

CHAPTER 1

1.0 GENERAL INTRODUCTION TO BIO-CONTROL TRAITS OF FLUORESCENT PSEUDOMONADS AND SCOPE OF THESIS

1.1 PLANT DISEASES AND BIOCONTROL

Plant diseases are an important constraint on worldwide crop production, accounting for losses of 10–30% of the global harvest each year (Strange and Scott, 2005). As a consequence, crop diseases represent a significant threat to ensuring global food security. To feed the growing human population it will be necessary to double food production by 2050, which will require the sustainable intensification of world agriculture in an era of unpredictable climate change (FAO, 2006). Controlling the severe plant diseases is one of the best means of delivering as much of the current productivity of crops as possible. Currently global expenditures on agricultural pesticide imports, summed across all nations of the world, have increased more than 1000% since 1960 (FAO, 2006). Faced with rising chemical use in many countries, food safety programs have introduced Good Agricultural Practices to diminish harm to human health. Food safety programs attempt to control the consumption of agrochemical residues, growth hormones, additives and naturally occurring toxins in foods by setting and enforcing Maximum Residue Levels for individual chemicals based on assessments of the risks that the chemicals pose to human health (International Assessment of Agriculture Knowledge, Science and technology for development (www.agassessment.org; www.islandpress.org/iaastd, 2008).

The negative effects of chemicals biofungicide / biopesticide include a decrease in biodiversity of the soil-inhabiting microorganisms, the non-target environmental impacts along with the development of resistance to fungicides by pathogens (Gerhardson and Wright, 2002), acute health problems resulting from exposure of farmers to chemical pesticides (Arcury and Quandt, 2003), pesticides residues in many food crops including fruits and vegetables which endanger the health of the consumers; furthermore, the increasing cost of pesticides, particularly in low-income countries of the world (Gerhardson and Wright, 2002). Health concerns and environmental hazards associated with the use of chemical fungicides have resulted in an increasing interest in the use of microbes to control plant disease and is an environment-friendly approach. Some

naturally occurring soil bacteria and fungi have demonstrated great potential to antagonize crop pathogens, hence, biological control involving the use of such plant beneficial microorganisms for plant protection is being considered as a viable substitute to reduce the use of chemical pesticides/fungicides. Biological control is a strategy that was proposed half a century ago, as a result of several negative effects that the increasing use of agro-chemicals had on the environment, farmers (applicators of the chemicals) and the consumers. According to current definitions, organisms and procedures involved in biological control include: (1) avirulent or hypo-virulent individual or population within the pathogenic species, (2) antagonistic microorganisms, and (3) effective resistance of the pathogen by the host plant through manipulation by biotic agents. The most abundant soil and plant-associated bacterial genera having biocontrol traits are *Burkholderia*, *Bacillus*, *Pseudomonas*, *Serratia* and *Streptomyces* (Abd-Allah, 2001; Berg et al., 2002; Nair et al., 2002; Costa et al., 2006; Mark et al., 2006). Fravel, 2005 estimated the number of biocontrol products in the market as 1% of agricultural chemical sales. There is need to search for more reliable and consistent biocontrol agents to replace the increasing demand for chemical residue-free agricultural products. The possibility of developing effective biocontrol agents may be achieved by isolating biocontrol strains from the same environment in which they are to be used.

Disease-suppressive soils are exceptional ecosystems in which crop plants suffer less from specific soil-borne pathogens than expected owing to the activities of other soil microorganisms. For most disease-suppressive soils, the microbes and mechanisms involved in pathogen control are unknown. Mendes et al., 2011 identified key bacterial taxa and genes involved in suppression of a fungal root pathogen by coupling PhyloChip-based metagenomics of the rhizosphere microbiome with culture-dependent functional analyses. More than 33,000 bacterial and archaeal species were detected, with Proteobacteria, Firmicutes, and Actinobacteria consistently associated with disease suppression. Members of the γ -Proteobacteria were shown to have disease-suppressive activity governed by nonribosomal peptide synthetases. Mendes et al, 2011 indicate that upon attack by a fungal root pathogen, plants can exploit microbial consortia from soil for protection against infections.

In the past 30-40 years, many research groups have focused on the modes of action of biocontrol and several mechanisms have been described that are responsible for the disease suppressive capacity of these pseudomonads. Hence, two widely used approaches to select for potential bio-

control agents focus first on isolation of antagonists from soils that are naturally suppressive to a particular pathogen (suppressive soils); the second approach comprises isolation from intended environment of use, such as soils, seeds or roots. The study of bacterial screening has a future that is characterized by many technical and conceptual challenges (Pliego et al, 2011)

Over the years, many bacterial isolates have been evaluated as potential biocontrol agents against soil borne fungal phytopathogens. However, few of them were ultimately successful after evaluation in field trials. One of the major reasons for this failure is the lack of appropriate screening procedures to select the most suitable microorganisms for disease control in diverse soil environments. For this reason, the study of bacterial screening has a future that is characterized by many technical and conceptual challenges. Many biocontrol organisms have been isolated from soils and identified as root-colonizing bacteria of the genus *Pseudomonas*.

1.2 PLANT GROWTH PROMOTING RHIZOBACTERIA

About 2 to 5 % of rhizobacteria exert a beneficial effect on plant growth termed as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR are defined as “beneficial free-living soil bacteria, which improve plant health or increase yield (Kloepper and Schroth, 1978) also referred to as “Yield Increasing Bacteria (YIB)” (Tang, 1994). PGPR have first been used for the agricultural purposes in former Soviet Union Russia and India in 20th century and are now being tested worldwide. The application of PGPR is one of the most promising methods for increasing agricultural productivity and reduction if not replacement of chemical fertilizers (Lugtenberg et al., 2002). Most of the PGPRs are present in the rhizoplane and the rhizospheric soil. PGPR are free-living bacteria (Kloepper, 1993), and some of them invade the tissues of living plants and cause unapparent and asymptomatic infections (Sturz and Nowak, 2000).

Bacterial genera generally considered as PGPRs include *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces* and *Thiobacillus* (Weller, 1988).

Based on their activities, PGPR are classified as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic and other pollutants or reducing their adverse effects) and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites). PGPR may induce plant growth by direct or indirect modes of action (Beauchamp, 1993; Kloepper, 1993; Lazarovits & Nowak, 1997). The direct mechanisms that are

involved in this process include production of phytohormones like Indole Acetic Acid (IAA), supply of biologically fixed nitrogen, phosphate and potassium solubilization, production of volatile growth stimulants e.g. ethylene and 2,3 butanediol (Vessey, 2003). Indirect mechanisms are those that suppress the effect of pathogenic or deleterious microbes and include production of siderophores, production of HCN, stimulation of Induced Systemic Resistance (ISR) and/or by competing with pathogens for nutrients or for colonization space (Glick, 1995). Indirect effects not only occur when PGPR act like biocontrol agents reducing diseases, but also when they stimulate other beneficial symbioses, or alleviate the toxicity to the plants. Furthermore, in most studied cases, a single PGPR often reveal multiple modes of action including biological control (Jetiyanon et al, 2003; Vessey, 2003).

Over the past four decades studies on the use of beneficial microorganisms as biocontrol agents have increased greatly. Several strains have been reported to show good performance *in vitro* and in specific trials, nonetheless, only few have demonstrated consistent and effective biocontrol in different field situations as a result very few get to the market (Kiely et al., 2006).

