 1994). Most of the inoculation experiments using the strains Cd or Sp7 were successful in increasing plant growth and yield of many crops. Nevertheless, negative or poor responses of different crops with strains Cd and Sp7 were reported (Baldani *et al*., 1987; Boddey *et al*., 1986; Smith *et al*., 1984; Wani *et al*., 1985). Furthermore, clear differences were reported between strains of *Azospirillum* in their ability to promote plant growth in greenhouse and field trials (Saric, *et al*., 1987; Venkateswarlu and Rao, 1983), and local *Azospirillum* strains often performed better than introduced ones such as Cd or Sp7 (Caballero-Mellado *et al*., 1992; Paredes-Cardona *et al*., 1988). The importance of the *Azospirillum* strain appears to be controversial with respect to promotion of plant growth (Okon and Labandera-Gonzalez, 1994), however, a single strain cannot be universally successful under all soil conditions, and with all hosts. A useful alternative is the formulation of multi-strain biofertilizers. For successful experiments with *Azospirillum*, researchers generally paid special attention to the optimal number of cells in the inoculant (Vande Broek *et al.*, 2000; Okon and Labandera-Gonzalez, 1994) and physiological state of cells (Okon and Labandera-Gonzalez, 1994), e.g., a high content of poly-β-hydroxybutyrate (PHB), formation of cysts and cell aggregation (Caballero-Mellado and Mascarúa-Esparza, unpublished results; Neyra *et al*., 1995; Okon and Itzigsohn, 1995; Sadasivan and Neyra, 1985), characteristics which are known to differ according to the culture conditions. Obviously, changes in pH, dissolved oxygen, and temperature affect the replication rates of the bacteria and their physiological state. Aggregating *Azospirillum* cells accumulate high amounts of PHB, but it depends on the carbon and nitrogen sources and their concentration in the culture media (Burdman *et al*., 1998; Fallik and Okon, 1996). Importance of cyst formation and cell aggregation as well as a high content of PHB are particularly relevant features with *Azospirillum*, because cells are more resistant to

Table 2. Field inoculation of traditional and commercial maize cultivars with a mixture of *A. brasilense* strains in different regions of México during 1999 and 2000.

Treatment	No. of evaluated sites	Evaluated area (ha)	Positive effects (%)	Yield increase* (%)	Average increase (%)
SUMMER OF 1999					
Without N-fertilization					
Traditional maize	14	56	96	12-98	42
Commercial maize	31	124	94	7-76	26
N-fertilization less than 100 Kg ha^{-1}					
Traditional maize	16	64	54	8-78	34
Commercial maize	32	128	60	6-56	30
N-fertilization higher than 110 Kg ha^{-1}					
Commercial maize	34	136	55	6-19	12
Total	127	508			
Average			72		29
SUMMER OF 2000					
Without N-fertilization					
Traditional maize	6	12	94	11-99	44
Commercial maize	8	16	92	4-44	31
N-fertilization less than 100 Kg ha^{-1}					
Traditional maize	7	14	58	12-79	41
Commercial maize	10	20	62	6-67	24
N-fertilization higher than 110 Kg ha^{-1}					
Commercial maize	12	24	50	8-16	10
Total	43	86			
Average			71		30

Positive effect (%) is defined as the percentage of experiments where beneficial effects were observed.

*Range of grain yield increase above non-inoculated plants.

desiccation, ultraviolet light and starvation stresses, and PHB can be used as an internal carbon and energy source for growth (Tal and Okon, 1985), which positively affects their survival in soil. These features also are relevant when peat is used for *Azospirillum* formulations. In Mexico, large-scale use of *Azospirillum* multi-strain (selected local strains) biofertilizers

with maize, wheat, sorghum and barley, gave better and consistent results when using peat inoculants formulated with PHB-rich cells. For instance, grain yields of modern and traditional maize cultivars were evaluated at 127 sites and in 254 ha with diverse soil and climatic conditions as well as different levels of nitrogen fertilization during 1999. Similarly, grain yields of maize were evaluated at 43 sites and in 86 ha during 2000. When nitrogen fertilizers were not applied to traditional and modern maize cultivars, the inoculation with *Azospirillum* exerted beneficial effects in 95 and 93% of the sites evaluated during 1999 and 2000, respectively. However, when fertilizers were applied in levels higher than 110 kg N/ha, the positive responses on the maize yield were observed only in 55 and 50% of the sites evaluated in 1999 and 2000, respectively. Although the yields evaluated in 1999 and 2000 were from different sites, with different cultivars and levels of N fertilizers, the inoculation of maize with *Azospirillum* showed consistent average yield increases in the production of grain of about 30% (Table 2). Considering the magnitude of these evaluations, the results show a very acceptable consistency and reflect that the large-scale use of *Azospirillum* biofertilizers is possible, but it requires previous well-focused strategies of field experimentation (Fages 1994). Recommendations, formulation and application technology of *Azospirillum* inoculants have been described (Fages, 1992; Okon and Itzigsohn, 1995).

Finally, when developing PGPR biofertilizers, the strain(s), the inoculum production and, in general, the development of appropriate formulations as well as strategies of field experimentations are fundamental conditions for a successful application of PGPR species, at least in the case of *Azospirillum* inoculants.

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Chapter 6

ROLE OF CYTOKININS IN PLANT GROWTH PROMOTION BY RHIZOSPHERE BACTERIA

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Abstract: Plant growth regulators (PGRs) are organic substances that influence the physiology and development of plants at very low concentrations. Cytokinins are one of the five major groups of PGRs or phytohormones and regulate cytokinesis in plant cells. Soil microorganisms are capable of synthesizing PGRs such as cytokinins in pure culture, soil and in association with plant tissues. This chapter reviews the structure and function of cytokinins in plant tissues and their production by plant growth promoting rhizobacteria (PGPR). A role for microbially-produced cytokinins in plant growth and development is proposed. Cytokinin production by PGPR is an innovative alternative to enhance plant growth and may be a sustainable approach to improve the yield and quality of agricultural crops. However further research is necessary to understand the principles underlying cytokinin production by rhizobacteria and to develop cytokinin-producing inoculants for practical application by growers.

Key words: cytokinins; phytohormones; plant growth regulators; PGPR; rhizobacteria

1 INTRODUCTION

Plant growth regulators (PGRs) are organic substances that influence the physiology and development of plants at very low concentrations. They are often effective at internal concentrations lower than 1 μM, whereas amino acids, organic acids, sugars, and other metabolites necessary for growth and development are usually present at concentrations of 1 to 50 m*M*. Plant growth and development are likely to be governed by PGR

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 173–195.

concentration and tissue sensitivity to PGRs (Venis, 1987). PGRs also play a crucial role in controlling the way in which plants grow and develop. "While metabolism provides the power and building blocks for plant life, it is the hormones that regulate the speed of growth of the individual parts and integrate them to produce the form that we recognize as a plant" (Davies, 2005).

Auxins, gibberellins, cytokinins, ethylene and abscisic acid (ABA) are the five major groups of PGRs, usually called phytohormones, are synthesized endogenously by plants and have beneficial effects on plant growth and development (Salisbury and Ross, 1992; Arshad and Frankenberger, 1993). Brassinosteroids and polyamines are also PGRs endogenously synthesized by plant tissues (Sasse,1991; Galston and Sawhney, 1990). PGRs also include synthetic compounds that cause many physiological responses when they are exogenously applied to plant tissues (Salisbury and Ross, 1992).

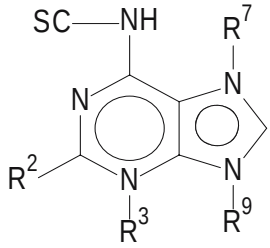
Soil microorganisms can promote plant growth by enhancing soil organic matter transformations, mobilizing inorganic nutrients, producing PGRs, acting as antagonists against pathogens and by several other mechanisms (Bolton *et al*., 1993). Scientists have attempted to alter the microflora of agricultural soils in order to favor plant growth and yield. Rhizobacteria that exert beneficial effects on plant growth and development have been termed plant growth-promoting rhizobacteria (PGPR) by Kloepper and Schroth (1978). Some PGPR are capable of synthesizing PGRs in pure culture and soil and these PGRs can be estimated by different techniques (Arshad and Frankenberger, 1993). In this chapter we shall discuss the structure, function and analysis of cytokinins. We shall also discuss PGRs in the rhizosphere, effects of PGPR on plant growth and development and PGPR and PGRs production.

2 CYTOKININS

2.1 Structure and function in plant development

Cytokinins are PGRs that regulate cytokinesis in plant cells (Skoog *et al.,* 1965). Following the discovery of kinetin (6-furfurylaminopurine), a compound isolated from autoclaved herring sperm DNA and exhibiting potent cell-division-promoting activity (Miller *et al.,* 1956), a naturally occurring kinetin-like compound was isolated from maize, sunflower fruits and soybean (Miller, 1961). The first naturally occurring pure crystalline cytokinin was isolated from *Zea mays* and named zeatin (Z) by Letham (1963). The structure of zeatin, (E)-4-(hydroxy-3-methyl-but-2-enyl)

aminopurine was confirmed by chemical synthesis (Shaw and Wilson, 1964) (Fig. 1). The natural occurrence of Z was confirmed both in *Z. mays* and in



SC1:-CH_2-CH=C(-CH_3)-CH_3

SC2:-CH_2-CH=C(-CH_3)-CH_2-OH

SC3:-CH_2-CH_2-CH(-CH_3)-CH_2-OH

Adenine (Ade) Structure

Modifications of the Adenine Structure

Form	**Abreviation**	**Type of Side Chain (SC)**	**Moiety attached at indicated N position**
Base	IPa	SC1	None
	Z	SC2	None
Riboside	IPA	SC1	Ribose at R^9
	ZR	SC2	Ribose at R^9
Nucleotide	IPNT	SC1	Nucleotide at R^9
	ZRNT	SC2	Nucelotide at R^9
N-Glucose (G) conjugate		SC1 / SC2	G attached at R^3, R^7 R^9
N-Alanine (A) conjugate		SC1 / SC2	A at R^9

Modifications of Side Chain

Form	**Abreviation**	**Type of SC**	**Moiety attached at indicated N position**
Dihydro-derivatives	DHZ	SC3	None
	DHZR	SC3	Ribose at R^9
O-Glucosyl derivatives	ZOG	SC2-G	None
	DHZOG	SC3-G	None
O-Acetyl (Ac) derivatives	OAcZR	SC2-Ac	Ribose at R^9
	OAcDHZ	SC3-Ac	None
O-Xylosyl (X) derivatives	OXZ	SC2-X	None
	OXDHZ	SC3-X	None

Fig. 1. Adenine cytokinins and derivatives after modifications of the purine structure and side chain. Compiled from Jameson (1994); Kaminek (1992), Brzobohaty *et al.*, (1994).

many other sources (Letham, 1978). Several new cytokinins have been isolated from natural sources and most of them were N^6-substituted adenine or substituted derivates and N- or O- glycosides and their phosphorylated derivatives (Letham, 1994; Shaw, 1994) (Fig. 1).

In addition to their widespread occurrence in plant tissues, numerous cytokinins have also been isolated from t-RNAs of virtually all organisms (Skoog and Armstrong, 1970). Possible functions of modified nucleosides of tRNA include influencing tRNA structure, providing recognition sites, affecting the efficiency and accuracy of translation, and having a regulatory role. Only modifications found in position 37 are the hypermodified, hydrophobic isopentenyl adenosine (IPA) derivatives (Fig. 1) known as cytokinins (Taller, 1994), and their distribution among organisms seems to show inter-kingdom differences (Skoog and Armstrong, 1970; Sprinzl *et al.*, 1991).

Cytokinins can be structurally classified into two categories: the adenine cytokinins (Fig. 1) and the diphenylurea cytokinins (Fig. 2) (Shaw 1994; Shudo, 1994). Both types of cytokinins have similar structure-relationships and similar biological activity suggesting that adenine and urea cytokinins are agonists and both types of cytokinins may act through a common receptor (Shudo, 1994).

Cytokinins have been found in roots, stems, leaves, flowers, fruits and seeds and are probably present in all living cells of intact higher plants (Salisbury and Ross, 1992). The extremely low levels of the endogenous cytokinins in plant tissues and the central role of the most likely precursors in cellular metabolism have made it difficult to determine the sites of cytokinin biosynthesis (Letham, 1994). However, strong evidence indicates that the root is the main site of cytokinin biosynthesis (Neuman *et al.*, 1990; Nooden and Letham, 1993). Cytokinins move from roots and embryonic axis to other tissues to control diverse aspects of development by interacting with other PGRs.

Elucidation of the metabolic pathways of cytokinins has been largely based on the use of exogenous radiolabeled cytokinins (Letham, 1994). Cytokinins exogenously applied to plant tissues usually are rapidly distributed among the respective nucleotide, nucleoside and base forms (Fig. 1), but are ultimately broken down either by side chain cleavage to the corresponding adenine derivates which are irreversibly inactived or by conjugation into storage or inactive forms (Jameson, 1994). Comprehensive reviews on cytokinin metabolism have been published elsewhere (Kaminek, 1992; Brzobohaty *et al.*, 1994; Frankenberger and Arshad, 1995). Extensive studies carried out on several plant species have revealed that metabolic differences exist even at the organ and tissue levels (Turner *et al.* 1985; Hocart and Letham, 1990) and specific studies of the biosynthesis and

metabolism of any particular system should be defined on the basis of this knowledge.

Cytokinins play a major or minor role throughout development, from seed germination to leaf and plant senescence and modulate physiological processes important throughout the life of the plant, including photosynthesis and respiration (Salisbury and Ross, 1992; Mok, 1994; Arshad and Frankenberger, 1993). The range of oxidative processes controlled by cytokinins includes senescence inhibition, cell growth, secondary-compound metabolism, respiration inhibition during senescence and stimulation of respiration during development (Musgrave, 1994). The variability of cytokinin effects suggests that these PGRs might have different mechanisms of action in different tissues, or that they have a common primary effect, which is followed by numerous secondary effects that depend on the physiological state of the target cells (Salisbury and Ross, 1992).

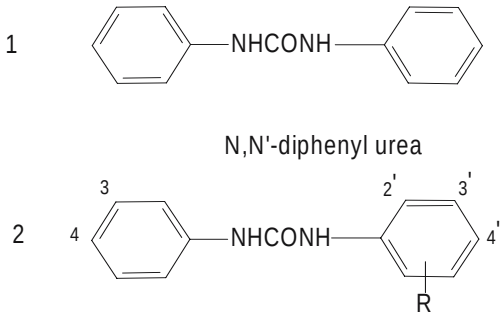
2.2 Analysis of Cytokinins

Following identification of kinetin and its effects on cell division and callus growth, research effort in the early 1960s was directed to development of cytokinin bioassays (Shaw, 1994). Bioassays have been indispensable for the detection of cytokinin activity and for evaluation of the activities of numerous compounds (Salisbury and Ross, 1992). A number of bioassays has been devised based on the various biological effects of cytokinins. The lettuce seed germination assay is based on the relationship between kinetin and red-light promotion (Miller, 1958). The *Funaria protonemata* (Hahn and Bopp, 1968) and pea lateral bud (Thimann and Sachs, 1966) bioassays are related to the ability of cytokinins to promote formation of new buds and release buds from apical dominance, respectively. The etiolated bean leaf disc (Miller, 1963), *Spirodela* frond expansion (Letham, 1967) and radish cotyledon (Letham, 1971) bioassays were based on the activity of cytokinins on leaf and cotyledon expansion. Senescence bioassays were devised with various plant species (Letham, 1967; Osborne and McCalla, 1961; Letham *et al.*, 1983). The *Amaranthus* bioassay measures the formation of betacyanin (Biddington and Thomas, 1973; Kohler *et al.*, 1987); and the cucumber cotyledon bioassay (Fletcher *et al.*, 1982) depends on the formation of chlorophyll.

Specificity, high sensitivity and detection of minute quantities are the essential attributes determining the efficacy of a bioassay (Skoog and Armstrong, 1970). In addition, quantitative bioassays allow comparisons to the activity of known standards (Letham *et al.*, 1983). Callus bioassays are generally specific, sensitive and quantitative but require a long assay time. A comparison of the activities of Z-derived cytokinins showed that tobacco

callus bioassays had the highest sensitivity (Letham *et al.*, 1983). Bioassays have been used to assess cytokinin activity of new compounds and cytokinin activity of plant extracts. The former demonstrate that a compound, either directly or indirectly, exerts cytokinin activity. The latter were essential in the earlier period of cytokinin research, but in recent years they have been replaced by more precise methods of cytokinin analyses, such as chromatography, immunoassays or quantitative mass spectra analyses.

Numerous analytical procedures have been used for the detection, isolation and identification of cytokinins, but few of them were developed specifically for cytokinins and they have been borrowed from the field of purine chemistry and biochemistry (Horgan, 1978). Most early procedures were extremely labor-intensive, time-consuming, imprecise and relatively insensitive (MacDonald and Morris, 1985). Several reports have demonstrated the advantages of high performance liquid chromatography (HPLC), for the separation of cytokinins in bioassays, over the more commonly used chromatographic methods of ion-exchange, paper and thin-layer, Sephadex LH20, and GLC (Challice, 1975; Kannangara *et al.*, 1978;



2a: *N*-3-fluorophenyl-*N'*-4-fluorophenylurea
2b: *N*-4-nitrophenyl-*N'*-4-phenylurea
2c: *N*-4-nitro-3-trifluoromethylphenyl-*N'*-phenylurea
2d: *N*-3, 4-dichlorophenyl-*N'*-phenylurea
2e: *N*-phenyl-*N'*-4-pyridylurea [(1)]

Fig. 2. Basic structures of the diphenylurea cytokinins

[Compounds (2a-2e) have consistently high or moderate activity in the initiation of cell division of tobacco pith but weak activity in the standard tobacco callus bioassay (Takahashi *et al.*, 1978). R: indicates possible substitutions of Cl, F, Br, CH_3O, OH or NH_2 on the *N'*-phenylurea ring which greatly enhance the activity. **(1)**: The pyridyl radical also can be attached to the positions 2, 3 of *N'*-phenylurea ring].

MacDonald *et al.,* 1981). Unfortunately, HPLC in association with bioassays is still labour-intensive and time consuming. The introduction of mass spectrometric methods in which the cytokinins were converted to volatile derivatives and subjected to gas liquid chromatography (GLC) prior to mass spectrometry (MS) improved precision but not sensitivity (Summons *et al.,* 1979). Detailed reviews about GC-MS and related methods for analysis of cytokinins have pointed out the necessity of several purification methods involving extraction, column separations, thin layer chromatography (TLC), HPLC and GC (Horgan, 1978; Palni *et al.,* 1986). The major challenge in the application of this technique is the purification of the complex plant extracts to a less-complex mixture where the mass spectrometric response may specifically correlate with cytokinin content (Hedden, 1986; Teller, 1994).

Radioimmunoassay (RIA), initially developed to quantify human hormones at physiological tissue concentrations**,** was adapted to determine cytokinins and became a highly specific and very sensitive analytical method (Weiler, 1980). The past thirty years have witnessed a marked growth in the use of specific antibodies in studies of cytokinin biology because cytokinins occur in many forms at relatively small concentrations and analytical procedures with high sensitivity and selectivity are required. Immunoassays were applied to the analysis of cytokinins after it was demonstrated that coupling of nucleosides to proteins gave immunogenic conjugates, which could be used to produce nucleoside-specific antisera (Constantinidou *et al.,* 1978; Milstone *et al.,* 1978). Quantification of cytokinins by immunoanalysis is based upon the competition of cytokinins present in a sample with a known quantity of labeled or immobilized cytokinin for binding to an anti-cytokinin antibody. The degree of competition is measured using either radiolabeled cytokinin (RIA) or enzyme-linked immunosorbent assays (ELISA). A standard curve based upon the addition of specific quantities of cytokinins to the assay is used to quantify cytokinins present in samples. Since the first report of an anti-cytokinin antibody by Hacker *et al.* (1970), antisera and monoclonal antibodies have been used to isolate and quantify endogenous cytokinins from a wide variety of plant tissues (Weiler, 1984; Hansen *et al.,* 1988; Saavedra-Soto *et al.,* 1988; Doumas *et al*., 1989) and microbial sources (Muller *et al*., 1988; Taller and Wong, 1989; Kraigher *et al.,* 1991; Morris *et al*., 1991; Upadhyaya *et al.,* 1991). As immunoassays have both high specificity and sensitivity with detection limits at the femtomole level, plant crude extracts can be used (Weiler and Ziegler, 1981; Weiler, 1984; Belding and Young, 1989; Young, 1989). Other advantages of the immunoassays are that at least one hundred samples can be completed in one day and the assay reproducibility is high with variation coefficients of triplicates of less than 5% (Weiler, 1984). Immunoassays have a wide field of application because they allow a much more detailed resolution in time and space of cytokinin levels within whole

plants and other plant systems, avoiding or simplifying complicated extraction procedures. However, the potential of immunoassay should not be overestimated and appropriate assay validation should always be considered (Banowetz, 1994). Extensive standardization procedures and repetitive dilution of the samples are standard for immunoanalysis and compared to other assays it has low sample capacity (Weiler, 1984; Banowetz, 1994).

Although the major analytical difficulties in physiological work, namely the rapid and reliable quantification of small amounts of physiologically relevant cytokinins as well as other PGRs in large series of samples, cannot be solved with the GC-MS technique, this analysis undoubtedly will remain as the method of choice for identification of cytokinin structures, elucidation of new structures in metabolic studies, and validation of immunoassay (Banowetz, 1994).

3 PGRs IN THE RHIZOSPHERE

Microflora able to produce PGRs *in vitro* are present in appreciable numbers in the rhizosphere of plants (Kampert *et al*., 1975; Barea *et al*., 1976; Arshad and Frankenberger, 1993). Veselov *et al*. (1998) isolated a high-molecular weight complex of polysaccharide and biologically active cytokinins in liquid cultures of *Bacillus* species commonly isolated from the rhizosphere of cultivated plants. The main cytokinins observed using enzyme immunoassay and thin-layer chromatography were ZR and a nucleotide. Ivanona *et al*. (2000) also found ZR in the liquid culture of the pink-pigmented facultative methylotroph (PPFM) *Methylobacterium mesophilicum* VKM B-2143 and the non-pigmented obligate methylotroph *Methylovorus mays* VKM B-2221. Trotsenko *et al*. (2001) reviewed the potential of aerobic methylotrophic bacteria as phytosymbionts and physiological, biochemical and molecular genetic aspects of their applications. Ashby (2000) suggested a role for PPFMs in cytokinin biosynthesis in plants.

Soils differ considerably in their PGR-synthesizing capacity, depending on their fertility status and organic matter content (Stevenson, 1986; Arshad and Frankenberger, 1993). Auxins in soil are derived from decomposition of carbonaceous materials from dead and living plant residues (Whipps and Lynch, 1983). The continuous release of root-derived organic carbon compounds in the rhizosphere stimulates an active rhizosphere microflora. Auxin- and gibberellin-like components were more abundant in the rhizosphere soil of maize than in non-rhizosphere soil, especially during seedling emergence while the highest amounts of cytokinin-like components were observed during anthesis (Rossi *et al*., 1984).

Van Staden and Dimalla (1976) have observed that cytokinin activity in acidic soils supporting *Acacia mearnsii* yielded high Z activity while soil supporting trees in association with mycorrhizal fungi, yielded an activity peak corresponding to ZR. However, it was not determined whether the cytokinins isolated from soils were leached from senescing leaves or were produced by the fungi or other microorganisms. Ho (1986) observed that phosphatase, nitrate reductase activities and PGR production differed considerably among different isolates of *Pisolithus tinctorius*. Thus, PGR production expressed as micromoles per gram of fungal dry weight was reported in the range of 22.1-271.4 for cytokinins**,** 56.0-1045.4 for IAA and 5.2-19.3 for gibberellins. Variability in ectomycorrhizal development among isolates of *P. tinctorius* has been reported (Molina, 1979; Marx, 1981) and was related to PGRs liberated by their mycelia (Navratil and Rochon, 1981). However, the significance of these *in vitro* variations to successful inoculation and desired host response needs to be experimentally determined in fungus-host-soil systems.

Although a higher percentage of microorganisms isolated from rhizosphere soil than from root-free soil are capable of synthesizing PGRs, they can influence plant growth only if the released PGR is taken up by the plant and is not metabolized by other microorganisms. Symbiotic associations such as mycorrhizal fungi, provide a direct route for PGR uptake by plants, establishing a bridge connecting the plant root with the surrounding soil microhabitats (Azcon-Aguilar and Bago, 1994). Although, mycorrhizae are widespread in the plant kingdom, the physiological effects of mycorrhizal PGRs need further study in order to improve their beneficial potential in specific plant associations (Arshad and Frankenberger, 1990). Moreover, changes in the root exudation patterns, PGR balance of the plant and PGR production by rhizosphere microorganisms affect the establishment of mycorrhizal fungi in the root cortex (Barea, 1997). Thus, microbe-microbe interactions are crucial to the understanding of events that occur at the root-soil interface (Lynch, 1990).

3.1 Effects of PGPR on plant growth and development

PGPR, such as *Azotobacter, Azospirillum* and *Rhizobium* induce alterations in plant physiology or produce metabolites such as PGRs that directly promote plant growth without interactions with native soil microflora. *Azotobacter* species have been extensively used as biofertilizers (Arshad and Frankenberger, 1993) and significant effects were found on several crops when established in the rhizosphere (Barea and Brown, 1974; Hussain *et al*., 1987). Although different mechanisms have been suggested, the beneficial effect of *Azotobacter* on plant growth is proposed to be due to

the production of PGRs (Brown, 1974; Barea and Brown, 1974; Azcon and Barea, 1975; Nieto and Frankenberger, 1989).

The seven species of *Azospirillum* isolated from the rhizosphere of forages, grain crops and other native and cultivated plants (Magalhaes *et al.*, 1983; Dobereiner and Pedrosa, 1987; Reinhold *et al.*, 1987; Khammas *et al*., 1989) are the most studied PGPR (Bashan and Holguin, 1997a; Bashan *et al.* 2004).Worldwide field experiments indicated 60-70 % success due to *Azospirillum* inoculation with yield increases of 5-30% (Okon and Labandera, 1994). This increased yield may be due to nitrogen fixation, improved mineral and water uptake, and production of PGRs which may act independently and in combination (Bashan and Holguin, 1997b). With respect to the two first mechanisms, extensive information is available (Kapulnik *et al.,* 1984; Boddey *et al.*, 1986; Dobereiner and Pedrosa, 1987; Murty and Ladha, 1988; Sarig *et al.*, 1988; Bashan and Levanony, 1990; García de Salamone *et al*., 1996). The effects of *Azospirillum* inoculation on root morphology can be mimicked by applying IAA (Morgenstern and Okon, 1987) or mixtures of auxin, GA_3 and kinetin (Hubbell *et al*., 1979; Tien *et al.,* 1979). Inoculation of maize seedlings with *Azospirillum* significantly increased root surface area and inoculated roots contained higher amounts of both free and bound IAA as compared to the control (Fallik *et al.,* 1989). Indole-3-butyric acid (IBA) was also identified and this pioneer work proved useful in the detection of changes in endogenous PGRs following PGPR inoculation.

PGR synthesis is involved in the highly specific *Rhizobium* – legume symbiosis in which as much as the 90% of the plant's requirements for nitrogen are supplied by nitrogen fixation (Phillips and Torrey, 1972; Puppo and Rigaud, 1978; Morris, 1986; Hirsch *et al.,* 1997, Ferguson and Mathesius, 2003). Auxin and cytokinin applications to roots in hydroponic media produced morphological changes in the roots, which were similar to those observed on plants inoculated with *Rhizobium* (Skoog *et al.,* 1965; Puppo and Rigaud, 1978). *Rhizobium* can stimulate cell division in the cortex and release auxins and cytokinins at the root surface or in the infection threads (Sequeira, 1973). Although, the production of active auxin might be limited *in situ*, cytokinin activity was detected in the medium of *Phaseolus vulgaris* plants inoculated with *Rhizobium phaseoli,* but not in medium containing the same amounts of non-inoculated roots or rhizobial cells alone (Puppo and Rigaud, 1978).

PGPR that produce beneficial effects on plant growth include *Serratia* (Zhang *et al*., 1997), *Pseudomonas* (Young *et al.,* 1990; Arshad and Frankenberger, 1993; Kloepper, 1993), *Burkholderia* (Pedersen and Reddy, 1996), *Agrobacterium, Erwinia* (Ryder and McClure, 1997) *Xanthomonas* (De Freitas *et al.,* 1997), *Arthrobacter* (Kloepper *et al*., 1990) and *Bacillus* (Turner and Blackman, 1991; Mariano *et al*., 1997)and among these genera,

Pseudomonas has received much attention (Schippers *et al*., 1987; van Loon *et al.*, 1997; Loper *et al*., 1997). Many strains of pseudomonads have traits that appear to aid in colonization of seeds and roots such as fast growth rates, motility, chemotaxis to root exudates and ability to catabolize diverse nutrient sources (Kloepper, 1993). A core collection of elite PGPR strains, including *Pseudomonas* and *Serratia* (Kloepper *et al*., 1988) were screened for PGR production and a relationship between induction of root elongation and production of threshold concentrations of some cytokinins was observed (Young *et al.*, 1990).

