CHAPTER 3

3.0 BIOSYNTHESIS AND REGULATION OF ANTIFUNGAL METABOLITES IN FLUORESCENT PSEUDOMONADS

3.1 INTRODUCTION

3.1.1 Biosynthesis of antifungal metabolites in fluorescent pseudomonads:

Strains of *Pseudomonas* spp. of worldwide origin have shown their abilities to suppress a wide variety of fungal root pathogens (Thomashow and Weller, 1988; Hammer et al., 1997; Mavrodi et al., 1998; Nowak-Thompson et al., 1999; Haas and Keel, 2003). The biocontrol ability of many strains is directly correlated with production of antibiotics such as 2, 4-diacetylphloroglucinol (2, 4- DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT) and hydrogen cyanide and the genetic loci for the biosynthesis of these antibiotics have been well characterized and the sequences are available (HCN) (Haas and Keel, 1997; Laville et al. 1998; Bangera et al., 1999; Raaijmakers et al. 2002; Haas and Keel, 2003).

3.1.1.1 2, 4- DAPG biosynthesis:

2,4-Diacetylphloroglucinol (2,4-DAPG), an antibiotic produced by *Pseudomonas fluorescens*, has broad-spectrum antibiotic activity, inhibiting organisms ranging from viruses, bacteria, and fungi to higher plants and mammalian cells.2,4-DAPG acts on multiple basic cellular processes. Three major physiological functions correlated with an increase in sensitivity to 2, 4-DAPG: membrane function, reactive oxygen regulation, and cell homeostasis (Kwak et al., 2011). 2, 4-DAPG in fluorescent *Pseudomonas*, is produced until the early stationary growth phase. Besides 2, 4- DAPG, other phlorogulcinol compounds isolated from fluorescent pseudomonads include monoacetylphloroglucinol (MAPG) and triacetylphloroglucinol (TAPG). 2, 4- DAPG is inhibitory to zoospores of oomycete, and causes membrane damage to *Pythium* spp. (Duffy and Defago, 1997). 2,4- DAPG appears to be degraded by the producing bacterium in to MAPG temporarily which accumulates as an intermediate product of the degradation process (Schnider-Keel et al., 2000; Abbas et al., 2004; Brodhagen et al., 2004; Baehler et al., 2005).2,4- DAPG

also appears to cause induced systemic resistance (ISR) in plants, thus Phil producing bacteria used in biocontrol can serve as specific elicitors of phytolexins and other similar molecules (Johri, 2003). The genes for the biosynthesis of 2,4- DAPG have been identified in *P. fluorescens* strains Q2-87, F113, CHA0 and Pf-5 (Bangera and Thomashow, 1999; Delany et al., 2000; Schnider-Keel et al., 2000; Paulsen et al., 2005). The 2,4- DAPG-biosynthetic locus includes the four biosynthetic genes *phl*ACBD and are transcribed as a single operon and required for the biosynthesis of both MAPG and 2,4- DAPG (Fig. 3.1) (Bangera and Thomashow, 1999; Delany et al., 2000; Schnider-Keel et al., 2000; Mavrodi et al., 2001). The divergently transcribed *phlF* gene located adjacent to *phlA* codes for a pathway-specific transcriptional repressor of the 2, 4- DAPG biosynthetic operon (Bangera et al., 1999; Schinder – Keel, 2000). 2, 4- DAPG also acts as signal that dissociates *phlF* from *phlA* promoter thereby autoinducing its own biosynthesis (Schnider-Keel et al., 2000; Bottiglieri and Keel, 2006). *Phl*D encodes a polyketide synthase (PKS) with similarity to plant genes encoding chalcone or stilbene synthases (Bangera and Thomashow, 1999). PhlD plays an essential role in the two proposed routes for the biosynthesis of the 2, 4- DAPG from MAPG (Bangera and Thomashow, 1999) and biosynthesis of 2, 4- DAPG from glucose (Achkar et al., 2005). Zha et al., 2006 demonstrated that PhlD bclongs to thc typc III PKS family and cxhibits a rclativcly broad substratc spccificity compared to other members of this family. In addition to its ability to produce phloroglucinol, it can produce a great diversity of products thereby expanding the existing reservoir of polyketides. The *phl*E gene located immediately downstream of the *phl*ACBD operon encodes a putative transmembrane permease (Bangera and Thomashow, 1999) which appears to be involved in 2, 4-DAPG resistance (Abbas et al., 2004). The *phl*H gene is located, at least in *P. fluorescens* strain CHA0 (*Pf* CHA0), downstream of *phl* F and is involved in pathway-specific control of 2, 4-DAPG biosynthesis. The available genetic data from other 2,4- DAPG-producing pseudomonad (Pf-5, Q2-87, F113) indicated that the *phl*G gene is not unique to strain CHΛ0, but appears to be commonly associated with the 2,4- DAPG biosynthetic locus(Schnider-Keel et al., 2000).The *phl*G gene has a size of 924-bp and is located between *phl*F and *phl*H (Fig. 3.1). It has been demonstrated that *phl*G encodes a hydrolase (PhlG) that catalyzes the conversion of 2, 4- DAPG into MAPG and acetate. The enzymatic activity of PhlG appears to be highly specific for its substrate 2, 4- DAPG (Bottiglieri and Keel, 2006).

3.1.1.2 Pyrrolnitrin biosynthesis:

Pyrrolnitrin (PRN) is a chlorinated phenylpyrrole antibiotic produced by several fluorescent and non-fluorescent pseudomonad. It was first isolated from *Burkholderia pyrrocinia* (Arima et al., 1964). *Pseudomonas* species such as *P. fluorescens, P. chlororaphis, P.aureofaciens,* and others such as *B. cepacia, Enterobacter agglomerans, Myxococcus fulvus* and *Serratia* spp. also produce PRN (Hammer et al., 1999). PRN was primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungus *Trichophyton* and subsequently PRN was developed as an agricultural fungicide (Ligon et al., 2000). PRN persists actively in the soil for 30 days and it does not readily diffuse as it is released after lysis of host bacterial cell, resulting in the slow release. It also has strong antifungal action against *R. solani* (El-Banna and Winkelmann, 1998). *P. fluorescens* strains producing PRN reduced take all decline of wheat (Tazawa et al., 2000). PRN producing strains have shown their activity against several bacteria and fungi, in particular *Rhizoctonia solani* (Hill et al. 1994), and this molecule is also effective against post-harvest diseases caused by *Botrytis cinerea* (Janisiewicz et al. 1988; Hammer and Evensen, 1993). The biocontrol agent, *P. fluorescens* BL915 contains four gene clusters involved in the biosynthesis of antifungal molecule PRN from the precursor tryptophan (Chang, 1981). Moreover a sequence from *B. pyrrocinia* DSM 10685 clustered closer to *Pseudomonas* than to other *Burkholderia* strains (de Souza et al. 2003). Genetic organization of pyrrolnitrin gene cluster includes 4 genes: *prnA, B, C, D* of 5.8 kb DNA (Hammer et al., 1997; Kirner et al., 1998). Product of *prnA* gene catalyses chlorination of L-trp to 7-chloro-L-trp to form aminopyrrolnitrin (Hammer et al.,1997).The *prnD* gene product catalyses oxidation of aminopyrrolnitrin to pyrrolnitrin (Nakatsu et al., 1995). Polymorphism was revealed in *prnD*, over a collection of *Pseudomonas* and *Burkholderia* of different origins.

3.1.1.3 Pyoluteorin biosynthesis:

Pyoluteorin (PLT) is an aromatic chlorinated polyketide with resorcinol ring linked to a bichlorinated pyrrole moiety. It was first isolated from *P. aeruginosa* (Takeda, 1958) followed by *P. fluorescens* Pf-5 and CHA0 (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). PLT is especially active against oomycetes like *Pythium ultimum*. PLT biosynthesis is initiated from proline or a related molecule, which serve as the precursors for dichloropyrrole moiety of PLT. It condenses with three acetate

equivalents coupled to chlorination and oxidation.The formation and cyclization of the C-skeleton proceeds by the action of a multienzyme complex (Nowak-Thompsan et al., 1999). PLT biosynthesis requires 10 genes; *pltLABCDEFG* and *pltRM* transcribed divergently (Nowak-Thompson et al., 1999). Among these, *pltB* and *pltC* encode type 1 polyketide synthetase. *Plt* G encodes a thio esterase; three halogenases are coded by *pltA*, *pltD* and *pltM*. The gene product of *pltR* is similar to LysR family of the transcriptional activators (Pierson et al., 1998; Nowak-Thompson 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).Until recently, PLT production seemed to be limited to a selected group of *Pseudomonas* spp. also producing 2, 4- DAPG and sometimes PRN, which are genotypically very similar (Keel et al. 1996; Sharifi-Tehrani et al., 1998; McSpadden gardener et al., 2000). Indeed, RFLP analysis of a *pltC* fragment showed no polymorphism among 12 *Pseudomonas* strains of different origins (de Souza et al., 2003).

3.1.1.4 Phenazine biosynthesis:

The phenazines (PHZs) are another important class of secondary metabolites produced by many strains of *Pseudomonas*. They cover a large family of heterocyclic nitrogen-containing, brightly colored pigments, with broad-spectrum antibiotic activity (Chin-A-Woeng et al., 2003). PHZs are low molecular weight secondary metabolites, produced by the bacterial genera *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). The implication of these antibiotics in biocontrol have been shown when bacterization of wheat and tomato seeds with *P. fluorescens* 2-79 and *P. chlororapis* PCL1391 provided protection against *Gaeumannomyces graminis* and *Fusarium oxisporum* f. sp. *radicis-lycopersici* (Thomashow and Weller,1988; Chin-A-Woeng et al.,1998). Priming the seeds with *P. chlororaphis* effectively controlled seed borne diseases of barley and oats. In addition, phenazines play an important role in bacterial competition and survival in the rhizosphere (Mazzola et al. 1992). More than 50 naturally occurring phenazine compounds have been identified. Few strains of PGPR produce 10 different phenazine derivatives at the 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). 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., 1992,1993). Seven genes *phzABCDEFG*, are involved in the synthesis of phenazine-1-carboxylic (PCA) and the *phz* biosynthetic loci are well conserved in *P. fluorescens* 2-79, *P. aeruginosa* PAO1 and *P. chlororapis* PCL 1394, with nucleotide homology ranging from 70 to 95 % between the different species (Chin-A-Woeng et al.,2003). Despite this homology, individual species differ in the range of compounds they produce. In all cases, PCA is the first compound formed in the biosynthetic pathway, but some species can have other genes coding for modifying enzymes. For example, in *P. aureofaciens* 30-84 PhzO is responsible for conversion of PCA in 2-OH-PCA (Delaney et al. 2001) (Fig. 3.3). And in *P. chlororapis, phzH* encodes an enzyme responsible for the conversion of PCA in phenazine carboxamide (PCN) (Chin-A-Woeng et al. 2001). 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 pyridoxaminc-5'-phosphatc oxidascs and scrvcs as a sourcc 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.

3.1.1.5 Hydrogen cyanide production:

HCN is a secondary metabolite produced by pseudomonads involved in disease suppression (Blumer and Haas , 2000). Production of HCN by biocontrol *Pseudomonas* has an implication in the suppression of diseases like black root-rot of tobacco caused by *Thielaviopsis basicola* (Laville et al., 1998). Genetic diversity is present in the genes responsible for cyanide synthesis leading to the classification of the HCN-producing *Pseudomonas* in 4 different groups based on *hcnBC* sequence, and it was shown that the different HCN groups differ in their HCN production level (Ramette et al., 2003)

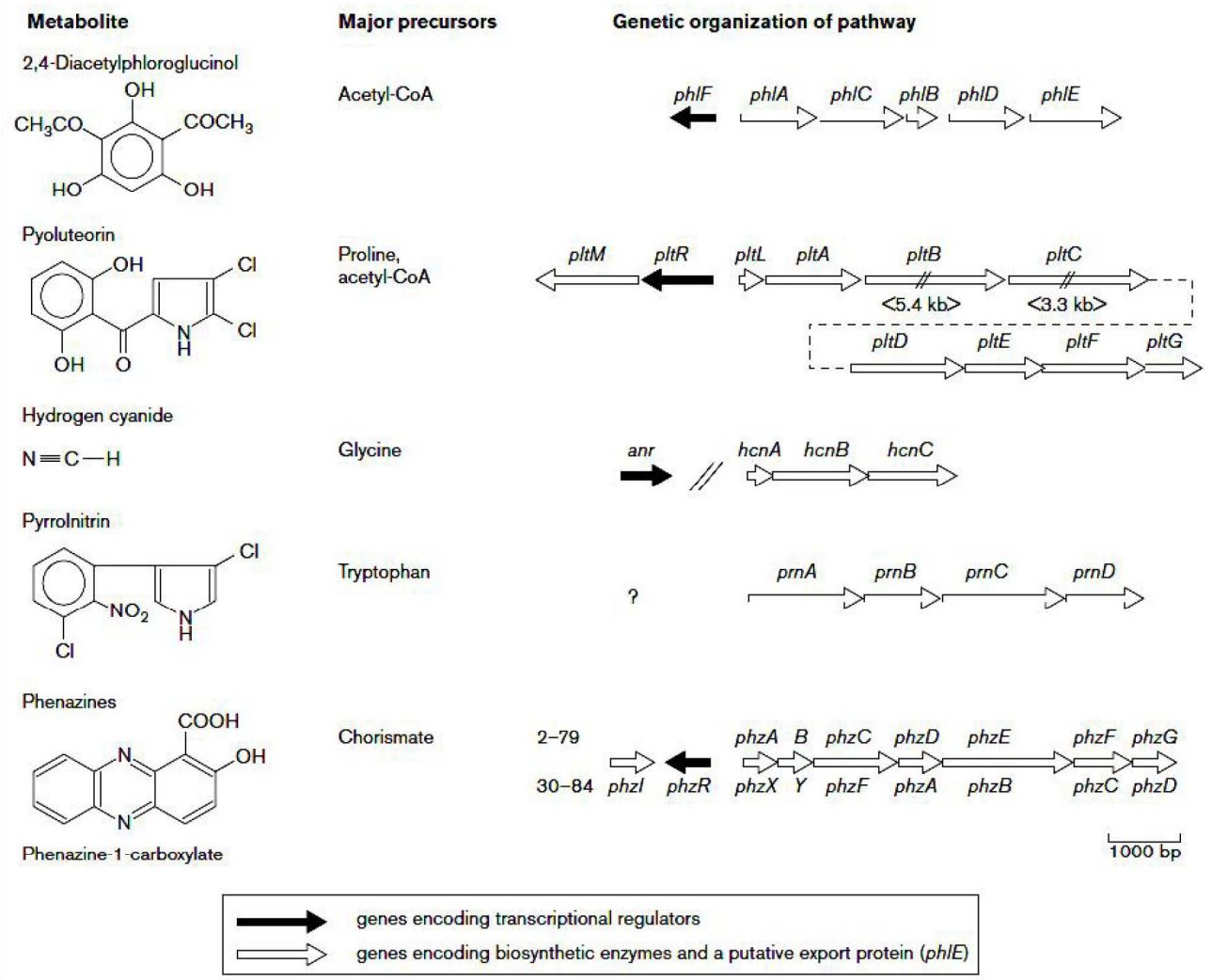


Fig.3.1: Genetic organization of 2, 4-DAPG, Pyoluteorin, HCN, Phenazine and Pyrrolnitrin biosynthesis genes (Haas et al., 2000)

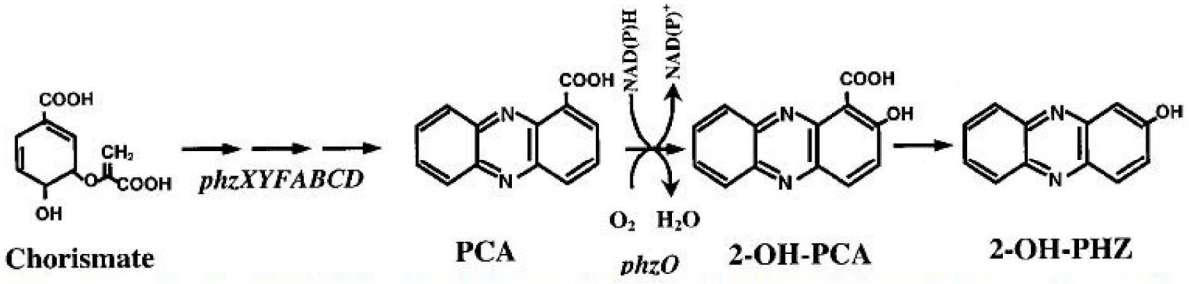


Fig.3.2 Biosynthetic pathway and genes involved for the biosynthesis of phenazines (Delaney et al., 2001)

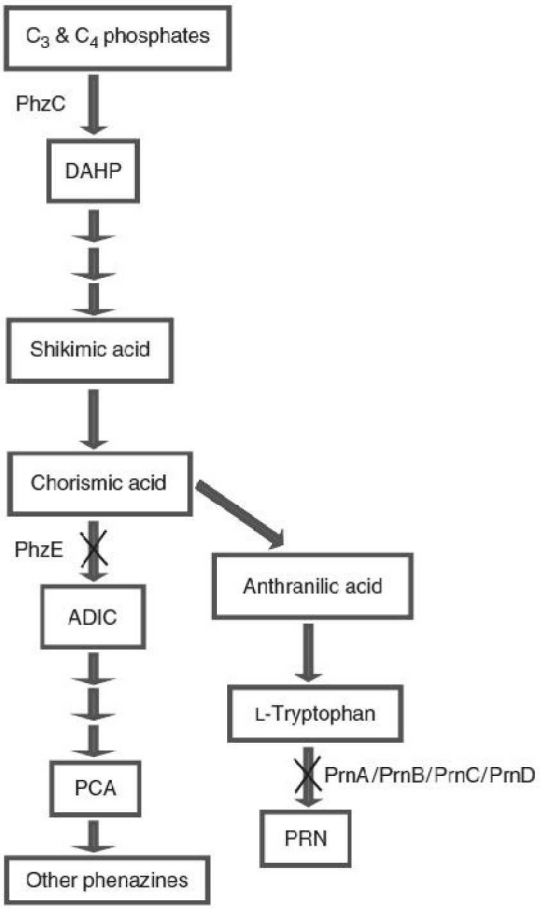


Fig.3.3: Biosynthesis of PRN and PHZ from shikimic acid in strain PA23-63 (Selin et al., 2012)

(C4- Erythrose-4-phosphate, C3-Phosphoenol pyruvate, DAHP- deoxyarabino-heptulosonate-7-phosphate; ADIC- 2-amino-4 deoxychorismic acid; PCA, phenazine-1-carboxylic acid; PRN, pyrrolnitrin)

3.1.2 Regulation of biosynthesis of antibiotics:

Regulation of secondary metabolites production in fluorescent pseudomonad involves environment dependent primary sensing, a secondary intermediate level for the regulation of antibiotic biosynthesis with other metabolic processes through global regulation and cellular homeostasis, a highly specific tertiary level regulation which requires the involvement of regulatory loci that are linked and divergently transcribed from structural genes for antibiotic biosynthetic genes (You et al., 1998; Duffy and Defago, 1999; Haas et al, 2000; Abbas et al., 2002). Numerous studies have been performed in order to identify traits and factors that contribute to successful establishment, spread and survival of bacterial inoculants in the

rhizosphere. These include (i) biotic and abiotic soil factors (Howie et al., 1987; Notz et al., 2001), (ii) host genotype factors (Weller and Thomashow, 1994; Smith and Goodman, 1999), (iii) rhizosphere-induced (rhi) genes (Rainey 1999) and colonization genes (Lugtenberg et al., 2001).