1.3 RHIZOSPHERE AND PGPR

Soil has a diverse ecosystem in which plant roots and micro organisms compete strongly for mineral and other nutrients. Lynch and Whipps, 1991 estimated that as much as 40% of the plant's primary carbon production may be released by plants through rhizodeposition, depending on plant species, plant age, and environmental conditions. Rhizosphere is defined as the layer of soil influenced by the root, and is much richer in microbial population than the surrounding bulk soil (Brimecombe et al, 2001). Generally the concentration of bacteria in the rhizosphere is 10 to 1000 times higher than that in bulk soil; it is still 100-fold lower than that in the average laboratory medium (Marschner H. et al, 1995). So, the lifestyle of rhizobacteria is considered as starvation. Rather than being static, the rhizosphere environment is typified by rapid changes in time and space, and allows succession of different groups of organisms and their functions (Handelsman and Stabb, 1996; Sorensen, 1997). During plant growth various materials that are deposited by roots into the rhizosphere, a phenomenon referred to as rhizodeposition (Whipps and Lynch, 1990), are divided into two main groups (Fig. 1.1). First group comprises a wide variety of water-soluble compounds including sugars, amino acids, organic acids, fatty acids, vitamins and enzymes (Dakora and Phillips, 2002) while second group comprises sloughed-off root cap cells and other debris and mucilage (polysaccharide) originating from the root cap or from lysates

released during autolysis. Most colonization occurs in the areas of maximum root exudation such as the elongation zone behind the root tip, junctions between epidermal cells, on root hairs, and at sites of lateral root emergence (Fig.1.1) (Chin-A-Woeng et al., 1997; Hansen et al., 1997; Lubeck et al., 2000). To exert their beneficial effects in the root environment, bacteria have to be rhizosphere competent, i.e., able to compete well with other rhizosphere microbes for nutrients secreted by the root and for sites that can be occupied on the root. 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 (Jetiyanon et al, 2003). These plant beneficial microorganisms are known to antagonize phytopathogens through competition for niches or nutrients (e.g. iron through siderophores synthesis); parasitism that may involve production of hydrolytic enzymes, for example, chitinase, glucanase, protease and cellulase that can lyse pathogen cell walls; inhibition of the pathogens by anti-microbial compounds (antibiosis); induction of systemic resistance in host plants (Whipps 2001; Compant et al., 2005).

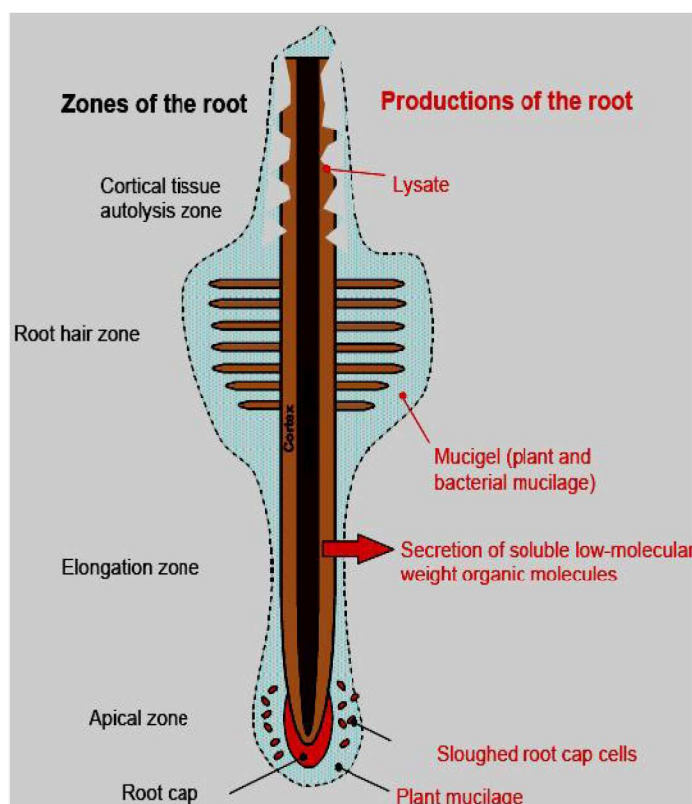


Fig. 1.1 Rhizodeposition root zones in the rhizosphere (Gobat et al, 2004)

Early colonization of the young rhizosphere is dominated by bacteria and especially *Pseudomonas* spp. proliferate in response to the simple carbohydrate exudates from the young plant roots (Sorensen 1997). Further rhizosphere is distinguished in to three fractions (Gobat et al., 2004) i.e., the endorhizosphere (interior of root), rhizoplane (surface of root) and rhizospheric soil (which remains attached to the soil).

1.3.1 Positive and negative interactions among rhizobacteria and plants:

Rhizosphere microbial communities significantly control the phytopathogen, nutrient acquisition, heavy metal resistance and ecological fitness of plants (Glick, 1995) Rhizobacteria can affect the plant development either negatively (deleterious bacteria) or positively (PGPR) as shown in fig.1.2, which depicts the negative and positive interactions between plant and rhizobacteria. The deleterious bacteria can produce phytotoxins and they compete with the plants and beneficial microorganisms for nutrient uptake (Fig 1.2) (Nehl et al., 1997). Competition for resources such as nutrients, oxygen and colonization site occurs generally in soil between soil-inhabiting organisms. Root inhabiting microorganisms compete for infection sites at the root surfaces (Alabouvette et al., 2006). For biocontrol purpose, it occurs when the antagonist directly competes with pathogens for these resources. Competition for nutrients, especially for carbon, is assumed to be responsible for the well-known phenomenon of fungistasis characterizing the inhibition of fungal spore germination in soil (Alabouvette et al., 2006). Couteaudier and Alabouvette, 1990 reported clearly that competition for carbon was one of the mechanisms used by the non-pathogenic *F. oxysporum* to suppress pathogenic *F. oxysporum*, the causal agent of fusarium wilt. Competition for trace elements, such as iron, copper, zinc, manganese etc., also occurs in soils. For example, iron is an essential growth element for all living organisms and the scarcity of its bio-available form in soil habitats results in a furious competition (Loper and Henkels, 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (also by most biocontrol agents) to solubilize and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow for lack of it (Loper and Henkels, 1997; Haas and Defago, 2005). Examples of siderophores produced by biocontrol agents are pyoverdin, salicylic acid and pyochelin (Haas and Defago, 2005). Pyoverdin (also called Pseudobactin), an extracellular diffusible pigment, produced by fluorescent *Pseudomonas* spp. is responsible for their fluorescence (Haas and Defago, 2005). Pyoverdin functions by binding to Fe^{3+} ion (the

insoluble and unavailable form of iron in the soil), which is subsequently transported into the cytoplasm of the producing organisms through interacting with a specific outer membrane receptor. After transportation into the cytoplasm, Fe^{3+} is converted to Fe^{2+} (Haas and Defago, 2005). Siderophore production favors rapid growth of the producing organisms.

