academicJournals

Vol. 8(15), pp. 1544-1561, 9 April, 2014
DOI: 10.5897/AJMR2013.6573
Article Number: FB69E5243798
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Fluorescent pseudomonads: Milestones achieved in the last two decades

K. Sarker[1,2]*, S. Dutta[2] and P. K. Das Mohapatra[1]

[1]Department of Microbiology, Vidyasagar University, Midnapore-721102, India.
[2]AICRP on Vegetable Crops, Directorate of Research, B.C.K.V., Kalyani-741235, Nadia, India.

Received 20 December 2013; Accepted 17 March, 2014

Environmental concerns have led to the need of sustainable use of natural resources. The conventional agriculture practice caused considerable pollution and unavoidable impacts on soil, water, animal as well as human health. Fluorescent pseudomonads, a group of root-associated bacteria that can colonize the roots of crop plants and produce antifungal metabolites represent a real alternative to the application of chemical fungicides. Fluorescent pseudomonads have been studied for decades for their plant growth-promoting effects through effective suppression of soil borne plant diseases. During root colonization, these bacteria produce antifungal antibiotics that can indirectly suppress fungal pathogens by scavenging iron in the rhizosphere environment through the release of siderophores, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors. Before engaging in these activities, the bacteria go through several regulatory processes at the transcriptional and post-transcriptional levels. Current genomic analyses of rhizosphere competence and biocontrol traits of fluorescent pseudomonads will likely lead to the development of novel tools for effective management of deleterious phytopathogens and a better exploitation of their plant-beneficial properties for sustainable agriculture. This review addresses the main findings on fluorescent pseudomonads for the last two decades. It summarizes and discusses significant aspects of this general topic, including taxonomic status, genetic diversity of fluorescent pseudomonads in India, different mechanisms of biological control and commercial use of fluorescent pseudomonads in agriculture.

Key words: Fluorescent pseudomonads, biological control agent, secondary metabolites, induction of systemic resistance, post-transcriptional regulation.

INTRODUCTION

This topic specifically will focus on fluorescent pseudomonads because they are present in many environments, especially in the plant rhizosphere. Many studies involve these bacteria that are able to improve plant growth and plant health and are implicated in the natural supressiveness of certain soils to many soil-borne

*Corresponding author. E-mail: subrata_mithu@yahoo.co.in or kanish.sarker@gmail.com.

diseases whereas others participate in the biodegradation of natural and man-made toxic chemical compounds. The γ-subclass of the Proteobacteria includes fluorescent pseudomonads (FLPs) along with several non-fluorescent species. The fluorescent *Pseudomonas* group includes: (1) phytopathogenic cytochrome *c* oxidase-positive species, viz. *Pseudomonas cichorii*, *Pseudomonas marginalis* and *Pseudomonas tolaasii*, (2) non-phytopathogenic, nonnecrogenic strains, viz. *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens* and *P. Pseudomonas aeruginosa* type species and (3) phytopathogenic necrogenic fluorescent *Pseudomonas* spp. without cytochrome *c* oxidase, viz. *Pseudomonas syringae* and *Pseudomonas viridiflava*. The non-fluorescent *Pseudomonas* group includes *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes* and *Pseudomonas seudoalcaligenes* (Palleroni, 1993). Phenotypic characterization has been employed to cluster and identify bacteria according to several features, viz. morphology, pigmentation and reaction to dyes and nutritional requirements. It was observed that *P. fluorescens* and *P. putida* are very heterogeneous. *P. putida* was subdivided into biotypes A and B, which became biovars A and B. *P. fluorescens* was also subdivided into 7 biotypes. The biotypes A, B, C, D and F were then called biovars (bv. I to V) and biotypes D and E became the *P. chlororophis* and *P. aureofaciens*, respectively which were then clustered as the *P. chlororaphis* (Jonhson and Palleroni, 1989). *P. fluorescens* bv. V includes strains that often cannot be classified because properties that are essential for the differentiation from other biovars are not identified. This system of multiple biovars reveals a high phenotypic heterogeneity, and probably reflects high genomic diversity. Phenotypic studies revealed this high variability with these two species, which resulted in subdivision in these biovars and sub-grouped of each one (Grimont et al., 1996; Janse et al., 1992; Latour et al., 1996; Lemanceau et al., 1995; Sorensen et al., 1991). To elucidate the real taxonomic condition of these subgroups it is important to characterize genotypically the species and biovars. DNA-rRNA hybridization studies led to the delineation of the genus *Pseudomonas* into five homology groups (Palleroni et al*.,* 1973). The genus *Pseudomonas sensu stricto* corresponds to the Palleroni *sensu* rRNA homology group I and contains all the fluorescent species (Palleroni, 1993). Different studies show very high genomic variability within biovars of *P. fluorescens* and *P. putida* and probably some biovars correspond to undescribed species (Champion et al*.,* 1980; Palleroni et al*.,* 1972). Bossis et al*.* (2000) also got similar results. The level of similarity of rhizosphere isolates identified as *P. fluorescens* or *P. putida,* by the phenotypic criteria of Palleroni was never greater than 55% as compared to the type strains of *P. fluorescens* and *P. putida* (Bossis et al*.,* 2000). Future studies on the ecology of fluorescent pseudomonads require reliable, fast and cheap methods to identify large numbers of isolates. The description of new species within the distinct species *P. fluorescens* and *P. putida* remains to be done in order to elucidate the taxonomic status of this group.

The current state of the pseudomonas taxonomy makes it difficult to assess the phylogenetic distribution of biocontrol agents within *P. fluorescens* and closely-related fluorescent pseudomonads (Bossis et al., 2000). However, it seems clear that these taxa include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. fluorescens* or also related fluorescent pseudomonads are considered (Sanguin et al*.,* 2008). It is important to note that *P. fluorescens* and neighbouring species are thought to include also strains with human pathogenicity potential (Wei et al., 2002; Bodilis et al., 2004), but the evidence to date is not fully convincing in the current taxonomic context and this issue deserves further clarification. *P. fluorescens* and closely-related fluorescent pseudomonads appear to be predominantly clonal (Frapolli et al., 2007). Yet, horizontal gene transfer may take place and such a possibility has been raised for genes involved in the interaction with the plant and/or phytopathogens (Ramette et al., 2003; Blaha et al., 2006). This includes also the hypothesis that genes involved in the synthesis of biocontrol compounds might have been acquired from the plant itself (Cook et al., 1995; Ramette et al., 2001).

GENETIC CHARACTERIZATION OF FLUORESCENT PSEUDOMONADS

The nucleotide sequences of various genes, especially those of small-subunit (SSU) rRNA have been widely used to identify and classify microorganisms (Woese, 1987). The study of SSU rRNA sequences resulted in many findings, such as the *Archaea*, a group of prokaryotes that is separated from *Bacteria*. This gene is most widely used. Its sequence is a mosaic of highly conserved regions interspersed with variable and hypervariable stretches that makes it convenient for PCR primer design (Gürtler and Stanisich, 1996). The 16S rRNA gene from *Pseudomonas* spp. contains 1492 nucleotide positions, of which 148 are variable and 65 positions of these are within three hypervariable regions (Moore et al*.,* 1996). The "*Pseudomonas* hypervariable (hv) regions" were defined as: hv 1, *Escherichia coli* 16S rRNA gene sequence positions 71- 95; hv 2, *E. coli* 16S rRNA gene sequence positions 455 - 475; and hv 3. *E.*

coli 16S rRNA gene sequence positions 998-1043 (Godfrey and Marshall, 2002; Moore et al., 1996). These positions are located, respectively, within the regions V1: helix 6, V3: helix 18 and V6: helices P35-1 and P35-2 (Godfrey and Marshall, 2002; Neefs et al., 1990). The regions hv1 is considered one of the most variable sequences in 16S rRNAs of bacteria across the phylogenetic spectrum (Gutell et al*.,* 1985; Woese, 1987) and is useful for differing intrageneric lineages and discerning the type strains of some species of *Pseudomonas* (Moore et al., 1996). Godfrey and Marshall (2002) used primers based in these regions to study the diversity of pseudomonads isolates from different parts of the world. The large database of 16S rRNA sequences is important for the analysis of environmental isolates and recognition of new sources of diversity (Moore et al*.,* 1996). Nevertheless, there are some pitfalls in the use of rDNA for studies of biodiversity (García-Martínez et al*.,* 1999). The gene size is constant and consequently different genes could not be easily separated by size. In addition, the 16S genes have hypervariable and extremely informative regions. They are often not divergent enough to separate in close relationships (García-Martínez et al., 1999; Normand et al., 1996). Other techniques are utilized to solve these limitations for characterizing isolates from the environment. The region located between the 16S and 23S (ITS1) rRNA genes is very variable in size and sequence even within closely related taxonomic groups (Gürtler and Stanisich, 1996). Size pattern can characterize different communities of *Eubacteria* or *Archaea* and the widely divergent sequence allows the detection of species-like units very precisely by PCR, oligo-probes or long DNA probe hybridization (García-Martínez et al*.,* 1999; Jensen et al*.,* 1993). Locatelli et al. (2002) designed a set of primers specific for *Pseudomonas* that allowed the amplification of ITS1 together with a significant part of 16S rDNA. The primers were conserved for all the *Pseudomonas* sequences tested and they showed specificity and efficiency for the amplification of 1100 to 1300 bp fragment to the *Pseudomonas* spp. Interspersed repetitive DNA sequence elements are present in prokaryote genomes and can be used as primers sites for genomic DNA amplification (Versalovic et al., 1991). Three families of repetitive sequences have been studied in most detail, including the 35 - 40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp, enterobacterial repetitive intergenic consensus (ERIC) sequence and the 154 bp BOX element comprised of three subunits (boxA, boxB and boxC) (Lupski and Weinstock, 1992). These sequences are located in distinct, intergenic positions all around the chromosome. Louws et al. (1994) demonstrated that REP, ERIC and BOX-PCR, referred to as rep-PCR collectively, were useful for the rapid molecular characterization of plant pathogenic bacteria, like *Xanthomonas* and *Pseudomonas*, especially at the pathovar level.

Many other approaches are used to analyze the diversity of the pseudomonads. Delorme et al. (2003) studied the membrane-bound nitrate reductase (*narG*) and nitrous oxide reductase (*nosZ*) genes diversity, implicated in the denitrification process. They compared the similarity indexes of the *narG* and *nosZ* genes in different strains and the genes led to the identification of two different groups of strains. The first group presented similarity between the genes suggesting similar evolutionary pathway. The second group, on the other hand showed higher diversity of the *nosZ* gene as compared to the *narG* gene, suggesting different evolutionary rates.