3.1.2.1 Abiotic factors mediated regulation:

Since abiotic environmental factors influence the type of antibiotic being produced, biocontrol activity may vary under different environmental conditions. Nutrient content, oxygen tension, osmotic conditions, phosphate, carbon and nitrogen sources as well as fungal, bacterial and plant metabolites can all differentially influence the production of 2,4- DAPG by *Pseudomonas*. 2,4-DAPG production can be modulated by abiotic and biotic factors, including carbon and nitrogen sources, metal ions and other minerals, and metabolites released by bacteria, fungi and plants (Duffy and Défago, 1999; Notz et al., 2001; Maurhofer et al., 2004). The impact of environmental factors on the production of 2, 4- DAPG has been studied both *in vitro* and *in situ* for a number of *Pseudomonas* strains (Shanahan et al., 1992; Duffy and Defago, 1999; Notz et al., 2001). For example, 2, 4- DAPG production was repressed in strain F113 by glucose, whereas in other strains of *P. fluorescens* glucose promoted 2, 4- DAPG production (Nowak-Thompson et al., 1994). Other carbon sources that promote 2, 4- DAPG production in *P. fluorescens* F113 were sucrose, fructose and mannitol, whereas sorbose repressed 2, 4- DAPG production (Shanahan et al., 1992). De La Fuente et al., 2007 has described the utilization of three carbon sources (trehalose, benzoate and valerate) by a collection of fifty five 2, 4- DAPG-producing *P. fluorescens* strains. Of the strains tested, 73%, 48% and 69% were able to utilize trehalose, benzoate and valerate as sole carbon sources, respectively. However, no correlation was found between a strains ability to utilize these carbon sources and superior rhizosphere competence on wheat and pea. The pH optimum for PCN production in *P. chlororaphis* PCL1391 is in the alkaline range (pH-7 to pH- 8) (Duffy and Defago, 1999), while in *P. aeruginosa* PCN production is optimal only at pH 7 and it is reduced at pH 8 (Slininger and Shea-Wilbur 1995). In *P. chlororaphis* PCL1391, PCN production is inhibited by salt stress (0.1 M NaCl), low temperature (16°C), and oxygen (10-20%). Under low oxygen conditions, PCN production increased and occurred at an earlier point in growth (van Rij et al., 2004). Glucose and glycerol exhibit a stimulatory effect on PCN production by *P. chlororaphis* PCL1391 (van Rij et al., 2004) and *P. aeruginosa* (Kanner et al., 1978), as well as PCA production by *P.*

fluorescens 2-79 (Slininger and Shea-Wilbur, 1995). Other antibiotics influenced by carbon source include PLT and 2, 4- DAPG (Fig.3.4) which show increased production in the presence of glycerol and glucose, respectively (Duffy and Defago, 1999). Dilution of growth media amended with glucose or glycerol had a stimulatory effect on 2, 4- DAPG but not on PLT (Duffy and Defago, 1999) (Fig.3.4). This differential effect on antibiotic production within the same organism may reflect the antibiotic specificity required under environmental changes in the ecosystem.

PRN production was stimulated by mannitol and fructose, and to a lesser magnitude, by glucose and glycerol (Duffy and Defago, 1999). In *P. chlororaphis* PCL1391, PCN production is stimulated by aromatic amino acids; tyrosine, phenylalanine, and to a smaller magnitude, tryptophan. Likewise, nitrogen sources like ammonia exhibit a stimulatory effect on PCN production by *P. aeruginosa* (Kanner et al., 1978) and *P. chlororaphis* PCL1391 (Slininger and Shea-Wilbur, 1995). Mineral-mediated regulation of antibiotic production is evident in *Pseudomonas* spp. In *P. fluorescens*, PCA production is increased in the presence of iron or magnesium (Slininger and Shea-Wilbur, 1995). The presence of inorganic phosphate has inhibitory effects on PLT, 2, 4- DAPG in *P. fluorescens* (Duffy and Defago, 1999) and phenazine production in *Pseudomonas* spp. (Turner and Messenger, 1986; Martin et al., 1994). Conversely, inorganic phosphate induced pyocyanine phenazine production in *P. aeruginosa* (Turner and Messenger, 1986).

The bacterial and plant metabolite salicylate as well as the fungal virulence factor fusaric acid inhibit 2, 4- DAPG production (Schnider-Keel et al., 2000; Baehler et al., 2005).When *Pseudomonas* is grown in batch culture, production of 2, 4- DAPG is typically observed at the end of the exponential growth phase during the transition to the stationary phase (Haas and Keel, 2003). Whereas in well-shaken liquid media the bacteria experience a uniform environment, there is a dynamic but more structured environment in the rhizosphere with a heterogeneous spatial distribution of nutrients.

3.1.2.2 Biotic factors:

Among the biotic factors, the resident microflora (Raaijmakers et al., 1999) as well as the plant can strongly influence the expression of antibiotic biosynthetic genes in pseudomonad. The composition of populations of fluorescent *Pseudomonas* spp. may vary significantly within the rhizosphere of different plant species cultivated in the same soil (Lemanceau et al., 1995), and

may change dramatically over time in response to agerelated physiological changes in plant species (Mazzola, 1999; Mazzola and Gu, 2000). It has been well demonstrated that *phlA* in strain CHA0 is expressed more strongly on maize and wheat roots than on bean and cucumber roots (Notz et al., 2001). This differential regulation is likely caused by differences in exudates composition between monocots and dicots (Notz et al., 2001). A major factor contributing to the inconsistent colonization by the bacterial inoculants remains their variable ecological performance (Weller, 1988). Although strains of fluorescent *Pseudomonas* spp. that produce 2, 4- DAPG are phenotypically similar (McSpadden et al., 2000; Mavrodi et al., 2001), they differ considerably in their ability to colonize the rhizosphere of particular crop species (Raaijmakers and Weller, 2001; Landa et al., 2002, 2003). In contrast, relatively few studies have addressed the effect of plant species on the dynamics, composition and activity of indigenous bacterial populations that share a specific antagonistic trait, such as the 2, 4- DAPG-producing *Pseudomonas* spp. Traits of both the plant and the bacteria undoubtedly contribute to the affinity between some bacterial genotypes and certain host plant species (Landa et al., 2003). In the same context, plant species variation was shown to significantly influence the community structure of *Pseudomonas* in the rhizosphere of two *Verticillium dahliae* host plants (Costa et al., 2006)

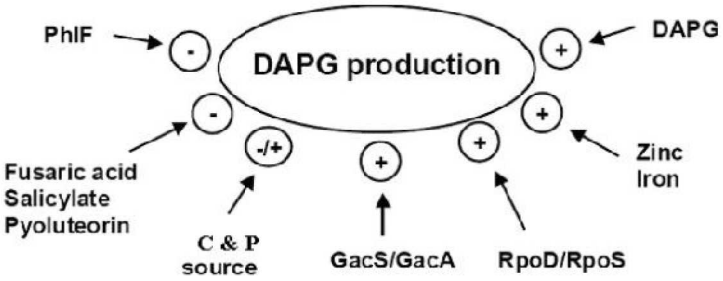


Fig 3.4 Abiotic factors influencing 2, 4- DAPG production (Schnider – Keel, 2000)

3.1.2.3 Global Regulatory Mechanisms:

GacS/GacA is a two-component signal transduction system found in many gram-negative bacteria including *Pseudomonas* spp and *Escherichia coli* and has been found in both pathogenic and nonpathogenic *Pseudomonas* spp.Unlike most two–component systems in bacteria, the genes encoding GacS and GacA are unlinked (Kinscherf and Willis, 1999; Nam et al., 2003).The non-pathogenic bacteria investigated include: *P. fluorescens* Pf-5, CHA0, BL915, *P. chlororaphis*

PCL1391, and *P. aureofaciens* strain 30-84. GacS (LemA) is a transmembrane sensor kinase first identified in *P. syringae* pv.*syringae* B728a (Willis et al., 1990). In response to an environmental signal, GacS is autophosphorylated and, subsequently, it transfers a phosphoryl group to its cognate cytoplasmic response regulator GacA. GacA was first identified in *P. fluorescens*, (Laville et al., 1992), and upon activation by phosphorylation; it controls its target genes either by a direct or indirect mechanism. GacS/GacA regulation of target gene expression occurs by positively regulating AHL synthesis (Reimmann et al., 1997; Chancey et al., 1999; Kinscherf & Willis, 1999), as well as acting independently of the AHL transduction pathway (Chancey et al., 1999; Kinscherf and Willis, 1999; Pessi and Haas, 2001). In *P. fluorescens*, no AHL signals have been detected (Heeb et al., 2002); however the GacS/GacA system positively regulates secondary metabolite and enzyme production in this genetic background. Since both pathogenic and nonpathogenic *Pseudomonas* spp. has GacS/GacA two-component regulatory systems, it is of importance to identify what signal(s) is commonly present and responsible for virulence or protection, depending on the bacteria-host association (Goodier and Ahmer, 2001). In *P. fluorescens* CHA0, gacS and gacA spontaneous mutations occur at high frequencies under nutrient-rich growth conditions and during stationary growth phase (Duffy and Defago, 2000). Figs.3.5 and 3.6 show the general characteristics of the Gac/Rsm signal transduction pathway in g-*Proteobacteria* (Rife et al., 2005) and the role of small RNA and regulatory sequences and their role in regulation of antifungal metabolites.

3.1.2.4 Sigma Factor Mediated transcriptional regulation:

Plant-associated *Pseudomonas* spp. encounter a multitude of environmental stress factors in response to which, the primary control of gene expression is mediated by the binding of specific sigma (σ) factors to the RNA polymerase core enzyme, necessary for promoter recognition. The expression of growth-related and housekeeping genes during exponential phase depends on RpoD (σ^{70}),whereas the expression of stationary phase genes is dependent on RpoS (σ^{s}).Throughout bacterial growth, σ factors are in competition for the binding of RNA polymerase (Jishage et al., 1996; Maeda et al., 2000) and the levels of RpoD (σ^{70}) and RpoS (σ^{s}) influence the expression of secondary metabolites. For example, in *P. fluorescens* CHA0, an increase in RpoD (σ^{70}) increases the production of pyoluteorin and 2, 4-DAPG both *in vitro* and

in the rhizosphere, ultimately increasing the biocontrol ability of this bacterium (Schnider et al., 1995). A mutation in *rpoS* increases the expression of PLT and 2, 4- DAPG biosynthetic genes. Conversely, an increase in the levels of RpoS (σ^s), functions to up regulate PRN, extracellular protease(s) and HCN production in the *Pf* CHA0 and Pf-5 (Sarniguet et al., 1995; Whistler et al., 1998; Haas and Keel, 2003; Heeb et al., 2005).

3.1.2.5 Quorum Sensing:

Quorum sensing and AHL production is more common among plant-associated *Pseudomonas spp.* compared to soil borne *Pseudomonas* spp. (Pierson et al., 1998). In *Pseudomonas* spp., quorum sensing is involved in regulating secondary metabolism, including the production of metal chelators (pyoverdines), lytic enzymes (protease, chitinazes), volatiles (HCN), and antifungal antibiotics. *Pseudomonas* bacteria producing auto inducer signal molecules known as HSL regulates expression (Pierson et al., 1995). Wood et al., 1997 demonstrated that bacteria in the wheat rhizosphere are capable of positively influencing the expression of genes in a bacterial population inhabiting the same niche. PhzI is an AHL synthase responsible for generating the signal molecule N-hexanoyl-homoserine lactone (C_6-HSL) (Wood et al., 1997). Activation of phenazine biosynthetic genes is mediated through the direct binding of PhzR-AHL complex at the phz-box, located upstream of the phenazine biosynthetic genes (Fig.3.2) as found in *P. aeruginosa, P. chlororaphis, P. fluorescence, P. putida, Pseudomonas syringae, Pseudomonas cichorii, and Pseudomonas corrugata* (Elasri et al., 2001) (Fig 3.7). Upstream in the cascade, the sensor GacS is activated by a putative environmental factor. Subsequently, GacS stimulates its cognate kinase GacA. GacA activates a cascade of genes including PsrA and RpoS. Unknown factors are regulated by GacA, unknown factors, together with RpoS; activate the quorum-sensing system phzI/phzR, which in turn switches on expression of the phz operon. These findings indicated that quorum sensing and the regulation of secondary metabolite production occurs under environmental conditions involving bacteria-host interactions. To determine how quorum sensing (QS) affects the production of secondary metabolites in Pseudomonas chlororaphis strain PA23. QS and RpoS have opposing effects on PA23 biofilm formation (Selin et al., 2012). While both QS-deficient strains produced little biofilm, the rpoS mutant showed enhanced biofilm production compared with PA23. QS Controls diverse aspects of PA23

physiology, including secondary metabolism, RpoS and biofilm formation. QS is expected to play a crucial role in PA23 biocontrol and persistence in the environment (Selin et al., 2012)

3.1.2.6 Positive Autoregulation

From an ecological aspect, autoinduction may be essential in combating fungal diseases through a rapid increase in antibiotic production, only when there is a sufficient bacterial population to efficiently support the antifungal effect (Schnider-Keel et al., 2000). This biocontrol effect was proven to be amplified when positive autoregulation functions as a cross-communication mechanism among 2, 4-DAPG producing bacteria (Maurhofer et al., 2004). It is possible that these regulatory mechanisms serve as an evolutionary adaptation, especially when the production of antifungal compounds can induce fungal defense mechanism(s) (Morrissey and Osbourn, 1999). Overall, the biocontrol effect will be more efficient through the use of cross-communication among homogenous or mixed bacterial populations. The mechanism postulated is as follows; 2,4-DAPG forms a complex with a repressor protein, PhlF, thereby lifting repression of the phlA promoter region(Bangera and Thomashow 1999).Similarly, positive auto regulation of pyoluteorin has been identified in *P. fluorescens* Pf-5 and CHA0 (Haas and Keel, 2003; Brodhagen et al., 2004). Positive auto regulation has also been demonstrated in *P. aeruginosa* siderophore biosynthesis (Reimmann et al., 1998).At a transcriptional level, 2,4-DAPG positively controls the expression of its own biosynthesis genes via the pathway-specific regulator PhlF (Schnider-Keel et al. 2000; Abbas et al. 2002; Brodhagen et al. 2004; Maurhofer et al. 2004;Baehler et al. 2005). Pyoluteorin strongly represses biosynthesis of 2,4- DAPG in strains CHA0 and Pf-5 and vice versa, pointing to a mechanism of mutual feedback control that may help *P. fluorescens* to keep production of these compounds at balance and as exogenous signals mediate both intra- and interpopulation communication. In the genetically distinct biocontrol strains CHA0 and Q2-87, 2, 4- DAPG produced by either strain on wheat roots is perceived as a positive signal boosting *in situ* expression of *phl* genes in the other strain (Maurhofer et al. 2004; Dubuis et al., 2007).

3.1.2.7 Negative Regulation:

The ability of one antibiotic to negatively regulate expression of a different antibiotic has been demonstrated in *P. fluorescens* Pf-5, PLT and 2,4- DAPG inhibit each other's biosynthesis at the

transcriptional level. Thus PLT-deficient mutants were found to produce more 2, 4- DAPG than the wild type (Brodhagen et al., 2004). Additionally, PLT, 2, 4- DAPG and salicylate affect the production of other antibiotics produced by *P.fluorescens* (Haas and Keel, 2003), suggesting that depending on the environmental conditions, certain antibiotics may be preferentially favored for mediating biocontrol. Similarly, fungal toxins can negatively regulate antibiotic production e.g. *F.oxysporum* f. sp. *radicis-lycopersici* produces fusaric acid, that functions as a phytotoxin, as well as an inhibitor of 2,4- DAPG production in *P. fluorescens* CHA0 (Duffy and Defago, 1997) and PCN production, by repressing phzR expression in *P. chlororaphis* PCL1391 (Van Rij et al., 2004; 2005).