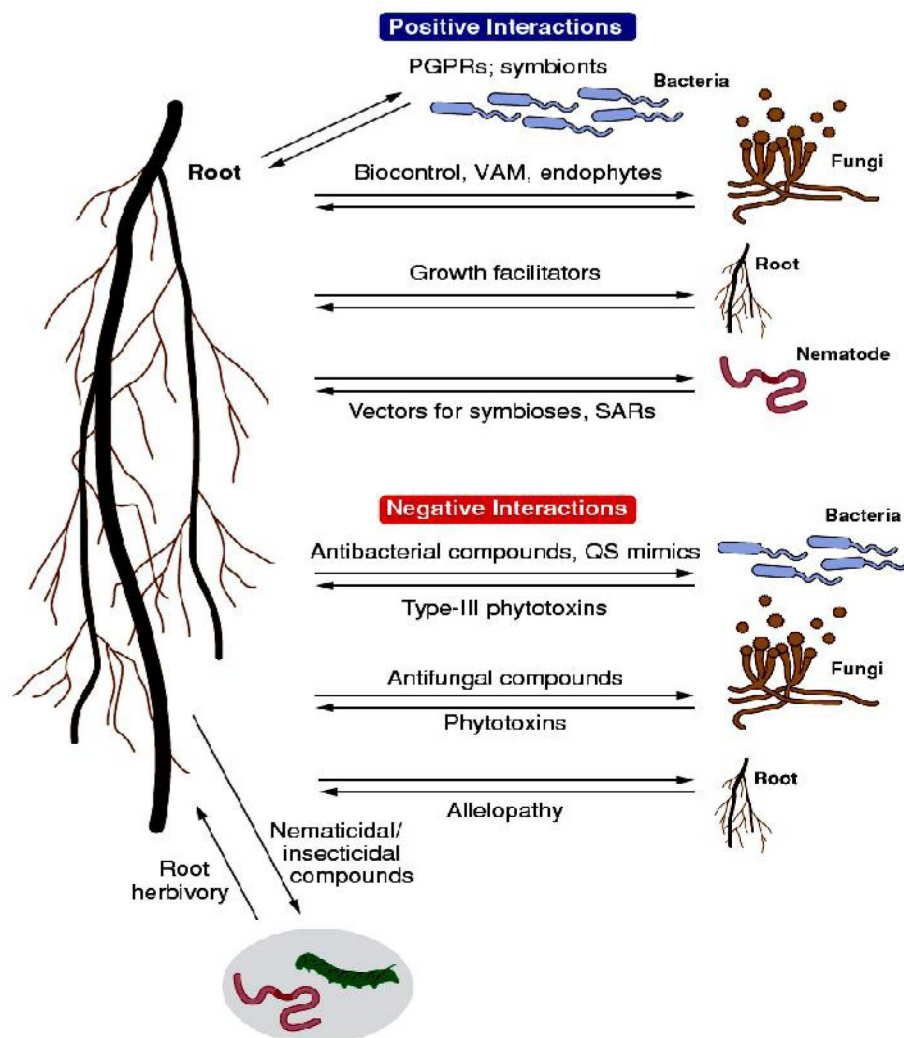


Fig.1.2 Positive and negative interactions among rhizobacteria and plants (Nehl et al, 1997)

Cross-talk and the molecular interactions during the establishment of PGPR strains in the rhizosphere involves the : 1. Pathogens establish contact with the susceptible plant cells or tissues, releasing a number of biologically active substances which affects host cell physiology, 2 .Microorganisms that produce biochemical active compounds against pathogens limit or suppress the disease, 3. Bacteria enable plants to protect themselves by inducing systemic acquired

resistance, 4. Pathogens produce toxins to defend themselves against bio-control or concurrent microorganisms, 5. Plant exudates serve as signals to induce secondary metabolite production in bacteria, 6. Introduced biocontrol bacteria may have an impact on resident soil microbiota, 7. Plants may develop resistance to a broad spectrum of pathogens (induced systemic resistance) or to specific ones (Fig 1.3) (Rezzonico, 2004; 2005)

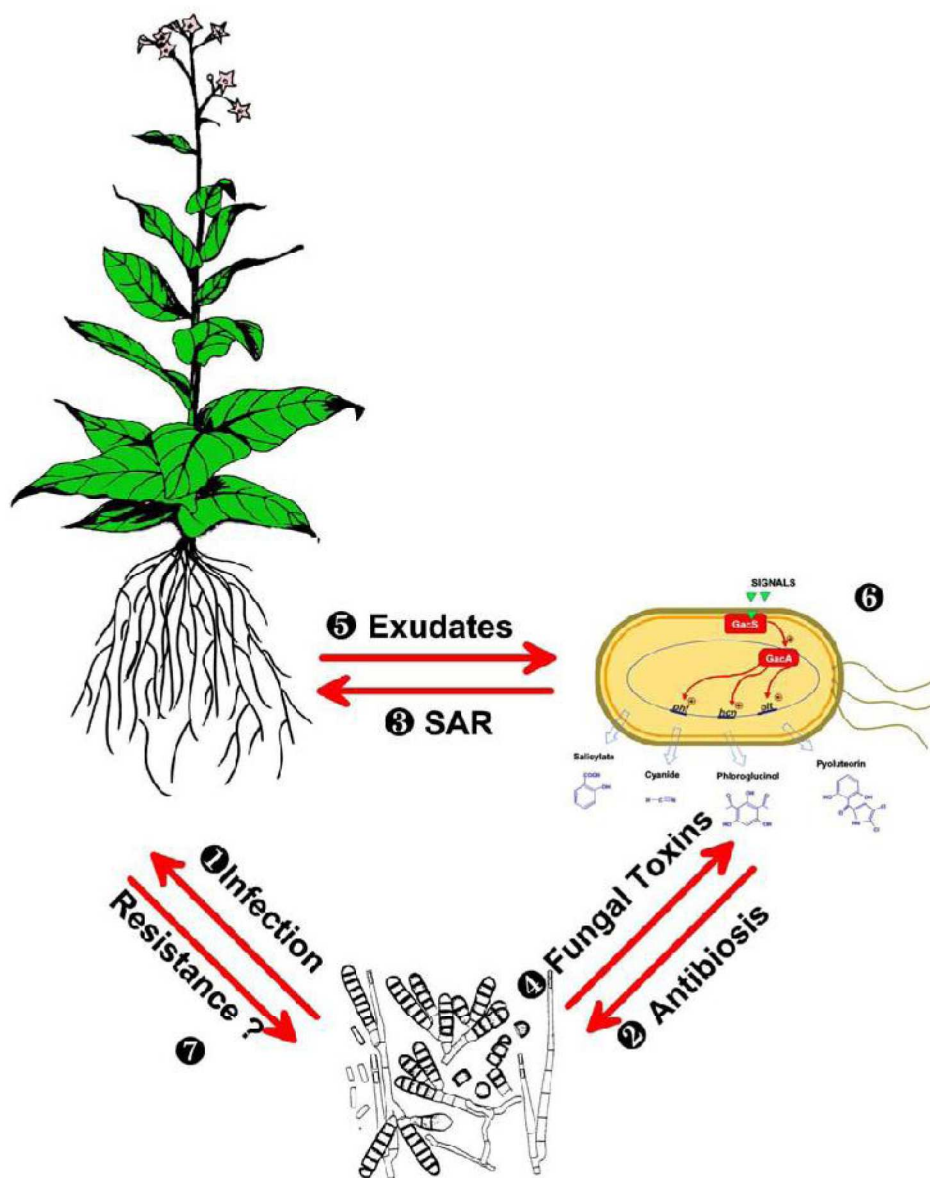


Fig. 1.3 Cross-talk in the rhizosphere and step wise the molecular interactions involved during the establishment of PGPR strains in the rhizosphere (Rezzonico, 2004)

