Chapter 3

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

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

Key words: antibiotics; biocontrol; PGPR.

1 INTRODUCTION

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

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

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

2 ANTIBIOTICS OF PGPR

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

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

Table 1. Antibiotics produced by rhizobacteria

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

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

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

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

2.1 Polyketides

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

2.1.1 Diacetyl phloroglucinol (DAPG)

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

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

2.1.1.1 Biosynthesis of DAPG

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

2.1.1.2 Phenotypes of DAPG producers

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

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

2.1.1.3 Genetic diversity of phlD among DAPG producers

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

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

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

2.1.1.4 Cross talk between DAPG producers

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

2.1.1.5 Positive cross talk

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

2.1.1.6 Negative cross talk

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

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

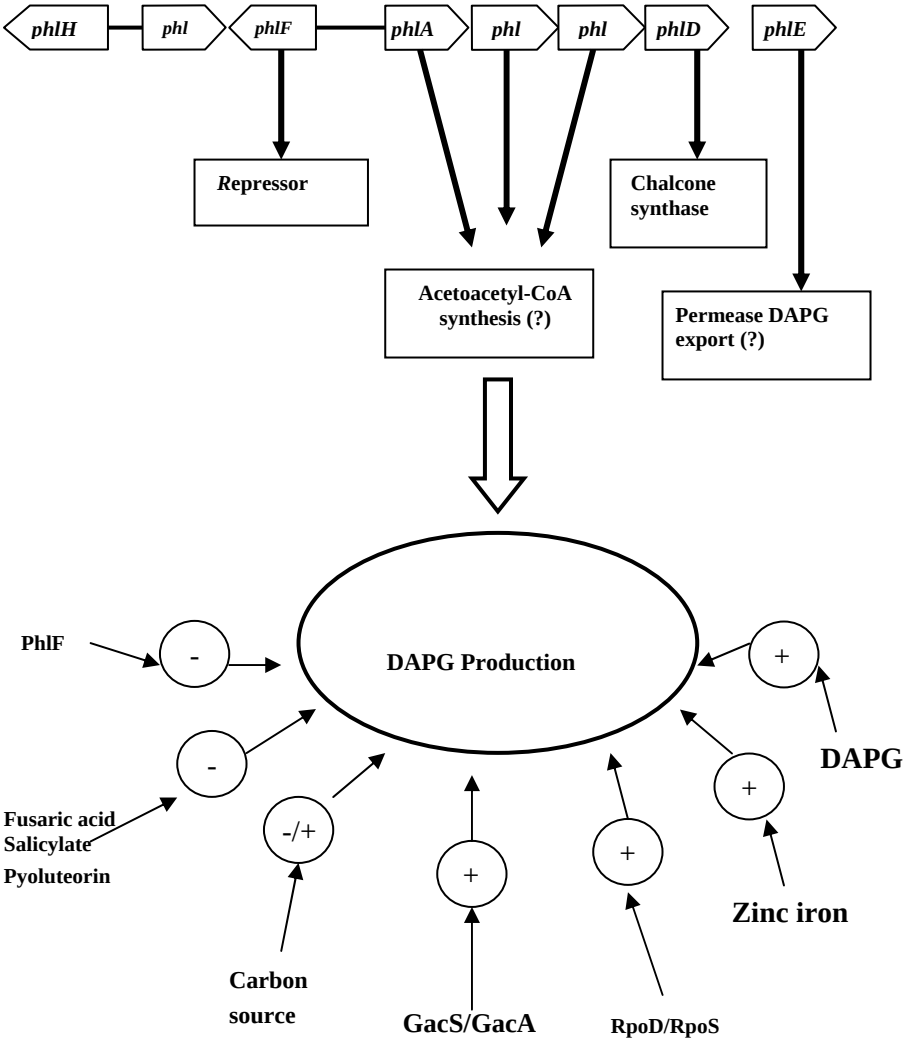

Fig. 1. Biosynthetic genes of DAPG and factors influencing its expression

2.1.1.7 Factors affecting DAPG production

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

2.1.2 Pyoluteorin

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

2.1.2.1 Gene locus for the biosynthesis of Plt

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

2.1.3 Mupirocin

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

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

2.2 Heterocyclic nitrogenous compounds

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

2.2.1 Phenazine

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

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

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

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

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

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

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

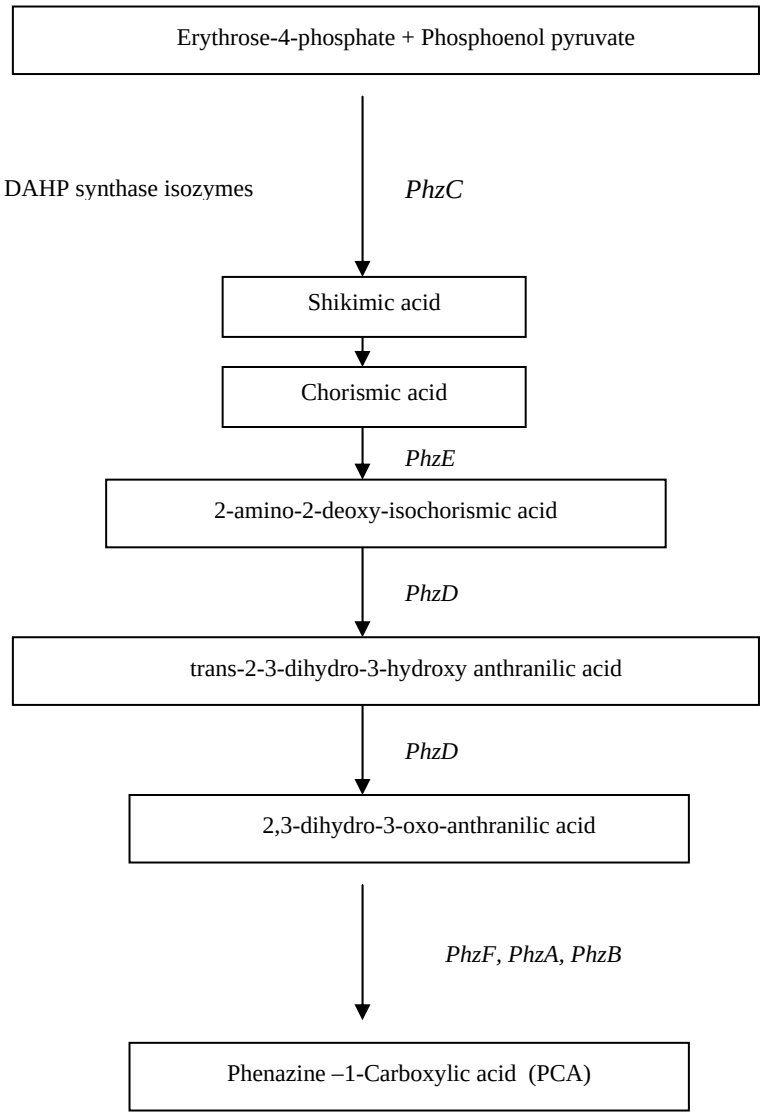

Fig. 2. Biosynthetic pathway of phenazine-1-carboxylic acid (PCA)

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

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

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

2.3 Phenylpyrrole antibiotic

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

2.3.1 Pyrrolnitrin

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

2.3.2 Genetic organization of pyrrolnitrin

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

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

2.3.3 Biosynthetic pathway of pyrrolnitrin

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

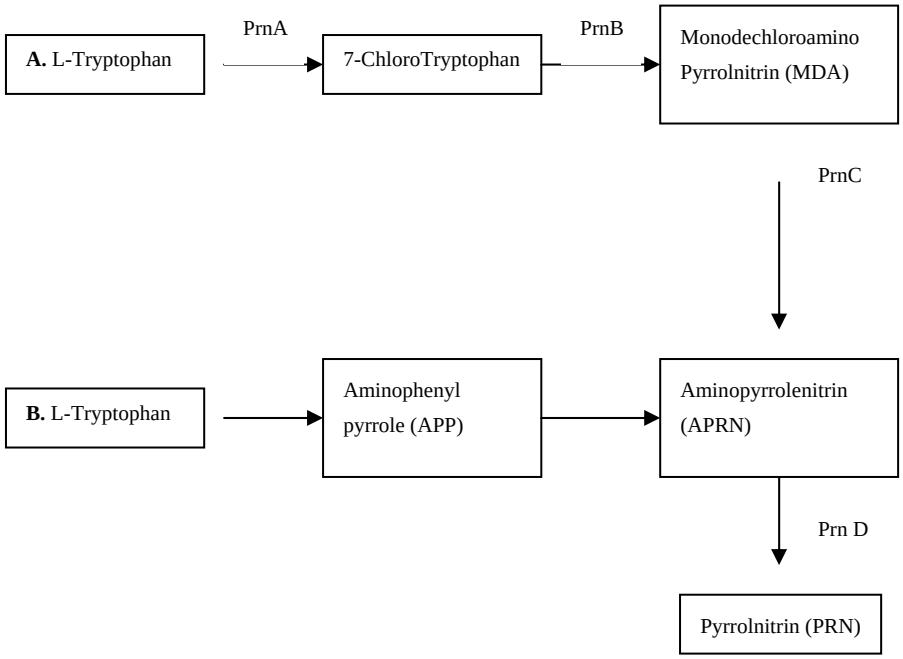

Fig. 3. Pathway for the synthesis of pyrrolnitrin

2.4 Cyclic lipopeptides

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

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

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

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

2.4.1 Durability of CLP in soil

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

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

2.5 Antifungal lipopeptide antibiotics

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

2.5.1 Iturins

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