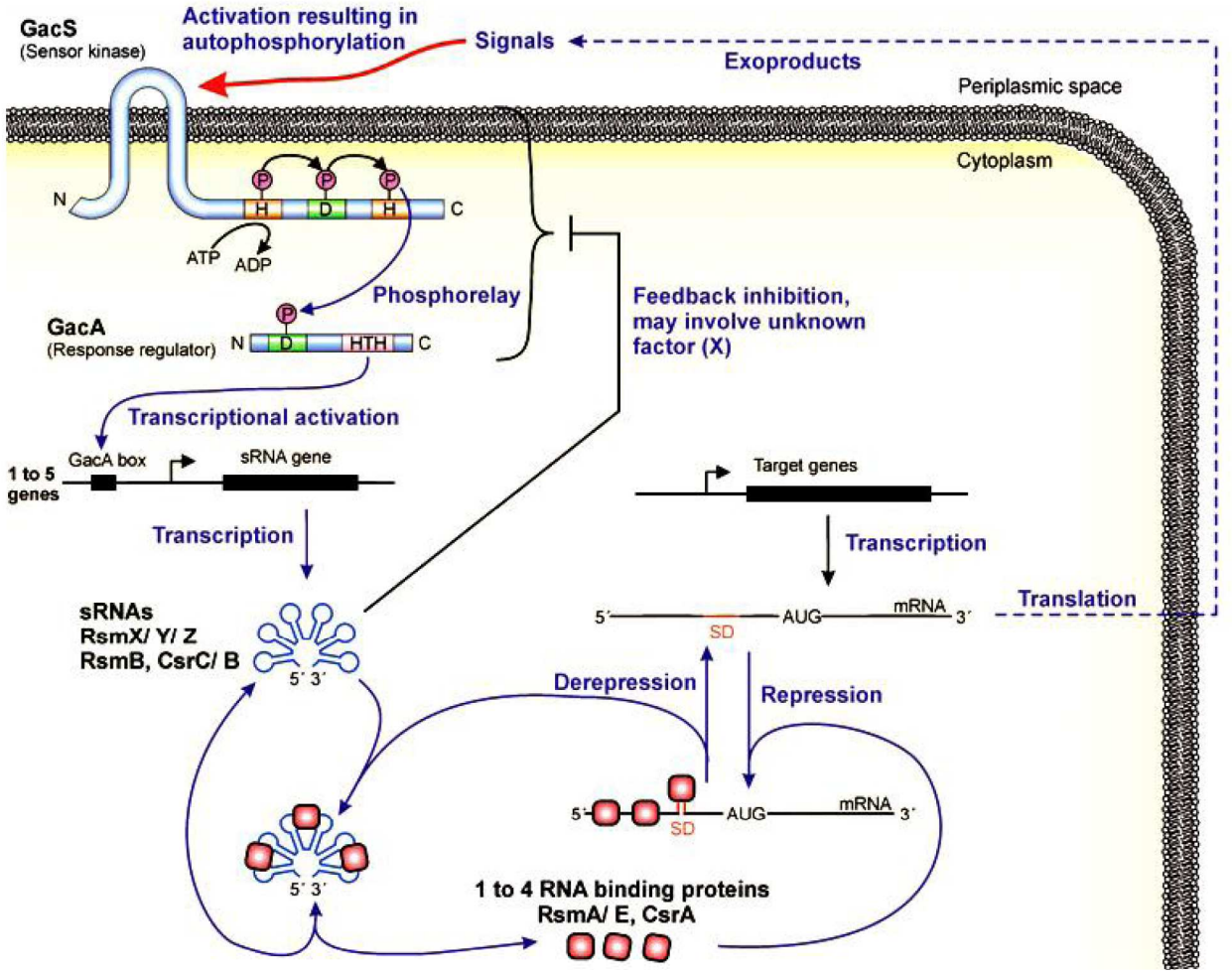


Fig.3.5: General characteristics of the Gac/Rsm signal transduction pathway in g-proteobacteria (Rife et al., 2005)

3.1.3 Functional and genetic diversity of antibiotic producing pseudomonas species:

The broad-spectrum antibiotic 2, 4- DAPG produced by biocontrol *Pseudomonas* is implicated in the suppression of several fungal root diseases and in suppressive soils (Keel et al. 1992; Bender et al. 1999; Mazzola 2002; Raaijmakers et al., 2002; Ramette et al. 2003;). Two main phenotypic groups have been differentiated based on 2, 4- DAPG producing *Pseudomonas* antibiotic production.One producing only 2, 4- DAPG and HCN and the other produces in addition pyoluteorin and, in some cases pyrrolnitrin (Keel et al. 1996). While members of this last group are known to be genetically very similar (Keel et al. 1996; Sharifi-Tehrani et al. 1998; McSpadden Gardener et al. 2000; De La Fuente et al. 2004), extensive genomic diversity was highlighted in DAPG + PLT- *Pseudomonas* by different fingerprinting methods such as ARDRA, RAPD and BOX-PCR (Keel et al. 1996; McSpadden Gardener et al. 2000; Mavrodi et al. 2001).

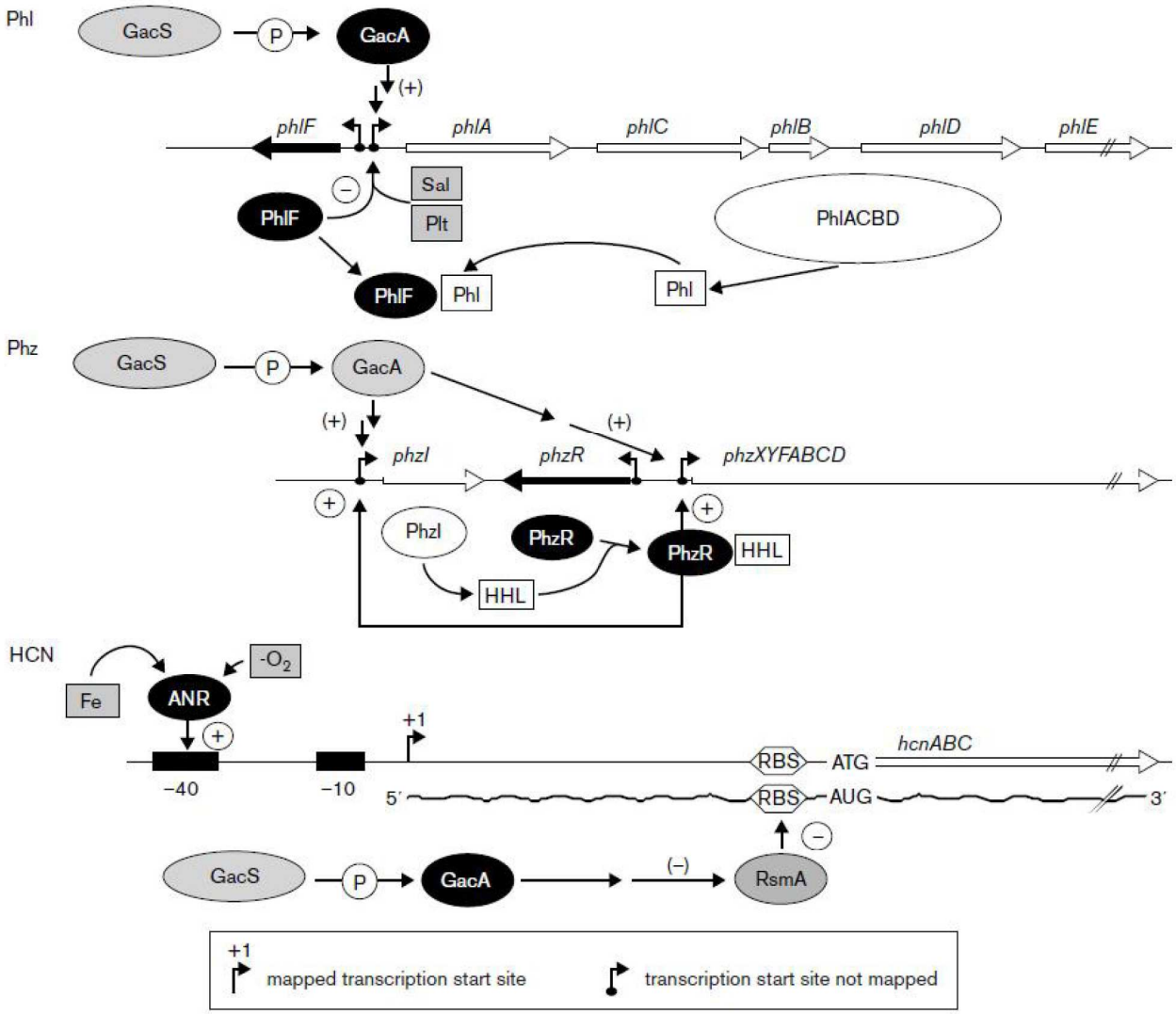


Fig.3.6 Global regulatory pathway for the 2, 4-DAPG, Phz and HCN biosynthesis regulation(Haas et al, 2000)

This led to the subdivision of 2, 4- DAPG-producing *Pseudomonas* in as many as 17 different genomic groups (Mc Spadden et al. 2000; Landa et al. 2002). The diversity is very important, since *phlD*, code for the key enzyme in 2,4- DAPG synthesis, displayed significant polymorphism, easily revealed by PCR-RFLP (Mavrodi et al. 2001; Ramette et al. 2001; Picard et al. 2003) and DGGE (Bergsma-Vlami et al. 2005b)

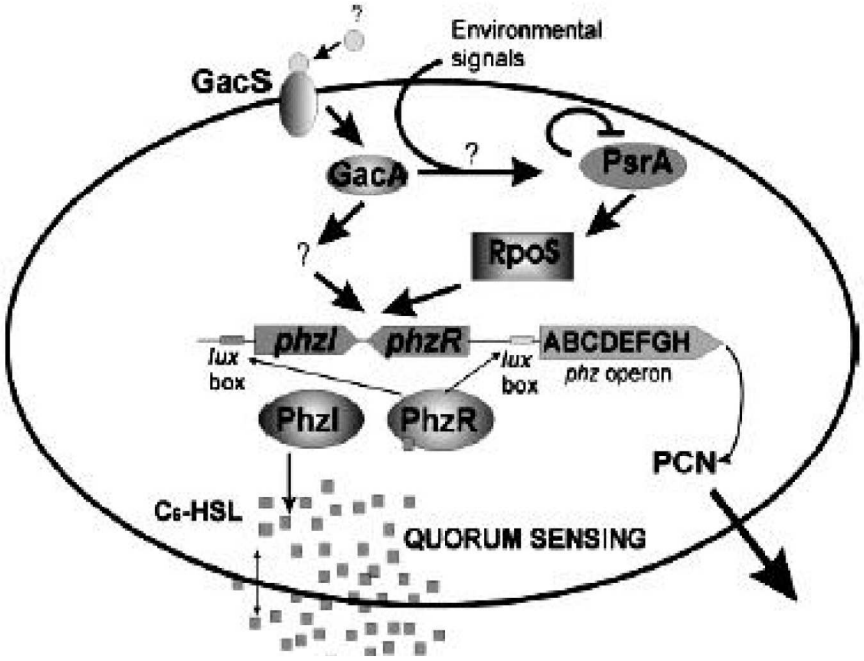


Fig.3.7 Model for the regulatory cascade governing PCN production in *P. chlororaphis* PCL1391 (Girard et al., 2006)

These *phlD* groups correlated very closely with those obtained by genomic fingerprinting (Mavrodi et al., 2001; Ramette et al., 2001; Landa et al., 2002). These observations led to the conclusion that the *phlD* gene evolved in concert with the rest of the bacterial genome. However, this was not confirmed on a collection of 144 *Pseudomonas* isolated from maize at different stages of plant growth which included 4 ARDRA and 59 RAPD groups but presented only one PCR-Hae III pattern (Picard et al., 2000, Picard and Bosco, 2003). This diversity among 2,4-DAPG producing *Pseudomonas* is relevant to biocontrol, because strains belonging to certain genotypes can have better biocontrol capacities, such as superior root colonizing abilities (Raaijmakers et al., 2001; Landa et al., 2002; Mavrodi et al., 2002) or higher antibiotic production (Ramette et al., 2001). To a finer scale, even a single base substitution in a gene involved in antibiotic synthesis can be associated with higher antibiotic production. It was highlighted that such a substitution at position 520 of the *phlD* gene can be a marker of good 2, 4- DAPG producers for the DAPG+ PLT- group (Picard and Bosco, 2003). Determination of the *phlD* genotype and analysis of the gene sequence can be very informative, and can help in the screening of large banks of potential biocontrol agent.

3.1.3.1 Detection and distribution of 2, 4- DAPG-producing Pseudomonas:

Based on whole-cell repetitive sequence-based PCR (rep-PCR), 18 genotypes (A-Q and T) could be distinguished by BOX-PCR of a worldwide collection of over 200 *phl* D+ isolates (McSpadden et al., 2000; Landa et al., 2002, 2005), and these correlated closely with groupings revealed by RFLP analysis of the *phlD* gene (Mavrodi et al., 2001; McSpadden et al., 2001; Landa et al., 2005). Additional genotypes have been described recently by Mazzola et al.,2004 (genotypes PfY and PfZ), Bergsma-Vlami et al. (2005) (genotypes PspC, PspD, PspF and PspZ) and by McSpadden et al.,2005 (genotypes R and S). Most genotypes distinguished by BOX-PCR produce similar quantities of 2, 4- DAPG *in vitro* (Mavrodi et al., 2001) and *in situ* (Raaijmakers and Weller, 2001), have similar substrate utilization profiles and do not considerably differ when compared by classical bacteriological tests (McSpadden et al., 2000; Raaijmakers and Wellller, 2001). However, they do differ significantly in their competitiveness in the rhizosphere and, at least on pea and wheat, the genotype of an isolate is predictive of its ability to establish and maintain population densities sufficient to suppress disease (Raaijmakers and Weller, 2001; Landa et al., 2003). Our current knowledge of the taxonomy, evolution and population structure of the 2, 4- DAPG-producing *Pseudomonas* spp. bacteria is mostly based on single-locus data (Ramcttc ct al., 2001; Ramcttc ct al., 2003; Rczzonico ct al., 2004). Thc gcnctic and cvolutionary relationship among 2,4- DAPG-producing pseudomonads was recently investigated in more detail by multilocus sequence typing (MLST) (Frapolli et al., 2007), a total of 65 pseudomonads consisting of 58 DAPG positive biocontrol strains of worldwide origin and seven DAPG negative representatives of *Pseudomonas* species were compared using 10 housekeeping genes (i.e. *rrs*, *dsbA*, *gyrB*, *rpoD*, *fdxA*, *recA*, *rpoB*, *fusA*, *rpsL* and *rpsG*). MLST differentiated 51 strains among 58 DAPG positive pseudomonad and was shown to be as discriminative as enterobacterial repetitive intergenic consensus (ERIC) PCR profiling. The topology derived from the phyllogenetic trees led to the identification of six main groups of 2, 4- DΛPG-producing *Pseudomonas* spp., which taxonomically could correspond to at least six different species. The usefulness of this approach can be assessed at several levels, i.e. in terms of (i) strain discrimination, (ii) phylogenetic analysis and definition of homogenous strain clusters, (iii) taxonomy and locality analysis (Frapolli et al., 2007). Different genetic traits that have evolved in microorganisms to compete successfully in diverse rhizosphere environments may allow maximizing root colonization and disease suppression (Weller et al., 2002). Knowledge of such

genetic traits involved in host preference of the antagonistic bacteria will help to identify strains that are adequately adapted to specific host-pathogen systems. Thus, it is not surprising that plant species and cultivars differ in their response to rhizosphere colonization (Landa et al., 2002, 2003; Mazzola et al., 2004; Okubara et al., 2003) and disease suppression by introduced and indigenous rhizobacteria (Maurhoffer et al., 1995). Plants also initiate and maintain sophisticated mutuality relationships with *phlD*+ isolates (Landa et al., 2005), and certain *phlD*+ genotypes have an affinity or preference for the roots of particular crops at the species and cultivar level (Landa et al., 2002, 2003, 2005; Mazzola et al., 2004;Bergsma-Vlami et al., 2005). Isolates of at least four genotypes (B, D, E and L) occur in take-all decline (TAD) fields in Washington State (USA) (McSpadden et al., 2000; Raaijmakers and Weller, 2001), but D-genotype isolates are most abundant, owing to the exceptional ability of isolates of this genotype to colonize the rhizosphere of wheat and barley. In Dutch TAD fields, however, genotypes M and F are abundant (de Souza et al., 2003) but they are absent in Washington State TAD soils. Multiple genotypes of 2, 4- DAPG producers are also found on other field-grown crops (i.e. pea, flax, corn and soybean) and, again one or two genotypes dominate depending on the geographic location (de Souza et al., 2003; McSpadden et al., 2000; Raaijmakers and Weller, 2001) and the host plant (Landa ct al., 2003, 2005; McSpaddcn ct al., 2005).

The broad spectrum antibiotic 2,4- DAPG is one of the most important antibiotics produced by biocontrol *Pseudomonas* and is implicated in the suppression of several fungal root diseases and in suppressive soils (Keel et al 1992; Bender et al 1999; Mazzola 2002, 2002; Ramnette 2003;). Several distinct groups of 2, 4- DAPG- producing *Pseudomonas* have been identified from various origins. Two main phenotypic groups can be differentiated based on their antibiotic production.One producing only 2, 4- DAPG and HCN and the other produces in addition PLT and in some cases PRN. This diversity among 2, 4- DAPG producing *Pseudomonas* is relevant to biocontrol, because strains belonging to certain genotypes can have better biocontrol capacities and/or superior root colonizing abilities. Genetic diversity was also present in the genes responsible for HCN synthesis. This diversity led to the classification of the HCN-producing DAPG + pseudomonas in 4 different groups based on hcnBC sequence and it was shown that different HCN groups differ in their HCN production level. These differences also correlated well with biocontrol in planta. The antimicrobial compounds produced by fluorescent pseudomonad other than 2, 4- DAPG, the diversity and phylogenetic relationships among PRN-

producing bacteria have also been assessed by analyzing the polymorphism of biosynthetic genes, in this case prnD (de Souza and Raaijmakers, 2003).