1.4 PSEUDOMONADS AS A PGPR:

Members of the genus *Pseudomonas* are rod-shaped, gram-negative bacteria that are characterized by their metabolic versatility, aerobic respiration, one or several polar flagella and a high genomic G+C content (59-68%) (Haas and Defago, 2005). *Pseudomonas* spp. can be found in diverse environments and are an important component of the plant-associated microflora (Rainey, 1999). The generation time of *Pseudomonas* spp. in the rhizosphere was found to be 5-14 hours, whereas it was found to be 77 hours in the bulk soil (Bowen and Rovira, 1973). The genus is divided into five ribosomal RNA homology groups on the basis of RNA and DNA hybridization among total of 27 different species. RNA group I contains important plant growth promoting members and has been further divided in various sub types depending on Poly Hydroxy Butyrate (PHB) production (Palleroni, 1984). This genus is heterogeneous and harbours plant, animal and human pathogenic species, including *P. aeruginosa*, *P. plecoglossicida*, *P. tolaasii*, and *P. syringae*.

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. 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 (Couteaudier and Alabouvette, 1990). 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 *Gaeumannomyces graminis* var. *tritici* (Weller et al., 2002). *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% (Martinez et al., 2002).

For many pseudomonads, production of metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) is the primary mechanism of biocontrol (Weller and Thomashow, 1993). Several lines of 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, Mavrodi et al, 2012). The antibiotics produced by bacterial biocontrol agents and their role in microbial interaction, were reviewed by Raaijmakers et al., 2002). 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. was developed for the biological control of *Pythium* diseases in hydroponics systems for greenhouses (Paulitz and Bélanger, 2001).

Pseudomonas spp. produce wide varieties of antibiotics, which confer a competitive advantage and microbial fitness to survive in most environments (Haas and Keel, 2003; Paulsen et al., 2005). Due to their ability to produce variable metabolites and to utilize several organic compounds most biocontrol pseudomonads are not specific for one pathogen or plant species only, but have a wide host range and suppress several pathogens. For instance, Siddiqui and Shaikat (2003) reported the suppression of four root-infecting fungi, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani* by the biocontrol strain, *P. aeruginosa* IE-6 both under laboratory and field conditions. Antagonistic *Pseudomonas* spp. have been isolated from agricultural soils as well as soils that were naturally suppressive to different plant pathogens, including *Gaeumannomyces graminis* var. *tritici*, *Fusarium oxysporum*, *Rhizoctonia solani* (de Souza et al., 2003; Garbeva et al., 2003; Bergsma-Vlami et al., 2005). Similar observations were made in a Swiss soil suppressive to *Thielaviopsis basicola* (Ramette et al., 2003). In addition, *Pseudomonas* spp. is common rhizosphere organisms and has been shown to be excellent root colonizers (Lugtenberg et al., 2001; Raaijmakers and Weller, 2001).

Among the variety of *Pseudomonas* spp. inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention because of their potential to control seed- and soilborne pathogenic fungi and oomycetes (Keel et al., 1992, 1996; Raaijmakers and Weller, 2001). These biocontrol strains, mostly classified as *P. fluorescens* and *P. putida*, are easy to culture *in vitro* and to manipulate genetically (Whipps, 2001). Their beneficial effects on plant health have been mainly attributed to active exclusion of pathogens from the rhizosphere through the secretion of a diverse array of antimicrobial metabolites (Thomashow and Weller, 1996; Handelsman and Stabb, 1996; Raaijmakers et al., 2002; Haas and Keel, 2003). Important antimicrobial compounds for which a major contribution to biocontrol has been demonstrated are 2,4-diacetylphloroglucinol, pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides, and the volatile hydrogen cyanide (Raaijmakers et al. 2002; Haas and Defago, 2005; Raaijmakers et al. 2006; Loper et al., 2007). In general, several of the effective biocontrol strains described to date

produce at least one of these diffusible or volatile antibiotics. Natural biological suppression to take-all disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* in fields cultivated to wheat was associated with the dominance of indigenous populations of root-colonizing fluorescent pseudomonads producing the antimicrobial metabolite 2,4-diacetylphloroglucinol (Raaijmakers and Weller, 1998; de Souza et al, 2003). Some strains, such as *P. fluorescens* CHA0 and Pf-5, produce multiple antibiotics with overlapping or different degrees of activity against plant pathogens (Paulsen et al., 2005; Bottiglieri and Keel, 2006).

In spite of numerous studies showing promising biocontrol activity in different host-pathogen systems, relatively few *Pseudomonas* strains have made it to the market as a commercial biocontrol product. To be effective in biocontrol of plant pathogens, *Pseudomonas* and other microbial inoculants have to meet several important criteria, including: i. effective and competitive colonization of the host plant (e.g. rhizosphere, spermosphere), ii. Stimulation of plant defence by induced systemic resistance (ISR) and/or systemic acquired resistance (SAR), iii. Direct antagonistic effects on the pathogen e.g., by antibiosis or by inactivation of virulence factors of the pathogen, and IV. Expression and/or production of the antagonistic traits need to occur at the right time and place (Lugtenberg and Bloemberg, 2004). Combining all of these traits into a single strain or mixture of strains is likely to produce a more consistent and effective level of plant protection (Haas and Keel, 2003). In this context, efficient exploitation of these bacteria in agriculture and horticulture requires more fundamental knowledge of traits that enhance their ecological performance (Rainey, 1999).

Most of the studies on PGPR are carried out on fluorescent pseudomonad because : they are common inhabitants of the rhizosphere, are easily isolated from natural environments, they have simple nutritional requirements and utilize a wide range of substrates, and are easy to culture and manipulate genetically, making them more amenable to experimentations. These organisms are ideally suited as inoculants also, because they can aggressively colonize the roots.

1.5 PGPR TRAITS OF FLUORESCENT PSEUDOMONADS

1.5.1 Siderophore production:

Iron is fourth most abundant element in Earth's crust, but it is largely non-available to life forms including microbes and plants. It occurs in ferric form (Fe^{3+}), which is sparingly soluble at pH

7.4, at concentrations of about 10^{-18} M. This is insufficient to support the growth of microorganisms as they require concentrations approaching 10^{-6} M, so availability of iron for microbial assimilation in rhizosphere is extremely limiting. Consequently, to survive in such environments, organisms secrete iron-binding ligands siderophores, which have been defined as "*low-molecular-mass, virtually ferric specific ligands, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell*" (O' Sullivan, 1992). The proposed mechanism for siderophore-mediated disease suppression by fluorescent pseudomonads is illustrated in Fig 1.4.