Direct growth promotion by *Pseudomonas* was first reported by Lifshitz *et al*. (1987). They observed that *P. putida* GR12-2 belonging to a collection of over 4000 cold-tolerant and nitrogen-fixing pseudomonads (Kloepper *et al.*, 1988) directly promoted root growth of *Brassica campestris* (canola) in the absence of either plant pathogens or deleterious microorganisms. Hong *et al*. (1991) observed that *P. putida* GR12-2 fixed N, produced fluorescent siderophores and synthesized IAA, concluding that any or all of these mechanisms could contribute to root elongation. Now, it has been reported that a small number of soil bacteria, including *P. putida* strain GR12-2, contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick, 1995). Wild type bacteria promoted root elongation of developing canola seedlings, but the ACC-deaminase mutants did not. ACC is a precursor of ethylene in plants. It has been proposed that *P. putida* strain GR12-2 might promote root elongation by hydrolyzing some of a plant's ACC, thereby lowering the endogenous ethylene concentration and allowing the roots to grow longer (Glick *et al*., 1994).

Specific PGPR strains stimulate plant growth and nodulation of leguminous crops when co-inoculated with both *Rhizobium* (Grimes and Mount, 1984, Chanway *et al.*, 1989) and *Bradyrhizobium* (Polonenko *et al*., 1987) in field experiments. Zhang *et al*. (1997) concluded that the improvement of plant growth, development and physiological activities of soybean seedlings after co-inoculation with *Bradyrhizobium* and certain PGPR strains was due to direct effects of PGPR on overall physiology rather than specific effects on nitrogen fixation. As the co-inoculation effects vary with PGPR and rhizosphere conditions, the mode of action of these nodule promoting rhizobacteria needs further research.

3.2 PGPR and PGRs production

The amounts of PGRs detected in the rhizosphere are generally less than the amounts that are actually synthesized (De Leij and Lynch, 1997). Appreciable accumulation of PGRs in the rhizosphere does not occur because of the heterotrophic activity of the microflora (Lynch, 1990). Arshad and Frankenberger (1993) pointed out that the production of PGRs

as microbial metabolites in soil has been linked to substrate availability, but the number of influencing factors is high. They have also shown that IAA and certain cytokinins can be produced in soil incubated with tryptophan and specific precursors such as adenine and/or isopentenyl alcohol. Bolton *et al.* (1993) reported tryptophan and adenine could be detected in plant root exudates. Gibberellin-like bioactivity has been detected in several microbial cultures, and it has been suggested that gibberellins, along with other microbial metabolites may affect plant growth and development (Arshad and Frankenberger, 1993). However, gibberellins are the least studied PGRs in soil systems. In contrast, more work has been done on ethylene than other PGRs because it is easy to detect. Ethylene has been identified as a common constituent of the soil atmosphere as a result of microbial activity. Primrose (1979) demonstrated that as little as 10 nL L^{-1} of an exogenous application of ethylene can cause a dramatic physiological response in plants and concentrations high enough to affect plant growth are found near the roots and could move rapidly from roots to shoots.

Gonzalez-Lopez *et al.* (1986) estimated the amounts of PGRs produced by *Azotobacter vinelandi* cultured in dialyzed soil media after 96 hours. Auxin activity was equivalent to 0.2-2 μg IAA mL^{-1} in oat coleoptile bioassay, gibberellin activity was 0.8-3.1 μg GA_3 mL^{-1} in a lettuce hypocotyl bioassay and cytokinin-like substance activity was 1.8-4.4 μg mL^{-1} in a radish cotyledon bioassay. They also observed that the production of auxins, gibberellins and cytokinins was influenced by growth and incubation time. PGRs were released continuously when *Azotobacter* was cultured in N-free medium and dialyzed soil medium. Cytokinin production was about three times higher in dialyzed soil medium than in a defined medium, after 15 days.

Three cytokinin-active fractions were detected and identified as Z, IPA and IPa in cell-free culture medium following growth of *Azotobacter vinelandi* OP to stationary phase (Taller and Wong, 1989). The total cytokinin activity equivalent was 0.75 μg of kinetin per liter. Nieto and Frankenberger (1989) also detected cytokinins in cultures filtrates of three *Azotobacter* species. The most prolific producer was *A. chroococcum* and the amount of cytokinin produced was 224 nmol of Z equivalents L^{-1} when 0.1 m*M* of both adenine and isopentyl alcohol were added to the medium as precursors of cytokinin biosynthesis. The growth of *A. chroococcum* was modified not only by the addition of both cytokinin precursors, but also by environmental conditions such as pH**,** carbon sources, N supply, temperature and aeration.

Azospirillum species also produced several PGRs in pure cultures, the amounts were highly variable and strain-specific (Hartmann *et al.*, 1983; Zimmer and Bothe, 1988). IAA specifically induced a number of proteins

and this bacterium could be used to enhance IAA concentrations in the rhizosphere to promote growth of inoculated crops. Muller *et al.* (1988) stated that the formation of IAA by *Azospirillum* is dependent on tryptophan in the medium, which has been detected in root exudates (Strzelczyk and Potojska-Burdziej, 1984). However, at least three routes for IAA biosynthesis were recently demonstrated in this bacterium (Dosselaere *et al.*, 1997).

Many strains of *Rhizobium* are capable of producing either IAA (Badenoch-Jones *et al*., 1982; García-Rodriguez *et al.,* 1984) or cytokinins (Wang *et al.*, 1982) or both (Phillips and Torrey, 1972; Newcomb *et al.*, 1977; Upadhyaya *et al.*, 1991) in pure culture at high cell densities. In pure cultures, certain fast-growing strains of *Rhizobium* can produce large quantities of the polyamine aminobutylhomospermidine, a tetramine not produced by slowly growing strains, but the physiological effects on roots and other plant parts have not been studied (Galston and Sawhney, 1990).

Effective nodulation of *Cajanus cajan* with *Rhizobium* strain IC3342 induces a systemic response which results in abnormal shoot development, with symptoms starting 25-30 d after sowing and inoculation (Letham *et al*., 1990). Plant symptoms include typical tip bending, followed by hyponasty, curling of leaves, release of apical dominance and proliferation of lateral buds. In grafting experiments, a leaf curl-inducing principle was produced in the root nodules and translocated to the growing shoots through the xylem. A continuous supply was essential for the manifestation of symptoms. They also observed that the riboside of the cytokinin BAP, supplied via the root system, induced some effects in the shoot, which are characteristic of the leaf curl syndrome, especially release of lateral buds from apical dominance and hyponasty. The main cytokinin metabolites in the xylem exudates of normal nodulated plants and leaf curl plants were the same, but the concentrations of the cytokinins in the latter plants were eight times higher than those in the former. It is particularly significant that plants inoculated with a mutant of IC3342, which did not evoke the leaf curl syndrome, contained cytokinin levels similar to those of normal plants. This is a novel intact plant system to study the role of endogenous cytokinins in shoot development.

Forty-eight hours after inoculation with *Bradyrhizobium*, Caba *et al.* (2000), compared the levels of different cytokinins in *Glycine max* [L.] Merr. Cv Bragg and its supernodulating mutant nts 382 and observed quantitative and qualitative differences in the amount of PGRs in the roots. They also observed that root IAA/cytokinin and ABA/cytokinin ratios were always higher in Bragg relative to its mutant and responded to inoculation. Fei and Vessey (2003) have reported that low concentrations of ammonium (<1.0 mM) stimulate nodulation and this stimulation is associated with higher levels of Z and lower levels of ZR than the control or nitrate-treated

Pisum sativum plants. There was no effect on IAA levels. These data support the theory that a high ratio of cytokinin: auxin in roots is favourable for nodule initiation and that the ammonium effect is mediated through increasing Z levels. This may be a mechanism by which cytokinin-producing PGPR could influence nodulation in legumes.

Blackman and Davies (1985) pointed out that a continuous supply of cytokinins from maize roots may be necessary to sustain maximal stomatal opening and that a decrease or an interruption of this supply due to soil drying may act as a signal of reduced root activity. Roots could communicate to the shoot some indications of a perturbation in the soil environment and because the roots are the major source of cytokinins to the plant, a drying or restrictive soil could reduce leaf cytokinin levels sufficiently to affect stomatal behavior (Zhang and Davies, 1989; 1991; Tardieu *et al.*, 1992).

Omer *et al*. (2004) reported a naturally occurring cytokinin containing 5´deoxyribose, a derivative of IPA, which is produced along with other cytokinins by the bacterium *Pantoea agglomerans* isolated from barley seeds and selected for inducing growth promotion in tomato. Serdyuk *et al* (2000) reported cytokinin activity of the 4-hydroxyphenyl alcohol (4-HPEA) isolated from the phototrophic purple bacterium *Rhodospirillum rubrum* and classified it as a phenolic non-purine cytokinin-like substance.

García de Salamone *et al.* (2001), characterized the production of three cytokinins by *Pseudomonas fluorescens* strain G20-18, one rifampicin resistant mutant and two Tn*pho*A-derived mutants with reduced capacity to synthesize these PGRs. Using immnunoassays and thin-layer chromatography, G20-18 was shown to produce higher amounts of IPA, ZR and DHZR than the three mutants during the stationary phase of growth in liquid medium. IPA was the major cytokinin produced. No differences were observed between strain G20-18 and the mutants in the amounts of IAA synthesized, nor were gibberellins detected in filtrates of any of the strains. Garcia de Salamone (2000) also detected Z7G, ZOG and an unknown metabolite at R_F =0.45 on the TLC chromatograms of 14-d-old cultures of strain G20-18 and its selected mutants. This indicated that the O-glucosyltransferase enzymes were active and competing for the same substrate with the Z-reductase and phosphorylase, which metabolize Z to produce DHZ and ZR, respectively. The unidentified compound or group of cytokinins is another indication that strain G20-18 can produce an array of cytokinin metabolites. These results are in contrast with those of Timmusk *et al*. (1999) who observed, using HPLC with on-line ultraviolet detection and a final step of GC-MS, that a strain of *Paenibacillus polymyxa* isolated from the rhizosphere of wheat produced the cytokinin IPa at a concentration of 1.5 nM and an unknown cytokinin compound in late stationary phase (20 d). García de Salamone (2000) has shown that strain G20-18 could colonize the

rhizosphere of wheat and radish and promote growth in contrast to the lack of responses obtained with its mutants (García de Salamone *et al.,* 1997). Cytokinin metabolism of radish plants was altered by inoculation with this PGPR strain resulting in significantly higher amounts of cytokinins in root and shoot tissues and different cytokinin ratios between sterile and inoculated radish rhizospheres. Garcia de Salamone and Nelson (2000) showed that cytokinin production by a *Pseudomonas* strain was linked to the promotion of tobacco callus growth. This is a useful tool for screening cytokinin-producing PGPR because a standardized tobacco callus bioassay is able to detect cytokinin concentrations as low as 5 pmol L^{-1}. In recent studies with *Arabidopsis thaliana*, the growth promotion response to PGPR was controlled by cytokinins and ethylene released in the colonized root systems of the treated plants (LiYan and Boland, 2004).

4 CONCLUSION

Under sub-optimal environmental conditions plants may not have the capacity to synthesize sufficient endogenous PGRs for optimal growth and microbial PGR production may have an important compensatory role. Thus, the stimulation of microbial biosynthesis of PGRs within the rhizosphere using specific PGPR strains may be an effective approach to improve plant growth and development.

Cytokinin production by PGPR is an innovative alternative to enhance plant growth and may be a sustainable approach to improve the yield and quality of specific crops. This direct mechanism of plant growth promotion by rhizobacteria is poorly understood and attempts to demonstrate consistent results in the field have been challenging. Therefore, additional research must be initiated in the laboratory, greenhouse and field to understand the principles underlying cytokinin production by rhizobacteria and to develop cytokinin-producing inoculants for practical application by growers.

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Chapter 7

PLANT GROWTH PROMOTING RHIZOBACTERIA: POTENTIAL GREEN ALTERNATIVE FOR PLANT PRODUCTIVITY

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Abstract: Use of plant growth promoting rhizobacteria (PGPR) for the benefits of agriculture is gaining worldwide importance and acceptance and appears to be the trend for the future. PGPR are bioresources which may be viewed as a novel and potential tool for providing substantial benefits to the agriculture. These beneficial, free-living bacteria enhance emergence, colonize roots, stimulate growth and enhance yield. PGPR are known to induce resistance against various plant pathogens in different crops ranging from cereals, pulses, ornamentals, vegetables, plantation crops, spices and some trees. Most studies have emphasized exploration and potential benefits of PGPR in agriculture, horticulture and forestry. The plausible mechanisms adopted by these rhizobacteria in growth promotion and resistance, though abundantly documented but still remains to be fully explored. Integrated use of PGPR allows the combination of various mechanisms thereby enhancing their beneficial abilities. However, their use has not been to the full potential due to inconsistency in their performance and their commercialization limited to few developed countries. Use of PGPR as bioinoculants, biofertilizers and biocontrol agents, advantages and disadvantages, practical potential in improved agriculture and future prospects are also discussed.

Key words: biocontrol agents; biofertilizers; bioinoculants; growth promotion; induced resistance; integrated pest management; rhizobacteria;

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 197–216.

1 INTRODUCTION

Many microorganisms are attracted by nutrients exuded from plant roots and this "rhizosphere effect" was first described by Hiltner (Hiltner, 1904)). He observed higher numbers and activity of microorganisms in the vicinity of plant roots. The rhizosphere and rhizoplane are colonized more intensively by microorganisms than the other regions of the soil. Some of these microorganisms not only benefited from the nutrients secreted by the plant roots but also beneficially influence the plants, resulting in a stimulation of their growth. For instance, rhizobacteria can fix atmospheric nitrogen, which is subsequently used by the plants, thereby improving plant growth in the soil deficient of nitrogen. Other rhizobacteria can directly promote the plant growth by the production of hormones. These rhizobacteria positively influence plant growth and health and often referred as plant growth promoting rhizobacteria (PGPR). However, their effects are complex and cumulative because of interactions of plants, pathogens, antagonists, and environmental factors (Schippers, 1992).

Genera of PGPR include *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia, Bacillus, Paenibacillus,* and some are members of the Enterobacteriaceae. Direct use of microorganisms to promote plant growth and to control plant pests continues to be an area of rapidly expanding research. Rhizosphere colonization is one of the first steps in the pathogenesis of soil borne microorganisms. It is also crucial for the microbial inoculants used as biofertilizers, biocontrol agents, phytostimulators, and bioremediators. *Pseudomonas* spp. are often used as model root-colonizing bacteria (Lugtenberg *et al*., 2001).

The beneficial effects of these rhizobacteria have been variously attributed to their ability to produce various compounds including phytohormones, organic acids, siderophores, fixation of atmospheric nitrogen, phosphate solubilization, antibiotics and some other unidentified mechanisms (Glick, 1995). Motile rhizobacteria may colonize the rhizosphere more profusely than the non-motile organisms resulting in better rhizosphere activity and nutrient transformation. They also eliminate deleterious rhizobacteria from the rhizosphere by niche exclusion thereby better plant growth (Weller, 1988). Induced systemic resistance has been reported to be one of the mechanisms by which PGPR control plant diseases through the manipulation of the host plant's physical and biochemical properties.

2 GROWTH PROMOTION OF CROP PLANTS BY RHIZOBACTERIA AND THE MECHANISMS

PGPR are beneficial for plant growth and also referred as yield increasing bacteria (YIB). They can affect plant growth and yield in a number of ways and enhancement of vegetative and reproductive growth is documented in a range of crops like cereals, pulses, ornamentals, vegetables, plantation crops and some trees. Treatments with PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields etc., (van Loon *et al*., 1998; Ramamoorthy *et al*., 2001). Though the exact mechanisms involved in growth promotion are still unclear, various mechanisms have been suggested to explain the phenomenon of plant growth promotion. These include increase in the nitrogen fixation, production of auxins, gibberellins, cytokinins, ethylene, solubilization of phosphorous, oxidation of sulfur, increase in availability of nitrate, extra cellular production of antibiotics, lytic enzymes, hydrocyanic acid, increases in root permeability, strict competition for the available nutrients and root sites, suppression of deleterious rhizobacteria, and enhancement in the uptake of essential plant nutrients etc.(Subba Rao,1982; Pal *et al*., 1999; Enebak and Carey, 2000). However, experimental evidence suggests that bacterially-mediated phytohormone production is the most likely explanation for PGPR activity in the absence of pathogens (Brown, 1974; Tien *et al*., 1979; Holl *et al*., 1988) while siderophore production by PGPR may be important for plants growth stimulation when other potentially deleterious rhizosphere microorganisms are present in the rhizosphere (Kloepper *et al*., 1980; Bossier *et al*., 1988).

3 DISEASE CONTROL MECHANISMS

3.1 Biocontrol

Plant pathogens such as fungi, bacteria, viruses, nematodes etc., which cause various diseases in crop plants are controlled by PGPR (Raupach *et.al*., 1996; Hasky-Gunther *et.al*., 1998; Vidhyasekaran *et.al*.,2001;Viswanathan and Samiyappan,2002). Mechanisms of biocontrol may be competition or antagonisms; however, the most studied phenomenon is the induction of systemic resistance by these rhizobacteria in the host plant (van Loon *et al*., 1998; Ramamoorthy *et al*., 2001). PGPR control the damage to plants from pathogens by a number of mechanisms including: out-competing the pathogen by physical displacement, secretion of

siderophores to prevent pathogens in the immediate vicinity from proliferating, synthesis of antibiotics and variety of small molecules that inhibit pathogen growth, production of enzymes that inhibit the pathogen and stimulation of the systemic resistance in the plants. PGPR may also stimulate the production of biochemical compounds associated with host defense. Enhanced resistance may be due to massive accumulation of phytoalexins, phenolic compounds, increases in the activities of PR-proteins, defense enzymes and transcripts, and enhanced lignification. Biocontrol may also be improved by genetically engineered PGPR to over express one or more of these traits so that strains with several different anti-pathogen traits can act synergistically (Glick and Bashan, 1997). Rhizobacteria-mediated ISR has been reported to be effective against fungi, bacteria and viruses, but appears to involve different signaling pathways and mechanisms.

3.2 Structural mechanisms

PGPR can induce structural changes in the host and these changes were characterized by a considerable enlargement of the callose-enriched wall appositions deposited onto the inner surface of cell wall in the epidermis and outer cortex (Benhamou *et al*., 1998), callose deposition (M'Piga *et al*., 1997) and lignification (Kloepper, 1993). A strain of *Pseudomonas fluorescens* functions as an activator of plant disease resistance by inducing callose synthesis in tomato (M'Piga *et al*., 1997). Bean roots bacterized with a saprophytic fluorescent pseudomonad, had higher lignin content than control (Anderson and Guerra, 1985).

Treatment of PGPR significantly reduced germination of sporangia and zoospores of *Phytophthora infestans* on the leaf surface of tomato than the leaves of the non-induced control. *Serratia plymuthica* strain R1GC4 sensitizes susceptible cucumber plants to react more rapidly and efficiently against *Pythium ultimum* attack through the formation of physical and chemical barriers at sites of fungal entry (Benhamou *et al.,* 2000). *Pseudomonas fluorescens* induced accumulation of lignin in pea roots (Benhamou *et al*., 1996a,b). *Bacillus pumilus* SE34 showed a rapid colonization of all tissues including the vascular stele in tomato and induced resistance against *Fusarium oxysporum* (Benhamou *et al.,* 1998). The main facets of the altered host metabolism concerned the induction of a structural response at sites of fungal entry and the abnormal accumulation of electron-dense substances in the colonized areas.

3.3 Biochemical mechanisms

PGPR are known to produce antibiotics, antifungal metabolites, enzymes, phenolics, signal compounds and other determinants of defense in response to pathogen attack. Various antibiotics like bacilysin, iturin-like lipopeptides, diacetylphloroglucinol and pyrrolnitrin, HCN, phenazine-1-carboxylate are produced by rhizobacteria (Thomashow *et al*., 1990). Rhizosphere colonization by *Pseudomonas aeruginosa* 7NSK2 activated phenlyalanine ammonia lyase (PAL) in bean roots and increased the salicylic acid levels in leaves (De Meyer *et al*., 1999). Increased activity of PAL was observed in *P. fluorescens* treated tomato and pepper plants in response to infection by *F. oxysporum* f. sp. *lycopersici* and *Colletotrichum capsici* (Ramamoorthy and Samiyappan, 2001). In bean, rhizosphere colonization of various bacteria induced peroxidase (PO) activity (Zdor and Anderson, 1992), The higher PO activity was noticed in cucumber roots treated with *Pseudomonas corruga*ta and inoculated with *Pythium aphanidermatum* (Chen *et al*., 2000). Foliar application of *P. fluorescens* increased chitinase and glucanase activities in rice (Meena *et al*., 1999). Groundnut plants, sprayed with *P. fluorescens* strain Pf1, showed significant increase in activities of PAL, phenolic contents, chitinase and glucanase 23-kDa thaumatin-like protein (TLP) and a 30-kDa glucanase (Meena *et al*., 2000). Earlier and increased activities of phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) were observed in *P. fluorescens* Pf1 pretreated tomato and hot pepper plants challenged with *P. aphanidermatum*. Phenolic compounds are toxic to pathogens in nature and may increase the mechanical strength of the host cell wall. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou *et al*., 1996a). Similarly, *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots following infection by *P. ultimum* (Benhamou *et al*. 2000). Moreover, *P. fluorescens* Pf1 isolate also induced the accumulation of phenolic substances and PR-proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato (Ramamoorthy *et al*., 2001) and *C. capsici* in pepper (Ramamoorthy and Samiyappan, 2001). The levels of a PR-protein increased in bean leaves following seed treatment with PGPR strains (Hynes and Lazarovits, 1989) while PR-proteins viz., PR-1a, 1b, 1c, endochitinase and b-1,3-glucanases were induced in intercellular fluid in the leaves of tobacco plants grown in the presence of *P. fluorescens* strain CHA0(Maurhofer *et al*., 1994). Increase in lignin content, peroxidase activity and 4-coumarate CoA ligase activity were observed after inoculation with *Xanthomonas oryzae* pv. *oryzae* in rice leaves pre-treated with *P. fluorescens* (Vidhyasekaran *et al*., 2001). Inoculation of PGPR can induce phytoalexin synthesis (Van Peer *et al*., 1991) and phenol accumulation

(M'Piga *et al*., 1997). Moreover, PGPR-mediated ISR triggered the hypersensitive reaction (HR), causing death of individual cell of leaves following inoculation with the pathogen. Analysis of H_2O_2 content, showed that H_2O_2 increased significantly in all treatments 12 h after pathogen inoculation, compared to non-induced control (Yan *et al*., 2002).

3.4 Molecular mechanisms

Mechanisms of rhizobacteria-mediated induced systemic resistance (ISR) to the large extent are unknown. ISR in *Arabidopsis* mediated by rhizobacteria is not associated with a direct effect on expression of known defense-related genes but stimulated the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Gene expression studies were performed with *Arabidopsis* gene-specific probes for the defense-related genes *PR-1., PR-2., PR-5., Hel., ChiB, Pdf*1.2, *Atvsp, Lox1, Lox2, Pal1*, and *Pin2*. Responsiveness of genes to the defense signaling molecules SA, ethylene, and jasmonate was verified by analyzing their expression in leaves treated with SA, the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), or methyl jasmonate (MeJA). Although variation in the expression of most genes was apparent, roots and leaves of *P. fluorescens* WCS417r-treated plants never showed an enhanced expression of any of the genes, at any time tested (van Wees *et al*., 1997).

PPO transcript levels increased in young leaves of tomato when mature leaflets were injured (Thipyapong and Steffens, 1997). Increase in mRNAs encoding PAL and chalcone synthase were recorded in the early stages of the interaction between bean roots and various rhizobacteria (Zdor and Anderson, 1992). ISR in A. *thaliana* by *P. fluorescens* WCS417r and subsequent inoculation of *Pseudomonas syringae* pv. *tomato* Dc3000(ISR) functions independently of salicylic acid but requires an intact response to the plant hormones jasmonic acid and ethylene. Rhizobacteria-mediated ISR is not based on the induction of changes in the biosynthesis of either JA or ethylene. ISR-expressing plants have the capacity to convert 1-aminocyclopropane-1-carboxylate (ACC) to ethylene providing a greater potential to produce ethylene upon pathogen attack (Pieterse *et al*., 2000). Fluorescent pseudomonads are also known to produce salicylic acid, which acts as local and systemic signal molecules in inducing resistance in plants (De Meyer and Hofte, 1997).

4 SIGNALING COMPOUNDS AND PATHWAYS

Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are involved in the regulation of basal resistance against different pathogens. These three signals play important roles in induced resistance as well. SA is a key regulator of pathogen-induced systemic acquired resistance (SAR) whereas JA and ET are required for rhizobacteria-mediated induced systemic resistance (ISR). Both types of induced resistance are effective against a broad spectrum of pathogens. Comparison of the effectiveness of SAR and ISR using a fungal, a bacterial, and a viral pathogen in non-induced *Arabidopsis* plants, these pathogens are primarily resisted through either SA-dependent basal resistance (*Perenospora parasitica* and Turnip crinkle virus (TCV)), JA/ET-dependent basal resistant responses (*Alternaria barssicicola*), or a combination of SA-, JA-, and ET-dependent defenses (*Xanthomonas campestris* pv. *armoraciae*). Activation of ISR resulted in a significant level of protection against *Alternaria brassicicola*, whereas SAR was ineffective against this pathogen. Conversely, activation of SAR resulted in a high level of protection against *Phytophthora parasitica* and TCV, whereas ISR conferred only weak and no protection against *P. parasitica* and TCV, respectively. Induction of SAR and ISR was equally effective against *X. campestris* pv. *armoraciae*. These results indicate that SAR is effective against pathogens that non-induced plants are resisted through SA-dependent defenses, whereas ISR is effective against pathogens in non-induced plants and resisted through JA/ET-dependent defenses. This suggests that SAR and ISR constitute a reinforcement of extant SA- or JA/ET-dependent basal defense responses, respectively (Ton *et al*., 2002).

Serratia marcescens 90-166 mediates induced systemic resistance to fungal, bacterial, and viral pathogens by producing salicylic acid (SA), using the salicylate responsive reporter plasmid pUTK21. High-pressure liquid chromatography analysis of culture extracts confirmed the production of SA in broth culture. Mini-Tn5phoA mutants, which did not produce detectable amounts of SA, retained ISR activity in cucumber against the fungus *Colletotrichum orbiculare*. Strain 90-166 induced disease resistance to *P. syringae* pv. *tabaci* in wild-type *Xanthi*-nc and transgenic NahG-10 tobacco expressing salicylate hydroxylase. Results of the study indicate that SA produced by 90-166 is not the primary bacterial determinant of ISR and the bacterial-mediated ISR system is affected by iron concentrations (Press *et al*., 1997).

Several genera of bacteria including pseudomonads are known to synthesize SA and SA is an intermediate in the biosynthesis of pyochelin siderophores (Ankenbauer and Cox, 1988). There are some indications that SA may be involved in bacterially mediated ISR since *Pseudomonas fluorescens* strain CHAO, which provides ISR in tobacco to tobacco necrosis

virus (Maurhofer *et al*., 1994), produces SA (Meyer *et al*., 1992; Visca *et al*. 1993). However, the role of SA production in CHAO-mediated ISR has not been reported. Leeman *et al*., (1996) reported that *P. fluorescens* strain WCS374, which provides ISR in radish against *F. oxysporum* f. sp. *raphani*, produced SA in quantities that were iron dose-dependent, and they suggested that ISR was due to bacterial SA production. Recently, the involvement of SA produced by *P. aeruginosa* 7NSK2 in the induction of resistance against *Botrytis cinerea* on *Phaseolous vulgaris* has been reported (De Meyer and Hofte,1997).

Root colonization of *A. thaliana* by the nonpathogenic, rhizosphere-colonizing bacterium *P. fluorescens* WCS417r has been shown to elicit induced systemic resistance (ISR) against *P. syringae* pv. *tomato* (Pst)(Knoester *et al*., 1999). Several ethylene-response mutants were tested and showed essentially normal symptoms of Pst infection. ISR was abolished in the ethylene-insensitive mutant *etr1-1*, whereas SAR was unaffected. Similar results were obtained with the ethylene mutants *ein2* through *ein7*, indicating that the expression of ISR requires the complete signal-transduction pathway of ethylene known so far. The induction of ISR by WCS417r was not accompanied by increased of ethylene production in roots or leaves, and neither by increases in the expression of the genes encoding the ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic (ACC) synthase and ACC oxidase. The *etr1* mutant, displaying ethylene insensitivity in the roots only, did not express ISR upon application of WCS417r to the roots, but did exhibit ISR when the inducing bacteria were infiltrated into the leaves. These results demonstrate that, for the induction of ISR, ethylene responsiveness is required at the site of application of inducing rhizobacteria (Knoester *et al*., 1999).

The *Bacillus amyloliquefaciens* EXTN-1 treated tobacco plants showed augmented, rapid transcript accumulation of defense related genes including PR-1a, phenylalanine ammonia-lyase, and 3-hydroxy-3methylglutaryl CoA reductase (HMGR) following inoculation with Pepper Mild Mottle Virus (PMMoV). Thus, their expression is associated with the development of both local and systemic resistance. All these results may indicate that EXTN-1 induces systemic resistance via salicylic acid and jasmonic acid-dependent pathways and timely recognition followed by rapid counter attack against the viral invasion is the key differences between incompatible interaction and compatible one (Ahn *et al*., 2002).