REPORTS OF GENETIC DIVERSITY OF FLUORESCENT PSEUDOMONADS FROM INDIA

The rhizosphere community structure of wheat and the influence of genotype on community structure have been studied extensively for the Indo-Gangetic region (Mittal and Johri, 2008). It was observed that the wheat genotype did not appreciably influence the total and pseudomonad populations. Population structure was only marginally different in the rhizosphere (RS) and rhizoplane (RP) fractions, which could be explained on the basis of a wheat genotype-dependent influence. Analysis of cultivable genetic diversity by employing ARDRA and rep- PCR showed that for any single variety, the distribution of various bacterial morphotypes was fairly even, although the RP fraction was generally more diverse than the RS fraction. Diversity indices showed var. UP2338 to be the richest (E), whereas var. HD2627 was most diverse (H′). Numerical analysis of phenotypic characters revealed that most of the isolates exhibiting greater similarity with *Pseudomonas* reference strains belonged to var. UP2338; this was later confirmed by 16S rDNA sequencing. Sequencing data also revealed that among γ -proteobacteria, pseudomonads were most prominent along with *Pseudoxanthomonas* and *Stenotrophomonas* (Mittal and Johri, 2008). In another study, the genetic diversity of plant growth promoting rhizobaterial fluorescent pseudomonads of sugarcane cultivated in Vagaikulam around Madurai, India has also been extensively studied. They showed using the 16s rDNA sequence similarity of the isolates that, they belonged to *Pseudomonas plecoglossicida, P. fluorescens, P. libaniensis* and *P. aeruginosa*. Further differentiation of the isolates was done through different genomic DNA finger printing techniques and it was observed that *Pseudomonas plecoglossicida* is a dominant species (Neelamegam et al*.,* 2012). Recently, *rpoB*-RFLP is becoming an emerging tool for determining

diversity indices of fluorescent pseudomonads. In a recent study with 543 isolates, collected from different parts of northern and eastern Indo-Gangetic plains showed that 26 different clusters were formed from 16S rDNA-RFLP whereas 27 clusters were generated by the *rpoB*-RFLP with similarity percent ranging from 3 to 100%. 16S rDNA sequencing showed 9 different species of *Pseudomonas,* whereas, *rpoB* sequencing showed 13 different species of *Pseudomonas*. Phylogenetic analysis based on 16S rDNA gene sequences generated 15 branches showing more than 70% of boot strap value, whereas 18 branches in the *rpoB* based phylogenetic tree were supported by bootstrap values above 70%. Diversity indices based on *rpoB* were higher than the ribosomal RNA gene (Yadav et al*.,* 2013).

INTERACTION BETWEEN PLANT AND FLUORESCENT PSEUDOMONADS: THE SELECTION MACHINERY OF HOST PLANT

Mechanism of recognition

Many plant-associated *Pseudomonas* promote plant growth by suppressing pathogenic microorganisms, synthesizing growth-stimulating plant hormones and promoting increased plant disease resistance. Others inhibit plant growth and cause disease symptoms ranging from rot and necrosis to developmental dystrophies such as galls. It is not easy to draw for the plant system a clear distinction between pathogenic and plant growth-promoting *Pseudomonas* (PGPP) as they colonize the same ecological niches and possess similar mechanisms for plant colonization. Over all, the net cost or benefit of interactions with PGPP is affected by the nutritional status of the soil, toxic effects of the bacterium and presence of fungal pathogens, further complicated by plant age, environmental factors, induced stress resistance and cross-talk between plant signal transduction pathways (Preston, 2004).

Recently, green fluorescent protein (GFP) and bioluminescence techniques have been employed effectively to investigate these issues. GFP technology, together with confocal laser scanning microscopy (CLSM), has facilitated the detection of the mechanism of recognition and colonization up to single cell level (Bloemberg et al*.,* 2000; Normander et al*.,* 1999).

Recognition machinery: Fllagelin and LPS

Plants have evolved the capacity to recognize and respond to a wide range of generic microbial molecules, the so-called pathogen-associated molecular patterns (PAMPs). Two of the most widely studied PAMPs produced by *Pseudomonas* are flagellins, subunits of the polar flagella produced by motile *Pseudomonas* and lipopolysaccharides (LPSs), constituents of the bacterial envelope.

Flagellin recognition in plants is mediated by flagellin-sensitive2 (FLS2), a membrane-associated kinase with an extracellular leucine-rich repeat (LRR) domain. FLS2 is a member of the Toll family of receptor kinases, which have been linked to developmental signaling and pathogen recognition in plants. Flagellin recognition by plants is host and strain-specific, e.g. the Ws-0 ecotype of *Arabidopsis* is insensitive to *Pseudomonas* flagellins, showing that flagellin recognition is not a universal characteristic of plants, even within a plant species (Gómez-Gómez et al*.,* 1999). Flagella are important for initial colonization of roots and leaf surfaces, but not for endophytic multiplication. Regulation of flagella expression could be an additional mechanism used to evade plant recognition of *Pseudomonas*.

A second commonly recognized factor is LPS. LPS recognition has mostly been studied in the context of plant pathogens, where it has been shown to induce plant synthesis of anti-microbial factors and to suppress the development of programmed cell death associated with the hypersensitive response (HR), an effect referred to as localized induced resistance or localized induced response (LIR) (Dow et al*.,* 2000).

Mechanism of root colonization: Preparing for the battle with phytopathogens

If a *Pseudomonas* strain cannot adequately compete within the environment of the rhizosphere and colonize the root surface then it will not be an effective BCA. Substantial efforts have been made to identify genes required for key rhizosphere function(s). Given that the rhizosphere is a complex and ever changing environment, it is not surprising that a diverse array of genes have been shown to play an important role in plant root colonization. To date, several genes involved in nutrient acquisition, motility, chemotaxis, adhesion, secretion and stress response have been implicated in the colonization ability of *Pseudomonas* strains.

One system that can play an important role in modulation of host defence responses by pathogens and PGPP is the type-III protein secretion system (TTSS). Pathogens such as *P. syringae* and *P. aeruginosa* use TTSSs to deliver ‘effector’ proteins into the cytoplasm of host cells. TTSS effectors are highly diverse, but their collective function appears to be to render the host more susceptible to infection, and to promote bacterial multiplication in host tissues (He and Jin, 2003). Plants have responded to the threat of bacterial hijacking by evolving surveillance mechanisms that detect the presence and activities of effector proteins. Recognition

of effectors triggers a pre-emptive defense response known as the HR during the early stages of infection, which generally manifests as localized programmed cell death and accumulation of anti-microbial compounds. Effectors that elicit the HR are referred to as Avr (avirulence) proteins. Recognition of Avr is generally conditioned by a single host protein, an R protein (Dangl et al*.,* 1996). The role of TTSSs in rhizobial symbioses appears to be similar to its role in pathogenesis: to modulate host defenses and promote growth in plant tissues. Preston (2004) reported that TTSS genes are present in many plant-colonizing and plant growth-promoting *P. fluorescens* and *P. putida* strains. Current evidence clearly suggests that plant cells can and do receive TTSS-secreted effectors from a wide range of plant-colonizing bacteria, including PGPP. However, further extensive analyses are needed to address the role of TTSSs in the ecology of plant colonizing bacteria (Preston, 2004).

MECHANISMS OF BIOLOGICAL CONTROL OF FLUORESCENT PSEUDOMONADS

According to the definition by Baker and Cook (1974) disease suppressive soils are “soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil”. This phenomenon, although rare, has been well characterized and there is strong evidence that disease suppression is the result of the presence of certain rhizobacteria with antifungal activity. Many biocontrol agents from *P. fluorescens* and closely related species are well characterized for their ability to produce antimicrobial compounds. The biosynthetic pathways involved in their production, as well as their regulation and the signals involved have received extensive attention (Baehler et al., 2006; Dubuis et al., 2007), and these bacteria have become prominent models for analysis of bacterial secondary metabolism. Three major mechanisms have been proposed to explain the suppressive and antagonistic effects of fluorescent pseudomonads. According to one, the pathogen is inhibited by competition for iron, according to the second mechanism, fluorescent pseudomonads inhibit phyto-pathogens by producing secondary metabolites with antibiotic activity, e.g. phenazines, pyrroles, acetylphloro-glucinols and cyanides (Davison, 1986; Défago and Haas, 1990) and thirdly induction of systemic resistance.

Competition for iron in the micro rhizosphere

Fluorescent pseudomonads owe their fluorescence to an extracellular diffusible pigment called pyoverdin (Pvd) or pseudobactin. This pigment has high affinity for Fe^{3+} ions (the association constant of the interaction (*K*ass) is ~1024 at pH 7) and is a siderophore (iron-carrier) of the producer strain (Meyer and Abdallah, 1978). Ferripyoverdin (that is, Pvd complexed with Fe^{3+}) interacts with a specific outer-membrane receptor, which is present in the producer but might also occur in some non-producers. Subsequently, Fe^{3+} is transported into the cytoplasm and reduced to Fe^{2+}. The resulting siderophore hypothesis postulates that PGPR exert their plant growth-promotion activity by depriving pathogens of iron (Adhikari et al., 2013). For example, under greenhouse conditions, *P. putida* strain B10 suppressed *Fusarium* wilt and take-all, but this suppression was lost when the soil was amended with iron, which repressed siderophore production in this strain (Kloepper et al*.,* 1980). A critical assessment of the siderophore hypothesis shows that in some, but not all, plant-pathogen systems tested under various environmental conditions, Pvd-negative (Pvd-) mutants of fluorescent pseudomonads protect plants less effectively than do the parental strains (Keel et al*.,* 1989; Loper and Buyer, 1991). It is important to point out that Pvd-mediated iron deprivation is a contingent biocontrol mechanism, which works much better at pH 8 than at pH 6; this reflects the increasing solubility of Fe^{3+} species with decreasing pH (Elad and Baker, 1985; Misaghi et al*.,* 1988). Another pseudomonad siderophore, pyochelin, has been identified as an antifungal antibiotic in a screening programme (Phoebe et al*.,* 2001). As pyochelin is a relatively weak Fe^{3+} chelator, but a good Cu^{2+} and Zn^{2+} chelator (Cuppels et al*.,* 1987; Visca et al*.,* 1992), it might be able to deprive some fungi of copper and/or zinc. Although siderophores are part of primary metabolism (because iron is an essential element), on occasion they also behave as antibiotics (which are commonly considered to be secondary metabolites).

Role of secondary metabolites in biological control of phytopathogens

Production of antibiotics in several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root pathogens. A number of disease suppressive antibiotic compounds have been characterized, including N-containing heterocycles such as phenazines, pyrrole-type antibiotics, pyo-compounds and indole derivatives as well as non- N- containing 2,4-diacetylphloroglucinol (DAPG). *In vitro*, these antibiotics inhibit fungal pathogens, but they can also be active against many bacteria and, in some cases, against higher organisms. The natural decline in ‘take-all’ disease (TAD) of wheat root caused by *Gaeummanomyces graminis tritici* (Ggt) during extended monoculture of wheat, is an interesting and extensively studied example of natural

biological control phenomenon and antibiotics are thought to be responsible for the reported biocontrol. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA) and 2,4-di-acetylphloroglucinol (Phl) have drawn great attention of research in biological control, since they help in competition within the rhizosphere milieu.