Under certain conditions, siderophores can function as a diffusible bacteriostatic or fungistatic antibiotic (Haas and Defago, 2005). De Boer et al., 2003) found that the role of siderophores was associated with the antagonistic properties of *P. putida* WCS358 in suppressing fusarium wilt of radish. Even though, various bacterial siderophores differ in their abilities to sequester iron, generally they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity. Kloepper et al., 1980 clearly demonstrated the inhibitory potential of pyoverdinin (Pvd)-producing *Pseudomonas* spp. towards bacteria and fungi with less potent siderophores in an iron-depleted medium. Although several authors have demonstrated the contribution of siderophores to disease suppression in some situations; it is believed that siderophores alone are not sufficient to account for suppression; if they were, it would be difficult to explain why most strains which produce siderophores, do not have biocontrol activity (Haas and Defago, 2005). Some siderophores are also good chelators of elements other than iron. For example, pyochelin is a good Cu^{2+} and Zn^{2+} chelator (Haas and Defago, 2005). Consequently, siderophores may directly stimulate the production of other anti-microbial compounds, when these elements are increasingly made available to the bacteria (Duffy and Defago, 1999).

1.5.2 Phosphate solubilization:

After nitrogen (N), phosphorus (P) is the most important macro-element required by both plants and microorganisms. P is found in soil, plants and in micro organisms in a number of organic and inorganic compounds. Although P is abundant in soils in both inorganic (originating mainly from applied P fertilizer) and organic forms (derived from microorganisms, animals and plants), it is still one of the major plant growth limiting nutrients. On average, most nutrients in the soil solution are available in millimolar amounts, but P is available only in micromolar or lesser quantities (Ozanne, 1980). The low availability of P to plants is because majority of soil P is

found in insoluble forms. Most of the P is found in form of Calcium, Iron or Aluminum phosphates (Ca-P, Fe-P, or Al-P). Plant roots can only absorb P in two soluble forms, the monobasic (H_2PO_4^-) and the dibasic (HPO_4^{2-}) ions (Vessey, 2003). To circumvent the problem, chemical phosphate fertilizers are used. However, due to its high reactivity, almost 75-90% of added P fertilizer is precipitated by Fe, Al and Ca complexes present in the soils, creating a demand for suitable alternatives to mobilize this fixed fraction of the important bioelement (Stevenson, 1986).

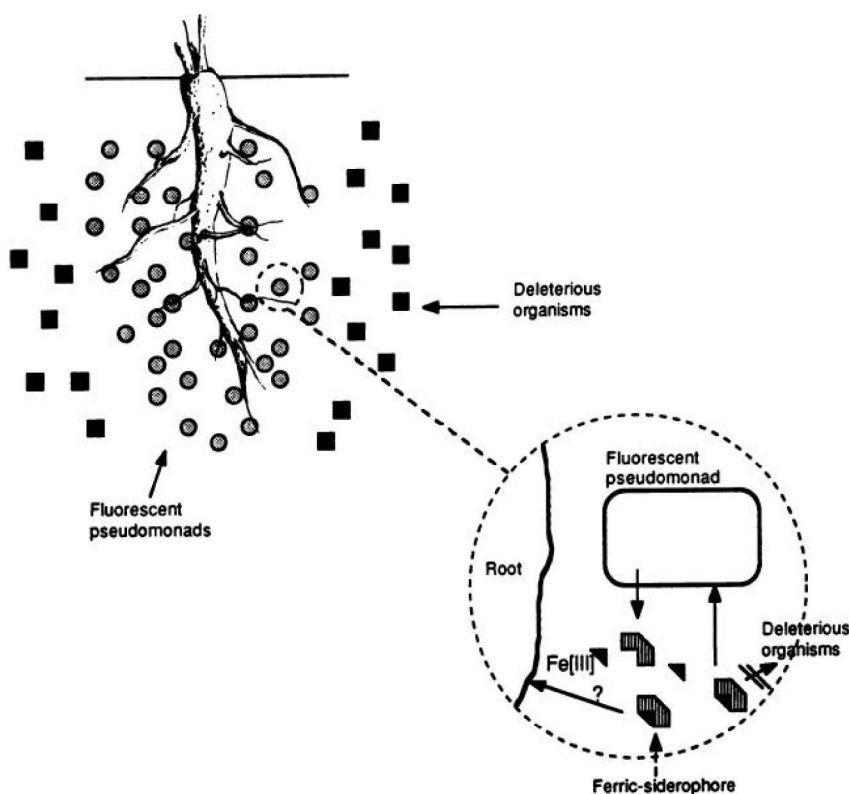


Fig. 1.4 Model for suppression of root pathogens by siderophores of fluorescent pseudomonad (O'Sullivan, 1992).

Indian soils contain about 0.01 to 0.2% of P which is not readily available to plants (Gaur et al, 1973). Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability (Nautiyal, 1999).

Phosphate solubilizing microorganisms (PSMs) including bacteria and fungi, mobilize Ca-P, Fe-P and Al-P complexes (Kucey et al., 1989) and thus help in increasing the availability of fixed phosphates for plant growth. Phosphate solubilizing bacteria (PSB) are common in the

rhizosphere and secretion of organic acids and phosphatase enzyme are common method of facilitating the conversion of insoluble forms of P to plant-available forms. The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants (Vessey, 2003). Phosphate solubilizing micro organisms mainly belong to *Bacillus* and *Pseudomonas* among the bacteria and to *Penicillium*, *Aspergillus* among the fungi.

Several *Pseudomonas* isolates are able to solubilize sparingly soluble inorganic and organic phosphates (Chabot et al., 1993). The fluorescent *Pseudomonas* strains exhibited marked phytase activity and liberated up to 81% of P from inositol hexaphosphate, an organic source of P. 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).

1.5.3 Lytic enzyme production:

Mycoparasitism is a process initiated by physical destruction of the fungal cell wall mediated by the action of hydrolytic enzymes produced by a biocontrol agent. Chitin and β -1, 3-glucan are the two major structural components of most plant pathogenic fungi. Antagonists invade pathogens by excretion of extracellular enzymes that can lyse pathogen cell walls or cause degradation of chlamydospores, oospores, conidia, sporangia, and zoospores. Such extracellular enzymes include chitinases, cellulases, proteases and β -1, 3-glucanases. Dunne et al., 2000 showed that overproduction of extracellular protease in the mutant strains of *Stenotrophomonas maltophilia* W81 resulted in improved biocontrol of *Pythium ultimum* (Mavrodi et al, 2012). Excretion of chitinases and glucanases by species of *Trichoderma* and *Streptomyces* has also been shown to play an important role in mycoparasitism of phytopathogenic fungi (Whipps, 2001).

1.5.4 Induction of plant resistance mechanisms:

Expression of natural defense reaction against stresses from biotic or abiotic origin is exhibited by all plants, such as (i) physical stresses (heat or frost), (ii) inoculation by pathogenic or non-pathogenic organisms, (iii) chemical molecules from natural or synthetic origins (Alabouvette et al., 2006). Early recognition of the aggressor by the plant is one of the mechanisms involved in elicitation of plant defense reactions (Lugtenberg et al., 2002). Recognition of the aggressor

immediately initiates a cascade of molecular signals and the transcription of many genes, which eventually results in the production of defence molecules by the plant. Such defence molecules include phytoalexins, pathogenesis-related (PR) proteins (such as chitinases, β -1, 3-glucanases, proteinase inhibitors etc.) and reinforcement of cell walls (Whipps, 2001). Cell wall thickenings, wall appositions or rapid death of the injured plant cells resulting in necrosis of the immediate adjacent tissues are barriers which cut the pathogen off its nutrients and contribute to slowing down of the fungus progressive invasion (Lugtenberg et al., 2002; Alabouvette et al., 2006). A virulent pathogen inhibits resistance reactions, or circumvents the effects of active defenses. As a result of these natural defense mechanisms, plants are able to produce an immune response after a primary pathogen infection known as systemic acquired resistance (SAR). The host plant can also benefit directly from non pathogenic rhizobacteria and fungi through the production of metabolites that trigger the induction of systemic resistance (ISR) that is phenotypically similar to SAR (van Loon et al. 1998). SAR is a pathogen-induced resistance which requires accumulation of salicylic acid while ISR is a rhizobacteria-induced type that depends on responses to ethylene and jasmonic acid. A variety of soil and rhizosphere bacteria and fungal isolates can provide protection against viral, fungal, and bacterial plant pathogens by turning on ISR in plants (van Loon et al., 1998; Whipps, 2001). However, rhizobacteria differ in their ability to turn on ISR, some are active on particular plants and not on the other (Whipps, 2001).