PGPR strains *B. pumilus* SE34 and *P. fluorescens* 89B61, elicited systemic protection against the blight on tomato and reduced disease (Yan *et al*., 2002). Induced protection elicited by both PGPR strains was SA-independent but ethylene- and jasmonic acid-dependent. In *Arabidopsis*, selected bacterial strains trigger a SA-independent but JA and ethylene dependent pathway that nevertheless, is dependent on the regulatory factor

NPR1, which is also part of the SA-dependent pathway. Two non-inducible ecotypes of *Arabidopsis* are impaired in the same gene (ISR1) and have reduced sensitivity to ethylene, confirming the importance of ethylene sensitivity in ISR signaling (Hammerschmidt *et al*., 2001).

5 USE OF PGPR ON COMMERCIAL SCALE

The development of biological products based on beneficial micro-organisms can extend the range of options for maintaining the health and yield of crops. As early as 1897 a "bacteriological fertilizer for the inoculation of cereals" was marketed under the proprietary name Alinit by Farbenfabriken vorm. Friedrich Bayer & Co." of Elberfeld, Germany, Today's Bayer AG. The product was based on a *Bacillus* species now known by the taxonomic name *Bacillus subtilis* (Kilian *et al*., 2000). In the mid-1990s in the USA, *B. subtilis* started to be used as seed dressing, with registrations in more than seven crops and application to more than 2 million ha (Backmann *et al*., 1994). This was the first major commercial success in the use of an antagonist. In Germany, FZB 24 *B. subtilis* has been on the market since 1999 and is used mainly as a seed dressing for potatoes (Kilian *et al*., 2000).

In response to environmental and health concerns about extended use of pesticides, there is considerable interest in finding alternative control approaches for use in integrated pest management strategies for crop diseases (Reuveni, 1995). It seems inevitable that fewer pesticides will be used in the future and that greater reliance will be placed on biological technologies including the use of microorganisms as biocontrol agents (Backman *et al*., 1997; Budge *et al*., 1995). However, microorganisms as biocontrol agents typically have a relatively narrow spectrum of activity compared with synthetic pesticides (Baker, 1991; Janisiewicz, 1988) and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Backman *et al*., 1997).

Commercial development has already been accomplished with two products marketed as Kodiak and Epic (Gustafson inc.), in which two different *Bacillus subtilis* biocontrol strains were combined with a fungicide (Carboxin-PCNB-metalaxyl) for use against soil borne diseases. During the 1996 season, approximately 5 million ha of crops were treated with these products, targeting diseases of roots caused by *Rhizoctonia solani* and *Fusarium* spp. plus promoting root mass and plant vigor through hormone-like responses and disease control.

Many root-colonizing bacteria are known to promote plant growth by producing gibberellins, cytokinins and indole acetic acid (Dubeikovsky *et*

al., 1993) and hence are called as PGPR. The application of five commercial chitosan-based formulations of carefully chosen PGPR developed at Auburn University, USA has previously shown demonstrable increase in the growth of nursery-raised plants such as cucumber, pepper and tomato among others. Later, seedlings of three indica rice cultivars, IR24, IP50 and Jyothi raised in rice field soil amended with each of the formulations in a 1:40 (formulation: soil) ratio have shown significant two-fold increase in root and shoot length, and grain yield. The observations do suggest that application of such commercial bacterial formulations can serve as microbial inoculants for the improvement of rice growth (Vasudevan *et al*., 2002).

6 INTEGRATION AND MIXTURES OF PGPR

In nature biocontrol results from mixtures of antagonists, rather from high populations of a single antagonist. Moreover, mixtures of antagonists are considered to account for protection of disease-suppressive soils (Lemanceau and Alabouvette, 1991; Schippers, 1992). Consequently, application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and may broaden the spectrum, enhance the efficacy and reliability of biocontrol (Duffy and Weller, 1995). Strategies for forming mixtures of biocontrol agents could be envisioned including mixtures of organisms with differential plant colonization patterns; biocontrol agents that control different pathogens; antagonists with different mechanisms of disease suppression; taxonomically different organisms and antagonists with different optimum temperature, pH and moisture conditions for plant colonization (Backman *et al*., 1997). Combination of various mechanisms of biocontrol is useful in achieving the goal without genetic engineering (Janisiewicz, 1996). PGPR strains INR 7 (*Bacillus pumilus*). GBO3 (*Bacillus subtilis*), and ME1 (*Curtobacterium flccumfaciens*) were tested alone and in combinations for biocontrol against *Colletotrichum orbiculare* (causing anthracnose), *Pseudomonas* syringae pv. *lachrymans* (causing angular leaf spot), and *Erwinia tracheiphila* (causing cucurbit wilt disease). Greater suppression and enhanced consistency was observed against multiple cucumber pathogens using strains mixture (Raupach and Kloepper, 1998). Studies on combinations of biocontrol agents for plant disease control have included mixtures of fungi (Budge *et al*., 1995; Datnoff *et al*., 1993, 1995; De Boer *et al*., 1997; Paulitz *et al*., 1990), mixtures of fungi and bacteria (Duffy *et al*., 1996; Duffy and Weller, 1995; Hassan *et al*., 1997; Janisiewicz, 1988; 1996; Leeman *et al*., 1996; Leibinger *et al*., 1997; Lemanceau and Alabouvette, 1991; Park *et al*., 1988) and mixtures of bacteria (De Boer *et al*., 1997; Janisiewiez and Bors., 1995; Johnson *et al*.,

1993; Mazzola al., 1995; Pierson and Weller, 1994; Raaijmakers *et al*., 1995; Roberts *et al*., 1997; Schisler *et al*., 1997; Stockwell *et al*., 1996; Sung and Chung, 1997; Waechter-Kristensen *et al*., 1994; Wei *et al*., 1996). Combinations of a strain of *Trichoderma koningii* with different *Pseudomonas* spp. isolates provided greater suppression of take-all disease than either the fungus or the bacterium alone (Duffy *et al*., 1996). Increased suppression of Fusarium wilt of carnation was observed by combining *P. putida* WCS358 with non-pathogenic *Fusarium oxysporum* Fo47 (Lemanceau *et al*., 1992, 1993). The enhanced disease suppression may be due to siderophore-mediated competition for iron by WCS358, which makes the pathogenic *F. oxysporum* strain more sensitive to competition for glucose by the non-pathogenic strain Fo47. Furthermore, strains of nonpathogenic *Verticillium lecanii*, *Acremonium rutilum* or *Fusarium oxysporum* with the fluorescent *Pseudomonas* spp. strains WCS358, WCS374 or WCS417 resulted in significantly better suppression of Fusarium wilt of radish compared to the single organism (Leeman *et al.* 1996). Mixtures of fluorescent pseudomonads were significantly more suppressive of take-all than either used alone (Pierson and Weller, 1994; and Duffy and Weller, 1995). Similarly, chitinase-producing *Streptomyces* spp. and *Bacillus cereus* isolates used in conjunction with antibiotic-producing *P. fluorescens* and *Burkholderia cepacia* isolates had a synergistic effect on the suppression of rice sheath blight (Sung and Chung, 1997). Limited numbers of compatible and effective mixtures of biocontrol agents are available. The majorities of mixtures have no benefit or detrimental effects on biocontrol activity. Further, a mixture that improves activity under one set of conditions may be antagonistic under another set of conditions. A biocontrol product composed of a mixture of strains has a potential economical constraint. Production and registration of such a product will be more costly than a product composed of single strain. Development of mixtures of biocontrol agents should be emphasized, because these may result in better plant colonization, better adapt to the environmental changes that occur throughout the growing season, have a larger number of pathogen-suppressive mechanisms and protect against a broader range of pathogens.

In few cases combinations of biocontrol agents do not result in improved suppression of disease (Hubbard *et al*, 1983; Sneh *et al*, 1984; Miller and May, 1991; Dandurand and Knudsen, 1993). Tomato seedlings were treated with the potential biocontrol agents such as nonpathogenic strains of *Fusarium* spp., *Trichoderma* spp., *Gliocladium virens*, *Pseudomonas fluorescens*, *Burkholderia cepacia*, and others in the greenhouse and transplanted into pathogen-infested field soil. Combinations of antagonists like multiple *Fusarium* isolates, *Fusarium* with bacteria, and *Fusarium* with other fungi, also reduced disease, but did not provide better

control than the nonpathogenic *Fusarium* (Larkin and Fravel, 1998). Use of a *T. harzianum* strain with a strain of *P. fluorescens* were able to suppress root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi* but did not result in better disease suppression (Dandurand and Knudsen, 1993). Positive and negative interactions of introduced microorganisms and indigenous microflora can influence their performance in the rhizosphere. For example, two groups of microorganisms that occupy the same ecological niche and have the same nutritional requirements are bound to compete for nutrients (Bakker *et al*., 1988; Fukui *et al*., 1994; Janisiewicz and Bors, 1995; Raaijmakers *et al*., 1995). Siderophore-mediated competition for iron between the two biocontrol agents *P. putida* WCS358 and *P. fluorescens* WCS374 decreased colonization of radish roots by the latter strain (Raaijmakers *et al*., 1995). Hubbard *et al*., (1983) described negative effects of endemic *Pseudomonas* spp. on *T. harzianum*. They suggested that negative effects were caused by effective competition for iron by the *Pseudomonas* spp. because addition of iron to naturally infested soil suppressed growth inhibition of *T. harzianum* and also suppressed *Pythium* seed rot of pea. Negative interaction between two biocontrol agents may also be due to detrimental effects of secondary metabolites produced by one organism on the other (Mew *et al*., 1994). Thus, an important pre-requisite for the desired effectiveness of strains appears to be compatibility of the co-inoculated microorganisms (Li and Alexander, 1988; Baker, 1990; Raaijmakers *et al*., 1995). Numerous biotic and abiotic factors contribute to this inconsistent performance of biocontrol agents (Weller, 1988). Inadequate colonization of the rhizosphere, limited tolerance to changes in environmental conditions and fluctuation in the production of antifungal metabolites are among the most important factors (Duffy *et al*., 1996; Pierson and Weller, 1994). Antagonism between the indigenous microbial population and biocontrol agent or mixture of biocontrol agents applied can also influence the performance of a biocontrol agent in the rhizosphere.

These results indicate that specific interactions of biocontrol agents influence disease suppression in combination. It is necessary, therefore to further investigate microbial interactions that enhance or detract biocontrol efficacy (Handelsman and Stabb, 1996) to understand and predict the performance of mixtures of biocontrol agents. Increasing the genetic diversity of biocontrol systems by the mixture of microorganisms may persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms (e.g. induction of resistance, production of antibiotics and competition for nutrients) under a broader range of environmental conditions (Pierson and Weller, 1994). Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action, a more stable rhizosphere community, and effectiveness over a wide range of environmental conditions. In particular combinations of fungi and bacteria

may provide protection at different times or under different conditions, and occupy different or complementary niches. Such combinations may overcome inconsistencies in the performance of individual isolates. Several researchers have observed improved disease control using combinations of multiple compatible biocontrol organisms (Duffy *et al*., 1996; Pierson and Weller, 1994; Lemanceau, 1991; Lemanceau and Alabouvette 1991; Leeman *et al*., 1996; Park *et al*., 1988) and have demonstrated enhanced biocontrol of Fusarium wilt by combining certain nonpathogenic strains of *F. oxysporum* with fluorescent strains of *Pseudomonas*.

7 DELIVERING PGPR: PROS AND CONS

Advantages of a seed treatment with PGPR in a biocontrol system are: 1) their saprophytic nutritional status makes large scale production feasible, 2) only small amounts of inoculum are required, 3) application is simple, 4) independence from energy sources for survival, 5) systemic spread along the surface of the developing root system, and 6) antagonistic activity on the root surface during the economically important phase of early root infection by the pathogens. Their versatile metabolism, fast growth, active movement, and ability to readily colonize the root surface make these rhizobacteria suitable for seed bacterization. Further, seed treatments provide targeted application of PGPR, allowing earlier protection than with foliar sprays. The additional plant growth-promotion by PGPR treatments in comparison to chemical pesticides adds another advantage. However, microorganisms as biocontrol agents have a relatively narrow spectrum of activity compared with synthetic pesticides (Baker, 1991; Janisiewicz, 1988) and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Backman *et al*., 1997). However, growing popularity of biocontrol is its record of safety during the past 100 years. No microorganism or beneficial insect deliberately introduced or manipulated for biocontrol purposes has, itself, become a pest and there is no evidence for negative effects of biocontrol agents on the environment. Effective biocontrol demands thorough knowledge of biological interactions at the ecosystem, organismal, cellular, and molecular levers. Biocontrol is also likely to be less spectacular than most physical or chemical controls but usually more stable and long lasting (Baker and Cook, 1974). Although biocontrol is having been used in agriculture for centuries, as an industry biocontrol is still in its infancy.

8 FUTURE PROSPECTS

Diseases are very common in plants and are responsible for the loss of approximately one third of the crop yield (Lugtenberg *et al*., 1994). Chemical pesticides that control plant diseases have become a threat to health and the environment and hence being banned worldwide. This has increased the interest in biocontrol of plant diseases. PGPR mediated agriculture is now gaining worldwide importance and acceptance for an increasing number of crops and managed ecosystems as the safe method of pest control. Biocontrol has untapped potential and is underused, under exploited, underestimated, often untried and therefore unproven. The new tools of recombinant DNA technology, mathematical modeling, and computer technology combination with a continuation of the more classical approaches such as importation and release of natural enemies and improved germplasm, breeding, and field testing should quickly move biocontrol research and technology into a new era. Although activity and effects of biocontrol have been reported for a number of antagonists, the underlying mechanisms are not fully understood. This deficiency in our knowledge often hinders attempts to optimize the biological activity by employing tailored application strategies. One can envision a number of different ways in which biocontrol efficacy of PGPR might be improved. Biocontrol efficacy of PGPR may be improved by genetically engineering them to over express one or more of these traits so that strains with several different anti-phytopathogen traits can act synergistically. More detailed studies are needed on the composition of the rhizosphere population, the effect of cultivar on bacterial population dynamics, the influence of inoculum density on antagonistic activity, the survival of inoculum under adverse conditions, and the role of environmental conditions in altering the activity of rhizobacteria. An attempt to overcome problems of varying efficacy may be attained by strain mixing, improved inoculation techniques, or gene transfer of active genetic source of antagonists to the host plant (Oostendorp and Sikora, 1986). The soil microbes are active elements for soil development and the basis of sustainable agriculture. Form the point of sustainable agricultural development and good eco-environment establishment, we propose a scientific fertilizer that is to apply organic, inorganic and microbial fertilizers in a balance and rational way to keep high and stable yield.

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Chapter 8

VISUALIZATION OF INTERACTIONS OF MICROBIAL BIOCONTROL AGENTS AND PHYTOPATHOGENIC FUNGUS *FUSARIUM OXYSPORUM* F. SP. *RADICIS-LYCOPERSICI* ON TOMATO ROOTS

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Abstract: The fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) causes foot and root rot of tomato, which can be controlled by various microbes including *Pseudomonas, Trichoderma* and non-pathogenic *Fusarium*. Microbes labeled with autofluorescent protein (AFP) markers can be visualized in live samples using confocal laser scanning microscopy (CLSM). This enables the simultaneous determination of both pathogen and biocontrol agent in the tomato rhizosphere and provides a better understanding of the biocontrol processes. Results of CLSM suggest that mechanisms of biocontrol of plant pathogens include inhibition of spore germination, competition for niches and nutrients, antibiosis, predation, parasitism, and induction of host resistance.

Key words: biocontrol; confocal laser scanning microscopy; *Pseudomonas;* rhizosphere; tomato foot and root rot; *Trichoderma.*

1 THE RHIZOSPHERE

The rhizosphere is the area in proximity to the root system by which it is influenced (Hiltner, 1904). The exudation of carbon sources such as organic acids, sugars and amino acids (Vancura and Hovadik, 1965) by the root creates a nutrient-rich rhizosphere which stimulates microbial activity. The composition of root exudates is influenced by multiple factors such as plant species, root region, and abiotic and biotic factors of the surrounding soil. Major soluble components of tomato root exudates include sugars, organic acids and amino acids (Lugtenberg and Bloemberg, 2004). The

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 217–231.

microbial community in the rhizosphere includes viruses, bacteria, fungi, nematodes and protozoa. The composition of the microflora and microfauna differs in soil types, plant species and surrounding soils where fewer nutrients are available. Moreover, interactions between plants and microorganisms can be classified as pathogenic, saprophytic and beneficial (Lynch, 1990).

2 PATHOGENIC INTERACTIONS

Pathogenic interactions can occur between microorganisms, such as parasitism of one fungus by another one (mycoparasitism) and the production of antibiotics by one organism that inhibit or kill other microorganism. Another pathogenic interaction involves microorganisms and plant roots resulting in plant diseases. Soil-borne plant diseases may be caused by nematodes, mites, bacteria, viruses, and fungi. The latter group causes the more damage to crop and its interaction with other plant pathogens generally has synergistic effect on plant disease. Plants can reject pathogens due to resistance and incompatibility and as a result non-host plants are not parasitized by the pathogens. Host-plants have a basic compatibility with the pathogen which is from the corresponding *formae speciales*. These pathogens produce a set of pathogenicity factors that allow successful parasitism. Generally plant diseases cause 10-20% loss in production (James, 1981).

3 BIOCONTROL OF PLANT DISEASES

Biocontrol of plant diseases is generally due to the presence of disease-controlling microorganisms collectively labeled biocontrol agents (Alabouvette *et al*., 1979; Schroth and Hancock, 1982; Schippers *et al*., 1987; Handelsman and Stabb, 1996). Factors such as soil pH and organic matter content contribute indirectly to the biocontrol of diseases by their effect on microbial activity. Microbial activity and their metabolites can act both directly and indirectly on the pathogen and/or on the plant, resulting in disease control (Mazzola, 2002).

Microbial analysis of these suppressive soils may contribute to the identification of potential biocontrol agents. Biocontrol agents are usually isolated from naturally suppressive soils (Montesinos, 2003). Since bacteria and fungi are natural enemies occurring in the soil, these organisms can proliferate in the plant rhizosphere while their effect on the environment is minimal. Different mechanisms of biocontrol have been described (Bloemberg and Lugtenberg, 2001; Thomashow and Weller, 1996; Whipps,

2001), depending on the biocontrol agent, pathogen, plant species, abiotic and biotic features of the soil.

4 TOMATO FOOT AND ROOT ROT

Fusarium oxysporum spp. are saprophytic fungi which grow and survive for long periods on organic matter, in soil and in the rhizosphere of many plant species (Garrett, 1970). Some *Fusarium* species cause wilting or root rotting whereas others are non-pathogenic. Both pathogenic and non-pathogenic *Fusarium* species can penetrate roots. In contrast to the non-pathogenic ones, pathogenic strains can penetrate the vascular tissues and cause disease (Olivain and Alabouvette, 1997). Wilt causing *Fusarium* spp. are highly host-specific and are classified in many different *formae speciales* based on the host plant species (Armstrong and Armstrong, 1981). The fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) causes tomato foot and root rot (TFRR) and is a serious constraint for field and greenhouse crops (Jarvis, 1988; Roberts *et al.*, 2000).

5 VISUALIZATION OF BIOCONTROL OF TFRR

The effects of different biocontrol agents on the pathogen *F.o.r.l.* were analysed. For visualization studies, the gnotobiotic sand system described by Simons *et al*. (1996) was used. This system was previously useful for visualizing interactions of roots with microbes labeled with autofluorescent protein (AFP) markers, such as *Pseudomonas* (Fig. 1A) (Bloemberg *et al.*, 1997; Bloemberg *et al*., 2000) and the pathogen *F.o.r.l.* (Figs. 1B through D) (Lagopodi *et al.*, 2002). Quartz sand was used because it could easily be removed from the roots by gentle washing, after which the roots can be examined using CLSM. In contrast, the removal of soil from the root is difficult and subsequent microscopy studies are hampered due to the autofluorescence of the soil particles. The quartz sand system focuses well on the interactions between the pathogen and biocontrol agent due to the absence of other rhizosphere microorganisms which are present in non-sterile soil systems.

Due to the absence of competing indigenous bacteria, the use of quartz sand results in a very high disease incidence (70-100%) and very efficient biocontrol (6-15% diseased plants in the presence of *Pseudomonas*

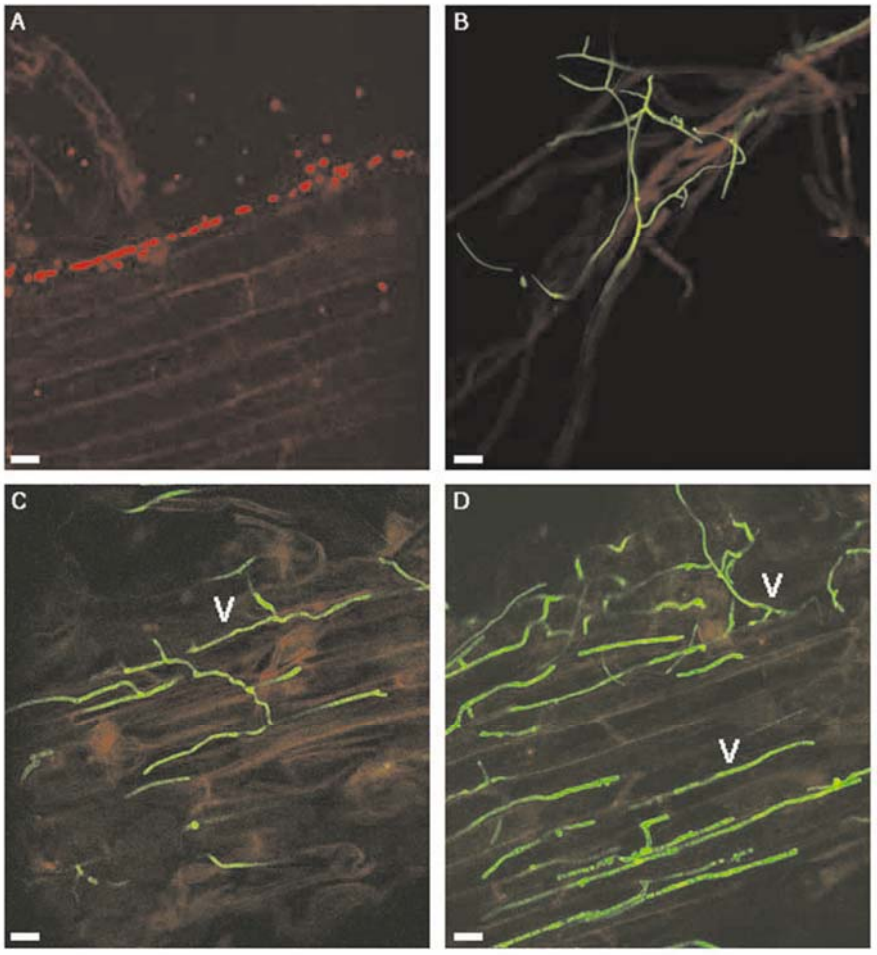

Fig. 1. Confocal laser scanning microscopical analysis of tomato root colonization by *Pseudomonas* and *Fusarium.*

Two-day-old tomato seedlings were inoculated at time zero with cells of *Pseudomonas* harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells (Panel A) and subsequently grown in a gnotobiotic sand system. Or two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of *F.o.r.l.* (FCL14) (Panel B-D). *F.o.r.l.* (FCL14) harbors a constitutively expressed *sgfp* gene and appears as green. Walls of tomato root cells appear as red due to autofluorescence.

Panel A, *Pseudomonas* cells colonizing the intercellular junctions of root cells of an inoculated seedling planted in sterile sand three days after planting. Panel B, Initial colonization of the tomato root by *F.o.r.l.* (similar for Fo47) 'attachment' to root hairs three days after planting. Panels C and D (picture taken by A. Lagopodi), *F.o.r.l.* hyphae growing along the intercellular junctions five days after planting. The size bar represents 10 µm in all panels.

chlororaphis PCL1391). For example, one of the mutants (*P. chlororaphis* PCL1119) which is impaired in biocontrol when tested in non-sterile potting soil, could significantly reduce disease incidence to 38-60% in the gnotobiotic system (Bolwerk *et al*., 2003). This is likely due to the absence of indigenous bacteria, which allows higher levels of the mutant strain. As a result strain PCL1119 can effectively compete with the pathogen for root colonization.

Using CLSM in combination with organisms differentially labeled with AFPs allowed the simultaneous visualization of both pathogens and the biocontrol agent on the root under disease controlling conditions in the gnotobiotic system. The biocontrol agents tested belong to *Pseudomonas*, *Fusarium oxysporum* and *Trichoderma*. For these analyses, tomato seedlings were grown for seven days in the gnotobiotic sand system and sand was infested with *F.o.r.l.*. The biocontrol agents were inoculated when (i) tomato seedlings were incubated with a bacterial suspension or a suspension of germinated *Trichoderma* spores (ii) fungi were mixed in the sand, either as spores (non-pathogenic *F. oxysporum*) or as mycelium (*Trichoderma* spp.).

To obtain more insight, *in vitro* studies were carried out on the effect of biocontrol agents at the initial stage of spore germination by *F.o.r.l.*. Spore germination was analysed in tomato root exudates and in culture supernatants of the biocontrol agents. These *in vivo* and *in vitro* studies contributed to our understanding of disease control by the biocontrol agents.

6. MECHANISMS OF BIOCONTROL OF TFRR

6.1 Antibiosis

Pseudomonas chlororaphis strain PCL1391 produces the antifungal metabolite (AFM) phenazine-1-carboxamide (PCN). Analysis of the PCN-biosynthetic mutant *P. chlororaphis* PCL1119 indicated that the production of PCN is required for biocontrol of TFRR in potting soil (Chin-A-Woeng *et al.,* 1998). Analysis of interactions of strains PCL1391 and PCL1119 or of purified PCN with the pathogen at the cellular level indicated that PCN causes stress on the pathogen, both on agar and in the tomato rhizosphere (Bolwerk *et al.,* 2003). On agar, in the absence of bacteria, *Fusarium* hyphae grew straight in radial orientation with a low frequency of branching (Fig. 2A). In the presence of either PCL1391 cells or of purified PCN growth directionality of the hyphae was altered; hyphae crossed each other while

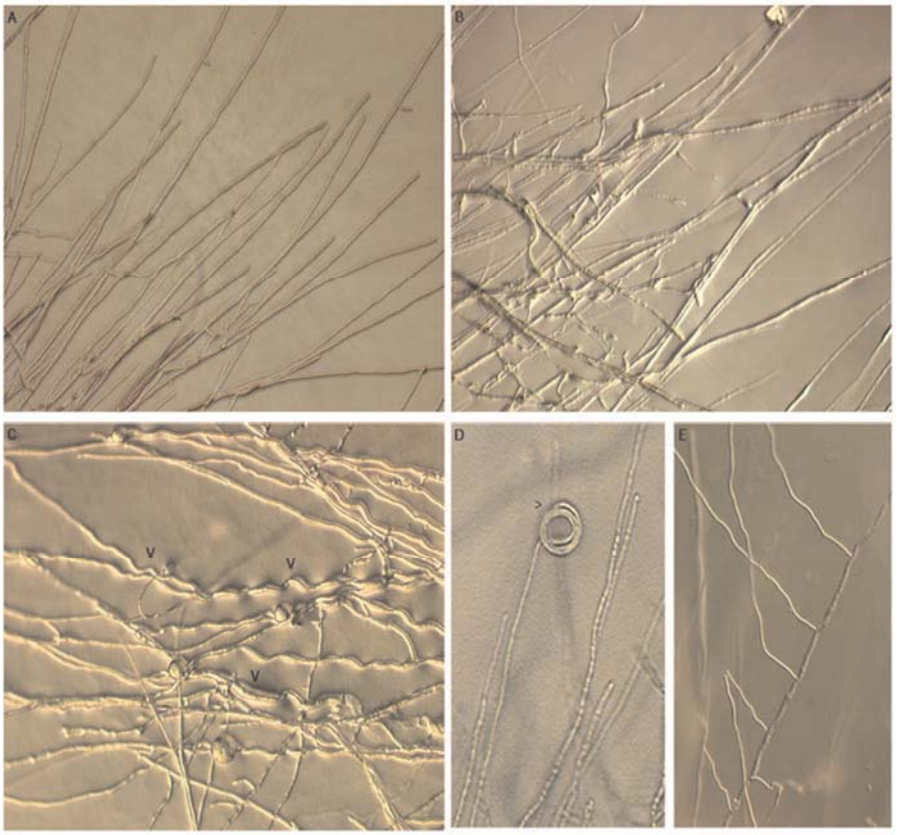

Fig. 2. Differential interference contrast microscopy analysis of *in vitro* effects of *Pseudomonas chlororaphis* PCL1391 on hyphal growth by and morphology of *F.o.r.l.*

F.o.r.l. was grown on microscopy glass slides covered with a thin layer of LB agar. Three days after growth *F.o.r.l.* hyphae were examined for effects of *P. chlororaphis* PCL1391 on growth and spore formation. Panel A, Growth of *F.o.r.l.* in the absence of bacteria. B through E, Growth of *F.o.r.l.* towards *P. chlororaphis* PCL1391, which is located (outside the picture) in the upper right corner (panel B, D and E) or at the left (panel C). B, Overview of the region close to the inhibition zone caused by PCL1391. B through D, Disturbance of hyphal growth directionality. C, Chlamydospores curly growing hyphae as well as thickening of hyphae was observed (arrow heads). D, Spiral growth of a hyphe. E, Increased branching frequency.

growing in different directions (Fig. 2B) and some hyphae showed curly growth (Fig. 2C) or formed spirals (Fig. 2D). Additionally, chlamydospore-like structures and swollen hyphae were observed (Fig. 2C, indicated by arrowheads) and branching frequency was increased (Fig. 2E). On agar, stress effects were not observed in the presence of cells of the PCN-biosynthetic mutant PCL1119, indicating that PCN is causing the stress in

the presence of PCL1391 cells. In the tomato rhizosphere, cells of PCL1391 and purified PCN were observed to cause comparable stress on the pathogen *F.o.r.l.* (Bolwerk *et al*., 2003). In the presence of either cells of PCL1391 or purified PCN, growth directionality was altered as indicated by the abrupt changes in growth direction (Fig. 3A) and by curly growth of hyphae (Fig. 3B). Hyphal morphology was affected as well; the number of vacuoles (Fig. 3C) and the diameter of some hyphae increased (Fig. 3D). In the presence of PCL1391 and purified PCN an altered branching pattern was observed: fork-like branching structures consisted of three branched hyphae, whereas in the absence of bacteria the structures consisted of two branched hyphae (Bolwerk *et al.,* 2003)

Chin-A-Woeng *et al*. (2000) demonstrated previously that efficient colonization of the tomato root system is essential for suppression of TFRR by strain PCL1391. Root colonization is considered to be the delivery system for AFMs, resulting in inhibition of the pathogen over the total root.