2, 4-di-acetylphloroglucinol (DAPG or Phl)

A broad-spectrum antibiotic, Phl is a phenolic molecule produced by many fluorescent pseudomonads and exhibits antifungal, antibacterial, antihelmenthic and phytotoxic activities (Abbas et al., 2002). In addition, it shows herbicidal activity resembling 2,4-dichlorophenoxyacetic acid (2,4-D). Phl is a polyketide synthesized by condensation of three molecules of acetyl CoA with one molecule of malonyl CoA to produce the precursor monoacetylphloroglucinol (MAPG), which is subsequently transacetylated to generate DAPG. This means that various biotic and abiotic factors associated with field location and cropping time affect the performance of fluorescent pseudomonads (Notz et al*.,* 2002). Complex biotic factors such as plant species, plant age, host cultivar and infection with the plant pathogen *Pythium ultimum*, can significantly alter the expression of the gene *phlA* (Notz et al*.,* 2001). Among abiotic factors, carbon sources and various minerals influence production of Phl. Fe^{3+} and sucrose have been reported to increase the levels of DAPG and MAPG in *P. fluorescens* F113, whereas in *P. fluorescens* Pf-5 and CHA0, Phl was stimulated by glucose (Nowak et al*.,* 1994; Duffy and Défago 1999). In *P. fluorescens* strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon. Microelements, such as Zn^{2+}, Cu^{2+} and Mo^{2+} have been found to stimulate Phl production in *P. fluorescens* CHA0 (Notz et al*.,* 2001). The exact mechanism of DAPG action is still unclear, although it is known that disease suppression by this antifungal molecule is a result of interaction of specific root-associated microorganisms and the pathogen. Phl also appears to cause induced systemic resistance (ISR) in plants.

The genetic constituents of 2, 4-di-acetylphloroglucinol

The sequences of the entire biosynthetic loci of *phl* are now available for *P. fluorescens* strain Q2-87 in the EMBL database (accession no. U41818). A total of five complete open reading frames (ORFs) and one partial ORF, within the 6.8 kb segment of DNA, is responsible for biosynthesis of DAPG. The genes *phlA*, *phlC*, *phlB* and *phlD* are contained in a large transcriptional unit transcribed in the same direction. This is similar to *phlE*, which is located downstream of *phlD* (Delany et al*.,* 2000). The gene *phlE* produces a red pigment that is involved in the transport of Phl out of the cell. In spite of these developments, the precise role of each gene in Phl biosynthesis is not clear. Another divergently transcribed gene, *phlF* is located 421 bp upstream of biosynthetic genes and consists of an ORF of 627 bp with a corresponding protein of 209 amino acids, with predicted molecular mass of 23,570 Da. PhlF is a repressor molecule that exhibits a helix-turn-helix DNA binding motif which regulates the Phl operon (Bangera and Thomashaw, 1996; Delany et al*.,* 2000). PhlF regulates the biosynthesis of Phl at the transcriptional level. The *phlA*-*phlF* intergenic region displays a complex organization wherein *phlA* is transcribed from a *s*32 RNA pol-dependent promoter that overlaps the promoter of the divergently transcribed *phlF* gene. Another specific sequence of 30 bp, known as *phlO*, is located downstream of *phlA*.

Interaction of PhlF repressor protein with this sequence results in repression. This signifies that the repression occurs by inhibition of promoter clearance (Bangera and Thomashaw, 1996; Schnider et al*.,* 1995). Two more regions of 7 and 9 bp are located in the intergenic region of *phlA*-p*hlF*, which not only bind the repressor but further strengthen the binding. The repression however occurs only during the early log phase, after which it is ineffective because of its interaction with the inducer Phl. Salicylate can interact with PhlF to stabilize its interaction with the *phlA* promoter, leading to tighter repression of Phl production. Thus, interactions of these molecules with PhlF contribute towards complex regulation of Phl biosynthesis (Corbell and Loper, 1995).

Phenazines

Phenazines (Phz) are N-containing heterocyclic pigments synthesized by *Brevibacterium*, *Burkholderia*, *Pseudomonas* and *Streptomyces* (Budzikiewicz, 1993; Stevans et al*.,* 1994). The intense colour of this molecule, its antibiotic property and involvement in pathogenic reaction have made it an interesting molecule for study. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism (Mavrodi et al., 1998). For example, *P. fluorescens* 2-79 produces mainly phenazine 1-carboxylic acid (PCA), whereas *P. aureofaciens* 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines. The major Phz synthesized by *P. aeruginosa* is pyocyanin (1- OH-5-methyl Phz). Almost all Phz exhibit

broad spectrum antibiotic activity against bacteria and fungi and they exhibit it by inhibiting the electron transport system of the pathogens.

Phz also play an important role in microbial competition in rhizosphere, including survival and competence (Mazzola and Cook, 1992). Phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid wherein the amide nitrogen of glutamine serves as the immediate source of N in the heterocyclic nucleus. PCA is the first Phz formed, which gets converted to PCA and acts as the key intermediate in the synthesis of other Phz in fluorescent pseudomonads (Mann, 1987). The broad-spectrum activity exhibited by Phz compounds against fungi and other bacteria is not well understood. However, it is believed that Phz can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (MnSOD) which causes enhanced production of - 2O (superoxide radical). There is a distinct possibility that the antibiotic action of pyocyanin is actually a result of toxicity of - 2O and H_2O_2 produced in increased amounts in its presence (Wood and Pierson, 1996).

The genetic constituents of phenazines

Structural and functional analysis shows that seven genes, *phzABCDEFG*, are involved in the synthesis of PCA. These are localized within a 6.8 kb fragment in *P. fluorescens* 2-79 (Mavrodi et al*.,* 1998). The Phz biosynthetic loci in *P. fluorescens* 2-79 (Mavrodi et al*.,* 1998), *P. aeruginosa* PAO1 and *P. chlororaphis* PCL 1394 are highly conserved (Chin-A-Woeng et al., 1998). Each *phz* locus contains a set of seven gene core operons, regulated in a cell density-dependent manner by homologues of LuxI and LuxR (Chin-A-Woeng et al., 1998; Latifi et al., 1995), which are found directly upstream of the Phz core. The core gene products, PhzC, PhzD and PhzE, which are homologous with PhzE, PhzA and PhzB in strain 30-84, are similar to enzymes of shikimic acid and chorismic acid metabolism (Delany et al*.,* 2001). PhzG is similar to pyridoxamine 5′ phosphate oxidase, which was found to be the source of cofactor for the PCA synthesizing enzyme(s).

Products of PhzA and PhzB genes are highly homologous and appear to be involved in the stabilization of a putative PCA-synthesizing multienzyme complex. The conversion of PCA to 2-OH-PCA in strain 30-84 is brought about by a gene *phzO* which is located immediately downstream of the biosynthetic operon in strain 30-84 (Mavrodi et al*.,* 1998). PhzO is a non-heme, flavin diffusible monooxygenase that adds a hydroxyl group to PCA at orthoposition relative to carboxyl group, which results in the synthesis of 2-OH-PCA.

Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chloro-phenyl) pyrrole] is an inhibitor of fungal respiratory chain and thus a broad-spectrum antifungal metabolite produced by many fluorescent and non-fluorescent strains of the genus *Pseudomona*s. It was first described by Arima et al*.* (1964). This highly active metabolite has been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, particularly members of the genus *Trichophyton.* A phenyl pyrrol derivative of Prn has been developed as an agricultural fungicide. Pyrrolnitrin persists actively in the soil for at least 30 days. It does not readily diffuse and is released only after lysis of host bacterial cell. This property of slow release facilitates protection against *Rhizoctonia solani* as the cell dies (Schnider et al*.,* 1995).

The genetic constituents of pyrrolnitrin

The *prn* operon has been completely sequenced; *prnABCD* spans 5.8 kb DNA which encodes Prn biosynthetic pathway in which four ORFs, *prnA*, *prnB*, *prnC* and *prnD* are involved. Two stem-loop structures, which are similar to *s*-independent transcription termination signals, have been identified in this sequence. One gene is located immediately 5′ to the beginning of ORF 1, while the other is located at the end of ORF 4. However, there are no *s*-independent transcription termination signals within or between the four ORFs (Gaffeny et al*.,* 1994). All four ORFs are located on a single transcriptional unit. The four genes encode proteins which are identical in size. Among these, *prnA* gene product catalyses the chlorination of L-trp to 7 chloro-L-trp (Hammer et al*.,* 1997). The *prnC* gene product chlorinates it at the 3-position to form an amino pyrrolnitrin. The *prnD* gene product catalyses the oxidation of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Kirner et al*.,* 1998). The organization of *prn* genes in the operon is identical to the order in which the reactions are catalysed in the biosynthetic pathway.

Pyoluteorin

Plt is an aromatic polyketide antibiotic consisting of a resorcinol ring, which is derived through polyketide biosynthesis. This in turn is linked to a bichlorinated pyrrole moiety, whose biosynthesis remains unknown (Kitten et al*.,* 1998; Nowak et al*.,* 1999). Biosynthesis of Plt is initiated from proline or amrelated molecule, which condenses serially with threemacetate equivalents coupled to chlorination and oxidation at yet unidentified stages. The formation and cyclization of the C-skeleton

has been reported to proceed by the action of a single multienzyme complex (Nowak et al*.,* 1999). Proline is the primary precursor of dichloropyrrole moiety of Plt.

The genetic constituents of pyoluteorin

Ten genes, *pltLABCDEFG* are involved in the biosynthesis of Plt. They span a 24 kb genomic region in *P. fluorescens* Pf-5. Among these ten genes, *pltB* and *pltC* encode type 1 polyketide synthetase and *plt*G encodes a thio esterase, three halogenases are coded by *pltA*, *pltD* and *pltM*37. Except for a 486 bp gap between the coding regions of *pltL* and *pltR*, contiguous *plt* genes are separated by less than 50 bp.

pltR and *pltM* are transcribed divergently from *pltLABCDEFG* gene cluster; a sequence within 486 bp intergenic region separates *pltRM* from the gene cluster. Among the *plt* gene products, PltR is similar to LysR family of the transcriptional activators (Nowak et al*.,* 1999; Pierson et al., 1998). Furthermore, PltR acts as a positive transcriptional activator linked to loci like *phzI* of the Phz biosynthetic locus. However, signals required for the transcription of *pltR* coinducer are yet to be identified (Chin et al*.,* 2003; Pierson et al., 1998).

Mechanism of regulation of biological control: The ambiguity slowly shown

Three levels of regulation

The regulation of the production of secondary metabolites such as antifungals is operated in bacteria through various mechanisms acting at transcriptional and posttranscriptional levels. An understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens, is a prerequisite for predicting the environmental conditions under which such bacteria are likely to perform optimally. Three levels of regulation have now been suggested; a primary sensing level which is dependent on the surroundings and a secondary or intermediate level that is responsible for regulation of antibiotic biosynthesis with other metabolic processes through global regulation and cellular homeostasis, and 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 (Abbas et al*.,* 2002; Haas et al*.*, 2000)

Regulation by typical bacterial two-component system

An environmentally regulated, two-component system is Now known to be essential for antibiotic production in various Gram-negative bacteria, including *Pseudomonas* (Meyer et al*.,* 2002). This prokaryotic, two-component regulatory system is a transmembrane protein that functions as a sensory kinase GacS and the cytoplasmic cognate response regulator GacA protein that mediates changes in gene expression in response to sensor signals. As a consequence of interaction with unknown signals, GacS sensor activates GacA response regulator by phosphorylation. The activator GacA, by virtue of its typical C-terminal helix-turn- helix motif, regulates the transcription of the target genes. While the direct GacA targets are not known, GacS/GacA system exerts a positive effect on cell density-dependent gene regulation; the latter is mediated by *N*-acylhomoserine lactone (AHL) in *P. aeruginosa*, *P. syringae* and *P. aureofaciens*. However, a similar system also effectively operates in other Gram-negative bacteria which do not produce AHL, e.g. *P. fluorescens* CHAO; here, GacS/ GacA strictly controls the expression of extracellular products such as exoenzymes, antibiotics and HCN when cells are in idiophase, that is, transition from exponential to stationary phase (Heeb and Haas, 2001).