1.5.5 Phytohormone production:

Indole acetic acid (IAA) production is a wide-spread phenotype among bacteria that inhabit the rhizosphere of plants. It has been reported that up to 80% bacteria isolated from rhizosphere can produce IAA. Diverse soil microorganisms including bacteria, fungi and algae are capable of producing IAA which exerts pronounced effects on plant growth. Early observations on the beneficial effect of seeds bacterization by IAA producing bacteria were first made with *Pseudomonas* spp. isolates, on root crops. By treating potato (*Solanum tuberosum* L.) seed pieces with suspensions of strains of *P. 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). Production of IAA by *P. 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 also produces ACC-deaminase (Jacobson et al., 1994), which degrades ACC, thus preventing plant production of inhibitory levels of ethylene.

1.5.6 Efficient Root colonization:

A good biocontrol agent should grow and persist or “colonize” the surface of the plant it protects (Weller, 1983). Along root surfaces there are various suitable nutrient rich niches attracting a great diversity of microorganisms, including phytopathogens. Competition for these nutrients and niches is a fundamental mechanism by which PGPR aggressively colonize plant roots (Kloepper, 1978). It has been shown that a mutant of *P. chlororaphis* PCL1391, impaired in colonization traits only, was no longer able to protect tomato from foot and root rot caused by *Fusarium oxysporum*. Thus, effective root colonization is a prerequisite to efficiency in the plant growth improvement by other traits including disease control by the production of antifungal metabolites, phytostimulation by production of phytohormones or biofertilization, by which microbes increase nutrient availability to plants.

1.5.7 Antibiosis:

It refers to the inhibition or destruction of the pathogen by the metabolic products produced during growth of the antagonist. These products may include volatile compounds, toxic compounds and antibiotics, which are deleterious to the growth or metabolic activities of other microorganisms at low concentrations. A number of disease-suppressive antibiotic compounds have been characterized chemically and include N-containing heterocyclic compounds such as phenazines, pyrrol-type antibiotics, pyo-compounds, and indole derivatives. Among the antimicrobial compounds released by plant-beneficial pseudomonads, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), phenazines, hydrogen cyanide (HCN) (Fig 1.5), have received

particular attention for their major contribution in biocontrol of root diseases that are caused by agronomically important fungal and oomycete pathogens including *Gaeumannomyces*, *Rhizoctonia*, *Thielaviopsis*, *Fusarium*, and *Pythium* species (Raaijmakers et al., 2006, Mavrodi et al, 2012). Most effective biocontrol pseudomonads produce at least one of the above-mentioned antibiotics and some strains, e.g. *P. fluorescens* strains CHA0 and Pf-5, produce multiple antibiotics. In general, these antibiotics have broad-spectrum toxic activity against fungi, bacteria, protozoa, nematodes, and sometimes also against plants or even viruses (Raaijmakers et al. 2002, 2006). Some of the antibiotic metabolites have remarkably diverse functions, besides their antifungal activity. DAPG, PLT, and phenazines can function as signal molecules that affect gene expression not only in the producer bacteria, but also in other organisms (Dubais & Keel, 2007) (Fig.1.6). DAPG has been described as an inducer of systemic plant resistance and as a stimulant of amino acid exudation from roots. Phenazines, in their reduced form, might enable the producing bacteria to mobilize micronutrients such as iron (Fe^{3+}) from the rhizosphere environment (Dubais & Keel, 2007). Table 1.1 gives the antibiotics produced by different strains of pseudomonas and their spectrum of action.

1.5.7.1 2, 4-Diacetylphloroglucinol (2, 4-DAPG)

2, 4-Diacetylphloroglucinol (2, 4-DAPG) is a polyketide compound which has received particular attention because of its broad-spectrum antiviral, antifungal, antibacterial, and antitumor activity and phytotoxic properties (Raaijmaker et al., 2002; Haas and Keel, 2003; Isnansetyo et al., 2003). Even in medical area, there has been increasing interest on the use of 2, 4-DAPG, due to its recently reported bacteriolytic activity against multidrug-resistant *Staphylococcus aureus* (Isnansetyo et al., 2003). 2, 4-DAPG is synthesized by several plant-associated fluorescent pseudomonads, and it plays a key role in the suppression of a wide variety of soil-borne diseases (Weller et al., 2002; de Souza et al., 2003; Haas and Defago, 2005; Ramette et al., 2006; Kang, 2012). 2, 4-DAPG inhibits zoospores of *Pythium* spp. and also damages the membrane of this Oomycetes fungus (de Souza, 2003,). 2,4-DAPG-producing pseudomonad are widespread worldwide and are commonly found in the rhizosphere of important crops such as maize, pea, and wheat (Picard, 2000; Raaijmakers and Weller, 2001; Landa et al. 2002; Bergsma-Vlami et al., 2005); they have also been shown as important biological components of the natural suppressiveness of certain agricultural soils to take-all disease of wheat (de Souza et al., 2003;

Weller et al., 2002), *Fusarium* wilt of pea (Landa et al., 2002) and black root of tobacco (Ramette et al., 2006).

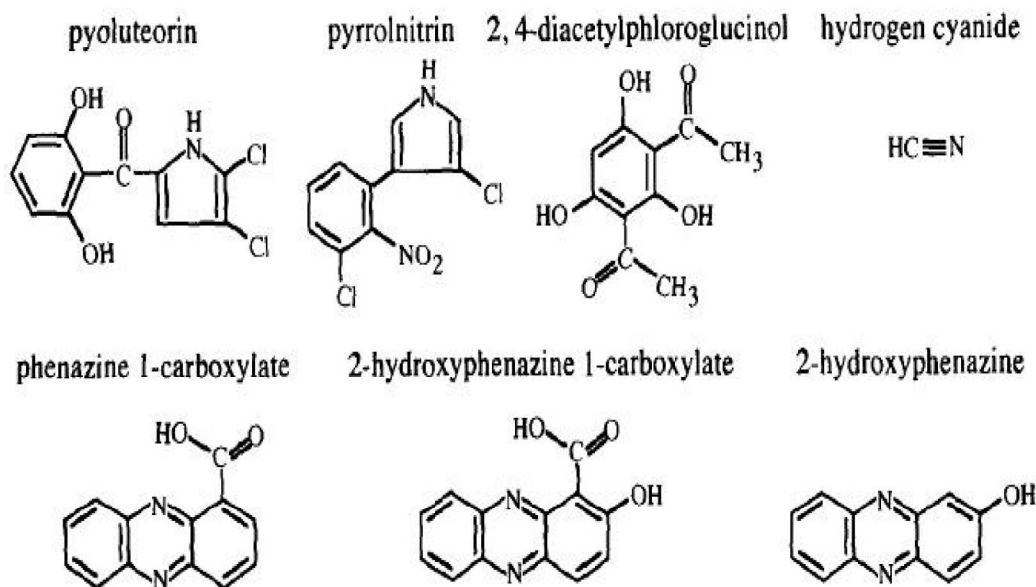


Fig. 1.5 Chemical structures of antifungal metabolites produced by fluorescent pseudomonads (Handel man, 1996)