6.2 Competitive spore germination limiting root colonization

Spore germination of a biocontrol agent *F. oxysporum* Fo47 is more efficient than *F.o.r.l.* in tomato root exudates and in solutions of glucose as well as citric acid (Bolwerk, 2005). Consequently, Fo47 utilizes more nutrients and subsequently proliferates stronger. In addition, the inoculum of Fo47 required for efficient biocontrol is fifty times higher than *F.o.r.l.*. As a result Fo47 hyphae reach the root earlier and in higher numbers compared to the hyphae of the pathogen and subsequent root colonization by *F.o.r.l.* is reduced in the presence of Fo47. A prediction which was confirmed by the CLSM visualization studies. In these studies tomato seedlings were grown in sand infested with spores of Fo47 and/or *F.o.r.l.*. Subsequently, whole roots (from crown to root tip) were analyzed for colonization by either fungi. Fo47 hyphae were observed at two to five sites per root whereas *F.o.r.l.* hyphae could not be observed or was observed at one site after three days of growth. The total root area colonized by *F.o.r.l.* was reduced up to 10 times in the presence of Fo47 during the seven days of the experiment. Additionally, the intensity of root colonization by *F.o.r.l.* was reduced as indicated by the absence of ‘heavy colonization’ in the presence of Fo47 (Bolwerk, 2005).

6.3 Secondary metabolites produced by biocontrol agents

The ability of biocontrol agents to inhibit spore germination of the pathogen *F.o.r.l.* can contribute to the biocontrol of TFRR. Pilot experiments indicated that culture supernatants of both *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391; grown in King’s medium B (KB; King *et al*., 1994),

limit spore germination of *F.o.r.l.* from 40% to 30% and 4%, respectively. When bacteria were grown in minimal BM medium (Meyer and Abdallah, 1978), the percentage of spore germination was lowered from 84% to 58% and 37% by strains WCS365 and PCL1391, respectively. Analysis of a GacS minus mutant of strain PCL1391 indicated that secondary metabolites, under the regulation of the two component system GacA/GacS, produced both in KB and BM media, contribute to the inhibition of spore germination by PCL1391.

Culture supernatant of *T. harzianum* T22 was shown to inhibit the germination of *F.o.r.l.* spores from 57 to 33% *in vitro*. Similarly, culture filtrate of *T. atroviride* P1 reduced germination of *F.o.r.l.* spores from 57% to 1-3% (Bolwerk, 2005).These observations indicate that these strains produce extracellular compounds which inhibit spore germination. Mutant analysis of strain P1 showed that both the CHIT42 endochitinase and CHIT73 exochitinase produced by P1 contribute to the inhibition of spore germination. It is likely that these extra-cellular compounds inhibit spore germination in the rhizosphere as well. As a result, the subsequent growth of *F.o.r.l.* towards the root, and colonization of the tomato root by *F.o.r.l.* is reduced. This mode of action is supported by CLSM analysis which showed that severity of root colonization and total area colonized by *F.o.r.l.* were significantly reduced in presence of the wild type strain P1 but not in the presence of the endo- and exochitinase mutants. These mutants were also impaired in their ability to control TFRR, both in non-sterile potting soil and in the gnotobiotic sand system (Bolwerk, 2005). CLSM analysis showed that like strain P1, strain T22 also significantly reduced the severity and total root colonization by *F.o.r.l.*. This effect is likely due to the production of chitinases and glucanases that limit spore germination of the pathogen *F.o.r.l.* (Bolwerk, 2005).

6.4 Competition for niches and nutrients on the tomato root

Analyzing roots of seedlings coated with bacteria grown in sand infested with spores of *F.o.r.l.*, showed that cells of strains PCL1391 and WCS365 colonize the root faster than the pathogen. Biocontrol agents occupy the same niche, i.e., the cellular junctions of the tomato root cells occupied by *F.o.r.l.* (Figs. 1A and 1D). At these sites root exudate is thought to be secreted (Chin-A-Woeng et al., 1997; Lagopodi et al., 2002). As a result *Pseudomonas* can effectively compete for both niches and nutrients and reduce root colonization of *F.o.r.l.* up to 80% (Bolwerk et al., 2003).

Analyses of tomato root colonization and disease development of seedlings grown in sand infested with spores of both *F.o.r.l.* and Fo47 showed that at least a 50-fold excess of biocontrol agent over pathogen was

required to obtain control of TFRR. Root colonization by Fo47 and *F.o.r.l.* involves the same niches at the root but root colonization by Fo47 is slower, less aggressive and occurs to a lesser extent than that of the pathogen despite its fifty-fold higher inoculum (Fig. 3E with 3F). High Fo47 inoculum is required to compensate for the difference in root colonization efficiency and is necessary to allow Fo47 to effectively compete for niches and nutrients when both fungi reach the rhizoplane after spore germination in the rhizosphere. This is confirmed by the decrease in root colonization by the pathogen at increasing concentrations of Fo47 (Bolwerk, 2005).

The growth of *T. atroviride* T22 strongly depends on the nutrient and mineral composition present in the gnotobiotic system. CLSM studies on root colonization by T22 showed differences in root colonization with increasing hyphal biomass in sand moisturized with (i) plant nutrient solution (PNS; Hoffland *et al.*, 1989), (ii) PNS with sucrose and (iii) hydroponic solution (HPS; Yedidia *et al.*, 1999). Comparison of the mineral composition of PNS and HPS showed that the latter contains more minerals (K, Ca and NH_4NO_3) and the concentration of trace elements is up to 5000 times higher. This indicates that growth of strain T22, and its ability to compete for niches and nutrients, strongly depends on the composition and concentration of minerals and/or trace elements.

6.5 Colonization of hyphae

Cells of *Pseudomonas* strains PCL1391 and WCS365 colonize hyphae in addition to root colonization (Fig. 3G). Several pilot experiments indicated that *Pseudomonas* could grow on the exudates and culture supernatants of *F.o.r.l.* (de Weert and Kamilova, *personal communication*). Consequently, bacteria that attach to the hyphae may feed on the hyphae, which could be the basis of the observed extensive hyphal colonization (Fig. 3G and 3H). The pathogenicity of the fungus might be negatively affected by feeding of bacteria.

6.6 Predation and parasitism

The PCN biosynthetic mutant *P. chlororaphis* PCL1119 and its parental strain PCL1391 cause comparable stress on pathogens in the gnotobiotic system, although the mutant does so with a delay of three days (Bolwerk *et al.*, 2003). We therefore hypothesize (a) that the production of extra-cellular metabolites other than PCN, such as chitinase, hydrogen cyanide and/or protease (Chin-A-Woeng et al., 1998) cause stress; (b) that PCN accelerates the occurrence of these stresses within the pathogen (Bolwerk *et al*., 2003); and (c) the production of chitinase and protease

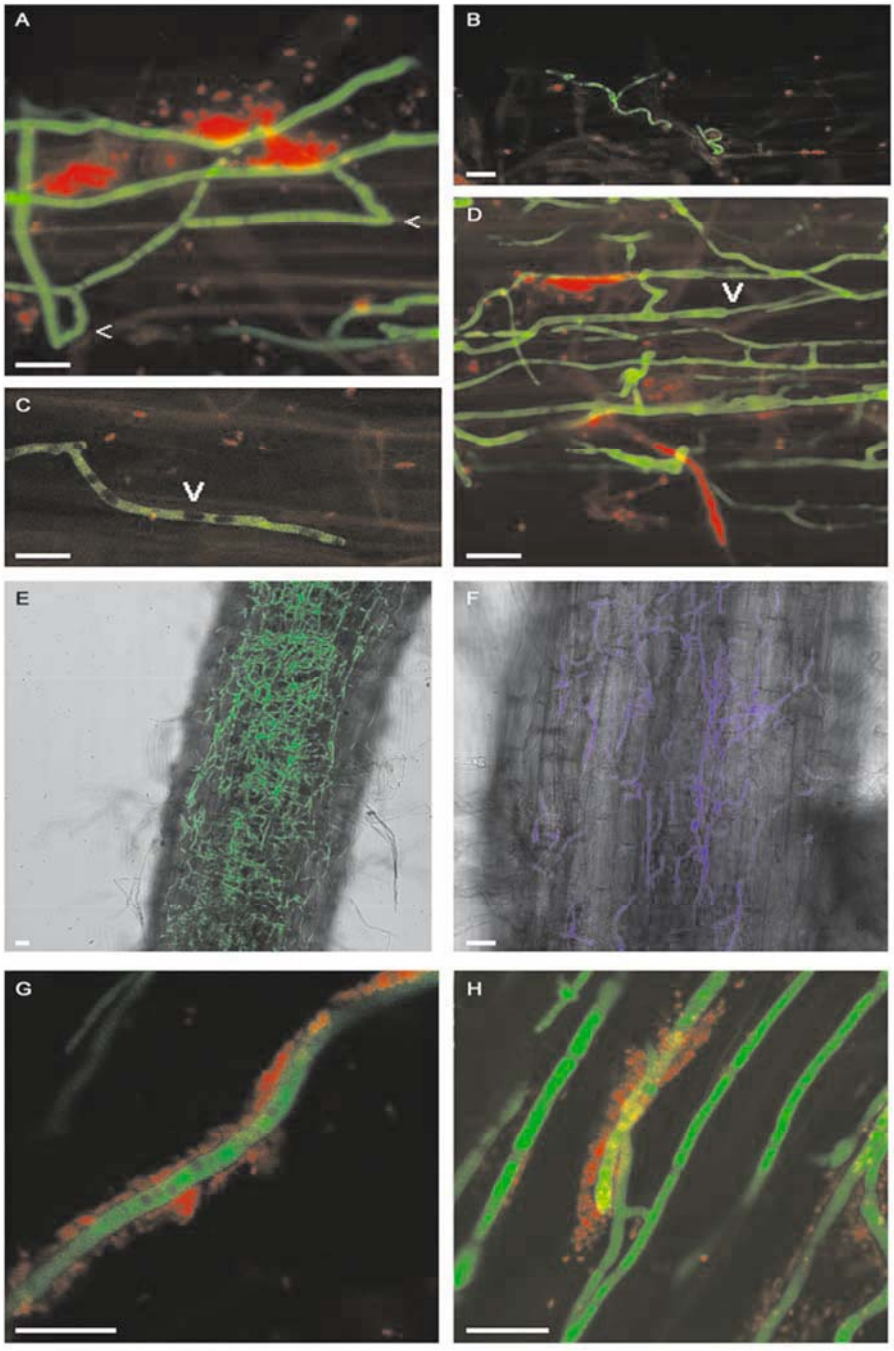

Fig. 3. Confocal laser scanning microscopical analysis of tomato root colonization by *Fusarium* and *Pseudomonas*.

Two-day-old tomato seedlings were coated with *Pseudomonas* bacteria harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells (panel A-D, G-H). Seedlings were grown in a gnotobiotic sand system containing spores *F.o.r.l.* (FCL14) (Panel A-E, G-H) or spores of Fo47 (FCL31) (panel F). *F.o.r.l.* (FCL14) harbors a constitutively expressed *sgfp* gene and appears as green. Fo47 (FCL31) harbors a constitutively expressed *ecfp* gene and appears as blue. Walls of tomato root cells appear as red due to autofluorence (panel A-D, G-H) or gray due to contrast light (panel E and F). Panel A-D, in presence of *P. chlororaphis* PCL1391 different stress effects are observed within *F.o.r.l.* hyphae. A, Abrupt changes in growth direction of *F.o.r.l.* hyphae (indicated by arrow heads). B, Curly growth of *F.o.r.l.* hyphe. C, Increased number of vacuoles in *F.o.r.l.* hyphae. D, Thickening of *F.o.r.l.* hyphae. Panel E and F, highest density of hyphal network of *F.o.r.l.* (E) and Fo47 (F) colonizing the tomato root. Panel G and H (picture taken by A.H.M.Wijfjes), Colonization of *F.o.r.l.* hyphae by *Pseudomonas* bacteria. The size bar represents 10 µm in all panels.

enables strain PCL1391 to attack the cell wall of the fungus and bacterial cells subsequently utilize the released compounds.

6.7 Induction of systemic resistance

Extensive root colonization by *P. fluorescens* WCS365 (Dekkers *et al*., 2000), in contrast to strain PCL1391 (Chin-A-Woeng *et al*., 2000), is not essential for biocontrol of TFRR. Apparently for WCS365 the presence of cells at the top of the root is sufficient to cause biocontrol. Gerrits and Weisbeek (1996) showed that strain WCS365 triggers induced systemic resistance (ISR) in *Arabidopsis thaliana.* Therefore, ISR is thought to be involved in the control of TFRR by *P. fluorescens* WCS365.

Analyses of tomato root colonization and disease development after coating the seedlings with Fo47 showed that, despite the lack of distribution of its hyphae over the root, Fo47 was able to reduce the disease incidence. This resembles the above observation of strain WCS365 and suggests that when Fo47 spores are coated on seedlings, competition for niches and nutrients plays a moderate role, if any, and that another mechanism (inducing systemic resistance) is more important for the reduction of the number of diseased plants. Fuchs *et al*. (1997) illustrated the ability of strain Fo47 to protect tomato plants against *F. oxysporum* f. sp. *lycopersici* Fol8 when inoculated separately in time or space, an observation which also indicates that *F. oxysporum* Fo47 is able to induce systemic resistance in tomato.

Incubation of seedlings in suspensions of *T. atroviride* P1 and *T. harzianum* T22 induced altered root formation: a new main root emerged from the original seed-generated root, which did not grow further and developed a brown color. In addition, this newly emerged root was generally shorter than the corresponding root structure of non-treated seedlings (Bolwerk, 2005). Interestingly, the mutants D11 and P1ND1, impaired in the production of chitinases (Woo *et al*., 1999; Brunner *et al.*, 2003), did not

cause the development of new roots (Bolwerk, 2005). In tomato, chitinases and glucanases have been described as pathogenesis-related proteins (Duijff *et al*., 1998; Fuchs *et al.*, 1997; Joosten and De Wit, 1988). Other studies showed that chitinase deposition was correlated with pathogen distribution in *F.o.r.l.*-infected tomato roots (Benhamou *et al.*, 1990). Possibly, chitinases and glucanases produced by *T. atroviride* P1 and *T. harzianum* T22 could enhance plant defense responses by a positive feedback mechanism. The induction of plant defense responses could be a mechanism of biocontrol of TFRR by *T. atroviride* P1 and *T. harzianum* T22. The ability of *Trichoderma* spp. to induce a defense response within tomato plants was described by De Meyer *et.al.* (1998) who showed that *T. harzianum* T39, spatially separated from *Botrytis cinerea*, reduced disease symptoms in tomato.

7 CONCLUDING REMARKS

Biocontrol is an attractive alternative to chemical control of plant diseases. Unfortunately, biological control is not always effective, especially in field trials. To improve the consistency of biocontrol, an extensive fundamental knowledge of various steps of biocontrol of disease is needed. Visualization studies of interactions between plant root, pathogen, and biocontrol agent deepened our insight in the following aspects: (i) The ecology of a biocontrol agent with respect to its survival and fitness in different growth substrates and conditions. The development of *T. harzianum* T22 in the tomato rhizosphere was shown to be strongly dependent on the mineral composition of the gnotobiotic system. (ii) The biocontrol traits of the agents that contribute to disease control. Hyphal colonization (this chapter, 6.5) and parasitism (this chapter, 6.6) by *Pseudomonas* may represent an efficient mechanism of biocontrol by these bacteria. (iii) Our studies indicated that the preferential spore germination by the non-pathogenic *F. oxysporum* Fo47 is likely to contribute to the control of TFRR. Inhibition of spore germination of *F.o.r.l.* due to the production of extra-cellular enzymes is considered to be the main mechanism of biocontrol by both *Trichoderma* strains analyzed. (iv) The interactions between plant root, pathogen and biocontrol agent and their visualization studies in the rhizosphere contributed to a better understanding of the temporal/spatial mechanisms in which the biocontrol agents affect the pathogen *F.o.r.l.*.

The complexity of the interactions between plant, biocontrol agent and pathogen influence the efficiency of biological control of plant diseases. Analysis of gene expression in the pathogen and the biocontrol agent on the tomato root will provide more information on the regulation of pathogenicity and biocontrol traits. To explore the role of hyphal colonization in the

biocontrol of TFRR by *Pseudomonas*, attachment and subsequent colonization of the hyphae should be studied in more detail, focusing on gene expression profiles, stress responses of the pathogen and biocontrol ability of the bacteria.

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Chapter 9

A PROTEOMICS PERSPECTIVE ON BIOCONTROL AND PLANT DEFENSE MECHANISM

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Abstract: Plants are invaded by a large number of pathogens and they resist pathogen attacks with preformed defenses and by inducing defense responses. Nature is bestowed with many biocontrol agents including plant growth promoting rhizobacteria (PGPR) and *Trichoderma* species. PGPR colonise the rhizosphere and regulate plant growth by inducing defense responses in plants via an induced systemic resistance (ISR) and/or a systemic acquired resistance (SAR), increase the availability of nutrients to plants, produce growth hormones, suppress phytopathogens, release volatile compounds, secrete anti-microbial metabolites and decrease phytotoxic microbial communities in the rhizosphere. *Trichoderma harzianum* controls phytopathogenic fungi by secreting cell wall-degrading enzymes, antibiosis and stimulating plants to produce their own anti-microbial compounds. Though genome sequencing has already been done for some symbiotic and phytopathogenic bacteria, genome sequencing of five PGPR has been only established recently. *Agrobacterium radiobacter* K84 and four strains of *Pseudomonas fluorescens*, Pf0-1, Pf-5, Q8r1 and SBW-25, are being sequenced. The utilization of proteomics to explore biocontrol agents and their mechanisms in plant disease management is in the stage of infancy. It has the potential to revolutionize the way research is conducted on the biocontrol agents and plant defense mechanisms. The interaction between a biocontrol agent, a phytopathogen and a plant brings significant changes to the plant proteome and metabolism. Recently, globular and organellar proteomics approaches have been employed to study the changes in plant proteome after treating with biocontrol agent. In addition to biocontrol agents, proteomics studies on plant defense mechanisms against fungal, bacterial and viral pathogens are also discussed.

Key words: biocontrol; defense proteins; globular proteomics; HPLC; induced resistance; mass spectrometry, organellar proteomics; PGPR; phytopathogen; *Trichoderma*; two-dimensional electrophoresis.

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 233–255.

1 INTRODUCTION

1.1 Induced resistance

Plants are attacked by many pathogens and they resist pathogen invasion both by inducing defense responses and with preformed defenses such as anti-microbial secondary compounds. Localised and systemic induced resistance occurs in most plants in response to attack by pathogenic microbes, physical damage due to insects or other factors, treatment with various chemical inducers and the presence of rhizobacteria (Harman *et al*., 2004a). There are three generally recognised pathways of induced resistance in plants. The production of pathogenesis-related (PR) proteins takes place by the attack of pathogenic micro-organisms in the first pathway. In the second pathway, PR proteins are generally produced as a result of wounding or necrosis-inducing organisms such as herbivory by insects. Typically, the pathogen-induced pathway relies on salicylic acid (SA) produced by the plant as a signalling molecule, whereas the herbivory-induced pathway depends on jasmonic acid (JA) as a signalling molecule. The latter pathway is designated as induced systemic resistance (ISR). This term is also used to refer resistance induced by plant growth promoting rhizobacteria (PGPR), non-pathogenic root-associated bacteria. Recently, rhizobacteria-induced systemic resistance (RISR) was classified as the third pathway (Harman *et al*., 2004a). Different pathways are induced by different challenges, although there seems to be cross talk or competition between pathways (Pieterse and van Loon, 1999; Harman *et al*., 2004a). The JA- and SA-induced pathways are characterised by the production of a cascade of PR proteins. These include antifungal chitinases, glucanases and thaumatins, and oxidative enzymes such as peroxidases, polyphenol oxidases and lipoxygenases. Low molecular weight compounds with anti-microbial properties (phytoalexins) can also accumulate. Any plant-wide process that results in the direct accumulation of PR proteins or phytoalexins is referred as systemic acquired resistance (SAR), which requires SA and confers long-lasting protection in both local and systemic tissues against a broad spectrum of micro-organisms (Durrant and Dong, 2004; Harman *et al*., 2004a).

Induced defense can be activated upon recognition of elicitors of pathogen. Plant disease resistance (R) proteins detect the disease-causing organisms by recognising specific pathogen effector molecules produced during infection process (Martin *et al*., 2003). Based on the combination of structural motifs, five classes of effector-specific R proteins are known, and their sequences suggest roles in both effector recognition and signal transduction. Although some R proteins may act as primary receptors of

pathogen effector proteins, most appear to play indirect roles in this process. The functions of various R proteins require phosphorylation, protein degradation or specific localisation within the host cell. Some signalling components are shared by many R gene pathways whereas others appear to be pathway specific. Readers can refer the comprehensive review written by Martin *et al*. (2003) for details about five classes of R proteins and other proteins that do not fit into five classes. New technologies arising from the proteomics revolution will greatly expand our ability to investigate the role of R proteins in plant disease resistance (Martin *et al*., 2003).

1.2 Biocontrol

Three types of bacteria-plant interactions are found in nature, which are symbiotic, pathogenic and associative (Puhler *et al*., 2004). The symbiotic interaction results in the formation of root nodules in plants. The physiological and biochemical status of nodules and symbiotic relationship change depending on the environmental conditions (Gurusamy *et al*., 1999; 2000; Chinnasamy and Bal, 2003a,b,c; Chinnasamy *et al*., 2003a,b). Genes from both the plant and rhizobia play a major role in the establishment and maintenance of symbiosis, in which the plant supplies reduced carbon to the bacteroid in exchange for fixed nitrogen (Perret *et al*., 2000). Phytopathogens produce diverse interactions. They employ specific methods to attack plant cells and to use plant substances for their growth. In the associative interaction, both the bacteria and plant benefit each other. Many of these associative bacteria act as biocontrol agents. Fungi, viruses and other micro-organisms also form different kinds of association with plants, which may be harmful or beneficial to plant growth. Recently, many micro-organisms are increasingly used as inoculants for biocontrol, biofertilisation and phytostimulation, though some inoculants such as *Rhizobium* and *Bradyrhizobium* have been successfully marketed for more than a century (Bloemberg and Lugtenberg, 2001; Ping and Boland, 2004).

Biocontrol is the process by which a pathogenic organism is maintained at low inoculum density or controlled or eradicated by beneficial organisms. Several microbes and insects present in the natural environment serve as potential biocontrol agents. They are non-pathogenic, environmental-friendly, cheaper to produce and easy to handle, and may create long-lasting effects. Biocontrol agents such as PGPR and *Trichoderma harzianum* act as attractive alternative to pesticides. Kloepper and Schroth (1978) first defined PGPR, which include soil bacteria that colonise the roots of plants following inoculation onto seed and enhance plant growth. PGPR regulate plant growth and suppress pathogen ingress into the plant system by various mechanisms, which include: induction of

defense responses in plants through an ISR and /or a SAR, increase the availability of nutrients, and produce growth hormones, volatile compounds and anti-microbial metabolites (Rodriguez and Fraga, 1999; Bloemberg and Lugtenberg, 2001; Walling, 2001; Persello-Cartieaux *et al*., 2003; Mew *et al*., 2004; Ping and Boland, 2004). These processes can cause substantial changes to the plant proteome and metabolism. Root colonisation by antagonistic fungi *T. harzianum* enhances root growth and development, crop productivity, resistance to abiotic stress, and uptake and use of nutrients (Harman, 2000; Yedidia *et al*., 1999, 2000; Harman *et al*., 2004a). *Bacillus subtilis* GB03, MBI205, MBI600, *B. amyloliquefaciens* GB99 (IN037a), *B. cereus* UW85, *B. pumilis* GB34 (INR-7), BacJ, *Burkholderia ambifaria* AMMDR1, *Methylobacter extorquens*, *Pantoea agglomerans* C9-1, *Pasteuria penetrans*, *Pseudomonas fluorescens* A506, Pf0-1, Pf-5, Q8r1, SBW-25, *P. aureofaciens* 30-84, *Streptomyces griseoviridis* K61, *S. lyticus*, *Aspergillus flavus* and *T. hamatum* T382 are also used as biocontrol agents (Fravel, 1988; Stohl *et al*., 1999; Knox *et al*., 2000; Ryu *et al*., 2003, 2004; Puhler *et al*., 2004). Most of these strains have been recently recommended as prioritised biocontrol agents for genome sequencing.

1.3 Proteomics

Proteomics is a leading field of science with huge potential. Wilkins and co-workers conceptualised the term 'proteome' to define the expressed complement of a genome (Wasinger *et al*., 1995; Wilkins *et al*., 1995). Proteomics is defined as the systematic analysis and documentation of all protein species and their post-translational modifications in an organism or a specific type of tissue or a cell or an organelle at a given time (Wasinger *et al*., 1995; Blackstock and Weir, 1999; Cahill *et al*., 2000; Pandey and Mann, 2000; Graves and Haystead, 2002; Patterson and Aebersold, 2003; Phizicky *et al*., 2003; Simpson, 2003). Proteomes are modified in function of biotic and abiotic factors. Though proteomics is advanced in animals and micro-organisms, it is still at the initial phase in plant science. Some of the important factors that influence the plant proteomes are disease states, insect damages, developmental stages, cell and tissue types, environmental stresses and soil conditions. The possibility to monitor alterations in protein profiles through the cutting edge proteomics technology is valuable for a deeper understanding of plant defense strategies against diseases and molecular mechanisms behind the biocontrol.

Proteomics predominantly employs the classical techniques of two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS) (Westermeier and Naven, 2002; Simpson, 2003, 2004). In 2-DGE, proteins are separated by isoelectric focusing (IEF) in the first dimension based on their charge and then resolved by sodium dodecyl sulphate-polyacrylamide

gel electrophoresis (SDS-PAGE) in the second dimension based on their molecular weight (Graves and Haystead, 2002; Salekdeh *et al*., 2002). Separated proteins have been visualised by numerous staining methods or by autoradiography. They can be identified by comigration with known proteins, immunoblotting, N-terminal sequencing, peptide mass determination by MS, peptide sequencing by tandem MS, and correlating the mass and sequence data with information in protein, genome and expressed sequence tag (EST) databases (McDonald and Yates III, 2000; Graves and Haystead, 2002; Patton, 2002). Alternative approaches that are becoming more popular are based on separation of proteins using multidimensional liquid chromatography followed by identification of proteins using MS or tandem MS (Palfree *et al*., 2003; Simpson, 2003; Apale *et al*., 2004).

Research on biocontrol agents, phytopathogens and plant defense mechanisms is changing dramatically in the advent of genomics and proteomics technologies. Most of the genome sequencing works have been directed towards phytopathogenic and symbiotic organisms (Kaneko *et al*., 2000; Simpson *et al*., 2000; Galibert *et al*., 2001; Goodner *et al*., 2001; Wood *et al*., 2001; da Silva *et al*., 2002; Kaneko *et al*., 2002; Salanoubat *et al*., 2002; Van Sluys *et al*., 2002; Buell *et al*., 2003; Weidner *et al*., 2003; Puhler *et al*., 2004). Though many genome sequencing projects are completed for plant pathogenic and symbiotic organisms, no genome project is yet finished for associative PGPR and other biocontrol agents. Currently, *Agrobacterium radiobacter* K84 and four strains of *Pseudomonas fluorescens* namely Pf0-1, Pf-5, Q8r1 and SBW-25 are being sequenced (Table 1).

Table 1. Genome sequencing projects undergoing for root colonising plant growth promoting rhizobacteria.