Regulation by quorum sensing

Bacterial populations in their natural habitats have now been reported to communicate with each other through chemical signals that are released in a cell density-dependent manner. This is referred to as quorum sensing (QS),that is, a minimum cell number, and operates through two broad categories of molecules, viz. amino acids and short peptide hormones commonly involved in Gram-positive bacteria and fatty acid-derivatives such as AHLs, in Gram-negative bacteria. On the root surface, many biofilm forming bacteria are present, where they can attain high population densities and accumulate the concentration of such signal molecules and regulate various physiological processes (Chin et al*.,* 2003). For example, a large family of regulatory systems has now been described that closely matches the LuxI and LuxR proteins of *V. fischeri*. QS relies on the fact that LuxI-type proteins synthesize AHLs (also known as autoinducers), which diffuse from bacteria that produce them either passively or by means of active efflux and accumulate at high population densities. AHL binds to and activates LuxR-type receptor proteins. These function either as cytoplasmic transcriptional factors or as repressors (Zang et al*.,* 2002).

Regulation by sigma factors

Regulation of antifungals operates at another level as well. This requires involvement of sigma factors that are otherwise an integral component of regulation of antifungals like Phl and Plt, e.g. *P. fluorescens* Pf-5. The

overexpression of activator gene *rpoD* or mutation deletion of suppressor gene *rpoS* increases Phl or Plt production (Kitten et al*.,* 1998). These genes encode sigma-factor σ^{32} and stationary-phase σ^{38} respectively, required during transcription. This suggests that σ factors compete for RNA pol, and any imbalance either due to excess of σ^{32} or lack of σ^{38} might enhance the expression of genes; the expression is driven by weak σ^{32}-dependent promoters (Schnider et al*.,* 2000).

Regulation via small RNA binding protein

In *P. fluorescens* CHA0, this system tightly controls the expression of several biocontrol factors including Phl, HCN, pyoluteorin, pyrrolnitrin and exoprotease (Haas and Keel, 2003). The GacS sensor kinase has an autophos-phorylation domain around His294, a phosphoacceptor domain around Asp717 and a histidine phosphotransfer domain around His863. On interaction with bacterial signal molecules, GacS is autophos-phorylated and a phospho-relay mechanism transfers a phosphate residue to the acceptor domain of the response regulator GacA159, 160; this then activates, directly or indirectly, the transcription of the three small RNA genes *rsmX*, *rsmY* and *rsmZ*. Titration of these RNAs by the RsmA and RsmE proteins relieves the translational repression exerted by these proteins at, or near, the ribosome binding site (RBS) of the target mRNAs (for example, *hcn* for HCN synthase, *apr* for exoprotease and *phl* for Phl synthase). Currently, the involvement of GacS/GacA two-component regulatory system in the production of secondary metabolites such as phenazines has been extensively studied for biological control activity in *Pseudomonas chlororaphis* 30-84 (Wang et al*.,* 2013).

Regulation via microbial metabolites

Microbial metabolites also play an important role in the regulation of antifungal molecules, e.g. synthesis of DAPG is autoinduced and repressed by other bacterial extracellular metabolites of strain CHAO (Schnider et al*.,* 2000). Furthermore, salicylate and secondary metabolites (fusaric acid and Pln) have negative effect on Phl production (Abbas et al*.,* 2002). Salicylate interacts with repressor PhlF and stabilizes its interaction with *phl*A promoter. This results in tighter repression of Phl production.

ROLE OF INDUCTION OF SYSTEMIC RESISTANCE IN BIOLOGICAL CONTROL: THE HOST IMMUNIZATION

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al*.,* 1998). In 1991, two research groups independently described induced systemic resistance (ISR) as the mode of action of disease suppression by nonpathogenic rhizosphere bacteria (Van Peer et al., 1991; Wei et al., 1991). Since then, the involvement of ISR in disease suppression has been studied for a wide range of biological control microorganisms and, in many cases, ISR was found to be involved. Phenotypically, ISR is similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens. Although the terms SAR and ISR are synonymous, to distinguish between pathogen- and rhizobacteria-induced resistance, SAR is used for the pathogen-induced type and ISR for the rhizobacteria-induced type. SAR requires accumulation of salicylic acid (SA) in the plant (Sticher et al*.,* 1997), ISR does not and, instead, is dependent on intact responses to ethylene and jasmonic acid (JA) (Pieterse et al*.,* 1998). When these different signal transduction pathways are triggered simultaneously in *Arabidopsis thaliana*, disease suppression is enhanced (Van Wees et al*.,* 2000). This suggests that combining bacterial traits that trigger either the SA, or the ethylene or JA dependent response can improve biological control. To manipulate this phenomenon effectively for practical applications, knowledge on bacterial traits such as flagella, lipopolysaccharides, siderophores, salicylic acid, antibiotics, which are involved in the triggering of ISR is essential.

Bacterial traits that induce systemic resistance

Flagella

Bacterial flagellins, the main protein component of flagella, can elicit defense responses in plants (Gomez-Gomez and Boller, 2000; Zipfel et al*.,* 2004). For *P. putida* strain WCS358, the involvement of flagella in ISR was studied in *Arabidopsis*, bean and tomato by applying isolated flagella and by using non-motile mutants that lack flagella (Meziane, 2005). In *Arabidopsis*, application of WCS358 flagella triggered ISR against *P. syringae* pv. *tomato*, whereas in bean or tomato, their application did not lead to induced resistance.

Lipopolysaccharide

Lipopolysaccharides (LPS) have been implicated in ISR triggered by fluorescent pseudomonads in carnation (Van Peer and Schippers, 1992), radish (Leeman et al*.,* 1995), and *A. thaliana* (Van Wees et al*.,* 1997). In the case of ISR against *Globodera pallida* (Stone) Behrens on potato

by *Rhizobium etli,* the LPS also plays a major role (Reitz et al*.,* 2002). In these studies, either purified LPS was used to induce systemic resistance, or mutants that lack part of the LPS were compared with the parental strain regarding their abilities to induce resistance.

Role of siderophores

Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron from the environment (Höfte, 1993), and their modes of action in suppression of disease were thought to be solely based on competition for iron with the pathogen (Duijff et al*.,* 1999). Interestingly, siderophores can induce systemic resistance (ISR) (Leeman et al*.,* 1996). A clear cut role for siderophores in ISR was reported for *P. putida* WCS358 in suppression of bacterial wilt in *Eucalyptus urophylla*, caused by *Ralstonia solanacearum* (Ran et al*.,* 2005). For *P. fluorescens* WCS374, the situation was different in that wild type bacteria, the pseudobactin mutant, and the purified pseudobactin were all effective in suppression of the disease, indicating redundancy of ISR-triggering traits of WCS374 in this system. Pseudobactin mediated ISR was also found to be effective against *Tobacco necrosis virus* in tobacco. A pseudobactin-minus mutant of *P. fluorescens* CHA0 was less effective in reducing numbers of viral lesions and lesion diameter than the parental strain (Maurhofer et al., 1994).

Salicylic acid

A rhizobacterial metabolite that was suggested to trigger the SA-dependent signal transduction pathway is SA itself (De Meyer and Höfte, 1997; Maurhofer et al*.,* 1998). Exogenous application of SA, even at the extremely low dose of 100 fg, to radish roots significantly reduced Fusarium wilt (Leeman et al*.,* 1996). Salicylic acid production has been observed for several bacterial strains, and exogenously applied SA can induce resistance in many plant species. For example, *P. fluorescens* strain WCS374 produces relatively large quantities of SA under conditions of iron limitation. Moreover, WCS374 is more effective, in radish, in ISR against fusarium wilt under iron-limited conditions when compared with conditions of sufficient iron availability (Leeman et al*.,* 1995). Possibly, the iron-regulated ISR by WCS374 in radish is mediated by the SA-containing siderophore pseudomonine (Mercado-Blanco et al*.,* 2001). Thus, for several bacterial strains, a role of bacterially produced SA in induced resistance has been suggested, but upon careful examination, SA seems not to be involved directly (Audenaert et al*.,* 2002).

Role of antibiotics

Production of antibiotics has been described as a powerful mode of action in disease suppression by which development and (or) activity of the pathogen is thought to be directly inhibited (Handelsman and Stabb, 1996). A role for DAPG in ISR was recently demonstrated in *Arabidopsis.* In this plant system, DAPG produced by *P. fluorescens* CHA0 is the key compound in ISR against *Peronospora parasitica* (Iavicoli et al*.,* 2003). Also in *Arabidopsis*, ISR against *P. syringae* pv. tomato by *P. fluorescens* Q2-87 depends on the production of DAPG (Weller et al*.,* 2004). In tomato, *P. fluorescens* CHA0 induces resistance against the root-knot nematode *Meloidogyne javanica*. Also, in this case, DAPG appears to be the bacterial trigger of ISR, since a DAPG-minus mutant was not effective and effectiveness was restored by complementation of the mutant (Siddiqui and Shoukat, 2003). Finally, in *P. aeruginosa* 7NSK2 the phenazine antibiotic pyocyanin is involved in ISR against *B. cinerea* in tomato (Audenaert et al*.,* 2002).

Role of β-aminobutyric acid

Currently, β-aminobutyric acid (BABA), non-protein amino acid, is thought to be important component of the signaling pathway regulating ISR response in plants. Knowledge about how the resistance is induced by BABA against biotic factors is still not fully understood. In the first step of defense, the plant produces reactive oxygen species (hypersensitivity response), and induces physical barrier by callose deposition and lignin accumulation in the cell walls. In addition, biochemical mechanisms of response to the stress are also initiated, for example biosynthesis of secondary metabolites (phenols, anthocyanin, phytoalexins) and induction activity of enzymes associated with active oxygen species, lignifications and plant secondary metabolism (Justyna and Ewa, 2013).

Role of population density

In a dose-response study of ISR mediated by *P. fluorescens* WCS374 in radish, it was revealed that population densities of 105 colony forming units per gram of root are required for significant suppression of disease (Raaijmakers et al*.,* 1995). In situations where no bacteria are introduced, population densities of one single bacterial genotype probably never reach such high densities, making it unlikely that, in the field, plants are triggered sufficiently by bacteria to express ISR. In dose-response studies, it was demonstrated that RS111a was effective in suppressing fusarium wilt of both radish and

flax at much lower initial population densities than RS111. These results suggest that the level of disease control through ISR by a specific strain can be improved. Further studies to elucidate the determinants of ISR of these strains are ongoing.