In this chapter, following study has been carried out,

1. Detection of antifungal metabolite gene in isolates and functional characterization by RP-HPLC quantification for each metabolite production in isolates.
2. How the various nutritional factors affects the biosynthesis pattern of the antifungal compounds 2,4- DAPG, PLT and PRN metabolites, which are the major determinants for the biocontrol activity by Pf CHA0.
3. How the nutritional factors regulate the phenazine derivative production in phenazine producer strains.
4. Optimum conditions claim to be optimal for model strain, was tested for other local isolates to understand strain to strain differences in the regulation. How nutritional factors play important role in the biosynthesis of antifungal metabolites in fluorescent pseudomonad.

3.2 MATERIALS AND METHODS:

3.2.1 Bacterial Strains maintenance and growth conditions:

Table 3.1 lists the bacterial strains used in this study. *Pf* CHA0 was routinely maintained on King's B medium plates (Hi-media Limited, India) at 28°C and stored for long term in 0.8% nutrient broth with 0.5% yeast extract (NBY) broth with 40% glycerol at -80°C. *Rhizoctonia bataticola* causal organism of dry root rot of *Vigna radiata* was maintained on potato dextrose agar slants (PDA, Hi-media, India).

Table 3.1 Bacterial strains used in the study

Strains	Strains used	References
Standard strains	*P. fluorescens* CHAO ,Pf-5, Q287	Dr. Fabio Rezzonico, Agroscope Changins-Wädenswil,Zurich, Switzerland
Fluorescent pseudomonad isolates	G1 ,G3, G5, G6, G8, G9 ,G13 ,G14, G16 ,G20, G22 ,G25 ,G29, G30, G36, G38, G44, G45, G46	This study, Chapter 2
	H4,H9	This study, Chapter2
	P1	This study, Chapter 2

3.2.2 PCR based detection of *phlD, hcnBC, pltC,* and *prnBC* in fluorescent pseudomonads:

3.2.2.1 PCR amplification to detect the presence of *phlD* gene:

Primers, nucleotides were obtained from Sigma Aldrich India (Table 3.2). PCR amplification was performed in a thermo cycler (Eppendorf, India) as described in Table 3.3 .Amplicon size of 726 bp was expected. PCR amplification was done in an eppendorf thermo cycler. Amplified fragments were resolved on 1% agars gels at 60 mV by standard protocol (Sam brook et al, 2001). A 100 bp DNA marker, Fermentas, India, was used as a size standard. The system used for PCR amplification is shown in Table 3.3.

Table 3.2 Primers used for the detection of antibiotic biosynthesis gene in fluorescent pseudomonads

Antibiotics	Primers	Primers	Tm	Amplicon size(bp)	References
2,4-DAPG (Phl)	Phl2a	Phl2a:5'- GAGGACGTCGAAGACCACCA- 3';	73.0	726	Raaijmakers et al,1997
	Phl2b	Phl2b:5'- ACCGCAGCATCGTGTATGAG- 3'	72.0		
Pyoluteorin (Plt)	PLTC1	PLTC1:5'-	74.2	438	De Souza et al, 2003
	PLTC2	AACAGATCGCCCCGGTACAGAACG- 3'	73.8		
		PLTC2:5'- AGGCCCGGACACTCAAGAAAACTCG- 3'			
Pyrrolnitrin (Prn)	Pyr1	Pyr1 5'-AAGCGCAACTTCGGCTTC-3'	59.9	812	Allaire et al, 2005
	Pyr2	Pyr2 5'- GTCGCGCAGCGCCTTGAT- 3'	64.5		
HCN	Aca	Aca: 5' –ACTGCCAGGGGCGGATGTGC- 3';	68.6	586	Ramette et al, 2003
	Acb	Acb 5' –ACGATGTGCTCGGCGTAC- 3'	62.2		
Phenazine (Phz)	PCA2a	PCA2a-5'-TTGCCAAGCCTCGCTCCAAC 3';	79.0	1150	Raaijmakers et al,1997
	PCA3b	PCA3b-5'-CCGCGTTGTTCCTCGTTCAT 3'	76.0		

Table 3.3 PCR protocols used for the detection of *phlD, hcnBC, pltC, prn BC* and *phzCD.*

	phlD	*hcnBC*	*pltC*	*prnBC*	*phzCD*
Initial Denaturation	95ºC 5 min	95ºC 5 min.	95ºC 5 min.	95ºC 5 min	95ºC 5 min
Denaturation	94ºC 40 s	94ºC 40 s	94ºC 40 s	94ºC 40 s	94ºC 40 s
Annealing	58ºC 45 s	59ºC 35 s	69ºC 45 s	56ºC 45 s	71ºC 45 s
Extension	72ºC 90 s	72ºC 90 s	72ºC 90 s	72ºC 90 s	72ºC 90 s
Final Extension	72ºC 10 min	72ºC 10 min	72ºC 10 min	72ºC 10 min	72ºC 10 min
Number of PCR cycles	32	35	35	35	35
Expected PCR product size (bp)	726 bp	586 bp	438 bp	812 bp	1150 bp

3.2.3 Quantification of antifungal metabolites produced by fluorescent pseudomonads:

3.2.3.1 Extraction and quantification of 2, 4- DAPG:

2,4- DAPG extraction was carried out using protocol discussed by Duffy and Defago, 1999).Bacterial culture (100 ml) was grown for 3 d in modified King′s B medium which was specifically used for 2,4-DAPG production, at 28ºC at 130 rpm. The 100ml grown culture was acidified using 1.5 ml of 1N HCL to a pH below 3 and 150 ml ethyl acetate was added. Vigorous shaking was done for 30 s and the two phases were allowed to separate. The 2,4-DAPG is separated in upper ethlyacetate phase. Ethlyacetate was separated and allowed to evaporate and the dried extract was dissolved in 1 ml methanol, filtered through 0.22 µm nylon type filters and stored in -20°C for high performance liquid chromatography (HPLC) analysis. At the time of HPLC, stored samples were thawed and filtered through 0.22 µm nylon type filters (Advanced Microdevices Pvt Ltd, India) and 20µl of filtered samples were injected in the HPLC with the help of a Hamilton syringe. To know the peak of interest pure 2,4- DAPG was injected and its retention time was obtained. To reconfirm the peak obtained in various isolates pure 2,4- DAPG was injected along with the sample and spiking was done whereby increase in area of the peak on addition of pure 2,4- DAPG was monitored. For quantification of 2, 4- DAPG a calibration curve was prepared using the pure DAPG in range of 1- 100 µg/ml. A graph of pure DAPG concentration against area of peak was obtained. This was used for calculating the concentration of DAPG from the peak area obtained with the 2, 4- DAPG extracted from the bacterial cultures. HPLC instrument from Shimadzu and software from Winchrome were used to carry out the analysis of 2, 4- DAPG. A mixture of 30% Acetonitrile: 25 % Methanol: 45% MQ water was used as mobile phase with the stationary phase as a C18 reverse phase silica-column. Flow rate was kept constant at 1.0 ml/min and absorbance observed at 272 nm. Using this protocol, 2, 4- DAPG levels was detected and compared in different isolates as well as the effect of different nutrient on 2, 4- DAPG production was studied.

3.2.3.2 Bioassay for 2, 4- DAPG:

Among other antifungal metabolites which are produced by fluorescent pseudomonas like pyrrolnitrin, pyoluteorin, phenazine and 2,4- DAPG, only 2,4- DAPG has inhibitory action on staphylococcus so in order to screen 2,4- DAPG producers from those isolates which are showing some antifungal acitivity, such a bioassay was done. *Staphylococcus aureus ATCC 6538*

was used as sensitive strain for bioassay of 2, 4- DAPG. Here the overnight grown *S. aureus* was placed on Luria-Bertanni Agar plates. Wells (2mm) were bored using cup borers and 80 µL of overnight grown cultures were poured in the wells. The plates were incubated at 37°C for 24 h. After incubation the plates were monitored for the zone of inhibition around the wells.

3.2.3.3 Extraction of PRN:

Two methods were followed for the extraction of PRN from producer strains.

1. Extraction using Amberlite XAD-4: *P. fluorescens* cultures were grown for 3 d in 50ml of minimal media containing Amberlite XAD-4 resin (Rohm and Haas Company, Philadelphia, U.S.A) (5% [vol/vol]) at 28°C. By the third day, a significant number of the cells had died and had been lysed, releasing PRN into the medium and would be readily bound to the resin, which was collected in a sieve and washed extensively with water. PRN was eluted from the resin by two consecutive extractions with isopropanol (0.5 volumes). The two extracts were combined and desiccated under vacuum in a rotary evaporator at 40°C. The desiccated material was dissolved in 1 ml of methanol and filtered using 0.2 µm pore sized filter and further analyzed by HPLC and TLC.

2. Ethyl acetate extraction:

All representative isolates of fluorescent pseudomonad were grown in 20 ml minimal media supplemented with D-tryptophan, precursor amino acid of PRN.

Minimal medium:

Ingredients	(g/l)
Glycerol	30.0
K_2HPO_4	3.0
KH_2PO_4	0.5
NaCl	5.0
$MgSO_4.7H_2O$	0.5
D- Trptophan	0.61

$ZnSO_4$	0.35 mM
$Mo_7(NH_4)_6O_{24}.\ 4H_2O$	0.5 mM

Tryptophan was autoclaved separately at 5 psi for 5 min. Zinc and molybdate solutions were also autoclaved separately at 15 psi for 15 min. Also the rest media components at 15 psi for 15 min Bacterial culture was grown at at 25^{o}C for 24 h under shaking conditions at 180 rpm and subsequently incubated at 25^{o}C in dark for additional 4 day in static conditions. After incubation period, PRN was extracted using equal volume of ethyl acetate after sonicating the culture with sonicator with pulse of 30 s for 3 min. Ethyl acetate was evaporated in oven at 40^{o}C. The left out residual is dissolved in 1ml methanol (HPLC grade) and filtered using 0.2 µm filter.

3.2.3 4. TLC analysis of PRN:

Glass plates were overlaid with silica paste with 1:2 ratio of fluorescent silica G254 with alumunium oxide silica powder and distilled water to make 0.25 mm thick layer. Plates were dried by overnight incubation in oven at 50^{o}C. Samples, in volumes of 20µl, were applied to fluorescent silica G-254 with aluminum oxide on TLC plates and they were developed with methanol: isopropanol: water (60:40, v/v). After development plates were transferred to chamber saturated with iodine till brown bands were observed on plates which appeared clearly after half-an-hour. Bands with reported RF values were noted. Reported Rf value of PRN is 0.82.

3.2.3.5 Quantification of antifungal metabolites by HPLC:

Aliquots of 20 µl were injected into a reversed-phase column (4 X100 mm) packed with Nucleosil C18 and thermostatically controlled at 50°C. Samples were eluted with a three-step linear methanol gradient from 18 to 23% (0 to 5 min), from 23 to 53% (5 to 6 min), and from 53 to 68% (6 to 15 min) in 0.43% *o*-phosphoric acid. The flow rate was 1 ml/min. Maximum UV absorbance and approximate retention times for detection were, respectively, 270 nm and 11.4 min for 2,4- DAPG (molecular weight of 210), 313 nm and 9.4 min for PLT (molecular weight of 268), 254 nm and 12.1 min for PRN (molecular weight of 257.1),

3.2.4 Effect of carbon sources and phosphate levels on 2, 4- DAPG production by isolates:

3.2.4.1 Growth on medium containing various concentrations of phosphate:

Here the cultures were grown in modified King′s B (without glycine) medium and same protocol as mentioned above for 2, 4-DAPG extraction was used. But here the concentrations of Phosphate supplemented in the medium were changed. Phosphate concentrations of 0, 8, 12, 17, 50 and 100 mM were used. The medium itself contained 12 mM (1.5g) phosphate. HPLC was used to quantitate the amount of 2, 4- DAPG produced by various isolates at different concentrations of phosphate and HPLC was carried out in the same way as mentioned above. At the end of three days of incubation samples were collected and absorbance measured at 600 nm to monitor cell growth.

3.2.4.2 Growth on medium containing various C sources:

Again the cultures were grown on modified King′s B Medium and the protocol as mentioned above was used for the 2, 4- DAPG extraction. Here glycerol which is used in normal Media was exchanged for various C sources like glucose, sucrose, fructose, mannitol, arabinose (1%). HPLC was carried in above mentioned way, to quantitate the amount of 2, 4- DAPG produced by various isolates in presence of different C sources. Monitoring the Cell Growth at different Phosphate concentrations at the end of three days of incubation samples were collected and read at 600 nm, to monitor cell growth.

3.2.5 Studies on the effect of nutritional factors on antifungal metabolite production by fluorescent pseudomonad strains:

To find out the effect of the variables on the biocontrol physiology of fluorescent pseudomonad, Fractional Factorial Design (FFD) was used and Software Design Expert 7 (DE 7) was used for analysis. The total number of experiments to be carried out according to FFD is n + 1, where n is the number of variables. Each variable is represented at two levels, high and low denoted by (+) and (-), respectively. The number of positive and negative signs per experiment or trial are (n + 1)/2 and (n - 1)/2, respectively. Each column should contain equal number of positive and negative signs. A total of 7 medium components were screened in 16 runs (Table 3.4)

Starter cultures were grown in 10 ml dilute (1/5-strength) NBY broth in 20 ml test tubes for 12 h at 28°C at 150 rpm, yielding approximately 10^9 CFU/ml. For further growth, 20 ml of dilute (1/5-strength) NBY broth in 100-ml Erlenmeyer flasks was inoculated with 100 µl of starter culture Autoclaved medium was amended with different combinations of following seven components: filter-sterilized solutions of KNO_3 (10mM), KH_2PO_4 (Pi, 10mM), $FeSO_4.7H_2O$ (Fe^{2+}, 0.5mM), $Mo_7(NH_4)_6O_{24}.4H_2O$ (Mo^{2+}, 0.5mM), $ZnSO_4.7H_2O$ (Zn^{2+}, 0.35mM) and with autoclaved stock solutions of glucose or citrate to give final concentration of 100 mM. Chemical analysis indicated that NBY broth contained (mg/liter): total nitrogen, 1441.0; amino nitrogen, 604.0; total phosphate, 600.1; potassium, 597.9; sodium, 259.7; chloride, 121.7; sulfate, 54.9; magnesium, 22.9; calcium, 6.1; zinc, 0.5; and boron, cobalt, copper, iron, lithium, manganese, and molybdenum, <0.1. All the 16 flasks were incubated at 25°C in shaking condition at 150 rpm. After 48h, 20ml of the culture was taken in sterile oakridge tubes for metabolite extraction. Cultures were acidified to pH 2 with 400 to 700 ml of 1 N HCl and extracted with 20 ml of ethyl acetate for 30 min with vigorous shaking at 150 to 200 rpm. Phase separation was accelerated with 15 min of centrifugation at 4,500 rpm (2,790 3 *g*). The organic phase was transferred to a round-bottom glass flask and flashes evaporated and the residue was dissolved in1 ml of HPLC grade methanol. Growth of cultures was noted by measuring absorbance at 600 nm.

Table 3.4 Combination of nutritional factors obtained by fractional factorial design by DE 8

Run	Citrate (mM)	Glucose (mM)	Pi (mM)	KNO3 (mM)	Fe2+ (mM)	Zn2+ (mM)	Mo2+ (mM)
1	100	100	0	0	0	0.35	0.5
2	0	100	0	10	0.5	0	0.5
3	100	0	10	0	0	0.35	0
4	0	0	10	0	0.5	0.35	0.5
5	100	100	10	10	0.5	0.35	0.5
6	100	100	10	0	0.5	0	0
7	0	0	10	10	0.5	0	0
8	100	0	0	10	0.5	0.35	0
9	100	0	10	10	0	0	0.5

Run	Citrate (mM)	Glucose (mM)	Pi (mM)	KNO3 (mM)	Fe2+ (mM)	Zn2+ (mM)	Mo2+ (mM)
10	100	100	0	10	0	0	0
11	0	0	0	0	0	0	0
12	0	100	0	0	0.5	0.35	0
13	0	100	10	0	0	0	0.5
14	0	100	10	10	0	0.35	0
15	100	0	0	0	0.5	0	0.5
16	0	0	0	10	0	0.35	0.5

(Note: In the runs where in both glucose and citrate was amended, glucose was autoclaved separately. Zinc and Molybdate solutions were added separately to the autoclaved medium. (Zn^{2+} was filter sterilized and added.**).** Similar procedure was followed with 20ml aliquot of the culture after 5 day of incubation. Extracts of both day cultures were subjected to quantitation of the various antifungal metabolites using HPLC and 100µl was used to carry out antifungal plate assay.

3.2.6 Design of experiment for the combinations of nutritional factors

The study of the effect of combinations of nutritional factors for the enhancement of bio-control traits by *Pf CHA0* was carried out by using Stat ease 8.

3.2.6.1 Extraction, identification and quantification of antifungal metabolites*:*

Antibiotics were extracted from bacterial supernatants and quantified with high-performance liquid chromatography (HPLC, Shimadzu 10) as described by Duffy and Defago, 1997. Metabolites were identified and by comparison with the pure 2, 4- DAPG, PRN and PLT. Metabolite quantity was estimated from standard curves of reference compounds and normalized for the bacterial absorbance at 600nm prior to extraction. Briefly, liquid culture of 20 ml were acidified to pH 2 with 400 to 700 µl of 1 N HCl and extracted with 20 ml of ethyl acetate for 60 min with vigorous shaking at 200 rpm. Phase separation was accelerated by 15 min of centrifugation at 5000 rpm. The organic phase was transferred to a round-bottomed glass flask

flash evaporated, and the residue was dissolved in 1 ml of HPLC-grade methanol and quantified by established HPLC procedures (Keel et al, 1992).