Organism	Web-site
A. radiobacter K84	http://depts.washington.edu/agro/homeM.htm
P. fluorescens Pf0-1	http://genome.jgi-psf.org/draft_microbes/psefl/psefl.home.html
P. fluorescens Pf-5	http://www.ars-grin.gov/ars/PacWest/Corvallis/hcrl/Pf5genome/status.htm
P. fluorescens Q8r1	http://www.wsu.edu/~mavrodi/q8r1.htm
P. fluorescens SBW-25	http://www.sanger.ac.uk/Projects/P_fluorescens/

Accumulation of vast amount of genomic data paved the way to analyse and compare the gene products, proteins, using a powerful proteomics technology. In recent years, proteomics have been used to study the interactions of symbiotic (Natera *et al*., 2000; Saalbach *et al*., 2002; Rolfe *et al*., 2003; Wienkoop and Saalbach, 2003; Bestel-Corre *et al*., 2004; Djordjevic, 2004; Hoa *et al*., 2004) and pathogenic (Konishi *et al*., 2001;

Rep *et al*., 2002; Keon *et al*., 2003; Smolka *et al*., 2003; Ventelon-Debout *et al*., 2004) organisms with plants. However, application of proteomics strategies in elucidating the mechanisms of biocontrol and induced resistance is not yet common.

In this chapter, I focus on the current status of proteomics-based studies in biocontrol and plant defense mechanism against fungal, bacterial and viral pathogens.

2 PROTEOMICS RESEARCH IN BIOCONTROL

Grinyer *et al*. (2004b) first time reported the proteome of the biocontrol fungus *T. harzianum*, which is a soil-borne filamentous fungus that can prevent the growth of a range of pathogenic fungi on many types of crop plants, providing an environmentally benign alternative to chemical fungicides. Twenty five protein spots belonging to 22 different genes were identified from a whole-cell protein reference map of *T. harzianum* by employing a combination of 2-DGE, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), liquid chromatography tandem mass spectrometry (LC MS/MS) and *de novo* sequencing. Studying proteomes of target cell organelles termed as subcellular or organellar proteomics is a promising approach because they represent discrete functional units and their complexity in protein composition is reduced relative to whole cells (Taylor *et al*., 2003). Subcellular proteomics has the capability to screen not only previously unknown gene products but also to assign them, along with other known but poorly characterised gene products, to particular subcellular structures (Dreger, 2003). Grinyer *et al*. (2004a) mapped the mitochondrial proteins of *T. harzianum* using a systematic proteomic approach. The mitochondrial proteins identified include: several proteins of tricarboxylic acid cycle and electron transport chain, chaperones, other protein-binding and import proteins, ketol-acid reductoisomerase, probable elongation factor EF-Tu and ADP/ATP carrier protein. *T. harzianum* secretes a range of cell wall-degrading enzymes that break down the cell wall of phytopathogenic fungi, leading to death (Harman and Kubicek, 1998). Antibiotics are also secreted from *T. harzianum* during the attack on phytopathogenic fungi (Lorito *et al*., 1996). *Trichoderma* species produce a wide range of antibiotic substances, more than 100 different metabolites. They also strongly stimulate plants to produce their own anti-microbial compounds (Sivasithamparam and Ghisalberti, 1998; Harman *et al*., 2004a). Therefore, *T. harzianum* alone or in combination with chemical fungicides gives some plants adequate protection against phytopathogens (Lorito *et al*., 1994).

Proteome analysis of *Trichoderma* strain-22 (T-22) revealed proteins that are homologues of Avr4 and Avr9 from *Cladosporium fulvum*. Similar proteins were also produced by *T. atroviride* strain P1 (Woo, 2003; Harman *et al*., 2004a). Proteomes from five-day old maize seedlings grown from seeds either treated or not treated with T-22 were fractionated by 2-DGE. Approximately 40% of the proteins that were seen in the presence of T-22 were not visible in gels that contained proteins from untreated plants (Harman *et al*., 2004a, b). Similar results have been obtained using bean and T-22 (Harman *et al*., 2004a, b). These results indicate that biocontrol fungi strongly modify plant metabolism, which in most cases benefits the plant (Harman *et al*., 2004a). Sonawane *et al*. (2003) used 2-DGE, MS and N-terminal sequencing to identify number of enzymes and transporters involved in amino acid uptake and metabolism in *P. fluorescens* ATCC 13525 and *P. putida* KT2440.

In all ecosystems, micro-organisms have to compete with each other for space and nutrients. Numerous antibiotics produced by a variety of bacteria and other microbes play a prominent role in antagonistic interactions. Proteomics approaches were used to elucidate the complex cellular responses of *B. subtilis* (Bandow *et al*., 2003) and *Staphylococcus aureus* (Singh *et al*., 2001) to antibiotics. However, they have not yet been applied to study the antibiotic proteins and peptides produced by beneficial micro-organisms such as PGPR in relation to biocontrol.

Recently, the effect of an antibiotic (concanamycin A) produced by *S. halstedii* on protein levels in the filamentous fungus *A. nidulans* was studied (Melin *et al*., 2002). Proteins such as concanamycin induced protein A (CipA) [homologous to cadmium induced protein 1 (CIP1) in *Candida* sp.], CipB (homologous to LovC, an enoyl reductase involved in the biosynthesis of lovastatin, a secondary metabolite identified in *A. terreus*), CipC (homologous to an EST sequence from *A. niger*), and CipD were up regulated. At the same time, concanamycin repressed protein A (CrpA) was down regulated and it was homologous to cross pathway control B (CpcB) protein that controls the global amino acid synthesis and the initiation of sexual development in *A. nidulans*. CrpB protein, homologous to glyceraldehyde-3-phosphate dehydrogenase A (GpdA), was also down regulated, which may reflect a response to antibiotic induced stress with a concomitant change in intracellular conditions (Melin *et al*., 2002). It indicates that proteome analysis is an useful tool for studying effects on gene expression during competitive interactions between bacteria and fungi.

A proteomic approach involving two-dimensional differential gel electrophoresis, MS and function-based activity profiling has been used to examine changes in the gut proteins of Indian-meal moth larvae resistant to insecticidal proteins (Cry toxins) of *B. thuringiensis* (Candas *et al*., 2003). This approach found an increased glutathione utilisation, elevation in

oxidative metabolism, differential maintenance of energy balance, alteration in a low molecular mass acidic protein homologous to F_1F_0-ATPase and decrease in the level of chymotrypsin-like proteinase in the resistant larvae. Several *Streptomycetes* species have been shown to be effective biocontrol agents for plant diseases (Doumbou et al., 2001). During the interaction between aquatic plant (*Lemna minor*) and saprophytic *Streptomycetes coelicolor*, 31 proteins were either induced or repressed in *S. coelicolor* (Langlois *et al*., 2003). The induced proteins were involved in energetic metabolism (glycolysis, pentose phosphate pathway and oxidative phosphorylation), protein synthesis, degradation of amino acids, alkenes, or cellulose, tellurite resistance, and growth under general physiological or oxidative stress conditions. The repressed proteins were synthesised under starvation. This study suggests that carbon and energy are acquired through degradation of compounds found in plant exudates. Bacteria utilise this additional carbon source in adaptations to physiological and oxidative stress. These traits might be essential for rhizosphere competence (Langlois *et al*., 2003). The setup of two-dimensional protein reference map for three-way interaction between plant, pathogen and biocontrol agent will provide a solid understanding on the molecular mechanisms behind this interaction and the role of biocontrol agent in plant disease control. It will also help in the characterisation of plant defense proteins, PR proteins, antibiotics and post-translational modifications of proteins during the three-way interaction.

3 PROTEOMICS RESEARCH IN PLANT DEFENSE MECHANISM

3.1 Plant defense responses to fungal attack

Proteomic analysis using one-dimensional gel electrophoresis (1-DGE), immunoblotting, peptide mass finger printing and mass spectrometric sequencing identified two isoforms of PR protein (PR-1a and PR-1b), β-1,3-glucanases (PR-2 and PR-Q'b), chitinase (PR-3) and PR-5x in tomato xylem sap infected with the vascular wilt fungus *Fusarium oxysporum* during compatible or incompatible interactions (Rep *et al*., 2002). In an incompatible interaction, the fungus was apparently contained within the vessel it had invaded, where as in a compatible interaction, it invaded neighbouring parenchyma tissue and had spread laterally to other vessels, eventually colonising the entire vascular system (Gao *et al*., 1995; Mes *et al*., 2000). PR-5x is very closely related to basic vacuolar PR-5 proteins. It accumulates in the xylem sap of tomato relatively early after infection. It is

the only protein produced in high amounts in an incompatible interaction, whereas other PR proteins accumulate only in compatible interactions, concomitantly with the appearance of disease symptoms (Rep *et al*., 2002). Shepherd *et al*. (2003) employed proteomics to examine changes in the specific stages in the life cycle of *Phytophthora palmivora*, which causes black pod diseases in cocoa and other economically important tropical crops (Erwin and Ribeiro, 1996). Approximately 1% of proteins appeared to be specific for each of the mycelial, sporangial, zoospore, cyst and germinated cyst stages of the life cycle (Shepherd *et al*., 2003).

Twelve proteins appeared to change in leaf blades of rice plants that were grown under different levels of nitrogen nutrient and were infected with blast fungus *Magnaporthe grisea* (Konishi *et al*., 2001). PR proteins were pointed out as the reason for incompatible interaction in rice plants following blast fungus infection. A proteomics approach utilising polyethylene glycol prefractionation, 2-DGE and N-terminal sequencing or internal amino acid sequencing has enabled to identify 12 new pathogen- and elicitor-responsive proteins including low abundant proteins, from 6 different genes in rice cells in response to the rice blast fungus infection (Kim *et al*., 2003). Rice PR protein class 10 (OsPR-10), isoflavone reductase-like (IRL) protein, β-glucosidase and putative receptor-like protein kinase (RLK) were among those induced by rice blast fungus. Six isoforms of probenazole-inducible protein 1 (PBZ1) and two isoforms of salt-induced (SaIT) protein responded differentially to blast fungus, elicitor, and signal molecules such as JA and SA. Recently, two RLKs, two β-1,3-glucanases (Glu1, Glu2), thaumatin-like protein (TLP) and peroxidase (POX 22.3) were also identified as differentially displayed proteins in rice leaves inoculated with *M. grisea* (Kim *et al*., 2004). The induction of PBZ1, OsPR-10, SaIT, RLK and TLP in incompatible interactions was slightly stronger and faster than in compatible interactions (Kim *et al*., 2003, 2004). Callose deposition and hypersensitive response were observed in incompatible interactions but excessive invading of fungal hypha with branches were seen in compatible interactions (Kim *et al*., 2004). In the rice-blast fungus interaction, infection with an incompatible pathogen results in rapid cell death with limited hypersensitivity, whereas the compatible pathogen causes a slower reaction. In this type of pathosystem, many defense-related genes such as PBZ1 and PR-1b were induced more strongly and earlier by an incompatible pathogen than by compatible pathogen (Lee *et al*., 2001; Agrawal *et al*., 2002). Thus, proteome analysis can distinguish differences in the timing and amount of protein expression induced by pathogens and other signals in incompatible and compatible interactions (Kim *et al*., 2003).

Twenty four hours after treatment with two fungal pathogen elicitors, chitosan and extracts of *Fusarium moniliforme*, the extracellular matrix of *Arabidopsis thaliana* cell suspension culture displayed an increase

in the level of two classical cell proteins (a putative enodochitinase and a polygalacturonase inhibiting protein) and two novel proteins (a putative receptor-like protein kinase and a probable apospory-associated protein) (Ndimba *et al*., 2003). The level of an unknown protein and a hypothetical protein, which has some homology to serine carboxypeptidases, were decreased at the same time. Two pathogen elicitor responsive proteins namely a xyliglucan endo-1-4-β-D glucanases (XEG) and a peroxidase were identified in the culture filtrate extracts. The perception of microbial signal molecules is part of the strategy evolved by plants to survive attacks by potential pathogens. In most cases, phosphorylation of plant proteins is required to initiate responses to microbial signals. AtPhos43, a plant specific protein, in *Arabidopsis* and related proteins in tomato and rice are differentially phosphorylated after treatment with flagellin, a bacterial elicitor, and chitin fragments, an elicitor from fungal cell walls (Peck *et al*., 2001). The directed proteomics identified that phosphorylation of AtPhos43 after flagellin treatment but not chitin treatment is dependent on FLS2, a receptor-like kinase involved in flagellin perception. Induction by both elicitors is not dependent on SA or EDS1 (enhanced disease susceptibility), a putative lipase involved in defense signalling (Peck *et al*., 2001). Recently a number of additional proteins has been identified that are phosphorylated in response to microbial elicitors. One of these proteins, a syntaxin, is phosphorylated *in vitro* by a calcium-dependent kinase, indicating a link between elicitor-induced calcium fluxes and change in protein phosphorylation (Ramonell and Somerville, 2002). The classical proteomic work reported by Ndimba *et al*. (2003) also showed that putative receptor-like kinase, XEG and putative endochitinase possess phosphorylated tyrosine residues. The identification of phosphorylated *bona fide* cell wall proteins and a putative extracellular receptor-like kinase with no transmembrane domain implicates the existence of an extracellular phosphorylation network, which could be involved in intercellular communication (Ndimba *et al*., 2003).

3.2 Pathogenesis and plant defense responses to bacterial attack

The bacteria *Xylella fastidiosa* is the causative agent of a number of economically important crop diseases, including citrus variegated chlorosis (CVC) (Smolka *et al*., 2003). The complete genome sequencing of the CVC strain 9a5c was published in 2000 and represents the first complete genome sequence of a plant pathogen (Simpson *et al*., 2000). Using combined proteomics techniques for protein separation, the products of 142 genes were recently identified in a whole cell extract and in an extracellular fraction of *X. fastidiosa* (Smolka *et al*., 2003). Proteins putatively associated with three

different adhesion systems (type IV fimbriae, mrk pili and hsf surface fibrils) were found to be constitutively coexpressed. This suggests the presence of structures possessing different adhesive properties that may be important for *X. fastidiosa* to colonise hosts with different tissue structural organisations. Last two adhesion systems were detected as multimeric complexes, which may be related to their function in forming large adhesive structures. The extracellular fraction of *X. fastidiosa* revealed the presence of 30 proteins with varied functions, suggesting that intracellular space is a multifunctional microenvironment containing proteins related to *in vivo* bacterial survival and pathogenesis. They may be important for the bacteria to colonise and successfully parasitize the hosts. A codon usage analysis of the most expressed proteins from the whole cell extract of *X. fastidiosa* showed a low biased distribution, which is proposed as a reason to the slow growing nature of these bacteria and this weakness may be used in a strategy for disease control (Smolka *et al*., 2003).

Several bacterial pathogens of plants have been intensively studied for quorum sensing (QS), the abilities of bacteria to assess their local population density and/or physical confinement via the secretion and detection of small, diffusible signal molecules (von Bodman *et al*., 2003). QS critically controls genes for pathogenicity and colonisation of host surfaces. N-acyl-homoserine lactones (AHLs) are the most commonly reported type of QS signals. Proteome analysis showed that eukaryotic host, the model legume *Medicago truncatula*, was able to detect nanomolar to micromolar concentrations of AHLs from both pathogenic (*Pseudomonas aeruginosa*) and symbiotic (*Sinorhizobium meliloti*) bacteria (Mathesius *et al*., 2003). The host plant responded in a global manner by showing changes in the accumulation of over 150 proteins. The accumulation of specific proteins and isoforms depend on AHL-structure, concentration and time of exposure. Along with many traits, QS also regulate the production of antibiotics, degradative enzymes, Hrp protein and epiphytic fitness (von Bodman *et al*., 2003). In addition, exposure to AHLs was found to induce changes in the secretion of compounds by the plants that mimic QS signals, which have potential to disrupt QS in associated bacteria (Mathesius *et al*., 2003). As pointed out by von Bodman *et al*. (2003), proteomics has huge potential to expand our perspective on QS regulation in plant pathogenic species. AHLs produced by PGPR play a vital role in the production of antibiotics such as phenazine and diacetyl phloroglucinol. Hence, proteomics studies on the effect of beneficial and pathogenic bacteria on plant system will help in elucidation of differential display of proteins, which could aid in differentiation of proteins induced by both beneficial and inimical bacterial species.

Xanthomonas axonopodis pv. *passiflorae* is the casual agent of bacteriosis or premature death of passion fruit. 2-DGE and N-terminal

sequencing were used to identify differentially displayed proteins during the treatment of *X. axonopodis* pv. *passiflorae* in media containing leaf extract of the compatible (passion fruit) and incompatible (tomato) hosts (Tahara *et al*., 2003). The global expression of proteins was almost identical in cells grown in medium containing leaf extract of the incompatible host. However, four proteins including two induced and two up regulated were identified in cells grown in medium containing leaf extract of the compatible host. A putative membrane-related protein and a hypothetical protein were novel proteins induced specifically by the leaf extract of the compatible host, whereas an inorganic pyrophosphatase and a hypothetical protein, that showed similarity to the *yci*F gene of *Salmonella thyphimurium,* were up regulated in the same condition. *X. axonopodis* pv. c*itri* was cultured in the presence of leaf extracts from a susceptible host plant (sweet orange), a resistant host plant (ponkan) and a non-host plant (passion fruit). The protein profiles were analysed using 2-DGE and N-terminal sequencing (Mehta and Rosato, 2001). Five differentially expressed proteins (pseudouridine synthase, elongation factor P, large subunit of ribulose 1,5-biphosphate carboxylase/oxygenase, sulfate-binding protein and heat shock protein G) were sequenced and their functions assigned by homology searching. Recently, proteome reference map of the soft-rot disease-causing *Erwinia chrysanthemi* has been created using 2-DGE, mutant analysis, Western blotting and MALDI-TOF MS (Kazemi-Pour *et al*., 2004).

Protein phosphorylation is one of the pivotal processes that take place during plant-pathogen interactions, induced resistance and plant defense (Xing *et al*., 2002). It can alter intrinsic biological activity of a protein, subcellular location and half-life. It can be important for the extent and duration of defense response. Many protein kinases and phosphatases have been identified that connect signal perception mechanism to plant defense responses (Romeis, 2001; Xing *et al*., 2002). Genomics and proteomics have already identified new components and will continue to influence the study of phosphorylation profoundly in plant-pathogen interactions (Xing *et al*., 2002). The phosphorylation of a chloroplast protein, oxygen-evolving enhancer 2 (OEE2), was induced in *Arabidopsis* infected with avirulent *Pseudomonas syringae* (Yang *et al*., 2003). OEE2 is also suggested as a molecule downstream of AtGRP-3 (glycine rich protein)/WAK1 (cell wall-associated kinase), which may be involved in defense signalling against pathogen. The changes in protein phosphorylation pattern of tobacco cells were analysed by 2-DGE in response to elicitation with cryptogein (Lecourieux-Ouaked *et al*., 2000). Recently, a protein reference map for the bacterial plant pathogen *A. tumefaciens* was reported (Rosen *et al*., 2004). It contains more than 300 proteins with an isoelectric point (pI) between 4 and 7. Quantitative analysis pointed out that some of these proteins were subjected to post-translational modifications. Rapid

changes reflecting kinase signalling processes have been detected when *Arabidopsis* cells pulse-labelled with radioactive orthophosphate during a treatment with the flagellin elicitor (Peck *et al*., 2001).

Transcription factors control the expression of specific genes, which are crucial for a variety of essential processes such as plant defense responses to biotic and abiotic stresses, plant growth and development, and metabolic regulation (Singh *et al*., 2002; Chinnasamy *et al*., 2004). More than 5% of the genes in the *Arabidopsis* genome encode transcription factors. Research carried out in the past few years has been productive in identifying various plant transcription factors, namely ERF, bZIP, AP2/EREBP, WRKY, MYB and SA-inducible DOF proteins, and their responses to pest attacks (Liu *et al*., 1999; Riechmann and Ratcliffe, 2000; Singh *et al*., 2002). Most of the transcription factors have been characterised through traditional genetics (forward genetics), reverse genetics, RNA interference (RNAi), virus-induced gene silencing, mutagenesis, T-DNA tagging, yeast two-hybrid system, steroid-inducible CO in combination with suppression subtraction hybridisation, genomics-based DNA microarray and mRNA-profiling technology (Riechmann and Ratcliffe, 2000). Recently, proteomics is emerging as an efficient methodology to characterise plant transcription factors. Proteomics-based approach was employed to study the roles of redox-sensitive plastid transcription factor in mustard chloroplast (Loschelder *et al*., 2004) and 10 different transcription factors in wheat seed development (Chinnasamy *et al*., 2004). However, proteomics is not yet fully used to analyse roles of transcription factors in biocontrol mediated plant defense against pathogens. A comprehensive view on the activation, regulation, function and interaction of various transcription factors in induced resistance, plant defense, antibiosis and biocontrol will emerge in the near future from the proteomics studies.

3.3 Plant defense responses to viral attack

The identification of plant viruses remains cumbersome despite the existence of an abundance of procedures to facilitate the process (Matthews, 1991). Unlike other techniques, MS offers the promise to identify an unknown virus without performing numerous other experiments. Peptide mass fingerprinting has been shown to be successful in the direct identification of purified viral strains (Lewis *et al*., 1998; She *et al*., 2001). In a proof-of-concept proteomics experiment, Cooper *et al*. (2003) successfully identified tobacco mosaic virus proteins from total protein extracts of infected tobacco leaves through 2-DGE followed by high performance liquid chromatography tandem mass spectrometry (HPLC MS/MS). They also demonstrated that the proteomic approach could be used

to characterise unknown viruses in infected plants. A virus that had previously been tentatively identified as a tobacco rattle virus was proved as a strain of potato virus X (Cooper *et al*., 2003). This type of methodology with further developments may be of significant value in plant pathology and plant disease diagnosis as more genomic sequence data is deposited in the public domain.

Proteomic analysis of rice cellular suspensions infected with rice yellow mottle virus (RYMV) showed changes in 64 proteins that were involved in defense, pathogenesis, stress, metabolism, translation and protein turnover (Ventelon-Debout *et al*., 2004). Both PR protein (PR-10A) and salt stress-induced protein were non-specific responsive proteins and were induced late at RYMV infection in a susceptible cultivar. Proteins such as dehydrins and enzymes involved in glycolytic pathway have been suggested as more specific to RYMV infection. Dehydrins have potential phosphorylation sites and its activity might be dependent upon phosphorylation status under RYMV infection. Ethylene is one of the signal molecules important for defense against a variety of pathogens. A decrease in the relative abundance of ethylene-inducible protein has been correlated to the susceptibility of rice cultivar to RYMV attack (Ventelon-Debout *et al*., 2004). According to Zaitlin and Palukaitis (2000), genomics coupled with proteomics technology could lead to the elucidation of the pathways determining susceptibility versus resistance, and the molecular basis of pathogenicity. The information available in proteomics research indicates that it is a reliable technology to study infection process, disease development, resistant strategies, specific genes and gene products involved, and biocontrol mechanisms. Using specific and narrow range IEF strips would be helpful to observe a more precise picture of less abundant protein variations during plant-phytopathogen-biocontrol agent interactions.

4 CONCLUSIONS AND FUTURE DIRECTIONS

Preformed and induced defense mechanisms play a vital role in the survival of plants against a diverse group of pathogens. Nature is filled with plenty of microbes and some of them, for example PGPR and *Trichoderma harzianum*, act as biocontrol agents that can invoke the defense mechanisms in plants. PGPR enhance plant growth by inducing defense responses, increasing availability of nutrients, producing growth hormones, volatile compounds and anti-microbial metabolites, suppressing phytopathogens, and decreasing pathogenic microbes in the rhizosphere. *T. harzianum* controls phytopathogenic fungi by secreting cell wall-degrading enzymes, producing antibiotics and stimulating plants to produce their own anti-microbial compounds. Using biocontrol agents against pests and diseases will be

helpful to achieve efficient and sustainable agricultural production systems. While chemical pesticides have their place in disease control, there is a growing awareness that biologically-based pest management (BBPM) and integrated pest management (IPM) strategies provide more environmentally sound and economically viable alternatives for agriculture. Despite years of research and development, significant questions regarding the molecular, physiological and ecological constraints that limit biological controls remain unanswered.

The advent of proteomics is revolutionising the study of plant-pathogen interactions and plant defense mechanisms, and is revealing a complex web of signalling cascades involved in plant defense responses. Study of all proteins through proteomics from a given cell, organelle or tissue simultaneously with respect to properties, such as expression levels, post-translational modifications, interactions with other molecules, functions and structures, is ready to contribute valuable information about cellular processes and metabolic pathways. This will result in an integrated global view of disease developmental processes, cellular mechanisms and networks at the protein level.

Both proteomics and genomics are not yet vigorously applied to elucidate the roles of biocontrol agents in plant defense and disease resistance. All of the sequenced genomes belongs to symbiotic and plant pathogenic organisms (Puhler *et al*., 2004). Most of the on-going genome sequencing works are directed towards symbiotic and phytopathogenic organisms. Recently, genome sequencing projects for five biocontrol agents have been initiated. *A. radiobacter* K84 and four strains of *P. fluorescens* namely Pf0-1, Pf-5, Q8r1 and SBW-25 are being sequenced. Given the importance of biocontrol in environmental sustainability and other benefits, efforts should be taken in the near future to sequence genomes of many biocontrol agents. It would be of interest to investigate the pattern, function and efficacy of antibiotic proteins and peptides produced by PGPR and other biocontrol agents in free-living state and in the presence of plant and/or pathogen in laboratory and field conditions with the combination of different soil, nutrient, biotic and abiotic factors. Application of globular and organellar proteomic approaches in various plant organs and tissues at different developmental stages of plants and time of plant-pathogen-biocontrol agent interactions would be able to highlight proteome difference in whole plants as well as individual cell organelles. These findings will be useful to understand roles of proteins at both cellular and organellar levels in plants during the development of induced resistance and defense mechanisms mediated by biocontrol agents.

Proteomic and genomic tools offer new possibilities for improving the selection, characterisation and management of biocontrols. Systematic investigations of the molecular mechanisms by which biocontrol agents

colonise and protect plants from pathogens can now be done with proteomic and genomic tools. In addition, proteomic and genomic studies of biocontrol agents will provide fundamental insights into the microbial ecology of the phytosphere (the environment immediately surrounding and including the plant), which encompasses the primary loci of biocontrol. Whether acting by competitive exclusion, biochemical antagonism, or induction of host defenses, biocontrol agents must be well adapted for survival and functional activity in the phytosphere. The genetic modification of biocontrol agents to produce higher levels as well as higher release rates of plant growth promoting compounds, pathogen suppressing metabolites and resistance inducing substances might give promising benefits to the sustainable agriculture and environment. Recent improvements made to proteomics technology along with cellular, molecular, biochemical, genetic, genomic and bioinformatic advancements have capability to achieve many important tasks in future, which will enhance our knowledge on biocontrols and plant defense mechanisms.

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Chapter 10

PLANT GROWTH PROMOTING RHIZOBACTERIA FORMULATIONS AND ITS SCOPE IN COMMERCIALIZATION FOR THE MANAGEMENT OF PESTS AND DISEASES

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Abstract: The export oriented agricultural and horticultural crops depends on the export of residue free produce and has created a great potential and demand for the incorporation of biopesticides in crop protection. To ensure the sustained availability of biocontrol agent's mass production technique and formulation development protocols has to be standardized to increase the shelf life of the formulation. It facilitates the industries to involve in commercial production of plant growth promoting rhizobacteria (PGPR). PGPR with wide scope for commercialization includes *Pseudomonas fluorescens, P. putida, P. aeruginosa, Bacillus subtilis* and other *Bacillus* spp. The potential PGPR isolates are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies. They are delivered either through seed treatment, bio-priming, seedling dip, soil application, foliar spray, fruit spray, hive insert, sucker treatment and sett treatment. Application of PGPR formulations with strain mixtures perform better than individual strains for the management of pest and diseases of crop plants, in addition to plant growth promotion. Supplementation of chitin in the formulation increases the efficacy of antagonists. More than 33 products of PGPR have been registered for commercial use in greenhouse and field in North America. Though PGPR has a potential scope in commercialization, the threat of certain PGPR (*P. aeruginosa*, *P. cepacia* and *B. cereus*) to infect human beings as opportunistic pathogens has to be clarified before large scale acceptance, registration and adoption of PGPR for pest and disease management.

Key words: biocontrol; biopesticides; commercialization; formulations; PGPR.

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 257–296.

1 INTRODUCTION

Despite the use of available means of plant protection, about one third of the crops produced are destroyed by pests and diseases. The discovery of synthetic chemicals has contributed, greatly to the increase of food production industry by controlling pests and diseases. However, the use of these synthetic chemicals during the last three decades has raised a number of ecological problems. In the recent years, scientists have diverted their attention towards exploring the potential of beneficial microbes, for plant protection measures. Bio-control agents are easy to deliver, improve plant growth, and activate resistance mechanism in the host, and increase biomass production and yield. These antagonists act through antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes, parasitism and through competition for space and nutrients.

Though bio-control with PGPR is an acceptable green approach, the proportion of registration of biocontrol agents for commercial availability is very slow. In addition, the present day bio-products can be further improved to obtain greater levels of disease reduction. Development of formulations with increased shelf life and broad spectrum of action with consistent performance under field conditions could pave the way for commercialization of the technology at a faster rate.