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

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

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

2.5.2 Bacillomycin

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

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

2.5.3 Plipastatin

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

2.5.4 Surfactin

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

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

2.6 Aminopolyols (Zwittermicin A)

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

2.6.1 Biosynthesis

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

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

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

2.7 Volatile antibiotics

2.7.1 Hydrogen cyanide (HCN)

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

2.7.2 Aldehydes, alcohols, ketones and sulfides

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

3 REGULATION OF BIOSYNTHESIS OF ANTIBIOTICS

Regulation of secondary metabolites production involves:

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

3.1 Two-component regulatory system

3.1.1 GacS/GacA system

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

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

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

3.1.2 LuxI and LuxR proteins based regulation

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

3.2 Sigma factors based regulation

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

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

3.3 Microbial metabolites in antibiotic regulation

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

4 MOLECULAR DETECTION OF ANTIBIOTICS

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

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

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

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

5 BROAD SPECTRUM ACTION OF ANTIBIOTICS BY PGPR

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

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

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

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

Continued table 3…..

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

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

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

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

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

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

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

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

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

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

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

7 ISR MEDIATED BY BACTERIAL ANTIBIOTICS

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

8 FACTORS MODULATING ANTIBIOTIC PRODUCTION AND ITS EFFICACY

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

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

8.1 Physical factors

8.1.1 pH, temperature and soil moisture

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

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

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

8.1.2 Nutrients

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

8.2 Level of acetylation

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

8.3 Variation in sensitivity

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

8.4 Cell concentration / Growth stage

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

9 CONCLUSIONS

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

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

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

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

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

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

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