1.5.7.2. Pyoluteorin (PLT)

Pyoluteorin (PLT) is a chlorinated polyketide antibiotic (Fig 1.5). Its production by several *Pseudomonas* species and its inhibitory activity against oomycetes fungi, including the plant pathogenic *Pythium ultimum* has been documented. For instance, the biocontrol agents, *P. fluorescens* Pf-5 and *P. fluorescens* CHA0 suppressed *Py.Ultimum*-incited diseases through production of PLT, in addition to some other antibiotics (Bender et al., 1999). Apart from its established extracellular role as an antibiotic, PLT was also shown to function as an autoinducer and intercellular signal molecule which influences the spectrum of secondary metabolites produced by distinct populations of bacterial cells co inhabiting the rhizosphere. This was clearly demonstrated by Brodhagen et al., 2004 where PLT produced by co-inoculated *P. fluorescens* Pf-5 cells improved the expression of a PLT biosynthesis gene in a PLT-deficient mutant of *P. fluorescens* Pf-5.

1.5.7.3. Pyrrolnitrin:

Pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl) pyrrole] (PRN) is a broadspectrum antifungal metabolite(Fig 1.5) produced by strains of *Enterobacter agglomerans*, *Myxococcus fulvus*,

Corallococcus exiguous, *Cystobacter ferrugineus*, *Serratia* spp. and several strains of *Pseudomonas* and *Burkholderia* (Hammer et al., 1999). The antibiotic was first isolated from *Pseudomonas pyrocinia* (Hammer et al., 1999). PRN functions by inhibiting fungal respiratory chain (Tripathi et al., 1969). This highly active metabolite has been used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, and a phenyl pyrrol derivative of PRN as an agricultural fungicide (Dwivedi and Johri, 2003). Similarly in biocontrol studies, the role of PRN in suppression of plant pathogens has been demonstrated by many investigators. Mutant strains of *P. fluorescens* BL915 deficient in PRN production had a greatly reduced ability to control *R. solani* seedling disease of cotton. Also transfer of a gene region that has a role in PRN synthesis derived from *P. fluorescens* BL915 into two *Pseudomonas* strains that were non-PRN producers nor effective antagonists of *R. solani*, conferred on the strains the ability to produce PRN and to antagonize *R. solani* both in vitro and in vivo disease control assays with cotton (Hill et al., 1994).

1.5.7.4 Phenazines (PHZ)

Phenazines (PHZ) are also antibiotics with broad-spectrum activity and they comprise a large family of over 100 compounds tricyclic ring nitrogen-containing brightly colored pigments (Fig 1.5) (Chin-A-Woeng et al., 2000; Kavitha et al., 2005). Currently, more than 50 naturally occurring PHZ compounds have been described and are exclusively produced by bacteria, such as *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacteriu*, *Pantoea* and *Burkholderia* species (Turner and Messenger, 1986). PHZ and its derivatives have been implicated in virulence, competitive fitness of the producing strains, and they are well-known for their antifungal properties (Mazzola et al., 2002; Dwivedi and Johri, 2003). Production of PHZ derivatives by a considerable number of *Pseudomonas* strains with antagonistic activity has been reported; most often is the production of pyocyanin, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) (Turner and Messenger, 1986; Chin-A-Woeng et al., 2000; Haas and Defago, 2005).

Table 1.1 Antifungal spectrum of antibiotics produced by pseudomonads

(Dwivedi & Johri, 2003)

Antifungal metabolites	Target Pathogen	Target Disease	Producer strains
2,4- DAPG	<i>G. graminis tritici</i>	Take all disease	<i>P. fluorescens CHA0</i>
	<i>Pythium ultimum</i>	Damping off of sugarbeet	<i>P. fluorescens Q2-87</i> <i>P. fluorescens F113</i>
	<i>Rhizoctonia solani</i>	Sheath blight	<i>P. fluorescens Pf-5</i>
	<i>Thielaviopsis basicola</i>	Black rot of tobacco	<i>P. fluorescens CHA0</i>
Pyrrolnitrin	Dermatophytic fungi	Skin mycoses	<i>Fluorescent and non fluorescent pseudomonads</i>
	<i>Bipolaris maydis</i>	Southern maize leaf blight	<i>P. cepacia</i>
	<i>Sclerotinia homoeocarpa</i>	Dollar spot of turf grass	<i>P. fluorescens Pf-5</i>
	<i>Drechslera poae</i>	Spring disease of Kentucky bluegrass	<i>P. fluorescens Pf-5</i>
Pyoluteorin	Members of oomycetes, especially <i>Pythium</i>	Damping - Off	<i>P. fluorescens Pf-5</i>
Phenazines	Species of bacteria and <i>G. Graminis tritici</i>	Take all disease	<i>P. fluorescens 2-79</i> <i>P. aureofaciens 30-84</i>

Production of PCA in *P. fluorescens* strain 2-79 and PCN in *P. chlororaphis* strain PCL1391 was reported as the major mechanism of suppression to take-all disease of wheat and tomato root rot, respectively (Chin-A-Woeng et al., 1998). Biosynthesis of more than one PHZ derivatives can occur simultaneously in many strains. For instance, simultaneous production of pyocyanin, PCA and PCN was found in *Pseudomonas aeruginosa* strains (Chang and Blackwood, 1969). The growth conditions influence the number and type of PHZ synthesized by an individual bacterium (Dwivedi and Johri, 2003). PHZ are redox active compounds, and thus, the mechanism for their action is assumed to be due to their ability to engage in redox cycling in the presence of various

reducing agents and molecular oxygen, resulting in the accumulation of toxic superoxide ions, hydrogen peroxide (H_2O_2) which are harmful to the cell or can lead to the death of the cell (Mavrodi et al., 2012).