2 CHARACTERISTICS OF A SUCCESSFUL PGPR FOR FORMULATION DEVELOPMENT

To develop a successful PGPR formulation, rhizobacteria should possess

a. High rhizosphere competence
b. High competitive saprophytic ability
c. Enhanced plant growth
d. Ease for mass multiplication
e. Broad spectrum of action
f. Excellent and reliable control
g. Safe to environment
h. Compatible with other rhizobacteria
i. Should tolerate desiccation, heat, oxidizing agents and UV radiations (Jeyarajan and Nakkeeran, 2000).

3 FORMULATION DEVELOPMENT

Major research on biocontrol is centered with the use of cell suspensions of PGPR directly to seed. Technologies become viable only

when the research findings are transferred from the lab to field. Though PGPR have a very good potential in the management of pests and diseases, it could not be used as cell suspension under field conditions. Hence, the cell suspensions of PGPR should be immobilized in certain carriers and should be prepared as formulations for easy application, storage, commercialization and field use.

4 CHARACTERISTICS OF AN IDEAL FORMULATION

a. Should have increased shelf life
b. Should not be phytotoxic to the crop plants
c. Should dissolve well in water and should release the bacteria
d. Should tolerate adverse environmental conditions
e. Should be cost effective and should give reliable control of plant diseases
f. Should be compatible with other agrochemicals
g. Carriers must be cheap and readily available for formulation development (Jeyarajan and Nakkeeran, 2000).

5 CARRIERS IN FORMULATION DEVELOPMENT

Commercial application of PGPR either to increase crop health or to manage plant diseases depend on the development of commercial formulations with suitable carriers that support the survival of bacteria for a considerable length of time. Carriers may be either organic or non-organic. It should be economical and easily available.

5.1 Organic/Non-organic Carriers

The organic carriers used for formulation development include peat, turf, talc, lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust, and vermiculite, etc. Carriers increase the survival rate of bacteria by protecting it from desiccation and death of cells (Heijnen *et al*., 1993). The shelf life of bacteria varies depending upon bacterial genera, carriers and their particle size (Table 1). Survival of *P. fluorescens* (2-79RN10, W4F393) in montmorillonite, zeolite and vermiculite with smaller particle size increased the survival rate than in kaolinite, pyrophyllite and talc with bigger particle size. The carriers with smaller particle size have increased surface area, which increase resistance

to desiccation of bacteria by the increased coverage of bacterial cells (Dandurand *et al*., 1994).

5.1.1 Talc / Peat / Kaolinite / Lignite / Vermiculite based formulations

Formulations of fluorescent Pseudomonas were developed through liquid fermentation technology. The fermenter biomass was mixed with different carrier materials (Talc/ Peat/ Kaolinite/ Lignite/ Vermiculite) and stickers (Vidhyasekaran and Muthamilan, 1995). Krishnamurthy and Gnanamanickam (1998) developed talc based formulation of *P. fluorescens* for the management of rice blast caused by *Pyricularia grisea*, in which methyl cellulose and talc was mixed at 1: 4 ratio and blended with equal volume of bacterial suspension at a concentration of 10^{10}cfu/ml. Nandakumar *et al*. (2001) developed talc based strain mixture formulation of fluorescent pseudomonads. It was prepared by mixing equal volume of individual strains and blended with talc as per Vidhyasekaran and Muthamilan (1995). Talc based strain mixtures were effective against rice sheath blight and increased plant yield under field conditions than the application of individual strains. Talc and peat based formulations of *P. chlororaphis* and *B. subtilis* were prepared and used for the management of turmeric rhizome rot (Nakkeeran *et al*., 2004).

One school of thought explains that CMC is added as a sticker at 1: 4 ratio to talc. Though it is effective in disease management, it would lead to the increase in the production cost, which would prevent the growers to adopt the technology. More over another school of thought explain that CMC and talc should be used at 1:100 ratios. Hence feasibility of the technique and shelf life of the product has to be evaluated to make the technology as a viable component in disease management so as to promote organic farming.

5.1.2 Microencapsulation

Microcapsules of rhizobacteria consists of a cross linked polymer deposited around a liquid phase, where bacteria are dispersed. Microparticles are characterized based on the distribution of particle size, morphology and bacterial load. The process of microencapsulation involves mixing of gelatin polyphosphate polymer pair (81:19 w/w) at acidic pH with rhizobacteria suspended in oil (Charpentier *et al*., 1999). Though rhizobacteria has been formulated through microencapsulation method, its shelf life declines at a faster rate, since polymers serve as a barrier for oxygen. This was later improved by developing microcapsules by spray drying. The release of *P. fluorescens-putida* from the microencapsulated pellets occurred after 15

min immersion in aqueous buffer. It showed that water served as triggering material for the bacterial release (Charpentier *et al*., 1999).

Though, microencapsulation aids in formulating bacteria, still the technology has to be well refined for early release of bacterial cells and for the establishment in the infection court to counter attack the establishment of pathogens. Most of the experiments on microencapsulation have been restricted only to lab. The technology should be standardized for the industrial application so that the technical feasibility could be assessed to popularize the same for field use.

6 FORMULATIONS AND SHELF LIFE

6.1 Talc formulation

Talc is a natural mineral referred as steatite or soapstone composed of various minerals in combination with chloride and carbonate. Chemically it is referred as magnesium silicate (Mg3Si4O10(OH)2 and available as powder form from industries suited for wide range of applications. It has very low moisture equilibrium, relative hydrophobicity, chemical inertness, reduced moisture absorption and prevent the formation of hydrate bridges that enable longer storage periods (http://www.luzenac.com/food.htm). Owing to the inert nature of talc and easy availability as raw material from soapstone industries it is used as a carrier for formulation development.

Kloepper and Schroth (1981) demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria. The fluorescent Pseudomonads did not decline in talc mixture with 20% xanthum gum after storage for two months at 4°C. *P. fluorescens* isolate Pf1 survived up to 240 days in storage. The initial population of Pf1 in talc-based formulation was 37.5 x 10^7cfu/g and declined to 1.3 x 10^7cfu/g after 8 months of storage (Vidhyasekaran and Muthamilan, 1995). Amendment of sucrose (0.72M) in King's B medium increased population and shelf life of *P. fluorescens* (P7NF, TL3) in talc-based formulation up to 12 months (Caesar and Burr, 1991). *P. putida* strain 30 and 180 survived up to 6 months in talc based formulations. The population load at the end of 6^{th} month was 10^8 cfu/g of the product (Bora *et al*., 2004).

6.2 Peat formulations

Peat (Turf) is a carbonized vegetable tissue formed in wet conditions by decomposition of various plants and mosses. It is formed by the slow decay of successive layers of aquatic and semi aquatic plants, e.g., sedges,

reeds, rushes, and mosses. Peat soils are used as carrier materials to formulate PGPR. Though peat carriers are cheap to use, it harbors lot of contaminants. The quality of peat is variable and not readily available worldwide. Sterilization of peat through heat releases toxic substances to the

Table 1. Shelf life of formulations in different carrier materials.

Carrier	**Bacteria**	**Shelf life**	**Reference**
Talc	Rhizobacteria	2 months	Kloepper and Schroth (1981)
Talc	*P. fluorescens* (P7NF, TL3)	12 months (8.4 Log cfu/g)	Caesar and Burr (1991)
Talc	*P. fluorescens* (Pf1)	8 months (1.3 x 10^7cfu/g)	Vidhyasekaran and Muthamilan (1995)
Talc	*B. subtilis*	45 days (1.0 x 10^6 cfu/g)	Amer and Utkhede (2000)
Talc	*P. putida*	45 days (1.0 x 10^3 cfu/g)	Amer and Utkhede (2000)
Talc	*P. putida* strain 30 and 180	6 moths (>1 x 10^8cfu/g) (not estimated during subsequent months)	Bora *et al*. (2004)
Lignite	*P. fluorescens* (Pf1)	4 months (2.8 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Peat	*P. fluorescens* (Pf1)	8 months (7.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Peat supplemente-d with chitin	*B. subtilis*	6 moths (>1 x 10^9cfu/g) (not estimated during subsequent months)	Manjula and Podile (2001)
Peat	*P. chlororaphis* (PA23) and *B. subtilis* (CBE4)	6 moths (>1 x 10^8cfu/g) (not estimated during subsequent months)	Nakkeeran *et al*. (2004)
Vermiculite	*P. fluorescens* (Pf1)	8 months (1.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Vermiculite	*B. subtilis*	45 days (>1.0 x 10^6 cfu/g)	Amer and Utkhede (2000)
Vermiculite	*P. putida*	45 days (>1.0 x 10^3 cfu/g)	Amer and Utkhede (2000)
Farm yard manure	*P. fluorescens* (Pf1)	8 months (1.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Kaolinite	*P. fluorescens* (Pf1)	4 months (2.8 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)

bacteria and there by reduce bacterial viability (Bashan, 1998). Peat based formulation of *Azospirillum brasilense* had a shelf life up to 4 months. The population load after 4 months of storage was 10^7 cfu/g of the product (Bashan, 1998). This population was sufficient for successful plant inoculation (Garcia and Sarmiento, 2000). Vidhyasekaran and Muthamilan

(1995) reported that the shelf life of *P. fluorescens* in peat-based formulation was maintained up to 8 months (2.8 x 10^6cfu/g). Shelf life of *P. chlororaphis* (PA23) and *B. subtilis* (CBE4) in peat carriers was retained for more than six months (Kavitha *et al*., 2003; Nakkeeran *et al*., 2004).

6.3 Press mud formulation

Press mud is a byproduct of sugar industries. It was composted using vermin-composting technique and later used as a carrier for *Azospirillum* spp. This carrier maximizes the survival of *Azospirillum* spp than lignite, which is predominantly used as a carrier material in India (Muthukumarasamy *et al*., 1997).

6.4 Vermiculite formulation

Vermiculite is a light mica-like mineral used to improve aeration and moisture retention. It is widely used as potting mixture and used as a carrier for the development of formulations for harboring microbial agents. Vermiculite based formulation of *P. fluorescens* (PF1) retained shelf life for a period of 8 months. The viable load of bacteria in the formulation was 1x10^6cfu/g (Vidhyasekaran and Muthamilan, 1995). Shelf life of *Azospirillum* in vermiculite-based formulation was retained up to 10 months. The viable cells after 44 weeks of storage were 1.3 x 10^7 cfu/g (Saleh *et al*., 2001).

7 DELIVERY SYSTEMS

Plant growth promoting rhizobacteria are delivered through several means based on survival nature and mode of infection of the pathogen. It is delivered through seed, soil, foliage, rhizomes, setts, or through combination of several methods of delivery.

7.1 Seed treatment

Seed treatment with cell suspensions of PGPR was effective against several diseases. Delivering of *Serratia marcescens* strain 90-166 as seed dip before planting and soil application of 100 ml of the same at the rate of 10^8 cfu/ml to the sterilized soil less planting mix after seeding reduced bacterial wilt of cucumber and controlled cucumber beetles besides increasing the fruit weight (Zehnder *et al*., 2001). Transfer of technology for commercial use could be possible if PGPR strains are made available as a product. After

realization of the same, several carriers were used for formulation development. Talc based formulation of *P. fluorescens* Pf1 was coated on to seeds at the rate of 4g/Kg (10^7cfu/g) of chickpea seeds (cv.Shoba) for the management of chickpea wilt. Sowing of treated chickpea seeds resulted in establishment of rhizobacteria on chickpea rhizosphere (Vidhyasekaran and Muthamilan, 1995). Treatment of cucumber seeds with strain mixtures comprising of *Bacillus pumilus* - INR7, *B. subtilis* – GB03 and *Curtobacterium flaccumfaciens* – ME1 with a mean bacterial density of $5x10^9$cfu/seed reduced intensity of angular leaf spot and anthracnose equivalent to the synthetic elicitor Actigard and better than seed treatment with individual strains (Raupach and Kloepper, 1998). Treatment of pigeonpea seeds with talc based formulation of *P. fluorescens* (Pf1) effectively controlled fusarial wilt of pigeonpea under greenhouse and field conditions (Vidhyasekaran *et al.*, 1997). Soaking of rice seeds in water containing 10g of talc based formulation of *P. fluorescens* consisting mixture of PF1 and PF2 (10^8cfu/g) for 24h controlled rice sheath blight under field condition (Nandakumar *et al.*, 2001). Seed treatment of lettuce with either vermiculite or kaolin based carrier of *B. subtilis* (BACT-0) significantly reduced root rot caused by *P. aphanideramtum* and it also increased the fresh weight of lettuce under greenhouse conditions. Seed treatment with vermiculite based *P. putida* reduced fusarium root rot of cucumber and increased the yield and growth of cucumber (Amer and Utkhede, 2000). Treatment of tomato seeds with powder formulation of PGPR (*B. subtilis, B. pumilus*) reduced symptom severity of ToMoV and increased the fruit yield (Murphy *et al.*, 2000).

7.2 Bio-priming

A successful antagonist should colonize rhizosphere during seed germination (Weller, 1983). Priming with PGPR increase germination and improve seedling establishment. It initiates the physiological process of germination, but prevents the emergence of plumule and radicle. Initiation of physiological process helps in the establishment and proliferation of PGPR on the spermosphere (Taylor and Harman, 1990). Bio-priming of seeds with bacterial antagonists increase the population load of antagonist to a tune of 10 fold on the seeds thus protected rhizosphere from the ingress of plant pathogens (Callan *et al.*, 1990). Chickpea seeds treated with talc-based formulation of Pf1 was primed by incubating the treated seeds for 20h at 25°C over sterile vermiculite moistened with sterile water. Population of Pf1 increased up to 100% in the rhizosphere, indicating that it provides a congenial microclimate for proliferation and establishment of bacterial antagonist (Vidhyasekaran and Muthamilan, 1995). Drum priming is a

commercial seed treatment method followed to treat seeds with pesticides. Drum priming of carrot and parsnip seeds with *P. fluorescens* Pf CHAO proliferated well on the seeds and could be explored for realistic scale up of PGPR (Wright *et al*., 2003).

7.3 Seedling dip

PGPR is delivered through various means for the management of crop diseases based on the survival nature of pathogen. In several crops pathogens gain entry into plants either through seed, root or foliage. In rice, sheath blight incited by *Rhizoctonia solani* is a major obstacle in rice production. As the pathogen is soilborne, it establishes host parasite relationships by entering through root. Hence, protection of rhizosphere region by prior colonization with PGPR will prevent the establishment of host-parasite relationship. Delivering of *P. fluorescens* strain mixtures by dipping the rice seedlings in bundles in water containing talc based formulation of strain mixtures (20g/l) for 2h and later transplanting it to the main field suppressed sheath blight incidence (Nandakumar *et al*., 2001). Similarly dipping of rice seedlings in talc based formulation of *P. fluorescens* (PfALR1) prior to transplanting reduced sheath blight severity and increased yield in Tamil Nadu, India (Rabindran and Vidhyasekaran, 1996). Dipping of strawberry roots for 15 minutes in bacterial suspension of *P. putida* (2×10^9 cfu/ml) isolated from strawberry rhizosphere reduced *Verticillium* wilt of strawberry by 11% compared to untreated control (Berg *et al*., 2001). Dipping of *Phyllanthus amarus* seedlings in talc based formulation of *B. subtilis* (BSCBE4) or *P. chlororaphis* (PA23) for 30 minutes prior to transplanting reduced stem blight of *P. amarus* (Mathiyazhagan *et al*., 2004).

7.4 Soil application

Soil being as the repertoire of both beneficial and pathogenic microbes, delivering of PGPR strains to soil will increase the population dynamics of augmented bacterial antagonists and thereby would suppress the establishment of pathogenic microbes on to the infection court. Vidhyasekaran and Muthamilan (1995) stated that soil application of peat based formulation of *P. fluorescens* (Pf1) at the rate of 2.5 Kg of formulation mixed with 25 Kg of well decomposed farm yard manure; in combination with seed treatment increased rhizosphere colonization of Pf1 and suppressed chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceris*. Broadcasting of talc based formulation of strain mixtures (Pf1 and FP7) by blending 2.5 kg of formulation with 50 kg of sand after 30 days of

transplanting paddy seedlings to main field significantly reduced sheath blight and increased yield under field conditions (Nandakumar *et al*., 2001). Incorporation of commercial chitosan based formulations LS254 (comprising of *Paenobacillus macerans* + *B. pumilus*) and LS255 (comprising of *P. macerans* + *B. subtilis*) into soil at the ratio of 1: 40 (Formulation: Soil) increased bio-matter production by increasing both root and shoot length and yield (Vasudevan *et al*., 2002). Soil application of the strain mixture formulations LS256 and LS257 comprising of two different *Bacillus* spp., was better than seed treatment and suppressed downy mildew under greenhouse and field conditions (Niranjan Raj *et al*., 2003).

7.5 Foliar spray

The efficacy of biocontrol agents for foliar diseases is greatly affected by fluctuation of microclimate. Phyllosphere is subjected to diurnal and nocturnal, cyclic and non-cyclic variation in temperature, relative humidity, dew, rain, wind and radiation. Hence water potential of phylloplane microbes will be varying constantly. It will also vary between leaves or the periphery of the canopy and on sheltered leaves. Higher relative humidity could be observed in the shaded, dense region of the plant than that of peripheral leaves. The dew formation is greater in centre and periphery. The concentration of nutrients like amino acid, organic acids and sugars exuded through stomata, lenticels, hydathodes and wounds varies highly. It affects the efficacy and survival of antatognist in phylloplane (Andrews, 1992).

Delivering of *Pseudomonas* to beet leaves actively compete for amino acids on the leaf surface and inhibited spore germination of *Botrytis cinerea*, *Cladosporium herbarum* and *Phoma betae* (Blakeman and Brodie, 1977). Application of *B. subtilis* to bean leaves decreased incidence of bean rust (*Uromyces phaseoli)* by 75% equivalent to weekly treatments with the fungicide mancozeb (Baker *et al*., 1983). Application of *P. fluorescens* on to foliage (1kg of talc based formulation /ha) on 30, 45, 60, 75 and 90 days after sowing reduced leaf spot and rust of groundnut under field conditions (Meena *et al*., 2002). Preharvest foliar application of talc based fluorescent pseudomonads strain FP7 supplemented with chitin at fortnightly intervals (5g/l; spray volume 20l/ tree) on to mango trees from pre-flowering to fruit maturity stage induced flowering to the maximum, reduced the latent infection by *C. gloeosporioides* beside increasing the fruit yield and quality (Vivekananthan *et al*., 2004). Though seed treatment and foliar application of *P. fluorescens* reduce the severity of rust and leaf spot under field conditions, it is not technically feasible due to increased dosage and economy realized from the crop. Hence, dosage and frequency of application

has to be standardized based on the crop value, which could be as a reliable and practical approach.

7.6 Fruit spray

Pseudomonas syringae (10% wettable powder) in the modified packing line was sprayed at the rate of 10 g/l over apple fruit to control blue and grey mold of apple. The population of antagonist increased in the wounds more than 10 fold during 3 months in storage (Janisiewicz and Jeffers, 1997). Research on the exploration of PGPR have to go a long way to explore its usage to manage post harvest diseases.

7.7 Hive insert

Honey bees and bumble bees serve as a vector for the dispersal of biocontrol agents for the control of diseases of flowering and fruit crops (Sandhu and Waraich, 1985, Kevan *et al*., 2003). An innovative method of application of bio-control agent right in the infection court at the exact time of susceptibility was developed by Thomson *et al*. (1992). A dispenser is attached to the hive and loaded with powder formulation of the PGPR or with other desired biocontrol agent. The foragers when exit the hive, the antagonist get dusted on to bee and delivered to the desired crop, while attempting for sucking the nectar. *Erwinia amylovora* causing fire blight of apple infects through flower and develops extensively on stigma. Colonisation by antagonist at the critical juncture is necessary to prevent flower infection. Since flowers do not open simultaneously the bio-control agent *P. fluorescens* has to be applied to flowers repeatedly to protect the stigma. Nectar seeking insects like *Aphis mellifera* can be used to deliver *P. fluorescens* to stigma. Bees deposit the bacteria on the flowers soon after opening due to their foraging habits. Honey bees have also been used for the management of gray mold of strawberry and raspberry (Peng *et al*., 1992; Sutton, 1995; Kovach *et al*., 2000).

7.8 Sucker treatment

Plant growth promoting rhizobacteria also play a vital role in the management of soilborne diseases of vegetatively propagated crops. The delivery of PGPR varies depending upon the crop. In crops like sugarcane and banana rhizobacteria are delivered through sett treatment or rhizome treatment respectively. Banana suckers were dipped in talc based *P. fluorescens* suspension (500g of the product in 50 liters of water) for 10 min after pairing and pralinage. Subsequently it was followed by capsule

application (50 mg of *P. fluorescens* per capsule) on third and fifth month after planting. It resulted in 80.6 per cent reduction in panama wilt of banana compared to control (Raguchander *et al*., 2000).

7.9 Sett treatment

Red rot of sugarcane is a major production constraint in sugarcane cultivation. Usage of chemical fungicides for the management of red rot was less effective to protect the crop. Since, PGPR act as a predominant prokaryote in the rhizosphere, fluorescent pseudomonads were explored for the management of red rot under field conditions. Viswanathan and Samiyappan (2002) delivered fluorescent pseudomonads through sett treatment. Two budded sugarcane setts were soaked in talc formulation of *P. fluorescens* (20g/l) for one hour and incubated for 18h prior to planting. Planting of treated setts increased cane growth, sugar recovery and reduced red rot incidence under field conditions.

7.10 Multiple delivery systems

Plant pathogens establish host parasite relationships by entering through infection court such as spermosphere, rhizosphere and phyllosphere. Hence, protection of sites vulnerable for the entry and infection of pathogens would offer a better means for disease management. Seed treatment of pigeonpea with talc based formulation of fluorescent pseudomonads at the rate of 4g/kg of seed followed by soil application at the rate of 2.5 kg/ha at 0, 30, and 60 days after sowing controlled pigeonpea wilt incidence under field conditions. The additional soil application of talc based formulation improved disease control and increased yield compared to seed treatment alone (Vidhyasekaran *et al*., 1997). Delivering of *P. fluorescens* as seed treatment followed by three foliar applications suppressed rice blast under field conditions (Krishnamurthy and Gnanamanickam, 1998). Combined application of talc based formulation of fluorescent pseudomonads comprising of Pf1 and FP7 through seed treatment, seedling dip, soil application and foliar spray suppressed rice sheath blight and increased plant growth better than application of the same strain mixture either through seed, seedling dip or soil (Nandakumar *et al*., 2001). Application of strain mixture based formulation of Pf1 and FP7 with or without chitin through seed, seedling dip and foliar spray suppressed leaf folder damage and sheath blight in rice under field conditions (Radja Commare *et al*., 2002). Seed and foliar application of talc based fluorescent pseudomonas reduced leaf spot and rust of groundnut under field conditions (Meena *et al*., 2002). The increased efficacy of strain mixtures through combined application might be due to

increase in the population of fluorescent pseudomonads in both rhizosphere and phyllosphere (Viswanathan and Samiyappan, 1999). Delivering of rhizobacteria through combined application of different delivery systems will increase the population load of rhizobacteria and thereby might suppress the pathogenic propagules.

8 EFFICACY OF FORMULATIONS AGAINST PLANT DISEASES

Plant diseases are in association with crop plants since agriculture began and was managed through synthetic pesticides to increase food production. But continuous usage of pesticides has resulted in the outbreak of pathogens resistant to fungicides apart from environmental pollution. Introduction of PGPR for increasing plant growth promotion during 1950s from the research findings in Soviet Union and in Western countries (Backman *et al*., 1997) opened new vistas to use PGPR as an alternate to chemical pesticides for the management of soilborne pathogens (Dunleavy, 1955; Kloepper, 1993). Application of PGPR either as single strain or strain mixtures based formulations checked pest and disease spread besides increasing growth and yield (Table 2).

8.1 Individual strain based formulations

Plant growth promoting rhizobacteria has diverse applications for the management of plant diseases in agriculture, horticulture and forestry. In addition it also plays a vital role in environmental remediation (Lucy *et al*., 2004). Fluorescent pseudomonads were first developed as talc based formulation for the treatment of potato seed tubers for growth promotion (Kloepper and Scroth, 1981). Treatment of chickpea seeds with *P. fluorescens* (Pf1) through seed followed by root zone application after 30 days of sowing increased seedling emergence, reduced Fusarial wilt incidence caused by *Fusarium oxysporum* f.sp. *ciceris* and increased the yield under field conditions. In addition it also increased the population of Pf1 strain in the rhizosphere (Vidhyasekaran and Muthamilan, 1995). Talc based formulation of *P. fluorescens* strain Pf1 and Pf2 increased grain yield of pigeonpea besides the control of pigeonpea wilt (Vidhyasekaran *et al*., 1997). Seed treatment of groundnut and pigeonpea with peat based formulation of *B. subtilis* supplemented with 0.5% chitin or with 0.5% of sterilized *Aspergillus* mycelium controlled crown rot and wilt of groundnut and pigeon pea respectively. It also increased growth promotion even in the presence of inoculum pressure (Manjula and Podile, 2001). Chitin

supplementation enhances the biocontrol efficacy of formulations. But incorporation of chitin will increase the production cost of biopesticides. Hence, identification of cheap and easy available source of chitin is essential. Seed treatment and soil application of *P. aeruginosa* strain 78 reduced root knot incidence of mungbean besides the reduction in the population density of *Meloidogyne javanica* under field conditions (Ali *et al*., 2002). Seed treatment with wettable powder formulation of *P. putida* strain 30 and 180 suppressed wilt of musk melon to the extent of 63 and 50% after 90 days of transplanting muskmelon in the field. But seed treatments with strain mixtures were not as effective as that of individual strains (Bora, 2004). The decrease in efficacy might be due to the incompatibility of the isolates, which might suppress the genetic expression of defense genes in either bacterial strain.

8.2 Strain mixtures based formulations

Several research outcomes on formulations explain that a single biocontrol agent has the ability to combat a plant pathogen. But, usage of single biocontrol agent in disease management might be also responsible for its inconsistent performance under field conditions. A single biocontrol agent may not perform well at all times in all kinds of soil environment to suppress plant pathogens (Raupach and Kloepper, 1998). In addition application of single biocontrol agent based formulation might have resulted in inadequate colonization, inability to tolerate the extremes of soil pH, moisture and temperature and fluctuations in the production of antimicrobial substances (Weller and Thomashow, 1994). Inconsistent performance of biocontrol agents was overcome by the combined application of several biocontrol strains that mimic the natural environment (Schisler *et al*., 1997; Raupach and Kloepper, 1998). Development of cocktail formulation with compatible isolates will improve disease control through synergy in cross talk between the isolates that lead to increased production of antibiotics at the site of colonization and thereby could suppress the establishment of pathogenic microbes. Advantages of strain mixtures include, broad spectrum of action, enhanced efficacy, reliability and also allow combination of various traits without genetic engineering (Janisiewicz, 1996). Application of mixed PGPR strains based formulations to field might ensure at least one of the mechanism to operate under variable environment that exist under field conditions (Duffy *et al*., 1996).

Application of talc based strain mixture formulation of fluorescent pseudomonads through seed, root, soil and foliage to rice crop suppressed sheath blight under field conditions better than individual strains based formulations. The average disease reduction for mixtures was 45.1% compared to 29.2% for individual strains. In addition to disease reduction

strain mixtures increased biomatter production and yield compared to individual strains (Nandakumar *et al*., 2001). Combined application of *Pichia guilermondii* and *Bacillus mycoides* (B16) reduced the infection of *Botrytis cinerea* by 75% on fruits in strawberry plants grown commercially under greenhouse conditions. But the individual application of either antagonist resulted in 50% reduction of strawberry fruit infection. Population of yeast increased when applied as mixture rather than single application (Guetsky *et al*., 2002). Delivering of talc based strain mixtures of *P. fluorescens* strains (Pf1 and FP7) through seed, soil and foliar reduced sheath blight and leaf folder incidence in rice under greenhouse and field conditions. It also reduced the feeding behavior of leaffolder, reduced larval and pupal weight, and increased larval mortality. Besides, population of parasitoids and spiders also increased in PGPR treated plots (Radja Commare *et al*., 2002).

Table 2. Efficacy of PGPR formulations against plant disease and growth promotion.

Formulation	**Crop**	**Results**	**Reference**
Talc based *P. fluorescens*	Potato	Significant plant growth promotion.	Kloepper and Scroth (1981)
Talc based *P. fluorescens*	Winter wheat	Significant plant growth promotion.	De Freitas and Germida(1992)
Peat based *P. fluorescens*	Cotton	Significant reduction of cotton seedling diseases.	Hagedorn *et al*. (1993)
Talc based *P. fluorescens*	Chickpea	Significant increase in grain yields and controlled fusarial wilt under field conditions.	Vidhyasekaran and Muthamilan, (1995)
Talc based *P. fluorescens*	Pigeonpea	Control of pigeonpea wilt and significant increase in grain yield.	Vidhyasekaran *et al*. (1997)
Chitosan based *B. pumilus*	Tomato	Induced resistance against *F. oxysporum*.	Benhamou *et al*.(1998)
Methyl cellulose and talc based *P. fluorescens*.	Rice	Suppressed rice blast both in nursery and field conditions.	Krishnamurthy and Gnanamanickm (1998)
B. subtilis strain LS213(commercial product)	Watermelon and muskmelon	Increased plant growth, and improved yield.	Vavrina (1999)

Continued table 2.