3.2.6.2 In vitro tests of fungal antagonism*:*

The plate test screening for *in vitro* antagonism against the plant-pathogenic fungus *Rhizoctonia bataticola* was performed by placing an agar plug with fungus in the centre of a Potato Dextrose Agar (Hi-media, Mumbai, India) plate and by adding 100µl ethyl acetate extract in the well bored at four places equidistance from centre at plate periphery. The plates were incubated at 30°C for fungal growth and checked for zones of mycelia growth inhibition after day 2 and day 5, when the fungal mycelium had reached the edge of the plate. Percentage of fungal inhibition was calculated as: radial growth of fungus from centre -radial growth of fungus in presence of extract) / radial growths of fungus from centre X 100. All tests were performed 4 times, with new extract used each time.

3.2.6.3 Principal Component Analysis

Principal Component Analysis **(**PCA) was used to establish combinations of variables e.g. OD_{600}, PRN, 2, 4- DAPG and PLT biosynthesis to describe the principal tendencies i, e. antifungal activity on 2^{nd} and 5^{th} day. PCA yields the corresponding Eigen values, which were extracted for each factor, and the variance percentages (accounted for and accumulative) corresponding to the principal components by formula Fn=A1 X1+A2 X2+...+An Xn.

3.3 RESULTS AND DISCUSSION:

3.3.1 Detection of *phlD, hcnBC, prn D* and *pltBC* among fluorescent *Pseudomonas* strains:

3.3.1.1 Detection of *phlD* gene in fluorescent *Pseudomonas* strains:

Fluorescent pseudomonad producing 2, 4- DAPG, are important group of PGPR that can inhibit a broad spectrum of plant pathogenic fungi. To detect the genes required for the biosynthesis of this antibiotic, detection of *phlD* (726 bp) gene by a PCR was performed. A collection of fluorescent *Pseudomonas* spp., which have shown biological control activity against *Rhizoctonia bataticola in vitro* and known to produce secondary metabolites such as siderophore and HCN, were checked for the phlD. Strains Q287, Pf-5 and *Pf* CHA0 were used as positive controls for the detection *phl*D gene. PCR based detection well to amplify *phlD* in a majority of pseudomonads (including strain Q2-87, Pf-5 and *Pf* CHA0), but performed poorly with some strains e.g. strain G25 and P1 (Fig.3.8). A DNA fragment about 726 bp in size was obtained in all cases as predicted from known *phlD* sequences, whereas no PCR product was obtained from some isolates viz. G29.

3.3.1.2 Detection of *hcnBC* in fluorescent *Pseudomonas* strains:

Among prokaryotes, HCN production seems restricted to Proteobacteria, in which it has been evidenced in several *Pseudomonas* species (*P. fluorescens*, *P. aeruginosa*, *P. chlororaphis*, and *P. aureofaciens*), *Chromobacterium violaceum*, *Rhizobium leguminosarum*, and to certain cyanobacteria (Blumer and Haas, 2000).Production of HCN by biocontrol fluorescent pseudomonad is implicated in suppression of diseases caused by phytopathogenic fungi, such as *Thielaviopsis basicola* on tobacco, *Septoria tritici*, and *Puccinia recondita* f. sp. *tritici* on wheat (by recombinant HCN-producing *P. putida* strains) (Flaishman et al., 1996). Primers ACa and ACb which were used for the detection of hcnBC (586bp) were designed based on whole *hcnABC* sequences already available in *Pseudomonas* strains CHA0 and *P. aeruginosa* PAO1. In these two strains, the *hcnBC* fragment amplified (586 nucleotides) included 156 bp of 1,404 bp of *hcnB* and 430 bp of 1,251 bp of *hcnC*.

With our PCR conditions, a single product of about 586 bp in length was obtained for 9 HCN+ strains (Fig.3.9) whereas no amplicon was obtained from several HCN-negative fluorescent pseudomonad strains (data not shown).

3.3.1.3 Detection of *pltC* gene in fluorescent *Pseudomonas* strains:

Due to broad-spectrum activity, PLT have received considerable attention. The primers PLTC1 and PLTC2 amplified the predicted 438-bp fragment from DNA of model *P.fluorescens* strain Pf-5, *Pf*-CHA0 and Q287. A fragment of the same size was obtained in 15 fluorescent *Pseudomona*s isolates (Fig.3.10).

3.3.1.4 Detection of *prnC* gene in fluorescent *Pseudomonas* strains:

With primer pair Pyr1/Pyr2, 812 bp fragments corresponding to positions 324 to 1136 of the 1700 bp *prnC* gene from fluorescent *Pseudomonas* strains was amplified. Four isolated strains were found to be positive for the amplification of *prnC* as can be seen from Fig.3.11.

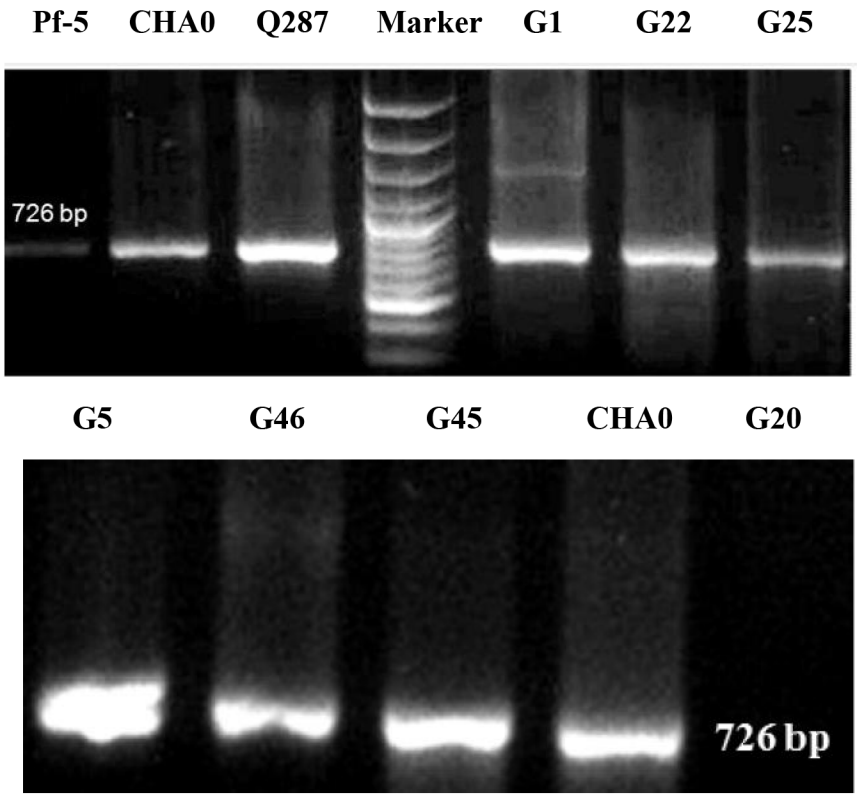


Fig.3.8 PCR amplification of *phlD* (726bp) gene in fluorescent *Pseudomonas* strains

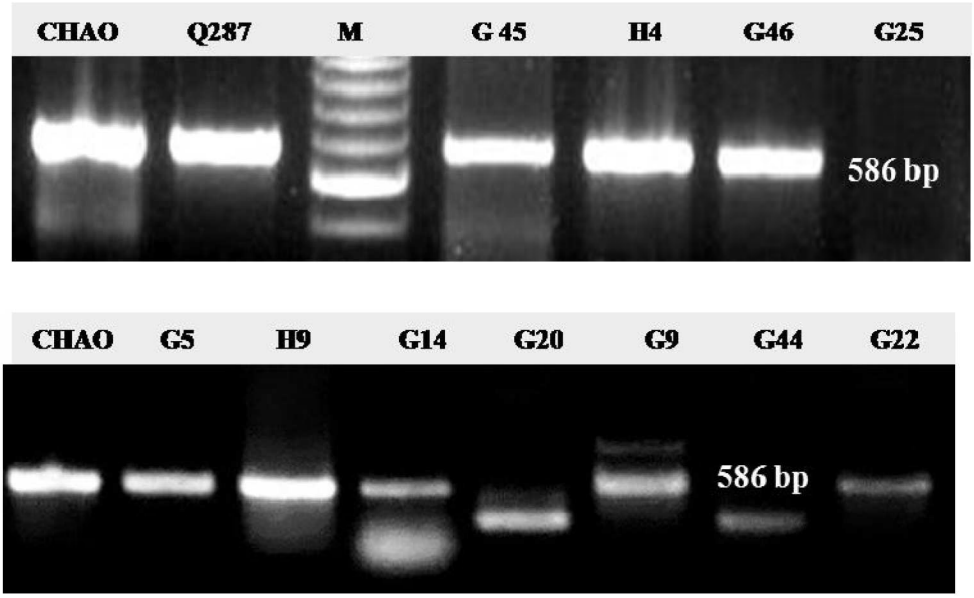


Fig 3.9 PCR amplification of *hcnBC* gene in fluorescent *Pseudomonas* strains

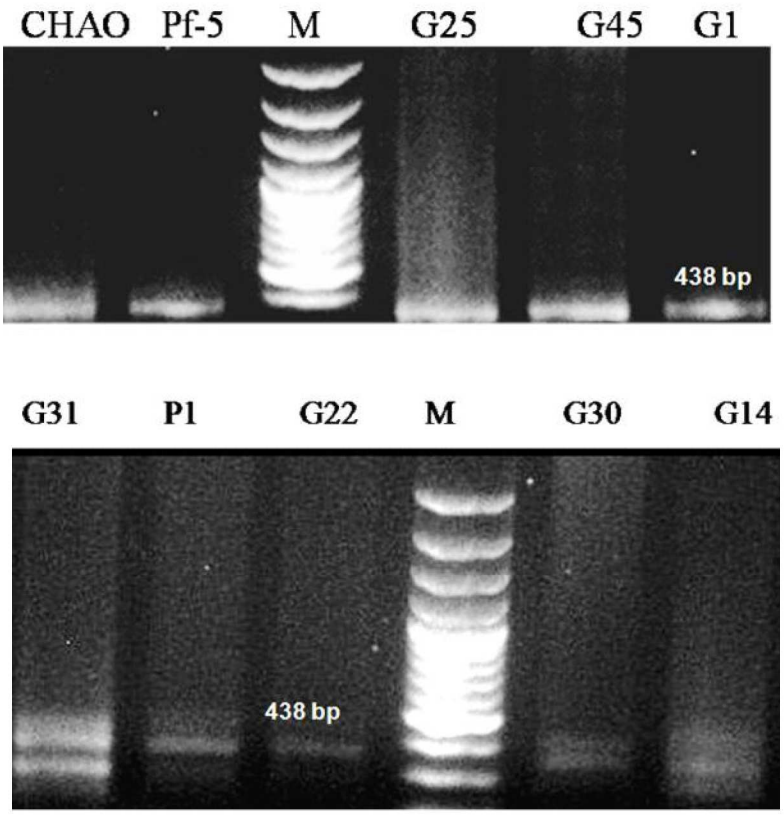


Fig. 3.10 PCR amplification of pltC(438bp) gene in fluorescent Pseudomonas strain

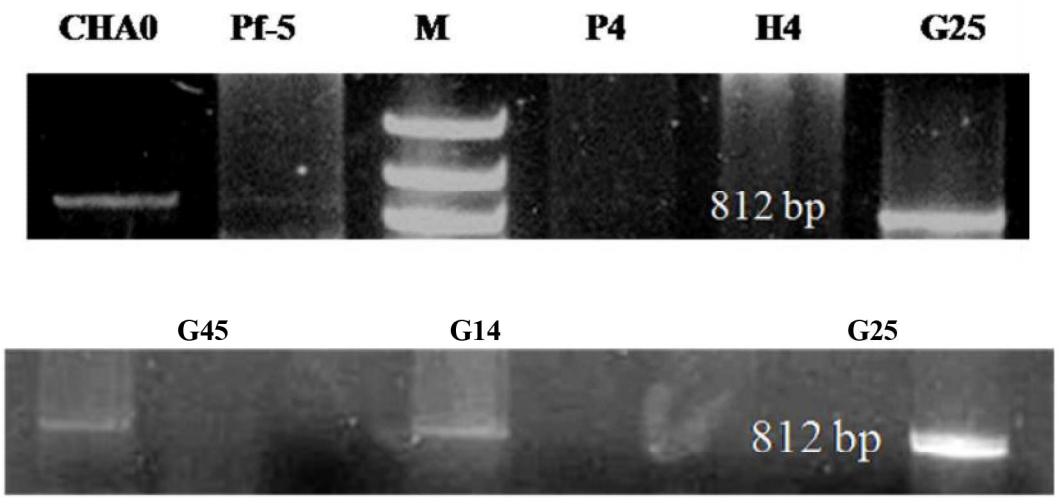


Fig. 3.11 PCR amplification of *prnC* gene from fluorescent *Pseudomonas* strains

Out of total 27 ITS positive fluorescent pseudomonas strains, 18 have shown positive amplification for *plt*C,15 for *phl*D,10 for *hcn*BC and only *5* for *prnC* (Table 3.5 and Fig 3.12). Strain G 45, G46 and G14 were found to be similar to standard strain *Pf* CHA0 in having the all antibiotic synthesis genes (Fig 3.12 and Fig 3.13). Similarly strains H4, H9, G5 and G22 were found to be similar to other standard strains Q287 and Pf-5, while other strains have shown positive combination for other pair of genes (Figs. 3.12 and 3.13).

Table 3.5 Summary of PCR amplification result for the detection of antibiotic synthesis genes in fluorescent *Pseudomonas* strains

Strain	ITS	phlD	hcnBC	prnC	pltC
CHAO	+	+	+	+	+
Pf-5	+	+	+	+	+
Q287	+	+	+	-	+
C2	+	-	-	-	+
G1	+	+	-	-	+
G3	+	-	-	-	-
G5	+	+	+	-	+
G6	+	-	-	-	+
G8	+	-	-	-	-
G9	I	I	-	-	-
G13	+	-	-	-	-
G14	+	+	+	+	+
G16	+	-	-	-	-
G20	+	-	-	-	-
G22	+	+	+	-	+
G25	+	+	-	-	+
G29	+	-	-	-	+
G30	+	-	-	-	+
G36	+	+	-	-	+
G38	+	-	-	-	-
G44	+	-	-	-	-
G45	+	+	+	+	+
G46	+	+	+	-	+
H4	+	+	+	-	+
H9	+	+	+	-	+
P1	+	+	-	+	+
Total	27	15	10	5	18

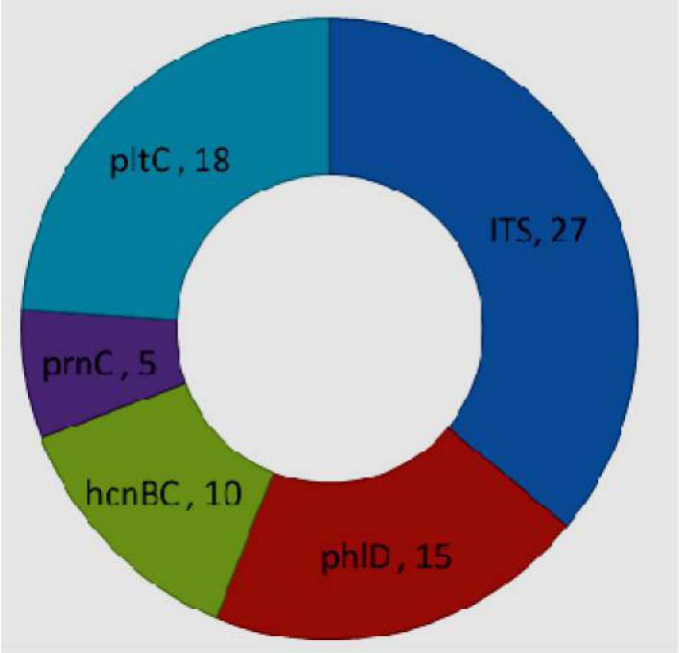


Fig.3.12 Pie graph for the number of ITS, *phlD, pltC, hcnBC and prnC* positive strains

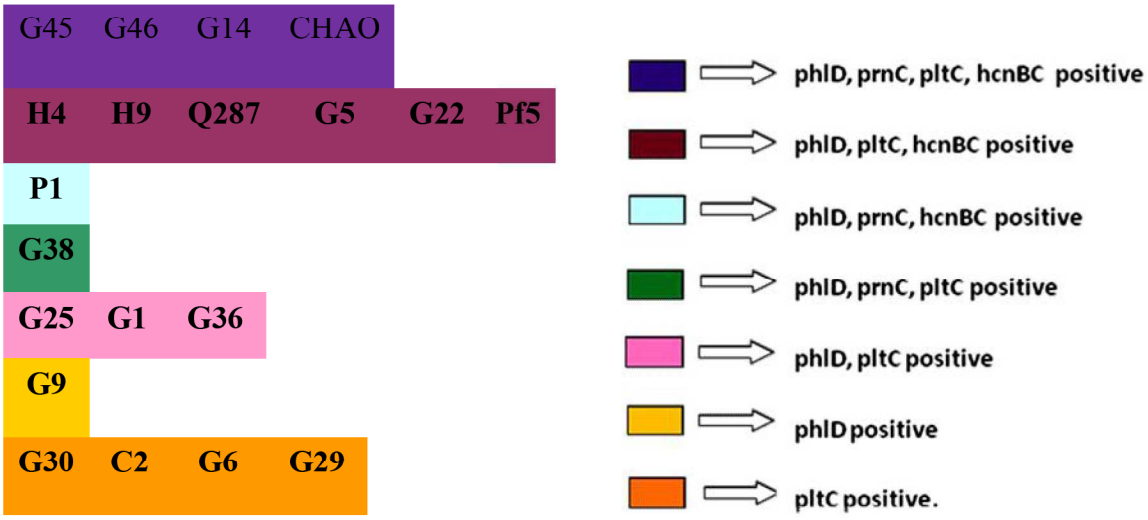
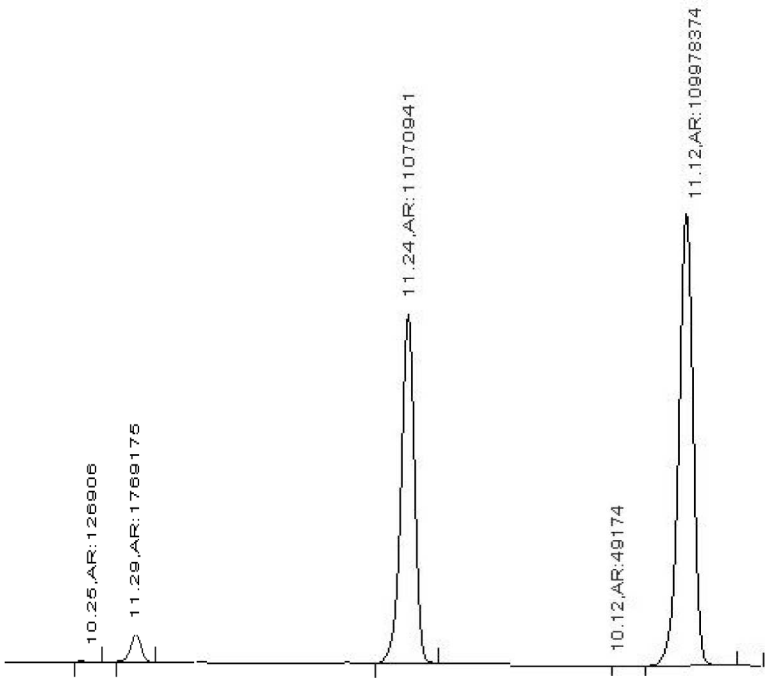


Fig.3.13 Distribution of antibiotic synthesis genes among fluorescent *Pseudomonas* strains.