Molecular mechanism of SAR and ISR

Early research on molecular mechanism involved in pathogen induced SAR showed that the onset of SAR is accompanied by a local and systemic increase in the endogenous levels of salicylic acid (Malamy et al*.,* 1990) and the concomitant up-regulation of a large set of genes (Ward et al*.,* 1991) including the pathogenesis related (PR) proteins (Van loon and Van strien 1999). Several PR proteins possess antimicrobial activity and are thought to contribute to the state of resistance attained. Genetic screens for SAR compromised Arabidopsis mutants revealed a series of mutants that all appeared to be affected in the same gene (Delaney et al*.,* 1995), the npr1 (non-expresser of PR genes) or nm1 (non-immunity). Mutant *npr1* plants accumulate normal levels of salicylic acid after pathogen infection, but are impaired in their ability to express PR genes and to mount SAR response, indicating that NPR1 (the gene product of *npr1*) functions downstream of SA in the SAR pathway. Recently, it has been demonstrated that, upon induction of SAR, NPR1 which possess ankyrin-like repeats, is translocated to the nucleus, where it activates the PR gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors (TGA transcription factors) that bind to promoter sequences required for SA- induced PR gene expression. Recent study has shown that the *Arabidopsis* NPR1 (nonexpresser of *PR* genes 1) protein is a master regulator of SAR. Salicylic acid directly binds to the NPR1 adaptor proteins NPR3 andNPR4, regulates their interactions withNPR1, and controls NPR1 protein stability. However, how NPR1 interacts with TGA transcription factors to activate defense gene expression is still not well understood. In addition, redox regulators, the mediator complex, WRKY transcription factors, endoplasmic reticulum-resident proteins, and DNA repair proteins play critical roles in SAR. SAR can even be passed on to progeny through epigenetic regulation (Fu and Dong, 2013).

Besides SA, the plant growth regulators jasmonic acid (JA) and ethylene (ET) have repeatedly been implicated in the regulation of primary resistance responses in plants (Pieterse et al*.,* 2001). Exogenous application of these compounds often results in an enhanced level of resistance. To investigate the role of JA and ET in rhizobactria mediated ISR the Arabidopsis JA response mutant jar1-1 and the ET response mutant etr1-1 were tested for their ability to express ISR. Both mutant were unable to mount resistance against Pst DC 3000 after colonization of the roots by *P. fluorescens* WCS417r (Pieterse et al., 1998), indicating that ISR requires responsiveness to both JA and ET. Furthermore, in the study of Pieterse et al. (1998) it was postulated that JA and ET induced ISR was SA- independent pathway and follows a signaling pathway in which components from the JA and ET response are successively engaged. Previously in several studies it was documented that NPR1 is an important regulatory factor in the SA dependant SAR response.

In 1998, Pieterse et al*.* demonstrated with Arabidopsis mutant npr1, that, like pathogen induced SAR, rhizobacteria mediated ISR is an NPR1 dependant defense response. Elucidation of the sequence of ISR signaling events revealed that NPR1 functions downstream of JA and ET in the ISR signaling pathway. NPR1 is able to differentially regulate defense genes expression depending on the signaling pathway that is activated upstream of it. In the year 2002, to study the association between induced resistance and basal resistance, the research group of Ton et al*.* (2002) used a collection of 11 Arabidopsis eds mutants with enhanced disease susceptibility (reduced basal resistance). Through this study they successfully demonstrated that EDS4 (product of eds4-1) for ET and EDS8 (product of eds8-1) for JA plays an important role downstream of JA and ET signaling pathway. Future research should reveal the exact role of these signaling components in the expression of ISR.

BIO CONTROL IN PRACTICE: THE PRACTICAL SCENARIO

This is the most important contexts in which biological control mediated by *P. fluorescens* strains and related pseudomonads has important practical implications. The first context corresponds to the use of biocontrol agents as inoculants of soil or plants, which has been successfully implemented in agronomic field trials (Amein et al*.,* 2008; Karthikeyan and Gnanamanickam, 2008). The use of P. fluorescens biocontrol agents is thought to have a limited ecological impact on indigenous saprophytic populations and to take place without negative side-effects on rhizosphere functioning (Mark et al*.,* 2006).

The principal reasons of inconsistency of biocontrol products under field condition

Although the vast body of research on *Pseudomonas* BCAs deals with their capacity to control soil-borne fungal

pathogens, there has been limited success developing commercially viable products.

Depletion of cell count

Many inoculation products are commercially available (Mark et al*.,* 2006), but strains of *Pseudomonas* spp. can mutate in culture and generally lose viability when stored for a period of several weeks (Haas and Défago, 2005). Any mutation or lack of viability may greatly affect performance and could be a catastrophic occurrence for the company selling the product. Efficient quality control will be definitely essential. Reliable assays to check the efficacy of the strains will be necessary. Methods for monitoring inoculants quality are being developed. Recent advances show that pseudomonas formulation can be improved for long term storage (Guo et al*.,* 2004) and efficient antagonistic activity (Wiyono et al*.,* 2008).

Root colonization

In addition, pseudomonas inoculants may perform inconsistently from one field to another and/or from one year to the next, as a consequence of variability in root colonization (Weller, 2007) or in expression of biocontrol traits (Mark et al*.,* 2006). Therefore, superior root colonization and effective functioning in the rhizosphere are key criteria when selecting strains, and research aims at better understanding the molecular basis of these traits (Mavrodi et al*.,* 2006) and the signaling processes regulating the ecology of *P. fluorescens in situ* (Dubuis et al*.,* 2007; Barret et al*.,* 2009).

Improving the biocontrol efficacy of pseudomonas biological control agents

Nowadays, many trading products for plant disease control are based on BCAs. Commercial development of this agricultural product must follow several criteria: large application on major crops, quality control, chemical effects and inoculums formulation. Product safety, production costs, effectiveness against target organisms and acreage and value of crops to be treated must all be considered in the development of biological control and PGPR.

Genetic modification to produce enhanced secondary metabolites

One strategy to develop improved biocontrol strains is to use genetic modification to enhance this activity. To a certain extent, this involves the construction of strains that produce increased levels of antimicrobial metabolites. More significant, however, is the recent focus on developing strains in which the timing of production is altered. This is crucial because, in general, *Pseudomonas* spp*.* only produce antifungal metabolites at high cell densities during the late logarithmic or stationary phase of growth. If the relevant biosynthetic genes are uncoupled from their regulatory controls, however, this may facilitate early production of antifungal metabolites, offering immediate protection to crop seeds/seedlings.

Control of gene expression at the transcriptional level is recognized as a primary mechanism for modulating the production of secondary metabolites. In this regard, the use of alternative σ factors has received considerable attention. Specific transcriptional activators/repressors may also regulate the transcription of secondary metabolite biosynthetic genes, for example, the PhlF protein, which is expressed from the Phl locus, represses transcription of the *PhlA-D* operon, which comprises genes encoding proteins that direct the synthesis of Phl (Delany et al*.,* 2000, Schnider et al*.,* 2000).

Mutation of *phlF* in a *P. fluorescens* strain increased Phl production *in vitro* during the early logarithmic phase of growth. Similarly, overexpression of *phlA-D* resulted in Phl overproduction and, concomitantly, enhanced biocontrol efficacy against *P. ultimum* in laboratory microcosm trials (Delaney et al*.,* 2001).

Enhancement of rhizosphere competence

Other studies have focused on the possibility of promoting microevolution of biocontrol strains to enhance their rhizosphere competence (de Weert et al*.,* 2004). Promising results were also obtained with the development of genetically-improved strains with higher plant protection ability, either by reprogramming the regulation of existing biocontrol traits (Mark et al*.,* 2006) or the introduction of novel mechanisms such as the degradation of pathogen quorum-sensing molecules (Molina et al*.,* 2003) or ACC deaminase activity (Wang et al*.,* 2000).

Sustainability at the crop micro-rhizosphere environment

The identification of factors that control the fate and performance of the inoculants, small-scale analyses are needed due to the heterogeneity characterizing the complex soil and rhizosphere environments (Sørensen et al*.,* 2001). Direct staining techniques and advanced microscopy had provided the first detailed single cell

images of root colonization by these bacteria using fluorescent antibodies, fluorescent *in situ* hybridization and marker gene technology. Fluorescence-labeled antibodies have been used with success for detection of root-colonizing *Pseudomonas* strains by immune-fluorescence microscopy (Kragelund and Nybroe, 1996, Troxler et al*.,* 1997). The rRNAtargeted fluorescent *in situ* hybridization (FISH) technique offers the possibility of non-extractive detection of target bacteria. More detailed *in situ* studies of single-cell distribution of *Pseudomonas* inoculants, using immunochemical methods and/or FISH have only been possible with the advent of confocal laser scanning microscopy (CLSM) (Lübeck et al*.,* 2000). Insertion of marker or report genes has been commonly used to distinguish the introduced pseudomonads from indigenous populations (Götz et al*.,* 2006). Some of the most popular genes used as markers or reporters encode enzymes are *luxAB, lacZ, luc, xylE* and *gusA* in which case a product of the reaction catalyzed by the marker gene is monitored (bioluminescence, color, etc.) (Sørensen et al., 2001). Gene product can also be detected by immunochemical techniques. Specific physical properties of the gene products may mediate their detection. It occurs to the product of *gfp* gene that is a protein, which emits green fluorescence when excited (Chalfie et al*.,* 1994).

Application of consortia

Another way to seek more effective biocontrol treatments is to inoculate consortia of *P. fluorescens* biocontrol agents, sometimes in mixture with other plant-beneficial microbes (Karthikeyan and Gnanamanickam, 2008). However, the compatibility of these inoculants despite possible bacteriocin-mediated competition (Validov et al*.,* 2005), their antimicrobial metabolites and their extracellular signals needs to be considered (Molina et al*.,* 2003; Dubuis et al*.,* 2007), especially when synergistic effects are sought. Recently, in a study conducted by Antonelli et al*.,* (2013) it was shown show that protection of melon against *Monosporascus cannonballus* has been successfully achieved by applying a consortia of *Bacillus sp.* BsCR and *Pseudomonas putida* PpF4.

Compatibility with residue chemicals

Another important point to be considered is the pesticide use in the cropping systems. Apparently the majority of fungicides do not affect PGPR. Fungicides used on potato seed pieces, like benomyl, captan, PCNB did not affect PGPR strains but mancozeb was inhibitory using *in vitro* tests. *Pseudomonas* spp. Have a biochemical machinery that confers many possibilities to degrade many compounds (Kononova and Nesmeyanova, 2002; Spiers et al*.,* 2000). However, further test will be necessary as new strains are found and new crops with various chemical needs are tested.