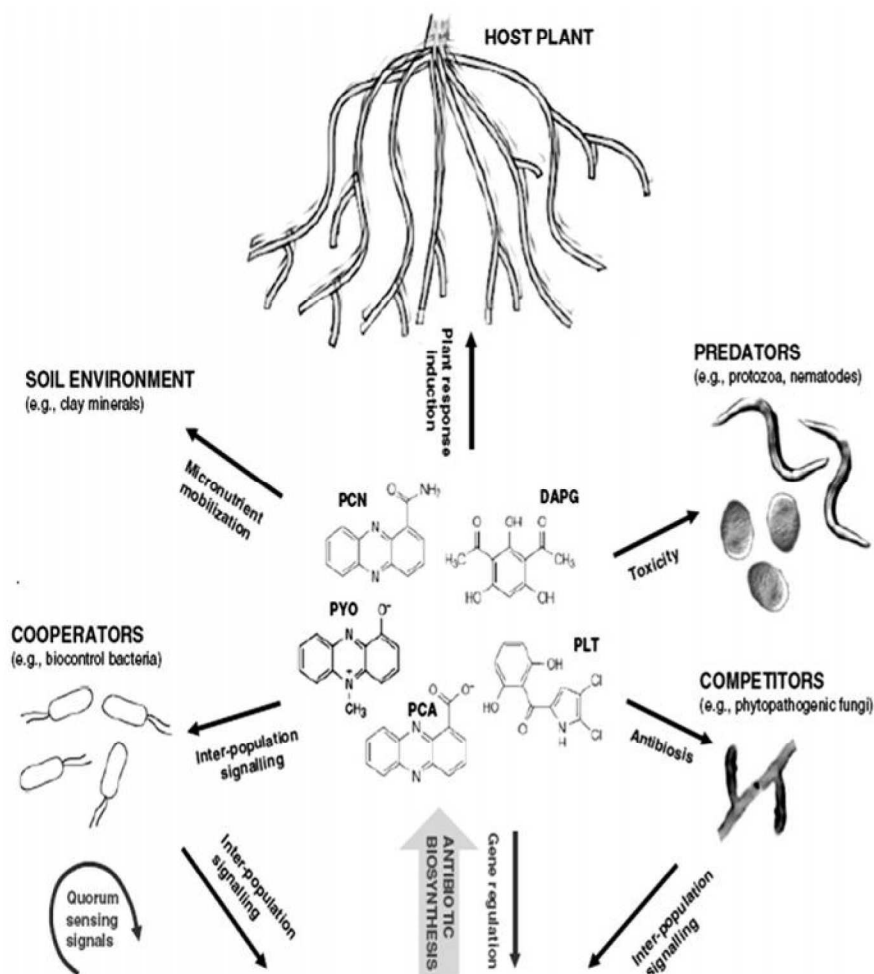


Fig.1.6 Overview of interactions between biocontrol strains, plants, pathogens, predators, cooperators and soil (Dubais & Keel, 2007)

1.6 SCOPE OF THE THESIS:

One of the main problems of PGPR inoculants in practice is that the applied microorganisms often fail to survive, or do not execute their specific function in soil environment. The positive effect of a PGPR strain to one soil plant environment does not guarantee its success in another soil sample or host plant genotype. Similar problems were reported for hydroponic and soilless systems of plant growth. These issues have been raised in many reviews and it has been suggested

to isolate the bio-control strains from the soil types and actual ecological niches, where it has to be applied for the commercialization.

The work presented in Chapter 2 deals with the isolation of fluorescent pseudomonad from Indian soil samples and crop types for the selection of strains that are well adapted and possess ecological fitness to Indian edaphic factors, plant types, seasonal variation and environmental fluctuations. The isolation method was designed in such way that it would be specific, stringent and non-laborious. Isolated strains were characterized for the presence of effective PGPR traits (e.g. siderophore production, antifungal activity, HCN production, ACC deaminase activity, P-solubilization ability, IAA production) required for their survival and competency in the rhizosphere.

Although ability of fluorescent pseudomonads to reduce severe diseases caused by soil borne fungal pathogens under laboratory conditions has been reported in several studies, but their inconsistent performance in field trials tends to be disappointing (Thomashow, 1997). Understanding the sources of variability is key to overcoming this obstacle. Because a primary mechanism of disease suppression available to fluorescent pseudomonad is antibiosis, it is thought that variable performance might result from variation in production of antimicrobial compounds like DAPG production. Much information about the mechanism and factors affecting the action of DAPG is available, but a more insight is needed about the ecological interactions taking place in the soil and root environments, which might influence production of DAPG. This will help to customize the biocontrol strains for use in particular environments, we can understand how to prepare the inoculums for optimal performance, the environment can be modified to be more favorable to strains, or strains could be constructed that are independent of environmental signals. Therefore the aim in Chapter 3 was to study the production and regulation of antibiotics in isolated fluorescent *Pseudomonas* isolates, which involved the detection of antibiotic biosynthesis genes, their genetic diversity, levels of different antibiotics and regulation of 2, 4-DAPG biosynthesis under different carbon sources and different concentrations of other nutrients. The effect of carbon sources on DAPG production by *phlD*⁺ strains was determined by High Pressure Liquid Chromatography (HPLC). To study the genotypic diversity among antibiotic-producing *Pseudomonas* species provides an enormous resource for identifying strains that are highly rhizosphere competent and superior in biological control of plant diseases.

Ecological interactions between rhizobia and other soil bacteria have been of interest in recent years because of their agronomical implications. As rhizobia and PGPR share common microhabitat in the root–soil interface, they must interact during their processes of root colonization. Although most of the times plant growth-promoting rhizobacteria may interact synergistically with root modulating rhizobia but some studies have also shown that there may be either no significant effect or deleterious effects of co inoculation of rhizobia and fluorescent pseudomonad on the plant in terms of their nodule number and other important aspects. It is already reported that the fluorescent *Pseudomonas* strains promote plant growth by suppressing plant pathogenic microorganisms through production of antibiotics but the action of these antibiotics is most often non-specific and thus may also harm the other plant beneficial microorganisms in the vicinity. Therefore the aim in Chapter 4 was to study of the inhibitory effect of antibiotics/antifungal metabolites of fluorescent *Pseudomonas* on rhizobia and interactions of fluorescent *Pseudomonas* and rhizobia under biocontrol supporting nutritional compositions in the presence/absence of fungal phytopathogen.

The objectives of Chapter 5 were to study the effect of the characterized strains in plant inoculation assays for plant growth promotion, plant assay for protection of mung bean from fungal phytopathogen *R. bataticola*. Plant assays was performed for the co-inoculation study of selected bio-control and PGPR strain of fluorescent pseudomonad and rhizobia to assess the bio-control potential of fluorescent pseudomonad strains toward *R. bataticola* under certain nutrient combinations.

The overall goal of this thesis project was to isolate the fluorescent pseudomonad from Indian soil and crop types and to study their antibiotic productions profiles especially with respect to the regulation of their biosynthesis vis-a-vis that of well-studied model strains e.g. *Pseudomonas fluorescens* CHA0, Pf-5 and Q-2 87. For e.g. 2, 4- DAPG biosynthesis ability under sucrose and at high inorganic phosphate (Pi) levels which are believed to inhibitory to 2, 4- DAPG biosynthesis in model strains. Further the study of the inhibitory effect of antibiotics/antifungal metabolites of fluorescent *Pseudomonas* on rhizobia, interactions of fluorescent *Pseudomonas* and rhizobia under biocontrol supporting nutritional compositions in the presence/absence of fungal phytopathogen and final confirmation by plant protection assay for protection of *Vigna radiata* from fungal diseases by fluorescent pseudomonas isolates was performed. Plant inoculation assays for the co-inoculation of selected bio-control and PGPR strain of fluorescent

Pseudomonas with *Rhizobium* spp was executed to assess the bio-control potential of fluorescent *Pseudomonas* strains toward *R. bataticola* under certain biocontrol supportive nutrient compositions. Ecological performance of antibiotic-producing *Pseudomonas* strains is an important aspect of biocontrol research and this work was aimed to contribute to the improvement of their rhizosphere competence, survival and biocontrol efficacy of biocontrol strains.

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