3.3.2 Production of 2, 4-DAPG by fluorescent pseudomonad:

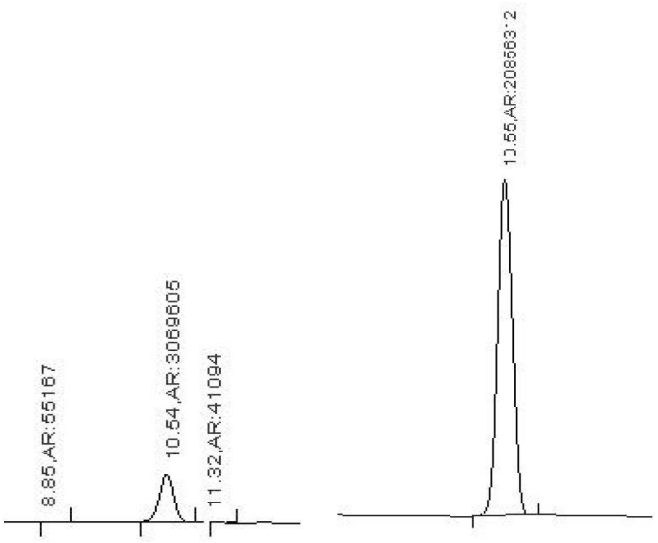
3.3.2.1 Quantification of 2, 4-DAPG:

To differentiate the 2, 4- DAPG production levels between isolates and to find out the best isolate, quantification of 2, 4- DAPG produced is necessary. 2, 4- DAPG production of rhizospheric isolates analyzed using HPLC and was compared to that of model strain *Pf* CHA0. Certain isolates like G1, G2, G8, G3 and C2 showed 2, 4- DAPG production better than *Pf* CHA0 (Figs. 3.14, 3.15 and Fig. 3.16) 2, 4- DAPG production by G1, G2, G8 and C2 was significantly high than *Pf* CHA0 and correlated well with bioassay with *S. aureus* and *R. bataticola* (Table 3.6).



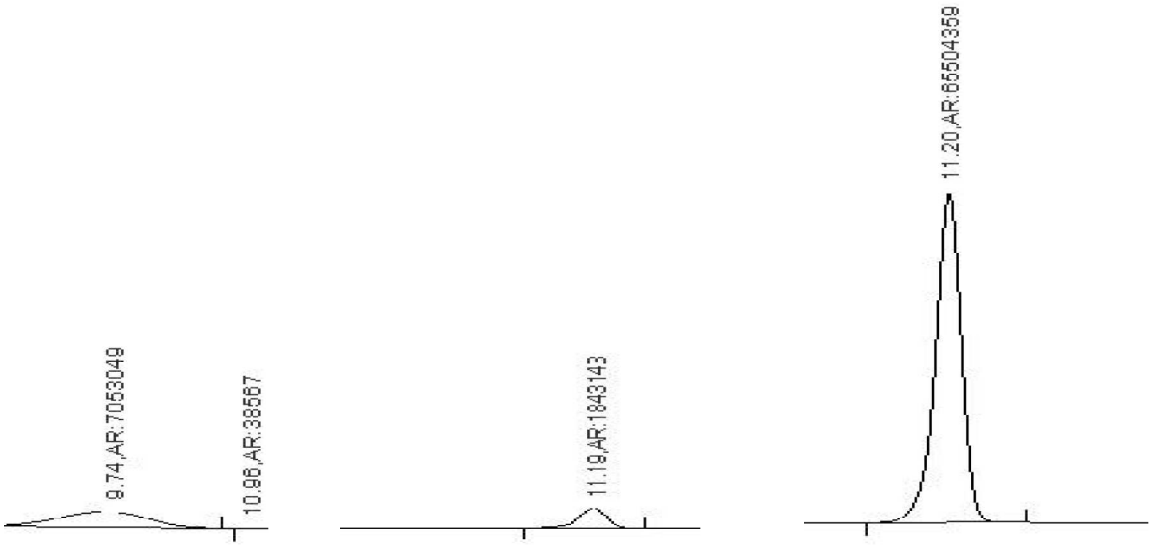
Pure 2,4- DAPG :	1 µg/ml	10 µg/ml	100 µg/ml
Peak R.T. (min.):	11.29	11.24	11.12
Peak area :	1769176	11070941	109976374

Fig.3.14: HPLC profile of pure 2, 4- DAPG



(a) (b)

Fig.3.15 HPLC profile of 2, 4- DAPG extracted from *Pf* CHA0 (a) only extract (b) extract with pure 2, 4- DAPG for spiking.



(a) (b) (c)

Fig. 3.16: HPLC profile for 2, 4-DAPG production by fluorescent *Pseudomonas* isolate

(a) Uninoculated medium (negative control) (b) 2, 4- DAPG extract in isolate (c) 2, 4- DAPG extract of isolate with pure 2, 4- DAPG for spiking.

Table 3.6 Quantification of 2, 4- DAPG and its bioassay

Strains	*R.bataticola* Inhibition (%)	DAPG production(ng/ml)	Bactericidal activity against *S.aureus*
Pf CHA0	38.4	740 ± 9.21	+++
B2	25.6	305 ± 37	+
C2	41.6	995 ± 25.13	+++
C7	44.8	640 ± 9.25	+++
G1	38.4	2340 ± 87	++++
G2	50	3350 ± 117.5	+++++
G3	28.8	1950 ± 98.5	++++
G4	38.4	160 ± 11	+
G5	32	503 ± 32	++
G6	39	185 ± 18	+
G7	28.8	215 ± 8	+
G8	57.6	965 ± 10.27	+++
H4	35.2	360 ± 10.79	++
H9	41.6	220 ± 38.28	+
P1	32	205 ± 17.25	+
M3	38.4	405 ± 15.43	++
G18	34	380 ± 8.27	++
G26	70	2040 ± 107	++++
G35	42	680 ± 12.21	+++
G46	60	1958 ±127.9	++++

3.3.2.2 Effect of C sources and P levels on 2, 4- DAPG production:

The rhizospheric fluorescent pseudomonads were isolated from diverse ecological niches. So it is possible that they may differ in their nutrient requirements and physiological properties. Out of all the known factors affecting the performance of 2,4- DAPG, P levels and C sources have long

been known to influence the activity of phytopathogenic microorganisms (Duffy and Defago, 1999), they contribute to the variability of biocontrol in different soils and on host crops that differ in root exudates composition.(Duffy and Defago, 1999), they have been reported to influence the production of other antibiotics like phenazine, zwittermicin etc. in biocontrol strains (Duffy and Defago, 1999), they are easy to provide during lab. Study or as fertilizer amendments to improve the activity of biocontrol strains and 2,4- DAPG production of *Pf* CHA0 is known to be repressed in presence of sucrose and at high phosphate levels (Duffy and Defago, 1999).2, 4- DAPG production of various isolates was checked in the presence of different C sources and P levels.

3.3.2.2.1 Effect of carbon sources on 2, 4- DAPG production:

It was seen that ecologically diverse isolates showed difference in their carbon utilization. Certain isolates preferred glucose for their growth, while some prefers sucrose but overall there was not much difference in their growth in presence of various carbon sources. In presence of different carbon sources, 2,4- DAPG production and growth ($O.D_{600}$) of strains was monitored to study which C sources directly affect the biosynthesis of 2,4- DAPG or indirectly due to difference in growth. C sources commonly found in plant root exudates (glucose, fructose, arabinose etc) had a differential influence on the 2, 4- DAPG produced by various isolates, irrespective of their effects on bacterial growth. The 2, 4- DAPG production by various isolates in presence of various carbon sources showed a very conspicuous behavior. The difference in 2, 4- DAPG production in presence of different C sources, and it could not be linked to their growth pattern. The 2, 4- DAPG production of isolates in presence of various carbon sources like glucose, fructose, sucrose, manitol, and arabinose was monitored and compared with *Pf* CHA0. It was observed that in some cases 2, 4- DAPG production was not high even when growth was good, and in some cases the production was excellent even at low growth (Fig. 3.17). For example, isolate G2 showed the less growth is in presence of manifold but the 2, 4- DAPG production in presence of manifold is higher than that in sucrose where growth is good. Isolate G8 showed significant increase in 2, 4- DAPG production, in presence of sucrose while its production in presence of other carbon sources is average. This signifies that evolutionary relations may exist between host plant and microorganisms, which affects their behavior (Duffy and Decagon, 1999). Plant specificity of biocontrol strains is generally due to influence of root

exudates and the effects of root exudates on bacterial growth and physiology have been reported (O'Connell, 1996). The present results suggest that plant specificity mediated by root exudates might also affects the antifungal metabolite secretion. As reported by Duffy and Defago, 1997 that *Pf* CHA0 show high 2, 4- DAPG in presence of glucose and less production in presence of sucrose. Certain isolates obtained in this study have shown better 2, 4- DAPG production than *Pf* CHA0. In presence of sucrose, isolates G8 and G1 showed better 2, 4- DAPG production than *Pf* CHA0. With arabinose as the sole C source, isolate G2 showed better 2, 4- DAPG levels than *Pf* CHA0 (Fig.3.17). Differential biocontrol physiology among G1, G2, G8 and C2 was clearly evident from the varied response to C sources (glucose, fructose, sucrose, arabinose and mannitol) in terms of 2, 4- DAPG production. Glucose but not glycerol and sucrose enhanced 2, 4- DAPG production in *P. fluorescens* Pf-5, CHA0 and many other strains whereas in only *Pf* F113 production of 2, 4- DAPG is stimulated by sucrose. Strain G8 has shown significantly high 2, 4- DAPG production in sucrose ($p = 0.018$) (Fig.3.17) so G8 could be considered physiologically different than other strains including *Pf* CHA0. Strain G1 has also shown 6 fold higher production in sucrose in compare to *Pf* CHA0 ($p = 0.02$) (Fig.3.17). Strain G2 has shown good 2, 4- DAPG production in presence of glucose as in case of *Pf* CHA0 ($p = 0.03419$). The effect of other C sources was not much significant (Fig. 3.17). Strain relatedness based on 2, 4- DAPG biosynthesis under different carbon sources is shown in Fig. 3.18.

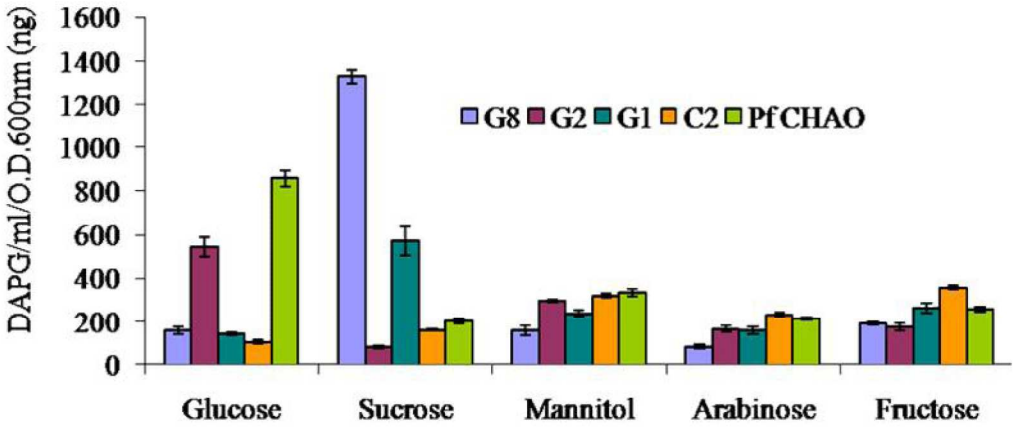


Fig 3.17 2, 4- DAPG production by fluorescent pseudomonad under different carbon sources

3.3.2.2.2 Effect of inorganic phosphate on 2, 4- DAPG production:

Because of diverse ecological niches of rhizospheric isolates, it is expected that they would have exposure to different levels of available P, depending on the physicochemical properties and composition of soil as well as environmental factors affecting soil properties of the location. So 2, 4- DAPG production of isolates was checked at different phosphate levels. It is reported that inorganic phosphate (Pi) represses the 2,4- DAPG production and in case of *Pf* CHA0 it get repressed at 10 mM inorganic phosphate level(Duffy and Defago,1999).

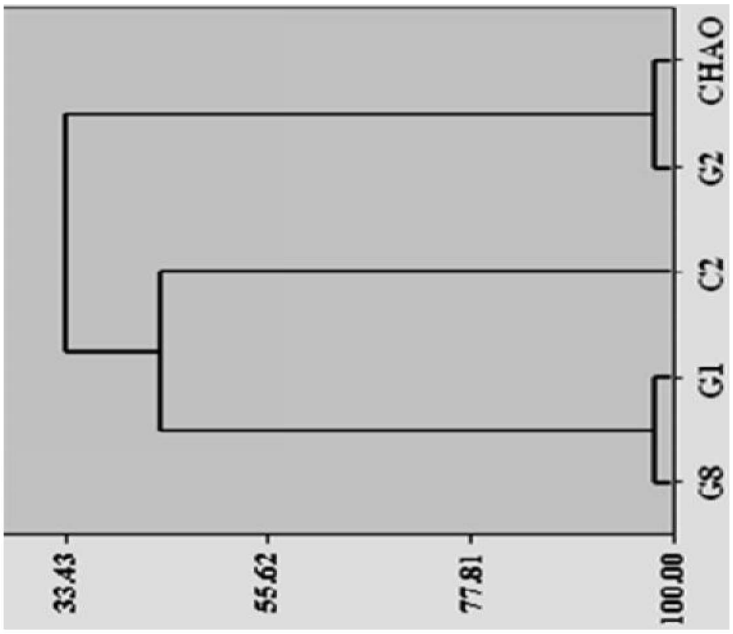


Fig.3.18 Relatedness of fluorescent *Pseudomonas* strains based on 2, 4- DAPG production under different C-sources

In the presence of different concentrations ranging from no phosphate amendments in medium to 100mM of phosphate, growth in terms of OD_{600} of isolates and 2, 4- DAPG production was checked and was compared to *Pf* CHA0. The 2, 4- DAPG production of *Pf* CHA0 decreased with increase in Pi concentrations, without major change in growth. Similarly there was no significant change in the growth of the isolates with increase in Pi concentrations. However, a striking effect of phosphate was observed on 2, 4- DAPG levels. The 2, 4- DAPG production by G2 was very similar to *Pf* CHA0 showing high production at low Pi concentrations. The isolates G1, G2, and

G8 showed higher 2, 4- DAPG production than *Pf* CHA0 in absence of any supplemented phosphate. While G8 and G1 showed comparatively good 2, 4- DAPG production even at high Pi levels (Fig. 3.19). Isolate G2 has minimum inhibitory effect of Pi on 2, 4- DAPG production and has shown good production up to 50 mM Pi ($p = 0.04$) (Fig. 3.19) while isolate G1 has shown gradual drop in 2, 4- DAPG production up to 50 mM Pi ($p = 0.006$) (Fig. 3.19).

In addition to repression of 2, 4- DAPG production in *Pf* CHA0 (Duffy and Defago, 1997), Pi is reported to repress other antibiotics (e.g., anthracycline and tetracycline), phenazines in *Pseudomonas* spp. (Duffy and Defago, 1999) and zwittermycin A and kanosamine in *Bacillus* (Milner, 1995). The bacterial isolates reported here however, differed in their sensitivity to Pi levels. In other bacteria, phosphate has been found to repress the antibiotic synthases, and interrupt transcription and promotion of biosynthetic genes, and also affect the pH and nutrient availability (Martin, 1994). But its mechanism of repression of antibiotic synthesis in *Pseudomonas* spp. is not very well understood. It has also been surmised that increased antibiotic synthesis is in response to nutritional stress e. g. phosphate starvation (Duffy and Defago, 1999), indicating this phenomenon to be starvation inducible rather than repression. This can explain the 2, 4- DAPG production behavior of some of the present isolates showing high production at low Pi levels (Duffy and Defago, 1999)

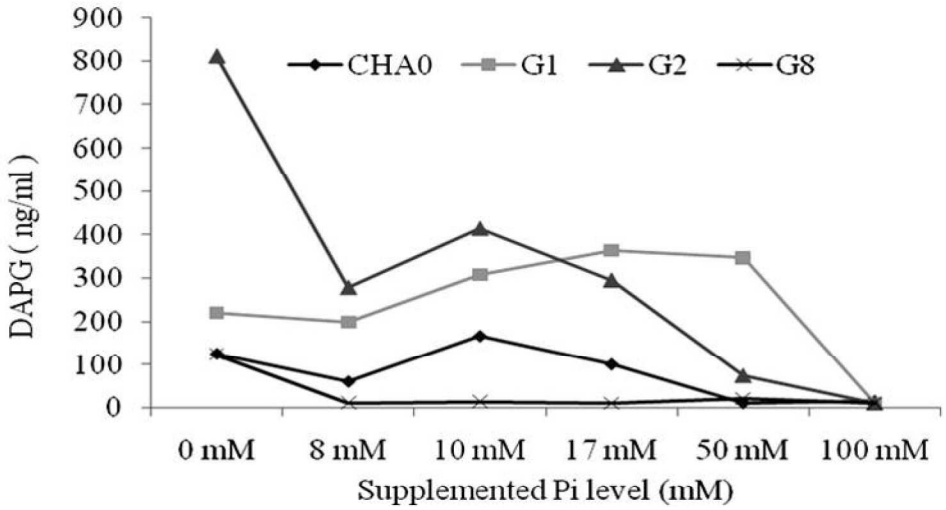


Fig. 3.19 Influence of phosphate levels on 2, 4- DAPG production in fluorescent *Pseudomonas strains*

3.3.3 Pyrrolnitrin production by fluorescent *Pseudomonas* strains:

Pyrrolnitrin production was found to be highest in G35 and followed by G25 and G26 (table 3.7). TLC results shows the spot of PRN in the ethyl acetate extract of G25, G26 and G35 as shown in Fig.3.20.