Development of bio-control formulation

An equally important, if not over-riding bottleneck, however, is the lack of suitable inoculants formulations that allow *Pseudomonas* cells to survive for long periods under storage at concentrations high enough to afford biocontrol (McQuilken et al*.,* 1998). Initially, bacterization of seeds utilized bacterial growth from culture media suspended in water. Although growth responses were noted in some cases, the problems with this system on a large scale are apparent. Strains may soon die in water suspensions and unless planting are done immediately after treatment, the inoculum dries and the populations of PGPR decline (Rainey, 1999). Powered formulations of PGPR have been developed and a number of commercially available gums were tested as suitable substrates for PGPR in comparison with methylcellulose. It was observed that PGPR establishment on roots and stimulation growth depends on the carrier. There is variability between crops that will be encountered when attempting to develop formulations for wide usage. Suslow and Schroth (1982) showed methylcellulose powder formulations were most suitable for pelleting onto sugar-beet. Powered formulations have benefit as the ease of storage, transport and handling. Also, by pelleting seed with a powder formulation is possible to concentrate a higher population of PGPR around the seed than by dipping in bacterial suspensions. The powder formulations also allow for storage for prolonged periods of time until planting. More studies are necessary to determine most efficient forms to apply PGPR to the plants. Furthermore, carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy (Duffy and Défago, 1999). Without doubt, however, further research is required on the development and optimization of microbial inoculants formulations, which will be compatible with current seed coating technologies. Furthermore, because survival during seed coating/pelleting and during storage at ambient temperatures is critical for the development of microbial inoculant products, it seems logical that these traits should form an integral part of any screening process for the selection of new *Pseudomonas* BCAs. In India, bio control market is regulated by Central Insecticide Board (CIB), under the section 9(3B), Insecticide act, 1968. A

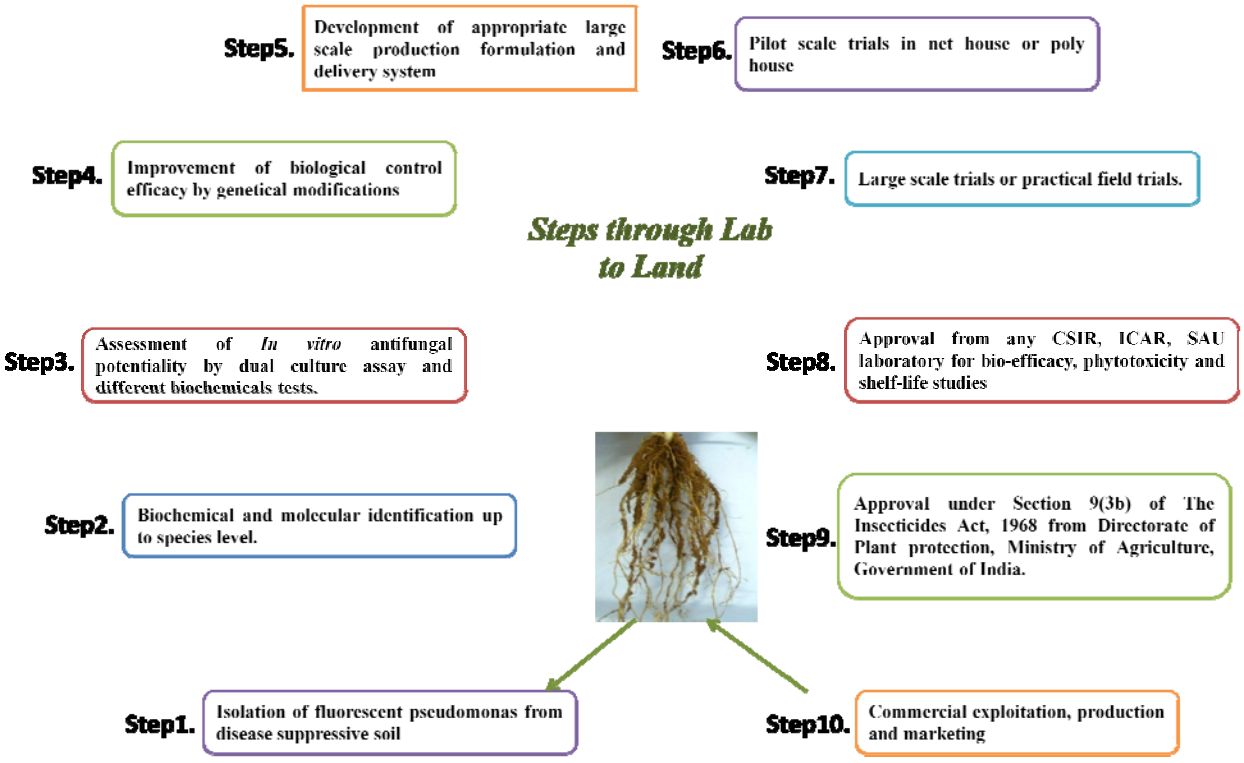

Figure 1. Schematic diagram showing the sequence of events from the isolation of fluorescent *Pseudomonas* from disease suppressive soils, through their development and improvement to their marketing as bio-control products in Indian markets.

schematic diagrammatic representation is given to understand how a potential bio control agent can be marketed in Indian agriculture (Figure 1)

CONCLUSION

Despite a century long history of rhizosphere research we are still at the beginning of understanding the complex plant-microbe interactions in this dynamic environment. The use of fluorescent pseudomonads as biological control agents (BCA) requires the precise understanding of the interactions between plant-bacteria, among bacteria-microbiota and how biotic and abiotic factors influence this relationship.

Exciting developments that will enable in-depth studies of the functioning of the rhizosphere microbiome include the use of phylo-chips to study the composition of microbial communities, development of metatranscriptomics and metabolic profiling of root exudates. In a few years, modern technologies, such as immunofluorescence microscopy, confocal laser scanning microscopy and reporter genes, have improved the study of *Pseudomonas* inoculants in soil and have markedly enhanced the knowledge about their behavior in this environment. Recent advances in studies of the intraspecies and interspecies signaling, quoram sensing are providing an important area for scientific research, as well as, relevant application. In recent years, many strategies to achieve the control of plant diseases using fluorescent pseudomonads as biological control agents (BCAs) have produced significant success, which are relevant since one of the goals of using BCAs, is to make them trustable and assessable product to the farmers. Consequently, continuous searching for new approaches to improve the field efficiency and delivery system of fluorescent pseudomonads as BCAs are strongly required to enable sensible applications to control diseases in a sustainable manner.

Conflict of interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We, hereby, express our sincere thanks to colleagues at the AICRP on Vegetable Crops, Directorate of Research,

B.C.K.V., Kalyani, West Bengal, India.

REFERENCES

Abbas A, Morrisey JP, Marquez PC, Sheehan MM, Delany IR, O'Gara F (2002). Characterization of interaction between the transcriptional repressor PhlF and its binding site at the *phlA* promoter in *Pseudomonas fluorescens* F113. J. Bacteriol. 184:3008-3016.

Adhikari A, Sarker K, De Roy M, Bhattacharya I, Mandal T, Das Mohapatra PK, Dutta S (2013) Siderophore mediated antagonism of fluorescent Pseudomonads against soil borne plant pathogenic fungi in West Bengal, India. Afr. J. Microbiol. Res. 7(39):4689-4700.

Amein T, Omer Z, Welch C (2008). Application and evaluation of *Pseudomonas* strains for biocontrol of wheat seedling blight. Crop Protect. 27:532-536.

Antonelli M, Reda R, Aleandri MP, Varvaro L, Chilosi G (2013). Plant growth-promoting bacteria from solarized soil with the ability to protect melon against root rot and vine decline caused by *Monosporascus cannonballus.* J. Phytopathol. 161(7-8):485-496

Arima K, Imanaka H, Kausaka M, Fukuda A, Tameera C (1964). Pyrrolnitrin a new antibiotic substance produced by *Pseudomonas.* Agric Biol Chem. 28:575-576.

Audenaert K, Pattery T, Cornelis P, Höfte M (2002). Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid pyochelin and pyocyanin. Mol Plant-Microbe Interact. 15:1147-1156.

Baehler E, de Werra P, Wick LY, Pe´chy-Tarr M, Mathys S, Maurhofer M, Keel C (2006). Two novel MvaT-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHA0. Mol. Plant- Microbe Interact. 19:313-329.

Baker KF, Cook R J (1974). Biological Control of Plant Pathogens WH Freeman San Francisco.

Bangera MG, Thomashaw LS (1996). Characterization of a genomic locus required for synthesis of the antibiotic 2 4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. Mol. Plant-Microb Interact. 9:83-90.

Barret M, Frey-Klett P, Boutin M, Guillerm-Erckelboudt AY, Martin F, Guillot L, Sarniguet A (2009). The plant pathogenic fungus *Gaeumannomyces graminis* var *tritici* improves bacterial growth and triggers early gene regulations in the biocontrol strain *Pseudomonas fluorescens* Pf29Arp. New Phytol. 181:435-447.

Blaha D, Prigent-Combaret C, Mirza MS, Moe¨nne- LY (2006). Phylogeny of the 1-aminocyclopropane- 1-carboxylic acid deaminase-encoding gene *acdS* in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol. Ecol. 56:455-470.

Bloemberg GV, Wijfjes AH, Lamers GE, Stuurman N, Lugtenberg BJ (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Mol Plant-Microbe Interact. 13:1170-1176.

Bodilis J, Calbrix R, Gue´rillon J, Me´rieau A, Pawlak B, Orange N, Barray S (2004). Phylogenetic relationships between environmental and clinical isolates of *Pseudomonas fluorescens* and related species deduced from 16S rRNA gene and OprF protein sequences. Syst. Appl. Microbiol. 27:93-108.

Bossis E, Lemanceau P, Latour X, Gardan L (2000). The taxonomy of *Pseudomonas fluorescens* and *Pseudomonas putida*: current status and need for revision. Agronomie. 20:51-63.

Budzikiewicz H (1993). Secondary metabolites: fluorescent pseudomonads. *FEMS* Microbiol Rev. 104:209-228.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994). Green fluorescent protein as a marker for gene expression. Science. 263:802-805.

Champion AB, Barrett EL, Palleroni NJ, Soderberg KL, Kunisawa R, Contopoulou R, Wilson AC, Doudoroff M (1980). Evolution in *Pseudomonas fluorescens*. J. Gen. Microbiol. 120(2):485-511.

Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGM, Schripsema J, Kroon B, Scheffer RJ, Keel C, Bakker Peter AHM, Tichy H-V, de Bruijn FJ, Thomas-Oates JE, Lugtenberg BJJ (1998). Biocontrol by phenazine 1-carboxamide producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f sp *radicis lycopersici.* Mol Plant-Microb Interact. 11:1069-1077.

Chin A, Woeng TFC, Bloemberg GV, Lugtenberg BJJ (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. New Phytol. 157:503-523.

Cook RJ, Thomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G, Kim DS (1995). Molecular mechanisms of defense by rhizobacteria against root disease. Proc Natl Acad Sci USA. 92:4197-4201Corbell N, Loper JE (1995). A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 177:6230-6236.

Cuppels DA, Stipanovic RD, Stoessl A, Stothers JB (1987). The constitution and properties of a pyochelin-zinc complex. Can. J. Chem. 65:2126-2130.

Dangl JL, Dietrich RA, Richberg MH (1996). Death do not have no mercy: cell death programs in plant-microbe interactions. Plant Cell. 8:1793-1807.

Davison J (1986). Plant beneficial bacteria. Bio. Technol. 6:282-286.

De Meyer G, Höfte M (1997). Salicyclic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. Phytopathol. 87:588-593.

De Weert S, Dekkers LC, Kuiper I, Bloemberg GV, Lugtenberg BJJ (2004). Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. J. Bacterio.186:3153-3159.

Défago G, Haas D (1990). *Pseudomonas* as antagonists of soilborne plant pathogens: modes of action and genetic analysis. Soil Biochem. 6:249-291.

Delaney TP, Friedrich L, Ryals JA (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resisitance. Pross. Nat. Acad. Sci. USA. 92:6602-6606.

Delany I, Sheenan MM, Fenton A, Bardin S , Aarons S, O'Gara F (2000). Regulation of production of the antifungal metabolite 2 4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113 genetic analysis of *phlF* as a transcriptional repressor. Microbiol. 146:537-543.

Delany IR, Walsh UF, Ross I, Fenton AM, Corkery DM, O'Gara F (2001). Enhancing the biocontrol efficacy of Pseudomonas fluorescens F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. Plant Soil. 232(1-2):195-205

Delaney SM, Mavrodi DV, Bonsall RF, Thomashaw LS (2001). *phzO* a gene for the biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaceins* 30-84. J. Bacteriol. 183:318-327.