B. subtilis Formulations	Cucumber, Watermelon, squash, ornamentals, vegetables, pepper, tobacco, loblolly pine and lodge pine.	Significant induction of resistance against various different pathogens.	Reddy *et al*. (1999); Kenney *et al*. (1999); Martinez- Ochoa *et al*. (1999) ; Ryu *et al*.(1999) ;Yan *et al*. (1999) and Zhang *et al* (1999).
Chitosan based *B. subtilis* strain LS213 (commercial product)	Tomato, tobacco, cucumber and pepper	Reduced the incidence of bacterial spot and late blight of tomato, angular leaf spot of cucumber and blue mold of tobacco.	Reddy *et al*. (1999)
Talc based formulation of *P. fluorescens* (CHAO and Pf1)	Sugarcane	Increased germination of sugarcane seeds, plant growth besides the supper-ssion of damping- off.	Viswanathan and Samiyappan (1999)
Vermiculite based *P. fluorescens*	Sugarbeet	Significant control of damping off	Moenne-Loccoz *et al*. (1999)
Talc based *P. fluorescens*	Rice	Significant reduction of sheath blight under field conditions.	Vidhyasekaran and Muthamilan (1999); Nandakumar *et al*. (2000).
Talc based *P. fluorescens*	Banana	Significant reduction of panama wilt of banana	Raguchander *et al*. (2000)
Vermiculite and Kaolin based *B. subtilis*	Lettuce	Suppressed root rot of lettuce caused by *P. aphanidermatum* and increased fresh weight of lettuce.	Amer and Utkhede (2000)
Vermiculite based *P. putida*	Cucumber	Significantly reduced root rot caused by *Fusarium oxysporum f. sp. cucurbitacearum*	Amer and Utkhede (2000)
Talc based *P. fluorescens* (Pf1)	Urdbean and Sesame	Increased growth promotion and reduced root rot caused by *M. phaseolina*.	Jayashree *et al*.(2000)
Talc based rhizobacterial mixtures of fluorescent pseudomonads	Rice	Significant plant growth promotion and suppression of rice sheath blight.	Nandakumar *et al*. (2001)
Peat based *B. subtilis* supplemented with chitin	Groundnut and pigeon pea	Significant control of groundnut root rot and pigeon pea wilt.	Manjula and Podile (2001)

Continued table 2.

Chitosan based mixed formulation of *Paenobacillus macerans* and *B. subtilis* (LS255)	Rice	Increased plant growth and yield in rice cultivars, IR24, IR50 and Jyothi.	Vasudevan *et al*. (2002)
Chitin based formulation of *B. subtilis* strain GB03+*B. pumilus* strain INR7(LS256) and *B. subtilis* strain GB03+*B. subtilis* strain IN937b	Tomato and Pepper	Increased yield of pepper and tomato.	Burelle *et al*. (2002)
Talc based *P. aeruginosa* strain 78	Mung bean	Reduced the incidence of root knot and population density of *Meloidogyne javanica* under field conditions.	Ali *et al*. (2002)
Talc based fluorescent Pseudomonads	Sugarcane	Significant increase in sett germination, increased cane growth and reduced red rot incidence.	Viswanathan and Samiyappan (2002)
Talc based *P. fluorescens*	Rice	Significant reduction of rice sheath blight, leaf folder and increased yield. Beside it also increased the population of insect parasites and predators.	Radja Commare *et al*. (2002)
Talc based *P. fluorescens*	Groundnut	Significant reduction of leaf spot and rust of groundnut.	Meena *et al*. (2002)
Talc based formulation of *B. subtilis* and *P. chlororaphis* (PA23)	Tomato	Increased growth promotion and significant reduction of damping off.	Kavitha *et al*. (2003)
Chitosan based mixed formulation of *B. subtilis* strain GB03+*B. pumilus* strain INR7(LS256) and *B. subtilis* strain GB03+*B. pumilus* strain T4(LS257)	Pearl millet	Reduced downy mildew and increased plant growth promotion in pearl millet.	Niranjan Raj *et al*. (2003)
Talc based *P. fluorescens* FP7 supplemented with chitin.	Mango	Significant reduction of anthracnose coupled with increase in fruit yield and quality.	Vivekananthan *et al*.(2004).

Continued table 2.			
Talc based *B. subtilis* (BSCBE4) and *P.chlororaphis* (PA23)	Turmeric	Significant reduction of rhizome rot and yield increase of rhizomes.	Nakkeeran *et al*. (2004)
Talc based *B. subtilis* (BSCBE4), *P. chlororaphis* (PA23) and *P. fluorescens* (ENPF1)	*Phyllanthus amarus*	Significant reduction of stem blight caused by *Corynespora cassicola* under field conditions.	Mathiyazhagan *et al*. (2004)
Talc based *P. putida*	Muskmelon	Effective control of wilt caused by *Fusarium oxysporum* f. sp. *melonis*.	Bora *et al*., (2004)

9 COMMERCIAL PRODUCTS

Research inventions from China, Russia and several other western countries during the early 1950 have proved the potential use of bacteria to be explored for plant disease management (reviewed by Backman *et al*., 1997). Owing to the potential of PGPR, the first commercial product of *B. subtilis* was introduced during 1985 for the use of growers by Gustafson,Inc. (Plano, Texas) in US (Broadbent,s *et al*., 1977). The strains of *B. subtilis* A-13, GB03, GB07 were sold for the management of soilborne pathogens under the trade names of Quantum@, Kodiak@ and Epic@ respectively (Broadbent,s *et al*., 1977). Release of *Bacillus* based products during 1985 has resulted in the increase in market size for the usage of bacterial products in crop disease management. Backman *et al*. (1977) stated that 60-75% of the cotton crop in US is treated with *B. subtilis* for the management of soilborne pathogens encountered in cotton ecosystem. Among several PGPR strains *Bacillus* based products gains momentum for commercialization. Because, *Bacillus* spp., produce endospores tolerant to extremes of abiotic environments such as temperature, pH, pesticides and fertilizers (Backman *et al*., 1997). Owing to the potentiality of *Bacillus* spp., 18 different commercial products of *Bacillus* origin are sold in China to mitigate soilborne diseases (Backman *et al*., 1997). The registered commercial products of PGPR are listed in Table 3. Details of registered products are in the web sites:
http://www.ippc.orst.edu/biocontrol/biopesticides/;
http://www.epa.gov/pesticides/biopesticides/.

10 IMPROVEMENT OF FORMULATION EFFICACY

In general, though biocontrol agents perform well in the management of plant diseases, they are highly sensitive to the fluctuations in environmental conditions and are inconsistent in their performance. The consistency of biocontrol agents could be enhanced through several means without going in for genetic engineering. Since nature is bestowed with millions of beneficial microbes, development of compatible cocktail of beneficial microbes would increase the efficiency of their performance. Strategies to enhance the efficacy of biocontrol organisms include

1. Development of compatible consortia.
2. Strains that induce synergistic expression of biocontrol genes.
3. Adjuvants, spreaders and stickers.
4. Genetic engineering of PGPR strains.
5. Formulations comprising of compatible PGPR strains and plant inducers of chemical origin.

Table 3. Commercial products of PGPR in plant disease management.

Product	**Target pathogens/diseases**	**Crops recommended**	**Manufacturer**
Bio-Save 10, 11, 100, 110, 1000 TM – *P. syringae ESC-100*	*Botrytis cinerea, Penicillium spp, Mucor pyroformis, Geotrichum candidum*	Pome fruit (Biosave 100) and Citrus (Biosave 1000)	Eco Science Corp, Produce Systems Div., Orlando
Blight Ban A506 – *P. fluorescens* A 506	*Erwinia amylovora* and russet - inducing bacteria	Almond, Apple, Apricot, Blueberry, Cherry, Peach, Pear, Potato, Strawberry,Tomato	Plant Health Technologies, USA
Cedomon TM – *P. chloroaphis*	leaf stripe, net blotch, *Fusarium sp,* spot blotch, leaf spot and others	Barley and Oats, potential for wheat and other cereals	Bio Agri AB, Sweden
Campanion – *B. subtilis* GB03	*Rhizoctonia, Pythium, Fusarium and Phytophthora*	Horticultural crops and turf	Growth products, USA
Conquer TM - *P. fluorescens*	*P. tolassii*	Mushrooms	Mauri Foods, Australia

Continued table 3.

Victus™ – *P. fluorescens*	*P. tolassii*	Mushrooms	Mauri Foods, Australia
BioJect Spot – less – *P. aureofaciens*	Dollar spot, Anthracnose and *P. aphanidermatum*	Turf and other crops	Eco Soil Systems, San Diego, CA
BioJet™ – *Pseudomonas* sp + *Azospirillum*	Brown batch and Dollar spot disease	Turf and other crops	Eco Soil Systems, San Diego, CA
Deny - *Burkholderia cepacia (Pseudomonas cepacia*)	*Rhizoctonia, Pythium, Fusarium* and diseases caused by lesion, spiral, lance, and sting nematodes.	Alfalfa, Barley, Beans, Clover, Cotton, Peas, Sorghum, Vegetable crops and Wheat	Stine Microbial Products, Shawnee, KS
Intercept ™- *P. cepacia*	*Rhizoctonia solani, Fusarium* sp., *Pythium sp.*	Maize, Vegetables, Cotton	Soil Technologies Corp, USA
Kodiak™, Kodiak HB™, Epic™, Concentrate ™, Quantum 4000 and System 3 ™ – *B. subtilis* GB03	*Rhizoctonia solani, Fusarium spp, Alternaria spp, and Aspergillus spp*	Cotton, Legumes	Gustafson, Inc., Dallas, USA
Bio Yield – Combination of *B. subtilis* and *B.amyloliquefaciens*	Broad spectrum action against greenhouse pathogens	Tomato, Cucumber, Pepper and Tobacco	Gustafson, Inc., Dallas, USA
Rhizo-Plus – *B. subtilis* strain FZB24	Against *R. solani, Fusarium spp., Alternaria spp., Sclerotinia* and *Verticillium.*	Greenhouses grown crops,forest tree seedlings, ornamentals, and shrubs.	KFZB Biotechnik GMBH, Berlin, Germany.
Serenade – *B. subtilis* strain QWT713. Available as wettable powder.	Powdery mildew, Downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight and others.	Cucurbits, Grapes, Hops, Vegetables, Peanuts, Pome fruits, stone fruits and others	AgraQuest, Inc., Davis, USA.

Continued table 3.			
Rhapsody – *B. subtilis* strain QST713. Aqueous suspension formulation	Powdery mildew, sour rot, downy mildew, and early leaf spot, early blight, late blight, bacterial spot, and walnut blight diseases.	Cherries, cucurbits, grapes, leafy vegetables,peppers, potatoes, tomatoes, and walnuts.	AgraQuest, Inc., Davis, USA.
Subtilex - *B. subtilis* MB1600	*Fusarium* spp., *Rhizoctonia* spp. and *Pythium* spp.	Ornamental and vegetable crops	Becker Underwood, Ames.
GB 34 Concentrate Biological Fungicide - *B. pumilus*	*Rhizoctonia* and *Fusarium*, which attack developing soybean roots	Soybean	Gustafson LLC1400 Preston Road TX 75093
SonataTM ASO *B. pumilus* strain QST 2808	Fungal pests such as molds, mildews, blights, rusts and to control Oak death syndrome	Used in nurseries, landscapes, oak trees and green house crops	Agra Quest, Inc.,Davis, USA
System 3 - *Bacillus subtilis GB03* and chemical pesticides	Seedling pathogen	Barley, Beans, Cotton, Peanut, Pea, Rice, Soybean	Helena Chemical Co.,Memphis USA
AtEze P. chlororaphis strain 63-28	*Pythium* spp., *Rhizoctonia solani, Fusarium oxysporum*	Ornamentals and vegetables	EcoSoil Systems, Inc., San Diego, CA
Pix plus plant regulator, *B. cereus* BPO1 technical, - *B. cereus* strain UW85	Used as growth regulator	Cotton	Micro Flo Company, Lakeland, FL 33807
Bio-save 10LP, 110 – *P. syringae*	*Botrytis cinerea*, *Penicillium* spp., *Geotrichum candidum*	Pome fruit, Citrus, Cherries and Potatoes	Eco Science Corp., FL 32779.

10.1 Development of compatible consortia

Biological control of plant pathogens in disease suppressive soil is due to the existence of mixture of microbial antagonists (Lemanceau and Alabouvette, 1991). Hence, augmentation of compatible strain mixtures of PGPR strains to infection court will mimic the natural environment and

could broaden the spectrum of biocontrol against different plant pathogens (Janisiewicz, 1988). Efficiency of biocontrol agents could be increased by the development of compatible strain mixtures of different biocontrol organisms by considering the following norms (Raupach and Kloepper, 1998).

1. Strain mixtures that differ in the pattern of plant colonization
2. Strain mixtures with broad spectrum of action against different plant pathogens
3. Strain mixtures with different modes of action
4. Strain mixtures with genetically different organisms having the capability to perform in different pH, moisture, temperature and relative humidity.

Vidhyasekaran and Muthamilan (1995) found that *P. fluorescens* - Pf1 was not inhibitory to nitrogen fixing bacteria, *Rhizobium* and *Azospirillum*. Development of strain mixtures with non-competitive nature of these bacterial strains will have an additive effect in increasing the yield and growth. Strain mixtures of Pseudomonads in combinations with other bacteria were found effective than the application of individual organisms (Duijff *et al*., 1999). Application of the mixture of phloroglucinol producers of *P. fluorescens* F113 and a proteolytic rhizobacterium suppressed sugar beet damping-off (Dunne *et al*., 1998). Combination of iron chelating Pseudomonas strains and inducers of systemic resistance suppressed Fusarium wilt of radish better than the application of individual strains (de Boer *et al*., 2003).

10.2 Strains that induce synergistic expression of biocontrol genes

Development of products with strains that induce the expression of biocontrol genes can also increase the bioefficacy of PGPR strains under field conditions. Combination of CHAO and Q287 of fluorescent pseudomonads enhanced the expression of the genes that code for diacetyl phloroglucinol. This would lead to the increase of DAPG pool in the rhizosphere and will suppress the disease causing organisms (Raaijmakers *et al*., 1999).

10.3 Adjuvants, spreaders and stickers

In general, the performance of PGPR formulations in controlling plant diseases is inconsistent. Since, disease suppression is the outcome of interactions between biocontrol agents, pathogen, plant and environment,

any fluctuations in growing seasons; environmental conditions and high inoculum pressure alter the efficacy of biocontrol formulations. Integrating the usage of formulations with other management strategies that aims at increasing the productivity of the crop could enhance the efficacy of formulations (Larkin *et al*., 1998). Performance of biocontrol agents in the formulations can be increased by the incorporation of water-soluble adjuvants, oils, stickers and emulsions. It increases the efficacy of biocontrol agents by supplying nutrients and by protecting the microbes from desiccation and death (Connick *et al*., 1991; Bateman *et al*., 1993; Barnes and Moore, 1997; Green *et al*., 1998; Ibrahim *et al*., 1999). Incorporation of carboxy methyl cellulose (CMC) in formulations serves as stickers in uniform seed coating of microbes. Though adjuvants and stickers increase the efficacy of bio-products it has its own demerits. Adjuvants/stickers in the formulations will be diluted when exposed to rain or heavy dew. It would alter the efficacy of formulations by reducing the establishment or colonization of PGPR onto the infection court. Sometimes spray application of emulsions or oil-based formulations may be toxic to plants. Hence, a thorough knowledge on the usage of adjuvants, stickers is essential for increasing the efficacy of formulations.

10.4 Genetic engineering of PGPR strains

Genomic tinkering of naturally occurring PGPR strains with genes that are beneficial to plants will lead to the accentuated expression of the genomic products which could alleviate the attack of both pests and diseases. This will facilitate for the introduction of a single bacterium with multiple modes of action to benefit the growers to save their crop with increased returns by reducing the inputs invested for plant protection measures. However, the release of genetically modified organisms is a policy decision to be made by the policy makers. Hence, appraising of policy makers about the safe usage of beneficial bacteria will be a boon to the farming community and environment.

10.5 Formulations of PGPR strains compatible with plant inducers of chemical origin

Plant inducers of chemical origin are used to trigger systemic resistance at very low concentrations against pests and diseases. The chemical inducer benzothiadiazole (Bion) is commercially used for inducing resistance in crop plants against pests and diseases (Gorlach *et al*., 1996). Hence, identification of PGPR strains that has compatibility with chemical inducers will have a synergistic action against pests and diseases.

11 COMMERCIALIZATION

Industrialization of biocontrol agents requires linkage between corporate and academic bodies. The success and commercialization of a scientific innovation depends on the availability of the technology to the end users. It depends on the linkages between the scientific organization and industries. Biocontrol technology could become as a successful component of plant protection only when it is commercialized.

11.1 Stages of commercialization

Stages of commercialization include isolation of antagonist, screening, pot test and field efficacy, mass production and formulation development, fermentation methods, formulation viability, toxicology, industrial linkages and quality control (Sabitha Doraiswamy *et al*., 2001).

11.1.1 Isolation of antagonist

Isolation of an effective strain plays a prime role in disease management. It is done from the pathogen suppressive soils either by dilution plate technique or by baiting the soil with fungal structures like sclerotia of pathogen. Consortium of biocontrol agents could be established by isolating the location specific and crop specific isolates. It could be used for the development of mixtures of biocontrol agents suited for different ecological niche.

11.1.2 Screening of antagonist

All the strains isolated from the different cropping system have to be ascertained for its virulence and broad spectrum of action against different pathogens causing serious economic threat to cultivation. Selection of an effective strain decides the viability of the technology. Hence a proper yardstick should be developed to screen the antagonistic potentiality of the biocontrol agents. *In vitro* screening of the antagonist through dual culture technique alone could not be an effective method for strain selection. To be an effective antagonist it should possess a high level of competitive saprophytic ability, antibiosis, should have the ability to secrete increased level of cell wall lytic enzymes (chitinases, glucanases and proteases), antibiotics and plant growth promotion. Hence the yardstick should be developed, comprising of above-mentioned components. Each component should be given weightage depending upon their role in disease management. This type of rigorous and meticulous screening will lead to

identification of an effective biocontrol strain suited for commercialization. Twenty rhizobacterial isolates from strawberry rhizosphere were evaluated for its antifungal action against *Verticillium dahliae*. The selection of best antagonistic bacterial isolate was done by screening for the antifungal action against different soilborne pathogens apart from the target pathogen. In addition it was also tested for the antifungal mechanism of the rhizobacteria for the production of lytic enzymes (chitinases, glucanases and proteases) and plant growth promotion. Collectively all these parameters were combined based on bonitur scale (28 points). The strain that had the highest score was selected for testing its efficacy under greenhouse. Among twenty strains, *P. putida* E2 had the maximum bonitur scale of 28 points and was highly effective in suppressing *Verticillium* wilt of strawberry under greenhouse conditions. It was found to perform better than the commercial product Rhizovit (Berg *et al*., 2001). This clearly explains that selection of bacterial antagonist plays a major role in commercialization of the bacteria for disease management. Initial mistake committed in strain selection will lead to complete failure of the technology.

11.1.3 Pot test and field efficacy

The plant, pathogen and antagonists are co exposed to controlled environmental conditions. Exposure of the host to the heavy inoculum pressure of the pathogen along with the antagonist will provide ecological data on the performance of the antagonist under controlled conditions. Promising antagonists from controlled environment are tested for its efficacy under field conditions along with the standard recommended fungicides. Since the variation in the environment under field condition influence the performance of biocontrol agent, trials on the field efficacy should be conducted for at least 15 – 20 locations under different environmental conditions to promote the best candidate for mass multiplication and formulation development (Jeyarajan and Nakkeeran, 2000).

11.1.4 Mass production and formulation development

The first major concern in commercial production systems involves the achievement of adequate growth of the biocontrol agent. In many cases biomass production of the antagonist is difficult due to the specific requirement of nutritional and environmental conditions for the growth of organism. Mass production is achieved through liquid and semisolid and solid fermentation techniques. The commercial success of biocontrol agents requires

- Economical and viable market demand
- Consistent and broad spectrum action

- Safety and stability
- Longer shelf life
- Low capital costs
- Easy availability of career materials (Jeyarajan and Nakkeeran, 2000)

11.1.5 Fermentation

Liquid and solid fermentation methods are used for the mass production of PGPR.

11.1.5.1 Liquid fermentation

This fermentation system has been adopted for the mass multiplication of fungal bacterial biocontrol agents. For mass multiplication the selected medium should be inexpensive and readily available with appropriate nutrient balance. Kings' B broth or nutrient broths are used for the mass production of *Pseudomonas* and *Bacillus* spp., through liquid fermentation technology (Kloepper and Schroth, 1981; Vidhyasekaran and Muthamilan, 1995; Manjula and Podile, 2001; Nakkeran *et al*., 2004).

11.1.5.2 Solid fermentation

In nature wide range of organic substrates could be used for the solid-state fermentation for mass multiplication. Solid fermentation media consisting of inert carriers with food bases was used for mass production of biocontrol agents (Lewis, 1991).The media with relatively low microbial content would be suited for solid-state fermentation and for the amendment of biocontrol agents. Solid substrates include straws, wheat bran, sawdust, moistened bagasse, sorghum grains, paddy chaff, and decomposed coir pith, farmyard manure and other substrates rich in cellulose for inoculum production. Siddiqui and Mahmood (1999) stated that bacteria had great potential to manage plant parasitic nematodes. But the practicality of the same could be done by incorporating the antagonistic bacteria to organic manures, followed by incubation at 35°C for 5-10 days coupled with frequent mixing under sterile environment along with water so as to maintain the organic manure under moist conditions, which aid in the proliferation of the bacteria. The enriched organic manure with biocidal value could be used for the management of nematodes and plant growth promotion.

11.1.6 Formulation viability

Shelf life of the formulations decides the commercialization of biocontrol agents. Formulations should support the viable nature of the product for the increased period of storage. Bio control product should have the minimum shelf life of 8-12 months for industrialization. Carrier material should not affect the viable nature of the biocontrol agent. Commercialization of the bioproducts is mainly hampered due to the poor shelf life. Hence research should be concentrated to increase the shelf life of the formulation by developing superior strains that support the increased shelf life, or the organic formulations that support the maximum shelf life with low level of contaminants must be standardized for making biocontrol as a commercial venture.

11.1.7 Toxicology

Safety and environmental considerations could not be taken for granted and it is crucial that biopesticides are regulated in an appropriate way to confirm the international standards. The regulatory environment is generally favorable for the bio-pesticides than the chemical pesticides. However the cost of carrying out the toxicological study for registration is still prohibitive. Toxicology includes information of antagonist on the safety to men, plants, animals and soil microflora. Cost incurred for the toxicological studies is high. These studies have to be done separately for each and every biocontrol organism separately. The huge investment on the toxicological studies warrants for the linkages between stakeholders and research organizations (Jeyarajan and Nakkeeran, 2000; Sabitha Doraiswamy *et al*., 2001).

11.1.8 Industrial linkages

The research institutes carry out the initial discovery of an effective organism, genetic manipulation of organisms to develop superior strains, and studies on mechanisms, field efficacy and protocols for the development of formulations. But to take this technology to entire country depends on the partnership between the stakeholders and institutes. Corporate resources are required for the large-scale production, toxicology, wide scale field-testing, registration and marketing. Entrepreneurship may be defined as the exchange of intellectual property for research grants, and a royalty stream, with the establishment of University – Industry partnership for the benefit of both. The first requirement for the entrepreneurship requires a patent application on the strain and the related technology, especially on the efficacy data, identity of the organism, toxicological data and delivery

system. Ideally the process of entrepreneurship will result in an academic corporate research team working towards a common goal.

11.1.9 Quality control

This is very much required to retain the confidence of the farmers on the efficacy of biocontrol agents. Being the living organisms their population in a product influences the shelf life. The population load of the antagonists decides the minimum level of requirement for bringing the effective biological control of the plant diseases. Depending on the type of the antagonist (bacteria or fungal), and formulation, the moisture content and population load varies. The other contaminating organisms should be also under the permissible limits.

12 CONSTRAINTS TO COMMERCIALIZATION

The success of microbial pesticides to suppress pests and diseases depends on the availability of microbes as a product or formulation, which facilitate the technology to transfer from lab to land. The constraints to biopesticides development and utilization mirror some of those factors that limit the development worldwide. Constraints include

- Lack of suitable screening protocol for the selection of promising candidate of PGPR.
- Lack of sufficient knowledge on the microbial ecology of PGPR strains and plant pathogens
- Optimization of fermentation technology and mass production of PGPR strains
- Inconsistent performance and poor shelf life
- Lack of patent protection
- Prohibitive registration cost (Schisler and Slininger, 1997; Fravel *et al*., 1998; Fravel *et al*., 1999)
- Awareness, training and education shortfalls
- Lack of multi disciplinary approach
- Technology constraints (Sabitha Doraiswamy *et al*. 2001).

12.1 Screening and selection of potential PGPR strain

Success of commercialization of PGPR strains depends on the selection of effective strains after adopting rigorous screening strategies. Because, any mistake during strain selection will be a costly mistake in product development (Schisler and Slininger**,** 1997). The potentiality of the

PGPR strain in the suppression of plant pathogen should be carried out at both lab and field conditions in different soil types with diversified microbial communities and climatic conditions (Roberts and Lohrke, 2003). It would lead to the development of a viable PGPR strain.

12.2 Microbial ecology and interaction

Suppression of plant disease is a four-way interaction of biocontrol agents, plants, pathogens and the environment. Hence, understanding of interaction between all these components is essential for developing a suitable biocontrol agent in disease management (Handelsman and Stabb, 1996; Larkin *et al*., 1998). Extracellular metabolites produced by PGPR strains interact with microbial community (plant pathogens and other microbes) and plant in rhizosphere or spermosphere or phyllosphere and result in the suppression of pathogenic propagules either by direct action of antibiotics or through elicitation of induced systemic resistance activated by the molecular determinants (lipopolysaccharide, salicylic acid), global regulators and siderophores of bacterial origin (Larkin *et al*., 1998; Thomashow *et al*., 1990; Thomashow and Weller, 1988; Loper and Henkels, 1999). However, knowledge on the influence of biotic and abiotic environment on PGPR strains to express its antimicrobial action has to be studied in depth under *in vivo* to improve the efficacy of PGPR strains. This will facilitate to identify bacterial strains that could perform well under diverse environmental conditions around the court of infection.

12.3 Fermentation technology and shelf life of formulations

Optimization of fermentation technology (Liquid or solid fermentation) with suitable medium (synthetic or semi-synthetic) for mass multiplication and identification of suitable carrier material (organic or inorganic) for formulation development with increased shelf life is a barrier in the commercial success of formulation development. Slininger *et al*. (1996) reported that liquid culture and formulation technologies has to be optimized for the commercial exploitation of *P. fluorescens* 2-79 for the management of take all disease of wheat. Commercial biomass production of bacterial antagonists requires large-scale fermenters. The biomass production and efficacy of biocontrol agents to suppress plant pathogens varies depending on the nutrient composition of the medium (Schisler and Slininger, 1997). Hence, the medium selected for biomass production should support the growth and efficacy of antagonist and the cost of medium should be economical so that the technology remains viable.

12.4 Patent protection and prohibitive registration cost

The environmental protection agency in developed and developing countries should relax the formalities and registration cost to promote registration of biocontrol agents either by universities or private companies. The patent protection rights for the effective products should be strengthened to encourage the organizations involved in identification and development of commercial biocontrol agents.

12.5 Awareness, training and education shortfalls

The general level of awareness among stakeholders about the potential value of biopesticides is lacking. There is a need for

- Awareness level among the policy makers of the potential for biopesticides, their efficacy and their effect in reducing the health and environmental problems.
- The opportunities offered by the commercialization in terms of generation of wealth and employment are to be promoted.
- Entrepreneurs and investors need to be informed about the opportunities that exist for establishing commercial companies to manufacture market and sell biopesticides.
- Government extension workers have to be trained in biopesticides and the communication between research and extension sectors have to be intensified.
- The nature and mode of action of biopesticides have to be explained to farmers who are used to chemical insecticides, which are often fast acting and are visibly effective (Sabitha Doraiswamy *et al*., 2001**).**

12.6 Lack of multidisciplinary approach

The process of biopesticides development to complete product requires research in areas of screening, formulation, field application, production, storage, toxicology as well as the steps necessary for commercialization, such as scale up production, registration and regulatory matters. Most of the research efforts undertaken with the use of biopesticides are confined only to the exploration, collection, isolation and identification of biocontrol agents combined with laboratory based bioassays. But in the process of product development the above research aspects shares only a fraction of work required to develop a complete product. Product development requires a multidisciplinary approach to biopesticides research

and development. Rarely a complete range of expertise exists in a single institute or organization.

12.7 Technology constraints

12.7.1 Delivery system

Success in biocontrol depends on understanding and use of delivery system. The research on delivery system is well below that of chemical insecticides. The attention on the application technology can improve biopesticides performance.

12.7.2 Biopesticides quality

The major problem in the field of biopesticides production is the product quality and stability. In small-scale production, contamination of inoculum is a common problem. The long -term shelf life of the product is highly essential to attract the multinational companies to invest on a large scale.

13 STRATEGIES TO PROMOTE COMMERCIALIZATION

Commercialization of biocontrol could be promoted by

- Popularization of biocontrol agents
- Industrial linkages

13.1 Popularization of biocontrol agents

Motivating the growers through

a. Publicity
b. Field demonstrations
c. Farmers days
d. Biovillage adoption
e. Conducting periodical trainings for commercial producers and farmers to increase / improve the supply.