Table 3.7 Pyrrolnitrin production by fluorescent pseudomonas strains

Strains	Pyrrolnitrin (ug/ml)
Pf CHA0	8.86
G1	0.20
G2	0.70
G8	0.11
C2	3.23
G26	51.13
G35	58.48
G18	0.50
G46	0.90
G25	48.06

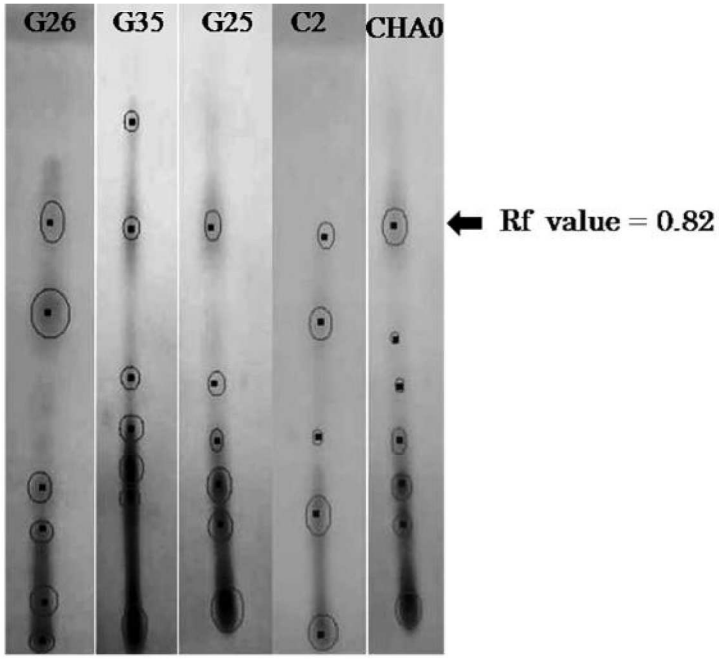


Fig. 3.20 TLC analysis for the separation and detection of PRN spot in the extract

3.3.4 Phenazine production by fluorescent *Pseudomonas* strains:

Phenazines are endogenous redox-active molecules with heterocyclic ring structures that are functionally similar to those of humic substances. They are produced by *Pseudomonas* spp. and a few other bacterial genera, including soil and clinical isolates.

3.3.4.1 Primary screening based on pigment production:

Primary screening of phenazine producer was done after the cultivation of strains in pigment production medium (PPM) and their potential of pigment formation. Since each phenazine derivative has a characteristic pigment due to substitution of different functional groups in the core phenazine ring scaffold, on the basis of the pigments formed by strains in PPM, they were sorted as putative phenazine producers (Table 3.8). Some strains e.g. G16, G 44, G36, G45, H4 showed a dark orange pigment that is characteristic of 2-OHPCA.Other strains like P4 developed a green pigment after 24 h. Pigment containing culture supernatants were tested for inhibition of pathogen *R.bataticola*, most strains that showed showed pigmentation also showed high fungal inhibition presumably due to phenazine production.

3.3.4.2 Screening based on MnO_2 reduction:

Chemical processes involving electron transfer are fundamental components of all metabolic processes and proteins involved in these pathways have been the focus of intensive investigation. In contrast, the importance of small-molecule-mediated extracellular redox in bacterial processes such as mineral respiration and survival in biofilms was realized later. In these situations, redox shuttling via diffusible small-molecules may allow bacteria access to terminal electron acceptors, without requiring physical contact with these substrates. Several classes of secondary metabolites capable of functioning as small-molecule redox shuttles have been identified in bacteria, including phenazines and quinone derivatives. Phenazines produced by soil bacteria can serve as electron shuttles, being reduced microbiologically and subsequently oxidized by poorly crystalline iron and manganese (hydro) oxides. Preliminary screen for extracellular redox molecules was accomplished using a layered MnO_2-based plate assay, which should allow for rapid assessment of redox behavior based on a strain's ability to convert insoluble Mn(IV) to colorless Mn(II) (Fig.3.21) (Balskus, 2009).

Each isolate was streaked on a portion of R5 medium agar plates with bottom layer of 3mM MnO_2 suspension. In this way, the cells would not directly contact the MnO_2, and any observed redox would presumably be due to a diffusible factor. All plates were incubated at 30 ºC and observed daily for 7 d. Strains on MnO_2 plates were scored for clearance of manganese oxides by assigning qualitative rankings based on both approximate radius of clearance and the extent of disappearance of the particulate Mn suspension. Some strains showed high amount of clearance on R5 plates indicating they reduce brown colored insoluble Mn (IV) to colorless Mn(II) (Fig.3.22)

Table.3.8 Characterization of isolates for phenazine production based on pigment production and antifungal activity

Strains	Pigment	Putative phenazine derivatives	Antifungal against *R. bataticola*
Pf-5	Orange	2-OHPCA	++
Q287	Yellow	PCA	++
G 5	Orange	2-OHPCA	++
G 16	Dark orange	2-OHPCA	+
G 22	Yellow	PCA	-
G 25	Orange	2-OHPCA	++++
G 29	Colourless	?	+
G 31	Colorless	?	+
G 35	Brown	?	+
G 36	Dark orange	2-OHPCA	++
G 44	Dark orange	2-OHPCA	+++
G 45	Dark orange	2-OHPCA	+++
G 46	Yellow	PCA	-
H 4	Dark orange	2-OHPCA	+
P4	Green	1-OHPCA	++

G44 showed the highest amount of clearance, while green pigmented P4 also showed high clearance. Strains G31, P33 G19, G20, G45, and H4 showed moderate clearance while C2, P31, Pf5, G16, G25, and P1 showed very little clearance. The strains were scored for manganese oxide clearance by assigning qualitative rankings based on the extent of clearance of the particulate Mn suspension (Table 3.9). Selected strains viz. G44, P4, G20 that showed higher amount of clearance were selected for further screening on individual R5 plates containing MnO_2 (Fig. 3.22). Clearance data for the pigmented strains indicated that the correlation of extracellular redox behavior with diffusible pigment production especially in the case of G44 and P4. Balskus, 2009 indicate that specific growth conditions have a dramatic influence on MnO_2 clearance and it may not be straightforward to ascertain that the same clearance results would be obtained in the environment.

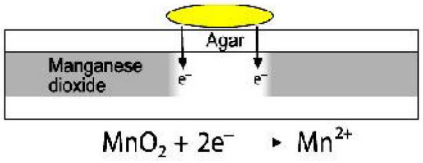


Fig.3.21 Mechanism of MnO_2 reduction (Newmann, 2004)

Table.3.9 MnO2 reduction by fluorescent *Pseudomonas* strains on R5 plates

Strains	MnO_2 reduction/color
Pf-5	+(orange)
C2	-
G 5	+++
G 16	+(dark orange)
G 19	++
G 20	++
G 25	+(orange)
G 31	++
G 36	++(dark orange)
G 39	+++
G 44	++++(orange)
G 45	++(dark orange)
H 4	++(dark orange)
P1	+
P31	+

3.3.4.3 Extraction and screening of MnO_2 reducing molecules from culture supernatants:

Further screening for phenazine producing strains based on the property of phenazine to reduce MnO_2, was done using chloroform extracts of PPM (3.23) grown cultures. Incubation of 200µl extracts directly with 100µl MnO_2 precipitates lead to reduction MnO_2 which could be observed by reduction in the brown precipitates (Fig. 3.23). Amount of clearance was semi-quantified (Table 3.10)

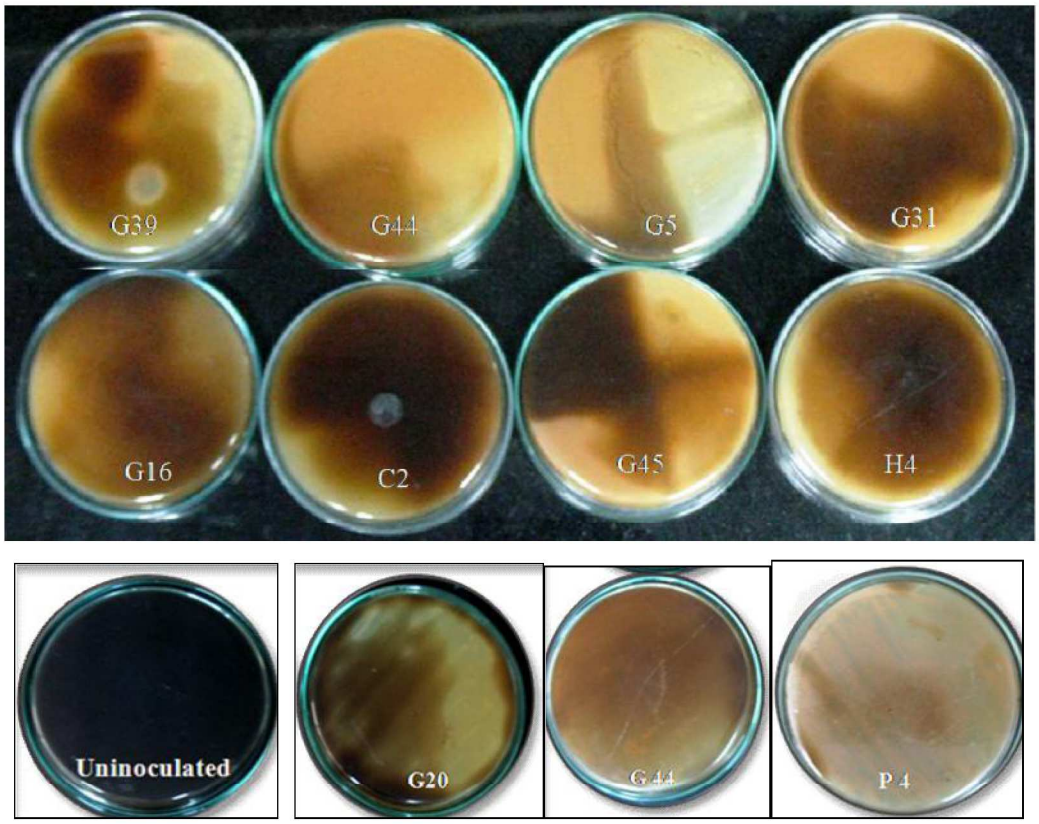


Fig.3.22 MnO_2 reduction by representative fluorescent *Pseudomonas* isolates

And found to be were in accordance with those obtained on R5 plates where strains G44, P4, showed high amount of clearance. This lead to the conclusion that it was phenazine indeed which was produced by these strains and which reduced MnO_2 precipitates.

3.3.4.4 Identification of phenazine derivative based on redox potential:

Electron shuttles are redox-active small molecules that are reduced within the bacterial cell and are oxidized outside the cell by terminal electron acceptors such as minerals and molecular

oxygen. Given enough terminal electron acceptors, one shuttle molecule can be recycled many times. This shuttling process is thought to be especially important in environment where terminal electron acceptors are not easily accessible, because they are either insoluble (e.g., Fe (III) minerals) or diffusion-limited (e.g., O_2).

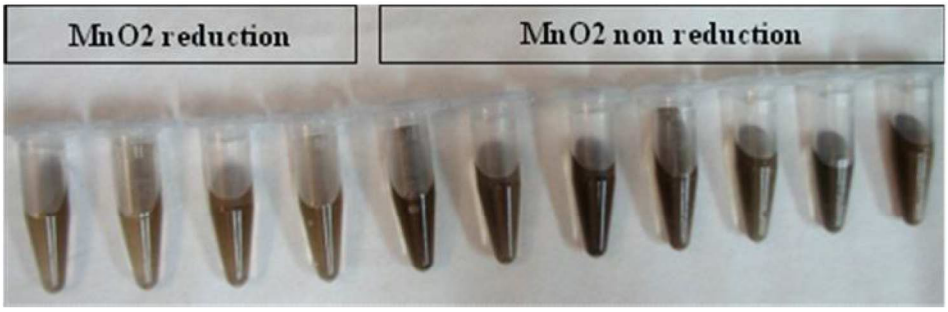


Fig. 3.23 Clearance observed due to reduction of MnO_2 precipitates in the presence of extracted phenazine

Table.3.10 Effect of phenazine extracts on MnO_2 precipitates:

Strains	MnO_2 Clearance
Q287	+
G 2	+++
G 5	-
G 16	+
G 21	++
G 22	+
G 25	-
G 29	++
G 31	++++
G 35	+++
G 39	+
G 44	++++
P4	+++++

Like other electron shuttles, phenazines often have reduction potentials sufficiently low under common physiological and environmental conditions so that the redox reactions between reduced phenazines and terminal electron acceptors such as Fe (III) are thermodynamically

feasible. The redox potentials of all PPM grown cultures were measured and compared with reported redox potentials of all phenazine derivatives (Table 3.11) (Wang *et. al.*, 2008,2009)

Table 3.11 Redox potential values for fluorescent *Pseudomonas* isolates grown in PPM

Strains	Redox potential (mV)
Q287	-116
C2	-98
C 7	-107
G 5	-116
G 6	-124
G 14	-114
G 16	-124
G 18	-90
G 19	-74
G 20	-81
G 22	-114
G 29	-50
P31	-149
G 35	94
G 36	-129
G 38	-124
G 39	22
G 44	-98
G 45	-126
H4	-124
P 1	-137
P33	-111

Based on the known redox potential values phenazine producing strains were sorted into: 1. Phenazine-1-carboxamide -135 mV $\geq E_{1/2}$ or 2. Phenazine carboxylic acid -135 mV $\leq E_{1/2}$ (Wang.Y. *et. al.*, 2008) Separation of strains into PCA and PCN was done according to their measured redox potential values Q287, C 7, G 5, G 36, G 38, G 45, H 4, P33 were characterized as PCA producers while G 14, G 16, G 44, P1, P4 as PCN producers.

3.3.4.5 Quantification of phenazine production:

Phenazine antibiotics extracted from strains were quantified by UV visible light spectroscopy as described by Whistler et. al., 2003. Quantification by OD $_{367nm}$ showed that extracts of strains G31 showed particularly high amount of phenazines (Fig. 3.24). On the basis of primary screening, strains G16, G 31, G44 and P4 were found to be a high PCN production. Strains G1, G27 and C2 showed high amount of phenazines production while strains C7, G25, G35 and Q287 have shown very little phenazine production.

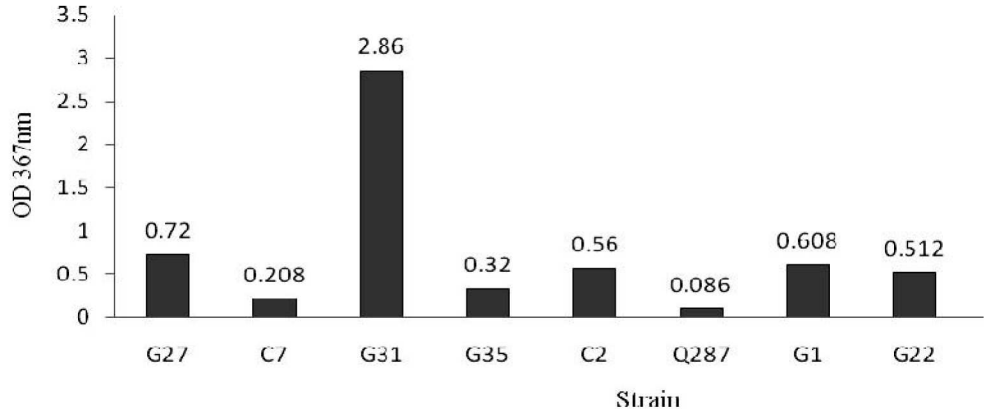


Fig. 3.24 Quantification of phenzaine production by fluorescent *Pseudomonas* isolates

3.3.4.6 Secondary screening method for putative PCN producers:

Five isolates were selected based on primary screening ,which were believed as high PCN producers were streaked on plates of Modified Vogel Bonner salts#1 supplemented with glucose and casamino acids containing overnight grown fungal pathogen *R. bataticola* to observe fungal growth inhibition (Fig.3.25 and Table 3.12). Since MVB1-glucose-CAS specifically favors PCN production, the strain G44 which showed maximum inhibition of fungal pathogen (Fig. 3.25) produces highest amount of PCN among all the strains.