Delorme S, Philippot L, Edel-Hermann V, Deulvot C, Mougel C, Lemanceau P (2003). Comparative genetic diversity of *narG narZ* and 16S rRNA genes in fluorescent Pseudomonads. Appl. Environ. Microbiol. 69(2):1004-1012.

Dow M, Newman MA, von RE (2000). The induction and modulation of plant defense responses by bacterial lipopolysaccharides. Annu. Rev. Phytopathol. 38:241-261.

Dubuis C, Keel C, Haas D (2007). Dialogues of rootcolonizing biocontrol pseudomonads. Eur. J. Plant Pathol.119: 311-328.

Duffy BK, Défago G (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 65:2429-2438.

Duijff BJ, Recorbet G, Bakker PAHM, Loper JE, Lemanceau P (1999). Microbial antagonism at the root level is involved in the suppression of fusarium wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358. Phytopathology. 89:1073-1079.

Elad Y, Baker R (1985). Influence of trace amounts of cations and

siderophore-producing pseudomonads on chlamydospore germination of *Fusarium oxysporum.* Phytopathol. 75:1047-1052.
Frapolli M, De´fago G, Moe¨nne-Loccoz Y (2007). Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp producing the antifungal compound 2 4- diacetylphloroglucinol. Environ. Microbiol. 9:1939-1955.
Fu ZQ, Dong X (2013). Systemic acquired resistance turning local infection into global defense. Annu. Rev. Plant Biol. 64:839-63.
Gaffney TD, Lam ST, Ligon J, Gates K, Frazelle A, Di Maio J, Hill S, Goodwin S, Torkewitz N, Allshouse AM, et al. (1994). Global regulation of antifungal factors by a *Pseudomonas fluorescens* biological control strain. Mol Plant Microb. Interact. 7:455-463.
García-García-Martínez J, Acinas SG, Antón AI, Rodríguez-Valera F (1999). Use of 16-23S ribosomal genes spacer region in studies of prokaryotic diversity. J. Microbiol. Meth. 36:55-64.
Godfrey SAC, Marshall JW (2002). Soil on imported shipping containers provides a source of new Pseudomonad biodiversity into New Zealand. New Zealand J. Crop Hortic. Sci. 30:19-27.
Gómez-Gómez L, Felix G, Boller T (1999). A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J. 18:277-284.
Götz M, Gomes NCM, Dratwinski A, Costa R, Berg G, Peixoto R, Mendonça-Hagler L, Smalla K (2006). Survival of *gfp*-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community. FEMS Microbiol. Ecol. 56:207-218.
Grimont PAD, Vancanneyt M, Lefèvre M, Vandemeulebroecke K, Vauterin L, Brosch R, Kersters K, Grimont F (1996). Ability of Biolog and Biotype 100 systems to reveal the taxonomic diversity of the Pseudomonads. Syst. Appl. Microbiol. 19:510-527.
Gürtler V, Stanisich VA (1996). New approaches to typing and identification of bacteria using the 16S - 23S rDNA spacer. Microbiol. 142:3-16.
Guo JH, Qi HY, Guo YH, Ge HL, Gong LY, Zhang LX, Sun PH (2004). Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. Biol Control. 29:66-72.
Gutell RR, Weiser B, Woese CR, Noller HF (1985). Compartive anatomy of 16S-like ribosomal RNA. Prog Nucleic Acid Res. Mol. Biol. 32:155-216.
Haas D, Keel C (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 41:117-153.
Haas D, Blumer C, Keel C (2000). Biocontrol ability of fluorescent pseudomonads genetically dissected importance of positive feedback regulation. Curr. Opin. Biotechnol. 11:209-297.
Haas D, Défago G (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3(4):307-319.
Hammer PE, Hill DS, Lam ST, van Pee KH, Ligon JM (1997). Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Appl. Environ. Microbiol. 63:2147-2154.
Handelsman J, Stabb EV (1996). Biocontrol of soilborne plant pathogens. Plant Cell. 8:1855-1869.
He SY, Jin Q (2003). The Hrp pilus: learning from flagella. Curr. Opin. Microbiol. 6:15-19.
Heeb S, Haas D (2001). Regulatory roles of GacS-GacA two component system in plant associated and other Gram-negative bacteria. Mol Plant-Microb Interact. 14:1351-1363.
Höfte M (1993). Classes of microbial siderophores *In* Iron chelation in plants and soil microorganisms Edited by LL Barton and BC Hemming Academic Press San Diego Calif pp 3-26.
Iavicoli A, Boutet E, Buchala A, Métraux JP (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant-Microbe Interact. 16:851-858.
Janse JD, Derks JHJ, Spit BE, Van DTWR (1992). Classification of fluorescent soft rot *Pseudomonas* bacteria including *P marginalis* strains using whole cell fatty acid analysis. Syst. Appl. Microbiol. 15:538-553.
Jensen MA, Webster JA, Straus N (1993). Rapid identification of bacterial on the basis of polymerase chain reaction amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. 59(4):945-952.
Jonhson J, Palleroni NJ (1989) Desoxyribonucleic acid similarities among *Pseudomonas* species. Int. J. Syst. Bacteriol. 39:230-235.
Justyna PG, Ewa K (2013). Induction of resistance against pathogens by β-aminobutyric acid. Acta Physiol. Plant. 35:1735-1748.
Karthikeyan V, Gnanamanickam SS (2008). Biological control of *Setaria* blast *Magnaporthe grisea* with bacterial strains. Crop Protect. 27:263-267.
Keel C, Voisard C, Berling CH, Kahr G, Défago G (1989). Iron sufficiency a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. Phytopathol. 79:584-589.
Kirner S, Philip EH, Steven DH, Annett A, Ilona F, Laura JW, Mike L, Karl-Heinz vP, James ML (1998). Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens.* J Bacteriol. 180(7):1939-1943.
Kitten T, Kinscherf T, McEvoy G, Willis DK (1998). A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae.* Mol. Microbiol. 28:917-929.
Kloepper JW, Leong J, Teintze M, Schroth MN (1980). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. Curr Microbiol. 4:317-320.
Kononova SK, Nesmeyanova MA (2002). Phosphonates and their degradation by microorganisms. Biochemistry Moscow. 67(2):184-195.
Kragelund L, Nybroe O (1996). Competition between *Pseudomonas fluorescens* Ag1 and *Alcaligenes eutrophus* JMP134 pJP4 during colonization of barley roots. FEMS Microbiol. Ecol 20:41-51.
Latifi AM, Winson K, Foglino M, Bycroft BW, Stewart GSAB, Lazdunski A, Williams P (1995). Multiple homologues of *LuxR* and *LuxI* control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PA01. Mol. Microbiol. 17:333-343.
Latour X, Corberand T, Laguerre G, Allard F, Lemanceau P (1996). The composition of fluorescent Pseudomonad populations associated with roots is influenced by plant and soil type. Appl. Environ. Microbiol. 62:2449-2456.
Leeman M, Den Ouden FM, Van Pelt JA, Dirkx FPM, Steijl H, Bakker PAHM, Schippers B (1996). Iron availability affects induction of systemic resistance to Fusarium wilt of radish by *Pseudomonas fluorescens.* Phytopathology. 86:149-155.
Leeman M, Van Pelt JA, Den OFM, Heinsbroek M, Bakker PAHM, Schippers B (1995). Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens.* Phytopathology. 85:1021-1027.
Lemanceau P, Corberand T, Gardan L, Latour X, Laguerre G, Boeufgras JM, Alabouvette C (1995). Effect of two plant species flax *Linum Usitatissinum* L and tomato *Lycopersicon esculentum* Mill on the diversity of soilborne populations of fluorescent pseudomonads. App Environ Microbiology. 61(3):1004-1012.
Locatelli L, Tarnawski S, Hamelin J, Rossi P, Aragno M, Fromin N (2002). Specific PCR amplification for the genus *Pseudomonas* targeting the 3' half of 16S rDNA and the whole 16S-23S rDNA spacer. Syst. Appl. Microbiol. 25:220-227.
Loper JE, Buyer JS (1991). Siderophores in microbial interactions on plant surfaces. Mol. Plant Microbe. Interact. 4:5-13.
Louws FJ, Fullbright DW, Stephens CT, de Bruijn FJ (1994). Specific genomic fingerprintings of phytopathogenic *Xantomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.
Lübeck PS, Hansen M, Sørensen J (2000). Simultaneous detection of the establishment of seed-inoculated *Pseudomonas fluorescens* strain DR54 and native soil bacteria on sugar beet root surfaces using fluorescence antibody and in situ hybridization technique. FEMS Microbiol. Ecol. 33:11-19.
Lupski JR, Weinstock GM (1992). Short interspersed repetitive DNA