13.2 Industrial linkages

a. Technical support should be made available to entrepreneurs on quality control and registration.
b. Regular monitoring is essential to maintain the quality.
c. Constant research support should be extended to standardize the dosage, storage, and delivery systems. Positive policy support from Government to use more of biocontrol agents in crop protection.

14 CONCLUSION

Increase in public concern about the environment has increased the need to develop and implement effective biocontrol agents for crop protection. An effective PGPR could be developed for disease control only after understanding its performance in the environment in which it is expected to perform. In nature agriculture crops are exposed to diverse environmental conditions and gambling of monsoons, which alter the microclimatic conditions existing around the infection court. A thorough knowledge on the mechanisms and performance related to disease control will help in the selection of promising candidates that suits industries to produce reliable commercial products (Collins *et al*., 2003).

Introduction of PGPR strains to phyllosphere, spermosphere or rhizosphere may be moderately effective or sometimes totally ineffective under field conditions to control plant diseases (Duffy *et al*., 1996)**.** Inefficacy of the strains under field conditions may be due to the variation in climatic conditions that suppress growth and survival of biocontrol agents (Guetsky *et al*., 2001)**.** In addition both pathogen and biocontrol agents does not have similar ecological niche for their growth and survival. Hence the efficacy of biocontrol agents could be improved through the usage of compatible mixed inoculum of biocontrol agents which could have a consistent performance under diverse environmental conditions (Guetsky *et al*., 2001; Janisiewicz, 1996)

PGPR formulations comprising of bacterial strain mixtures having the capability to induce chitinase in plant play an important role in hydrolyzing chitin, the structural component in gut linings of insects and would lead to better control of insect pest (Broadway *et al*., 1998). In addition certain PGPR strains also activate octadecanoid, shikimate and terpenoid pathways. This in turn alters the volatile production in the host plant leading to the attraction of natural enemies (Bell and Muller, 1993). Identification of entomopathogenic PGPR strains that have the capability to colonize phylloplane in a stable manner will be a breakthrough in the management of foliar pests (Otsu *et al*., 2004). Combined application of

entomopathogenic strains with compatible PGPR strains that have the ability to suppress plant diseases has to be developed for broad spectrum action.

On the contrary, certain studies explain that some strain mixtures perform even lower than that of individual strains. So, the basic knowledge on molecular signaling mechanisms between related strains and species has to be understood for the development of a better formulation that could suppress a broad spectrum of pathogens and pests besides plant growth promotion.

The formalities involved in registration of formulation are very stringent and the cost incurred for registration of individual strains is also high. At this juncture, the cost incurred for the registration of formulations with mixed strains should not be prohibitive to the industrialist to venture in to the field of commercialization of the organism. If it is found to be prohibitive than the research developments from the lab would not reach the end-users.

But one cannot compensate the quality and safety of the product for the use of farming community. The advocates of biocontrol also face a tough time to convince the environmental protection agencies about the safety of the organisms. Because, acceptance, registration, transfer of technology and adoption of the biocontrol agents at field level relies on the safety of the organism to be used. Biocontrol researchers cannot deny that several well known bacterial biocontrol agents have a threat to become as an opportunistic pathogen. Occurrence of immune compromising infectious diseases and tissue transplants has made opportunistic pathogens as a visible threat to human health.

Several potential biocontrol agents used for plant disease management behave as opportunistic human pathogens. Though *P. aeruginosa* is a potential biocontrol agent of gray leaf spot on turf, it is also a virulent opportunistic pathogen which infects wounds and severe burns. *P. cepacia,* which is used for the management of pea root rot, has the capability to infect lungs of the patients having cystic fibrosis. *Bacillus cereus*, being a potential candidate for the management of damping-off and root rot of soybean, it is also a food contaminant and closely mimics *Bacillus anthracis*, the causal agent of anthrax disease. The confusions involved in distinguishing between the related strains that turn as opportunistic pathogens for humans has to be solved to convince the policy makers and environmental protection agencies to promote acceptance, registration, transfer of technology and adoption.

Amidst these obstacles, since PGPR has its own potentiality in plant disease and pest management several products have been registered for the practical use of farming community. Sixty to 75% of cotton crops raised in U.S. are treated with commercial product of *B. subtilis* (Kodiak) effective against soilborne pathogens such as *Fusarium* and *Rhizoctonia*. It is also

used in peanut, soybean, corn, vegetables and small grain crops (Backman *et al*., 1997). In China, PGPR has been in commercial development for over than two decades and are referred as yield increasing bacteria (YIB). It is applied over an area of 20 million hectares of different crop plants (Chen *et al*., 1996; Kilian *et al*., 2000). In India, more than 40 stakeholders from different provinces have registered for mass production of PGPR with Central Insecticide Board, Faridabad, Haryana through collaboration with Tamil Nadu Agricultural University, Coimbatore, India for the technical support and information (Ramakrishnan *et al*., 2001). Though the market size for PGPR usage is increasing constantly under greenhouse and field conditions, finding solutions for the above obstacles will create a break through in the adoption of biocontrol agents for field applications.

Acknowledgement: Last author is thankful to Department of Science and Technology, Government of India, New Delhi for granting him a research project (No.SP/SO/A22/2001) on PGPR.

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Chapter 11

BIOCONTROL OF PLANT DISEASES BY GENETICALLY MODIFIED MICROORGANISMS: CURRENT STATUS AND FUTURE PROSPECTS

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Abstract: The biocontrol of plant diseases by microorganisms is a promising alternative to the chemical pesticides. *Serratia marcescens* strain B2 effectively controls fungal diseases of cyclamen and rice. Biocontrol by *S. marcescens* strain B2 is mediated by the combined effects of plural chitinases, antibiotic prodigiosin, induced systemic resistance. Activity of *S. marcescens* is often negatively affected by abiotic and biotic factors and antibiotic biosynthesis of this bacterium is reduced under the influence of rice-associated bacteria. A genetically modified rice-indigenous bacterium was developed by introducing genes encoding for antifungal factors. Disease inhibitory genes were isolated from *S. marcescens* and put under the control of several types of promoters, which were isolated from the recipient. These genetically modified microorganisms effectively suppressed rice blast disease caused by *Pyricularia oryzae* and are not affected by abiotic or biotic factors. Introduction of disease inhibitory genes controlled by promoters and derived from the recipient is a useful technology for the development of biocontrol agents.

Key words: biocontrol; counteraction; chitinases; environmental factors; genetically modified microorganisms; indigenous microorganisms; promoters

1 INTRODUCTION

Public concern about the impact of chemical fungicides on human health and the environment has intensified the search for alternative methods to control plant diseases. The biocontrol of plant diseases by antagonistic

Z. A. Siddiqui (ed.), PGPR: Biocontrol and Biofertilization, 297–312.

microorganisms such as bacteria and fungi holds great promise (Campbell 1989). Biocontrol activities of microorganisms include the production of antibiotics, lytic enzymes and induction of systemic resistance in the host plant (Figure 1). The effectiveness of biocontrol agents can be influenced by a myriad of environmental factors, both abiotic and biotic. This results in less disease suppression and insufficient reduction of pathogens. Therefore, biocontrol achieved is often unpredictable, and results are too variable for agricultural use. The molecular pathways by which abiotic factors such as nutrient conditions, temperature, humidity, light, etc., affect the expression of antagonistic activities of biocontrol agents have been studied under laboratory conditions. However, little is known about the mechanism by which biotic factors affect the activity of biocontrol agents on phytopathogens, particularly in the rhizosphere and on the plant surface.

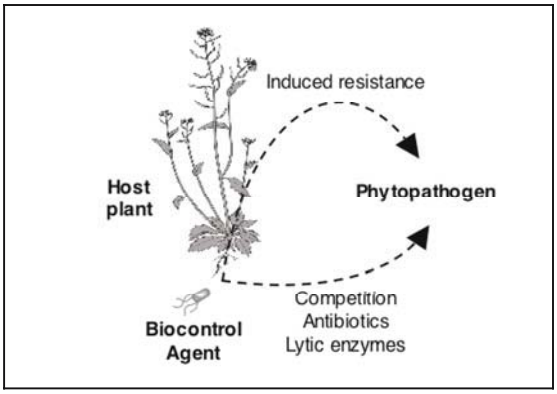

Fig. 1. Mechanisms of biocontrol for phytopathogens.

2 BIOCONTROL OF PHYTOPATHOGENS BY ANTAGONISTIC BACTERIUM *SERRATIA MARCESCENS*

Many bacteria including *Pseudomonas* spp., *Bacillus* spp., *Burkholderia* spp. and *Enterobacter* spp. etc. have been identified as biocontrol agents (Cook 1996, Cook *et al.* 1996, Desai *et al.* 2002, Mukohara 1998, Vidhyasekaran 2004). *Serratia* spp., has also been identified as a useful biocontrol agent and plant growth-promoting bacterium (Guo *et al.* 2004, Okamoto *et al.* 1998, Ordentlich *et al.* 1988, Raupach *et al.* 1996, Wei *et al.* 1996), as well as an opportunistic mammal or plant pathogen (Bruton *et al.* 2003, Grimont and Grimont 1978, Hejazi and

Falkiner 1997, Rascoe *et al*. 2003). One of its strains, *S. marcescens* strain B2, has been isolated from the tomato plant. Strain B2 colonizes the rhizosphere of cyclamen and rice plants, and effectively controls fungal diseases of cyclamen and rice (Iyozumi *et al*. 1996, Someya *et al.* 2000, 2005a).

2.1 Mechanisms of biocontrol by antagonistic bacteria

Antagonistic bacteria employ different mechanisms for biocontrol of phytopathogens: competition may lead to niche exclusion, production of antibiotics, lysis of the pathogen, or induction of systemic resistance in the host plants (Campbell 1989). *S. marcescens* produces several chitinolytic enzymes, including both exo- and endo-type chitinases. These chitinases are potentially useful inhibitors of phytopathogenic fungi that contain chitin as a major structural component (Akutsu *et al.* 1993, Gooday 1990, Herrera-Estrella and Chet 1999, Kobayashi *et al.* 1995, Ordentlich *et al.* 1988). Strain B2 produces at least four chitinolytic enzymes (Someya *et al.* 2001). Each chitinolytic enzyme inhibits the growth of phytopathogenic fungi and synergistic antifungal activity of endo- and exo-type chitinolytic enzymes has also been observed (Someya *et al*. 2001). *Serratia marcescens* also produces prodigiosin, a tripyrrole antibiotic that functions as an antifungal factor. In addition, some strains of the *Serratia* spp., including strain B2, induce systemic resistance in the host plant against various diseases (Liu *et al*. 1995, Raupach *et al*. 1996, Someya *et al.* 2002, Wei *et al.* 1996).

3 INFLUENCE OF ENVIRONMENTAL FACTORS ON THE ACTIVITY OF BIOCONTROL AGENTS

Abiotic factors can influence the activity of biocontrol agents and these factors include pH, temperature, moisture, light conditions, soil type, nutrients, components of the soil atmosphere, inorganic or organic soil constituents, and pesticide application (Adams and Wong 1991, Burpee 1990, Duffy and Défago 1999, Kredics *et al.* 2003, Landa *et al.* 2001, Ownley *et al*. 1992, 2003, Schmidt *et al.* 2004, Shanahan *et al*. 1992, Someya *et al.* 2004, van Rij *et al.* 2004). These factors have the impact on the interactions of phytopathogenic fungi and microbial antagonists. The combined effects of both abiotic and biotic factors (such as resident microflora) under field condition have an influence on the expression of antagonistic effects of biocontrol agents (Duffy *et al*. 2003, Haas and Keel 2003).

3.1 Influence of plant-associated indigenous microbes on biocontrol activity

Serratia marcescens suppresses various plant diseases but biocontrol of disease often fails when inoculation of pathogen preceded bacterial inoculation by a prolonged period, even though *S. marcescens* remains present in the plant rhizosphere soil (Someya *et al.* 2003a). This caused decline in antibiotic and chitinase production in the plant rhizosphere following inoculation. Although *S. marcescens* colonizes the rhizosphere, but the influence of indigenous bacteria apparently prevents antibiotic and chitinase biosynthesis by this bacterium. More than 75% of bacterial isolates from rice plants were able to inhibit antibiotic biosynthesis of *S. marcescens*, without inhibiting its growth (Someya *et al.* 2003a) (Figure 2). Some bacterial isolates were also capable of inhibiting chitinase biosynthesis in *S. marcescens* strain B2 (Someya *et al.* 2005b). This clearly demonstrates that plant-associated microbes can affect the expression of antagonistic traits of biocontrol agents.

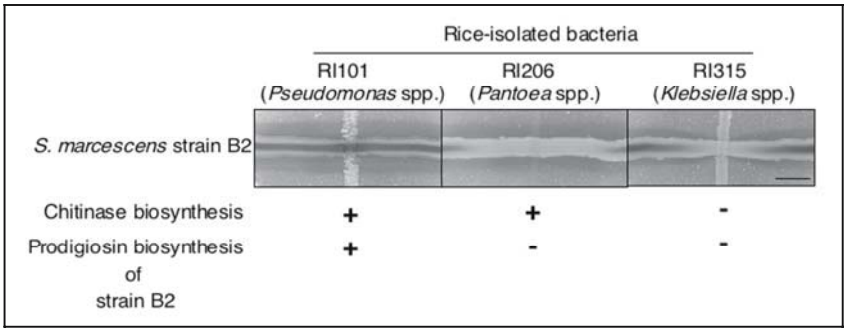

Fig. 2. Lytic enzymes and antibiotic biosynthesis by biocontrol agent *Serratia marcescens* strain B2 under the influence of rice-associated bacteria. Rice-associated bacterium RI206 (*Pantoea* spp.) inhibited the antibiotic prodigiosin biosynthesis by strain B2, and RI315 (*Klebsiella* spp.) inhibited both lytic enzyme chitinase and antibiotic prodigiosin biosynthesis. Scale = 10 mm.

3.2 Influence of phytopathogens on biocontrol activity

Some phytopathogens produce metabolites and influence the effectiveness of biocontrol agents. *Pseudomonas fluorescens* produces antibiotic 2,4-diacetylpholoroglucinol (2,4-DAPG) and suppresses various plant pathogens (Bonsall *et al.* 1997, Dowling and O'Gara 1994, Raaijmakers *et al.* 1997) while *Fusarium oxysporum* produces the phytotoxic fusaric acid, which counteracts the biosynthesis of 2,4-DAPG or

pyoverdine by *Pseudomonas* spp. (Duffy and Défago 1997, Duffy *et al*. 2003, Landa *et al*. 2002, Notz *et al*. 2002). *Fusarium oxysporum* interfere with autoregulation of 2,4-DAPG biosynthesis as a defense strategy to thwart antagonism. In addition, a phytopathogen metabolite, deoxynivalenol, produced by *F. graminearum*, acts as a negative signal resulting in reduced expression of a specific chitinase gene in the fungal biocontrol agent *Trichoderma atroviride* (Lutz *et al*. 2003). Expression of antibiotic 2,4-DAPG in *P. fluorescens* is also repressed by the bacterial extracellular metabolites salicylate and pyoluteolin in addition to fusaric acid (Schnider-Keel *et al*. 2000).

3.3 Influence of host plant on biocontrol activity

Plant host genotype, age, root exudates, and pathogen infection can affect bacterial antibiotic biosynthesis by *P. fluorescens* (Kraus and Loper 1995, Kravchenko *et al*. 2003, Notz *et al*. 2001). Moreover, higher plants secrete a variety of signal-mimic compounds that can stimulate or inhibit behaviors in bacteria, which are regulated by *N*-acyl homoserine lactone (AHL) signal molecules (Bauer and Teplitski 2001, Teplitski *et al*. 2000, Walker *et al*. 2003). Many of the mechanisms of biocontrol are important for the plant disease control by bacteria such as biosynthesis of antibiotics and lytic enzymes and regulated by quorum sensing via AHLs (Chernin *et al*. 1998, Horng *et al*. 2002, Pierson and Pierson 1996, Swift *et al*. 1996, Thomson *et al*. 2000, Zhou *et al*. 2003).

The success of biocontrol by antagonistic bacteria is dependent on complex interactions between plants, phytopathogens, and biocontrol agent under field conditions (Figure 3). The combined effect of these interactions can both suppress and enhance the activity of biocontrol agents. The application of a mixture of biocontrol agents or the restoration of biocontrol activity by AHLs, can result in synergistic interactions between the different components (De Boer *et al*. 2003, Fray *et al*. 1999, Fukui 2003, Fukui *et al*. 1999, Pierson and Weller 1994, Schisler *et al*. 1997, Wood *et al*. 1997). Our group developed a new biocontrol agent whose activity is not influenced by either biotic or abiotic factors to make biocontrol more predictable and reliable.

4 DEVELOPMENT OF GENETICALLY MODIFIED BIOCONTROL AGENT

The use of genetically modified microorganisms as biocontrol agents has been reported throughout last decade. Genetically modified

microorganisms with increased expression of biocontrol traits have been developed in certain cases to improve biocontrol potential (Delany *et al*. 2001, Giddings 1998, Maurhofer *et al.* 1992). Chitinolytic enzyme gene has been introduced into certain microbes for the control of phytopathogenic fungi (Chernin *et al*. 1997, Downing and Thomson 2000, Hirayae *et al.* 1996, Ikeda *et al*. 1996, Shapira *et al*. 1989, Sundheim *et al.* 1988, Toyota *et al*. 1994). This resulted in enhanced activity of the genetically modified microbe against phytopathogenic fungi but chitinolytic activities of such transformants are low, possibly because these chitinase genes are under the control of relatively weak promoters which were isolated from other organisms (Chernin *et al.* 1997, Fuchs *et al*. 1986). Later, we tried to add a biocontrol trait in *Erwinia ananas* by the introduction of the chitinolytic enzyme gene from *S. marcescens*, which was expressed under the control of *E. ananas* promoters.

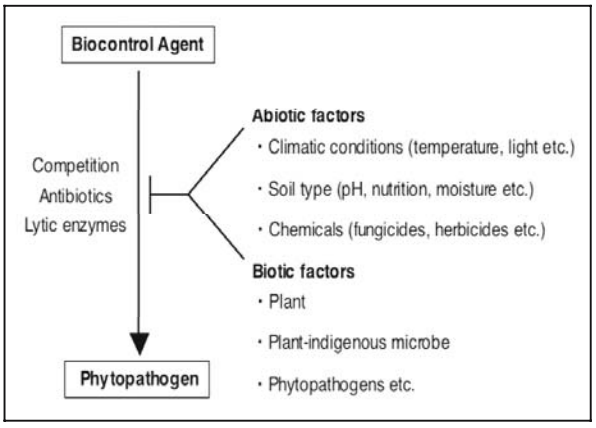

Fig. 3. Factors affecting the biocontrol of phytopathogens by biocontrol bacteria.

4.1 Promoter trapping from plant-associated bacterium

Rice epiphytic bacterium *E. ananas* NR1 colonizes rice leaves but NR1 does not produce antifungal factors such as lytic enzymes or antibiotics. First, we constructed a promoter-trap vector pEGFP-V1 for the recipient bacterium *E. ananas* NR1 (Figure 4). This plasmid contains a promoterless gene that encodes the enhanced green fluorescent protein, EGFP (Numata *et al.* 2004).

We trapped various promoters from the *E. ananas* genomic DNA by shotgun cloning. Approximately 3,500 clones were obtained, 300 of which exhibited fluorescence (Figure 5). The relative activity of the trapped promoters was compared with expression of EGFP placed under the control

of the *Escherichia coli lac* promoter also in pEGFP-V1. Nine clones (pcf1, pcf9, pcf10, pcf15, pcf51, pcf52, pcf53, pcf55 and pcf85) with particularly strong fluorescence were selected for further experiments. Sequence similarities of those fragments containing promoter activity are indicated in Table 1.

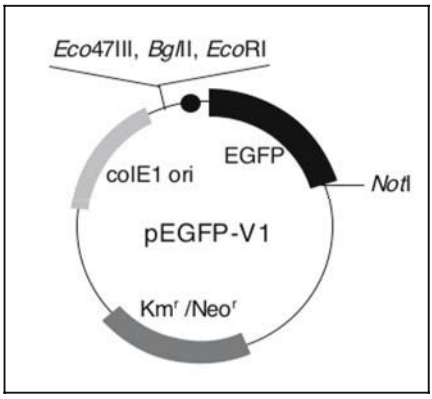

Fig. 4. Promoter-trap vector pEGFP-V1.

Fig. 5. Clones trapped fragments containing promoter activity using pEGFP-V1. 300 clones, which showed GFP expression, were obtained from approximately 3,500 clones. Arrows indicate GFP expressing clones.

4.2 Chitinolytic gene expression driven by entrapped promoters in rice epiphytic bacterium *E. ananas*

S. marcescens chiA, encoding the 58-kDa endochitinase ChiA, was cloned from strain B2. The endochitinase gene was then placed under the control of the promoters isolated from *E. ananas*, as described above, and a broad-host-range plasmid vector was used to introduce *chiA* into the rice epiphytic bacterium *E. ananas* NR1. The constructed vectors were

Table 1. Sequence similarity of promoter-containing fragments (pcf) isolated from E. ananas chromosomal DNA by promoter trapping.

Clone	Insert size (bps)	Sequence similarity (most similar protein, significance)
pcf1	411	The promoter region of *E. coli envA* gene encoding a lipopolysaccharide-synthesizing enzyme
pcf9	458	The promoter region of *E. coli fabA* gene encoding the D-3-hydroxydecanoyl dehydratase
pcf10	1,100	-
pcf15	214	The promoter and 5'-terminal region of R100 *pemI* plasmid stable inheritance protein
pcf51	800	The promoter and 5'-terminal region of *E. coli lysC* encoding the lysine-sensitive aspartokinase
pcf52	251	-
pcf53	154	-
pcf55	650	The promoter region of *Salmonella thphimurium smvA* encoding the methyl viologen resistance protein
pcf85	240	The promoter region of *Yersinia enterocolitica sodA* encoding the superoxide dismutase

-: no sequence homology

designated for their respective promoters, for example pchiA-V1pcf9 and pchiA-V1pcf53 (Someya *et al*. 2003b). One of the transformants, *E. ananas* NR1/pchiA-V1pcf9, did not produce lytic enzyme chitinase under low nutrient conditions; however, transformant *E. ananas* NR1/pchiA-V1pcf53 did produce chitinase under the same nutritional conditions (Figure 6). Therefore, depending on the promoter, chitinase expression by transformants is not necessarily influenced by nutrient conditions.

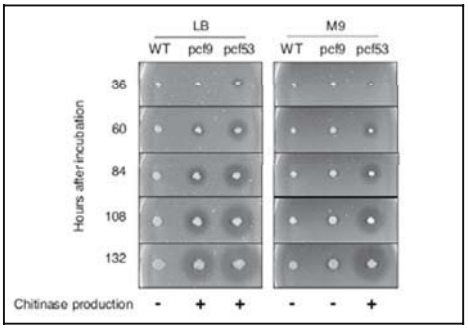

Fig. 6. Chitin degradation on chitin-supplemented *LB* and *M9* (low nutrient) agar plates by *E. ananas* NR1 (*WT*), *E. ananas* NR1/pchiA-V1pcf9 (*pcf9*), and *E. ananas* NR1/pchiA-V1pcf53 (*pcf53*), respectively.

4.3 Influence of rice-associated bacterium on chitinase production by genetically modified *E. ananas*

The influence of rice-isolated bacteria on the chitinase expression in *E. ananas* transformants was examined. Chitinase production was not inhibited by rice-isolated bacteria in the transformants, although the same rice-isolated bacteria efficiently inhibited chitinase biosynthesis in *S. marcescens* (Someya *et al*. 2005b) (Figure 7). The mechanism by which the rice-isolated bacteria inhibited chitinase production in *S. marcescens* was not evaluated. Our results clearly indicate that the genetically modified *E. ananas* escapes suppression of chitinase production by rice-indigenous microbes.

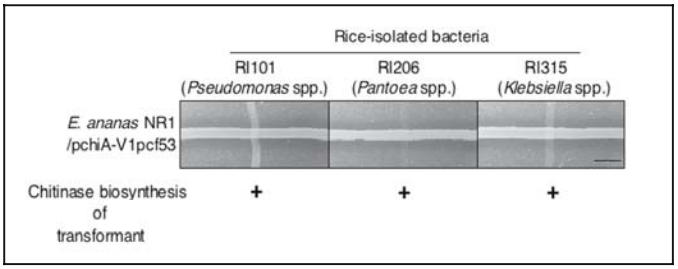

Fig.7. Chitinase biosynthesis by the genetically modified *E. ananas* NR1/pchiA-V1pcf53. Transformant produced chitinase under the influence of rice-associated bacterium RI315 (*Klebsiella* spp.), which inhibited the chitinase biosynthesis by *S. marcescens* strain B2 (in *Fig. 2*).

4.4 The biocontrol efficacy by transformants *in vivo*

The antifungal activity of ChiA produced by transformed *E. ananas* NR1 was demonstrated *in vitro* by the inhibition of hyphal growth of phytopathogens (Someya *et al.* 2003b). In addition, the transformed *E. ananas* suppressed the incidence of rice blast caused by *Pyricularia oryzae* under greenhouse conditions and the magnitude of the suppressive effect depended on the promoter used (Someya *et al.* 2003b) (Figure 8). Therefore, the introduction of antagonistic factor genes combined with several different promoters, derived from the recipient, is a useful technology for the development of new biocontrol agents.

4.5 Risk assessments for the agricultural use of genetically modified biocontrol agents

Genetic manipulation of microorganisms and their deliberate release into the environment have potential advantages, but certain associated risks

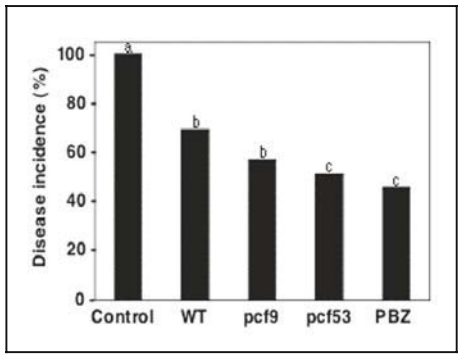

Fig. 8. Biocontrol effect of *E. ananas* NR1 (*WT*), genetically modified *E. ananas* NR1/pchiA-V1pcf9 (*pcf9*), and *E. ananas* NR1/pchiA-V1pcf53 (*pcf53*) on rice blast caused by *Pyricularia oryzae*. Each bacterium inoculated the rice foliage 1 hr before pathogen inoculation. As comparison, fungicide probenazole (*PBZ*) applied 3 days before pathogen inoculation. Disease incidence was calculated 1 week after pathogen inoculation. The letters indicate statistical significance as determined by the Tukey method ($p = 0.05$).

may also exist. Many scientists are convinced from the benefits that can be obtained by use of genetically modified microorganisms (GMOs) but general public are suspicious by use of a technology that they do not understand. They believe that GMOs, that they can not understand may pose a threat to their environment and health and meddles with the basis of life (Amarger 2002, Stephenson and Warnes 1996). However, no differences could be detected between GMOs and corresponding unmodified microorganisms in terms of survival, spread, persistence in the field, and ecological impact (De Leij *et al*. 1995, Natsch *et al*. 1997). Obviously, naturally occurring biocontrol agents can also affect the agricultural ecology (Gullino *et al.* 1995). In the biocontrol, biocontrol agent is either naturally occurring or genetically modified, it is based on promotion of a specific antagonistic microbe, and thus may interferes with the natural ecological balance. Regardless, the development of uniform and scientifically based guidelines for the release of genetically modified microorganisms in order to facilitate more routine screening in the environment is critical (Cook 1996, Cook *et al*. 1996, Giddings 1998, Ryder 1994, Wilson and Lindow 1993, Yoda 2004).

5 CONCLUSIONS

The commercial application of biocontrol agents is still subject to debate (Mathre *et al*. 1999, Stewart 2001, Utkhede 1996, Walsh *et al*. 2001). Over the past few decades, plant pathologists have made substantial progress

in the commercialization of biocontrol agents for plant disease control. There are numerous commercial products available worldwide (Desai *et al*. 2002). However, only one biopesticide, *Trichoderma*, which was developed in the 1950s, has been widely available in Japan. Recently, some newer biopesticides have been developed, including BioKeeper (Central Glass Co. Ltd., Tokyo, Japan), Botokiller, Biotrust (Idemitsu Kosan Co. Ltd., Tokyo, Japan) (Mukohara 1998), Eco-Hope (Kumiai Chemical Industry Co., Ltd., Tokyo, Japan) and Serunae-Genki (Taki Chemical Co., Ltd., Hyogo, Japan). A growing knowledge about biocontrol of plant diseases will support and lead to development of sophisticated and useful biopesticides. Proper use of biopesticides with restraint will play an important role in sustainable agriculture in the 21st century.

Many scientists share the belief that the artificial re-creation of suppressive soil (Schroth and Hancock 1982, Shipton *et al*. 1973) through biocontrol can play a part in disease control and may decrease the use of chemical pesticides. Although biocontrol is still in its infancy in reality, the growing understanding of the molecular mechanisms as well as the increased interest by biotechnology companies, will assure the future development and commercialization of biocontrol. The use of genetically modified microorganism is likely to play a significant role in this development.

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