sequences in prokaryotic genomes. J. Bacteriol. 174:4525-4529.
Malamy J, Carr JP, Klessig DF, Raskin I (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science. 250(4983):1002-1004.
Mann J (1987). *Secondary Metabolism* Clarendon Press Oxford UK 2nd edn.
Mark GL, Morrissey JP, Higgins P, O'Gara F (2006). Molecular-based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. FEMS Microbiol. Ecol. 56:167-177.
Maurhofer M, Hase C, Meuwly P, Métraux JP, Défago G (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: Influence of the *gacA* gene and of pyoverdine production. Phytopathology. 84:139-146.
Maurhofer M, Reimmann C, Schmidli-Sacherer P, Heeb S, Haas D, Défago G (1998). Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. Phytopathology. 88:678-684.
Mavrodi DV, Ksenzenko VN, Bonsall RF, Cook RJ , Boronin AM, Thomashaw LS (1998). A seven gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. J. Bacteriol. 180:2541-2548.
Mavrodi OV, Mavrodi DV, Weller DM, Thomashow LS (2006). Role of *ptsP orfT* and *sss* recombinase genes in root colonization by *Pseudomonas fluorescens* Q8r1-96. Appl. Environ. Microbiol. 72:7111-7122.
Mazzola M, Cook RJ, Thomashaw LS (1992). Weller D M and Pierson III L S Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. 58:2616-2624.
McQuilken MP, Halmer P, Rhodes DJ (1998). Application of microorganisms to seeds In Formulation of Microbial Biopesticides: Beneficial Microorganisms Nematodes and Seed Treatments Edited by HD Burges Dordrecht: Kluwer Academic Publishers 255-285.
Mercado-Blanco J, Van der Drift KMGM, Olsson P, Thomas- Oates JE, van Loon LC, Bakker PAHM (2001). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. J. Bacteriol. 183:1909-1920.
Meyer JM, Abdallah MA (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis purification and physicochemical properties. J. Gen. Microbiol. 107:319-328.
Meyer JM, Geoffroy VA, Baida N, Gardan L, Izard D, Lemanceau P, Achouak W, Palleroni NJ (2002). Siderophore typing a powerful tool for the identification of fluorescent and non-fluorescent pseudomonads. Appl. Environ. Microbiol. 68(6):2745-2753.
Meziane H, Van der SI, Van Loon LC, Höfte M, Bakker PAHM (2005). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Mol. Plant Pathol. 6:177-185.
Misaghi IJ, Olsen MW, Cotty PJ, Donndelinger CR (1988). Fluorescent siderophore-mediated iron deprivation: a contingent biological control mechanism. Soil Biol. Biochem. 20:573-574.
Mittal S, Johri BN (2008). Influence of management practices on the diversity of pseudomonads in rhizosphere soil of a marginal wheat cropping system. Biol. Fertil. Soil. 44:823-831.
Molina L, Constantinescu F, Michel L, Reimmann C, Duffy B, De´fago G (2003). Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiol. Ecol. 45:71-81.
Moore ERB, Mau M, Arnscheidt A, Böttger EC, Hutson RA, Collins MD, Van Der PY, De Wachter R, Timmis KN (1996). The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* sensu stricto and estimation of the natural intrageneric reletionships. Syst Appl Microbiol. 19:478-492.
Neefs JM, Van der Peer Y, Hendriks L, De Wachter R (1990). Compilation of small ribosomal subunit RNA sequences. Nucleic Acid Res. 18:2237-2317.
Neelamegam R, Ayyadurai N, Kayalvizhi N, Gunasekaran P (2012). Genotypic and Phenotypic Diversity of PGPR Fluorescent Pseudomonads Isolated from the Rhizosphere of Sugarcane *Saccharum officinarum* L. J. Microbiol. Biotechnol. 22(1):13-24.
Normand P, Ponsonnet C, Nesme X, Neyra M, Simonet P (1996). ITS analysis of prokariotes *In*: Akkermans DL Van Elsas JD, Bruijn FJ eds Molecular Microbial Ecology Manual Kluwer Academic Publishers Dordrecht p 1-12.
Normander B, Hendriksen NB, Nybroe O (1999). Green fluorescent protein-marked *Pseudomonas fluorescens*: localization viability and activity in the natural barley rhizosphere. Appl. Environ. Microbiol. 65:4646-4651.
Notz R, Maurhofer M, Dubach H, Haas D and Défago G (2002). Fusaric acid producing strains of *Fusarium oxysporum* alter 2 4-diacetylphloroglucinol biosynthesis gene expression in *Pseudomonas fluorescens* CHA0 *in vitro* and in the rhizosphere of the wheat. Appl Environ Microbiol. 68:2229-2235.
Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D, Défago G (2001). Biotic factors affecting expression of the 2 4-diacetylpholoroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathology. pp 873-881.
Nowak-Thompsan B, Chancey N, Wing JS, Gould SJ, Loper JE (1999). Characterization of pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 181:2166-2174.
Nowak-Thompsan B, Gould SJ, Kraus J, Loper JE (1994). Production of 2 4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. Microbiol. 40:1064-1066.
Palleroni NJ (1993). *Pseudomonas* classificassion: a new case history in the taxonomy of Gram- negative bacteria. Antonie van Leeuwenhoek. 64:231-251.
Palleroni NJ, Ballard RW, Ralston E, Doudoroff M (1972). Deoxyribonucleic acid homologies among some *Pseudomonas* Species. J. Bacteriol. 110:1-11.
Palleroni NJ, Kunisawa R, Contopolou R, Doudoroff M (1973). Nucleic acid homologies in the genus *Pseudomonas.* J. Syst. Bacteriol. 23:333-339.
Phoebe CH Jr, Combie J, Albert FG, Van Tran K, Cabrera J, Correira HJ, Guo Y, Lindermuth J, Rauert N, Galbraith W, Selitrennikoff CP (2001). Extremophilic organisms as an unexplored source of antifungal compounds. J Antibiot. 54 (1):56-65.
Pierson LS, Wood DW, Pierson EA, Chancey ST (1998). *N*-acyl homoserine lactone-mediated gene regulation in biological control by fluorescent pseudomonads: current knowledge and future work. Eur. J. Plant Pathol. 104:1-9.
Pieterse CMJ et al (2001). Cross-talk between plant defence signaling pathways: boost or burden? Ag Biotech Net. 3 ABN 068.
Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ and van Loon LC (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis.* Plant Cell. 10:1571-1580.
Preston GM (2004). Plant perceptions of plant growth-promoting *Pseudomonas.* Philos Trans R Soc London 359:907-918.
Raaijmakers JM, Leeman M, Van Oorschot MMP, Van der SI, Schippers B, Bakker PAHM (1995). Doseresponse relationships in biological control of fusarium wilt of radish by *Pseudomonas* spp. Phytopathology. 85:1075-1081.
Rainey PB (1999). Adaption of *Pseudomonas fluorescens* to the plant rhizosphere. Environ Microbiol. 1 :243-257.
Ramette A, Frapolli M, De´fago G, Moe¨nne-Loccoz Y (2003). Phylogeny of HCN synthase-encoding hcnBC genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. Mol Plant-Microbe Interact. 16:525-535.
Ramette A, Moe¨nne-Loccoz Y, De´fago G (2001). Polymorphism of the polyketide synthase gene *phlD* in biocontrol fluorescent pseudomonads producing 2 4- diacetylphloroglucinol and comparison

of PhlD with plant polyketide synthases. Mol Plant-Microbe Interact.14:639-652.
Ran LX, Li ZN, Wu GJ, Van Loon LC, Bakker PAHM (2005). Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur. J. Plant Pathol. 113:59-70.
Reitz M, Oger P, Meyer A, Niehaus K , Farrand SK, Hallman J, Sikora R (2002). Importance of the 0- antigen core-region and lipid A of rhizobial lipopolysaccharides for the induction of systemic resistance in potato to *Globodera pallida*. Nematology. 4:73-79.
Sanguin H, Kroneinsen L, Gazengel K, Kyselkova´ M, Remenant B, Prigent-Combaret C, Grundmann GL, Sarniguet A (2008). Development of a 16S rRNA microarray approach for the monitoring of rhizosphere *Pseudomonas* populations associated with the decline of take-all disease of wheat. Soil Biol. Biochem. 40:1028-1039.
Schnider-Keel U, Arnaud S, Monika M, Caroline B, Brion D, Gigot-Bonnefoy C, Cornelia R, Notz R, Geneviève D, Dieter H, Christoph K (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and suppression by the bacterial metabolite salicylate and pyoluteorin. J Bacteriol. 182(5):1215-1225.
Schnider-Keel U, Keel C, Blumer C, Troxer J, Défago G, Haas D (1995). Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. 177:387- 5392.
Siddiqui IA, Shoukat SS (2003). Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: Importance of bacterial secondary metabolite 2 4-diacetylphloroglucinol. Soil Biol Biochem. 35:1615-1623
Sørensen J, Jensen LE, Nybroe O (2001). Soil and rhizosphere as habitats for *Pseudomonas* inoculants: new knowledge on distribution activity and physiological state derived from micro-scale and single-cell studies. Plant Soil. 232:97-108.
Spiers AJ, Buckling A, Rainey PB (2000). The causes of *Pseudomonas* diversity. Microbiology. 146:2345-2350.
Stevans AM, Dolan KM, Greenberg EP (1994). Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the lux promoter region. Proc. Natl. Acad. Sci. USA. 91:12619-12623.
Sticher L, Mauch-Mani B, Métraux JP (1997). Systemic acquired resistance. Annu. Rev. Phytopathol. 35:235-270
Suslow TV, Schroth MN (1982). Rhizobacteria of sugar beets effects of seed application and root colonization on yield. Phytopathology. 72:199-206.
Ton J, De Vos M, Robben C, Buchala A, Métraux JP, Van Loon LC, Pieterse CM (2002). Characterization of *Arabidopsis* enhanced disease susceptibility mutants that are affected in systemic induced resistance. The plant J. 29:11-21.
Troxler J, Zala M, Natsch A, Moënne-Loccoz Y, Défago G (1997). Autecology of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the rhizosphere and inside roots at later stages of plant development. FEMS Microbiol. Ecol. 23:119-130.
Validov S, Mavrodi O, De La Fuente L, Boronin A, Weller D, Thomashow L, Mavrodi D (2005). Antagonistic activity among 2 4-diacetylphloroglucinolproducing fluorescent *Pseudomonas* spp. FEMS Microbiol Lett. 242:249-256.
Van Loon LC, Bakker PAHM, Pieterse CMJ (1998). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36:453-483.
Van Loon LC, Van Strien EA (1999). The families of pathogenesis-related proteins their zctivities and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55:85-97.
Van Peer R, Schippers B (1992). Lipopolysaccharides of plant-growth promoting *Pseudomonas* sp strain WCS417r induce resistance in carnation to fusarium wilt. Neth. J. Plant Pathol. 98:129-139.
Van Peer R, Niemann GJ, Schippers B (1991). Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp strain WCS417r. Phytopathology. 81:728-734.
Van Wees SCM, De Swart EAM, Van Pelt JA, van Loon LC, Pieterse CMJ (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. 97:8711-8716.
Van Wees SCM, Pieterse CMJ, Trijssenaar A, Van't Westende YAM, Hartog F, van Loon LC (1997). Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. Mol Plant-Microbe Interact. 10:716-724.
Versalovic J, Koeuth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application of bacterial genomes. Nucleic Acid Res. 19:6823-6831.
Visca P , Colotti G, Serino L , Verzili D, Orsi N, Chiancone E (1992). Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. Appl. Environ. Microbiol. 58(9):2886-2893.
Wang C, Knill E, Glick BR, De´fago G (2000). Effect of transferring 1-aminocyclopropane-1-carboxylic acid ACC deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can. J. Microbiol. 46:1-10.
Wang D, Lee SH, Seeve C, Yu JM, Pierson LS, Pierson EA (2013) Roles of the Gac-Rsm pathway in the regulation of phenazine biosynthesis in Pseudomonas chlororaphis 30-84. Microbiology open. 2(3):505-524.
Ward ER , Uknes SJ, Williams SC, Dincher SS Wiederhold, DL, Alexander DC, Ahl-Goy P, Metraux JP, Ryals JA (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. The plant cell. 3(10):1085-1094.
Wei B, Huang T, Dalwadi H, Sutton CL, Bruckner D and Braun J (2002). *Pseudomonas fluorescens* encodes the Crohn's disease-associated I2 sequence and T-cell superantigen. Infect Immun. 70:6567-6575.
Wei G, Kloepper JW, Tuzun S (1991). Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology. 81:1508-1512.
Weller DM, Van Pelt JA, Mavrodi DV, Pieterse CMJ, Bakker PAHM, Van Loon LC (2004). Induced systemic resistance (isr) in arabidopsis against pseudomonas syringae pv. tomato by 2,4-diacetylphloroglucinol (dapg)-producing pseudomonas fluorescens. APS Annual Meeting. Phytopathology 94: S108
Weller DM (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology. 97:250-256.
Wiyono S, Schulz DF, Wolf GA (2008). Improvement of the formulation and antagonistic activity of *Pseudomonas fluorescens* B5 through selective additives in the pelleting process. Biol. Control. 46:348-357.
Woese CR (1987). Bacterial evolution. Microbiol. Rev. 51(2): 221-271.
Wood DW, Pierson III LS (1996). The phzI gene of Pseudomonas aureofaciens 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. 168(1):49-53.
Yadav S, Yadav S, Kaushik R, Saxena AK, Arora DK (2013). Genetic and functional diversity of fluorescent *Pseudomonas* from rhizospheric soils of wheat crop. J. Basic Microbio. doi: 10.1002/jobm.201200384.
Zang Zhang RG, Pappas KM, Brace JL, Miller PC, Oulmassov T, Molyneaux JM, Anderson JC, Bashkin JK, Winans SC, Joachimiak A (2002). Structure of bacterial quorum sensing transcription factor complexed with pheromone and DNA. Nat. 417(6892):971-974.
Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nat. 428:764-767.