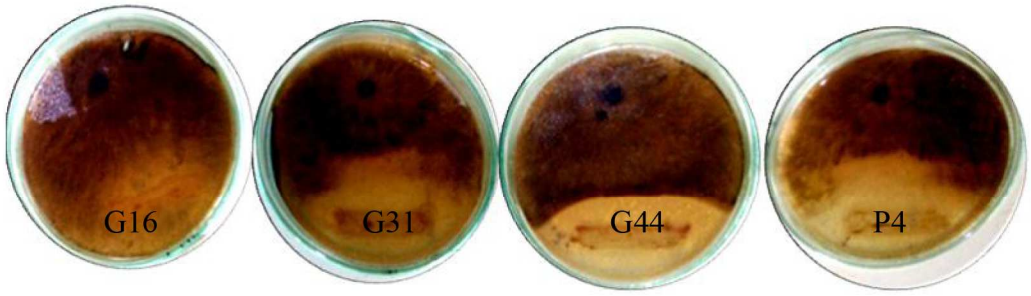


Fig. 3.25 Inhibition of *R.bataticola* by fluorescent *Pseudomonas* strains on MBV1-agar plates

Based on primary and secondary screening strategy used for the type of phenazine produced by strains, following the traits detections e.g. pigment production, high MnO_2 reduction, negative redox potential and antifungal activity exhibited by the strain G44, this strains was considered as a putative PCN producer (Fig.3.25 and Table 3.12).

Table 3.12 Inhibition of *R.bataticola* strains on MVB1 plates

Strain	Antifungal activity against *R. bataticola*
G 16	-
G 31	+
G 44	++++
P 4	+++

3.3.5 Effect of nutritional factors on the production of putative phenazine-1-carboxamide (PCN) in G44

3.3.5.1 Effect of nitrogen sources on putative PCN production potential:

Effect of different N-source on PCN levels were studied after replacement of N- source in MVB1-glucose-cas by other N-sources. Fungal inhibition assay was indirectly used to indicate the amount of PCN produced. Results of antifungal activity showed that 8mM NH_4Cl showed maximum antifungal activity (Table 3.13 and Fig. 3.26). To test whether NH_4^+ ions stimulated PCN levels, increased concentration of NH_4Cl was supplemented in the medium as well as different concentrations of $(NH_4)_2SO_4$ were used. Increase in NH_4Cl levels to 16mM and 32mM showed a marked decrease in PCN production. $(NH_4)_2SO_4$ did not significantly contribute to

PCN production at any concentration although an increase in concentration of $(NH_4)_2SO_4$ from 4mM to 8mM decreased PCN production by 33% but a further increase in concentration to 16mM did not show any change. Supplementing the original nitrogen source with urea or $NaNO_3$ did not show increased PCN production. The original medium contained 0.05% casamino acids and an increase in casamino acids concentration to 16mM lead to marked increase in PCN production (Fig. 3.26, Table 3.13). To test whether stimulatory effect of casamino acids on PCN levels can be ascribed to individual amino acids, casamino acids were replaced by individual amino acids. Aromatic amino acids, phenylalanine and tryptophan were supplemented as nitrogen source in order to check if they enhanced PCN production. However, results of antifungal activity of PCN extracts did not show marked increase compared to other nitrogen sources.

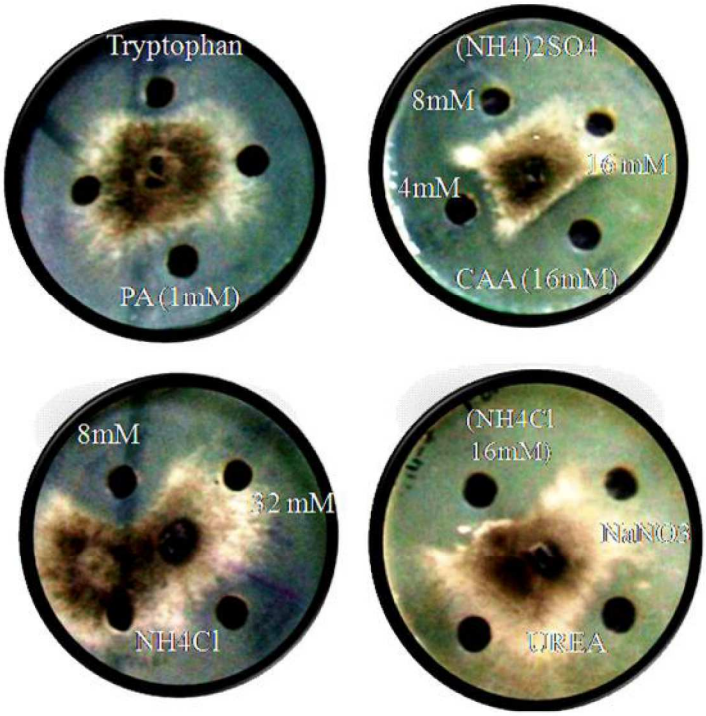


Fig. 3.26 Inhibition of *R.bataticola by* PCN extracts of strain G44 grown on MBV1-agar plates with different nitrogen sources

3.3.5.2 Effect of carbon sources on putative PCN production:

To study the effect of various carbon sources on the level of PCN production, G44 was grown in MVB1-cas supplemented individually with C-equivalent concentrations of different carbon sources. Amount of PCN production was determined idirectly on the basis of amount of inhibition of fungal pathogen *R.bataticola*. MVB1-cas supplemented with glycerol showed

maximum fungal inhibition (Table 3.13). Fig.3.27 shows the highest PCN production/ antifungal effect of CAS amino acid as nitrogen sources

Table 3.13 Fungal inhibition by PCN extracts of G44 grown on different N sources

N source	Concentration	Inhibition	Inhibition of extract (%)
NH_4Cl	8mM	++++	78.57
	16mM	++	64.28
	32mM	+	28.57
Casamino acids	16mM	++++	77.80
$(NH_4)_2SO_4$	4mM	++	42.80
	8mM	+	38.57
	16mM	+/-	27.50
Urea	8mM	+/-	27.50
$NaNO_3$	16mM	+	28.57
Phenylalanine	1mM	++	50
Tryptophan	1mM	+	28.57

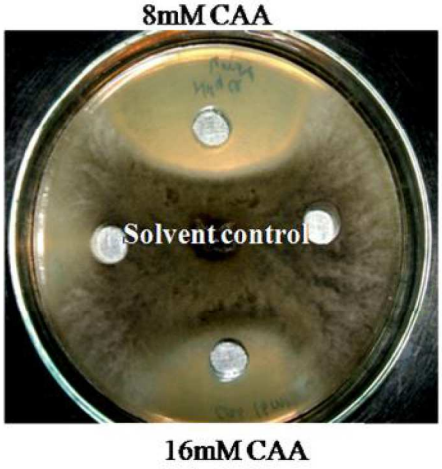


Fig. 3.27 Fungal growth inhibition by G44 under 8mM and 16mM casamino acids supplemented in MVB1-glucose-CAS.

While fructose, sucrose and glucose showed moderate inhibition (Fig. 3.28 and Table 3.14). Supplementing citric acid and malic acid as carbon sources in the original medium showed very little fungal inhibition and therefore minimum PCN production. Similar to our strain, the production of PCA by *P. fluorescens* 2-79 (Slininger and Shea-Wilbur, 1995) and PCN production by *P. aeruginosa* (Kanner *et al.* 1978) were found to be stimulated by glucose and glycerol. This suggests a similar response of phenazine-producing pseudomonad to these carbon sources.

Table 3.14 Fungal inhibition by PCN extracts of G44 grown on different C sources.

C-Source	**Concentration(mM)**	***R.bataticola* Inhibition**	***R.bataticola* Inhibition (%)**
Glycerol	60	++++	80
Fructose	30	+++	70
Glucose	30	++	40
Sucrose	15	++	40
Citric acid	30	+	20
Control	0	-	0

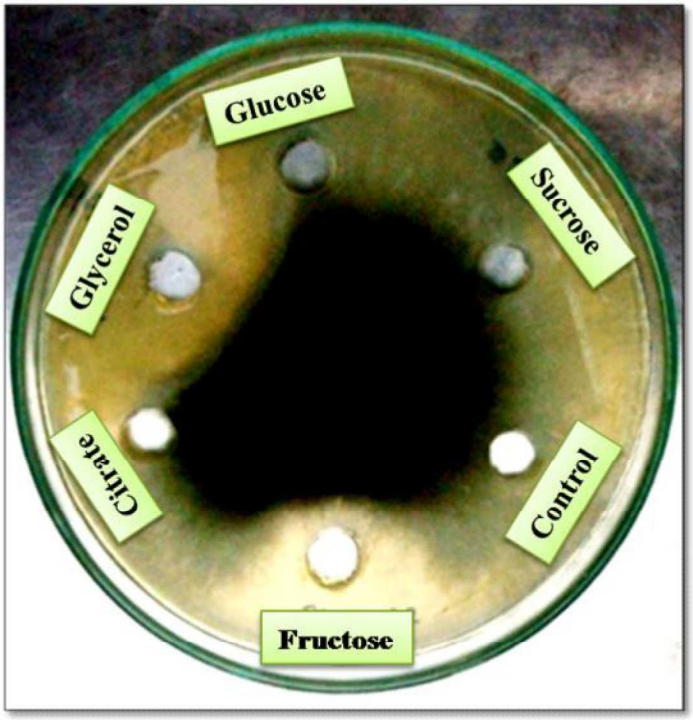


Fig. 3.28 Growth Inhibition of *R.bataticola* by PCN extracts of strain G44 grown on on MBV1-agar plates supplemented with different C-sources

3.3.5.3 Cumulative effect of highest producing factors:

To find synergistic effects of environmental factors which strongly influence PCN production in the strain G44, carbon and nitrogen sources which showed highest PCN production were combined. However, the results did not show any synergism and the cumulative effect of high producers was same as each individual supplementation (Fig. 3.29).

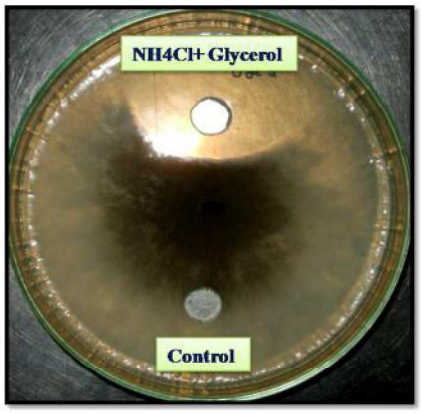
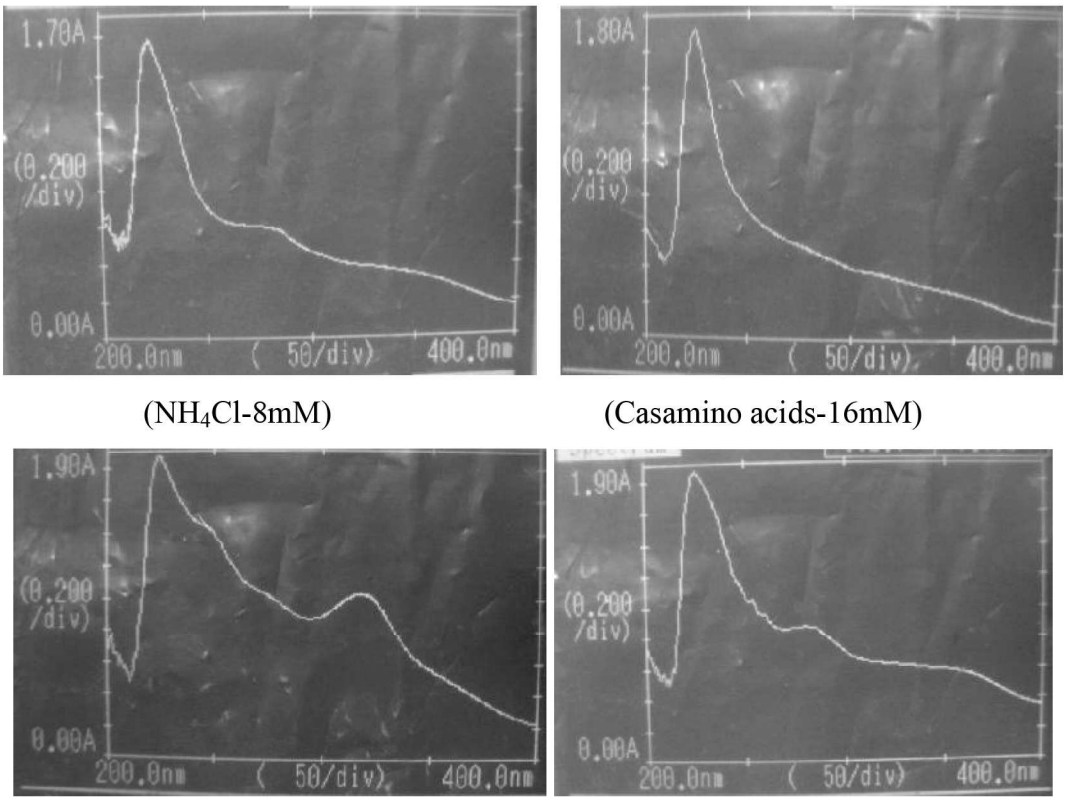


Fig. 3.29 Iinhibition by PCN extract of MVB1 supplemented with NH_4Cl & glycerol

3.3.5.4 UV-absorption spectra of phenazine derivatives:

G44 cultivated in medium supplemented with different carbon and nitrogen sources were scanned on UV spectrum for PCN extracts. Since each phenazine derivative has a specific UV maxima peak (Fig.3.30), peaks obtained for PCN extracts for all high producing C and N supplements were compared with standard peaks (Mavrodi *et. al.*, 2006, Mazurier et al,2009). Absorption maxima of PCN extracts of G44 in all the 5 extracts matched with that of the standard PCN peak. Peaks obtained for NH_4Cl 8mM, glucose and casamino acids showed the presence of a shoulder adjacent to the PCN peak this may be due to the presence of other derivatives of phenazine in smaller amounts in the extract apart from PCN which is in excess. Hence it can be concluded from the data that the extract obtained under these specific culture conditions for the strain G44 contains PCN. The nutritional factors that affect PCN production studied here are likely to affect biocontrol and can explain at least partially, the inconsistency of biocontrol in field experiments.



(NH_4Cl-8mM) (Casamino acids-16mM)

(Glycerol-60mM) (NH_4Cl-8mM + glycerol-60mM)

Fig.3.30 UV absorption maxima for PCN extracts of G44 on different carbon and nitrogen sources

3.3.6 Statistical analysis for the effect of nutritional factors on antifungal metabolite production in fluorescent pseudomonad:

Understanding the factors that regulate the biosynthesis of antimicrobial compounds by disease suppressive strains of *P.fluorescens* is an essential step towards improving the level and reliability of their biocontrol activity. In present study the attention was focused on mineral and C sources because they have long been known to influence the activity of phytopathogenic micro-organisms (Engelhard, 1989), they contribute to the variability of biocontrol in different soils and on host crops that differ in root exudates composition (Latour et al., 1996), and they have been reported to influence production of other antibiotics in biocontrol strains (Gutterson 1990; Milner et al., 1995; Duffy and Defago, 1999).Quantitative and/or qualitative differences in the sugar, nitrogen, phosphate and mineral components of root exudates could determine the effectiveness of bio-control in given crop-pathogen systems (Martin et al, 1994).

3.3.6.1 Effect of nutrient combinations on growth and antifungal activity of *Pf* CHA0:

The effect of seven nutritional factors (glucose, citrate, KNO_3, KH_2PO_4, $FeSO_4$, $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$ on the growth, antifungal activity and production of 2, 4- DAPG, PLT and PRN, by model biocontrol strain *Pf* CHA0 was studied using a novel approach of statistical design. Use of 1/5-strength NBY broth and amendment with different combinations of nutritional factors was studied for their effect on the growth, antifungal activity and antibiotic production by *Pf* CHA0. 2, 4- DAPG, PLT and PRN were extracted with ethyl acetate and quantified by established HPLC procedures. Effect of each factor and their combinations on 2, 4- DAPG, PRN, PLT and PHZ production was studied by regression analysis using DE 8.

A significant effect of nutrient combinations on growth of the bacterium was observed for both day 2 ($p=0.0026$) and day 5 ($p=0.011$). Effect on antifungal activity of the ethyl acetate extract was highly significant at day 2 ($p=0.008$) and day 5 ($p=0.0007$). The medium amended with a combination with Zn, Mo and nitrate only did not support growth possibly because of very low C/N ratio (C/N<1) and the stress effect of heavy metal Zn^{2+} and Mo^{2+} and it was not included in the further data analysis and graphs.

As described in Table 3.15 the combinations amended with citrate, $FeSO_4$, glucose and KH_2PO_4, with citrate, $FeSO_4$, KNO_3 and $ZnSO_4$ and the combination amended with citrate, glucose, $FeSO_4$, KH_2PO_4, KNO_3, $(NH_4)_6Mo_7O_{24}$ and ZnSO4 have shown highest antifungal

activity of 70% on day 5 and high growth (OD $_{600nm}$ = 3±0.5) (Fig 3.31). Combination amended with KNO_3, $(NH_4)_6Mo_7O_{24}$ and $ZnSO_4$ did not supported the *Pf* CHA0 growth. Combinations amended with citrate, $FeSO_4$, glucose and KH_2PO_4 and combination amended with glucose, KH_2PO_4, KNO_3 and $ZnSO_4$ have supported high growth on both the days of sampling with OD $_{600nm}$ value of 4±0.5. Combination amended only with all inorganic components viz., $FeSO_4$, KH_2PO_4, $(NH_4)_6Mo_7O_2$, and $ZnSO_4$ showed green fluorescent pigment biosynthesis and antifungal activity up to 40% on day 5.

Effect of nutritional factors and their contribution to day 2 and day 5 antifungal activities shown in Fig.3.32, Citrate has strong positive effect on antifungal activity on day 5 and contribution 29% and day 2 with contribution 14%. However, glucose showed more positive effect on day 2 than day 5 with contribution 33% on day 2 and only 3% on day 5. Citrate with glucose has positive effect and shown 16% contribution on day 2 and 12% on day 5 (Fig...3.33).

Although C sources differentially influence medium acidification during growth (Dekleva et al, 1987), which may then indirectly affect antibiotic biosynthesis (Slininger et al, 1995) and bio control activity (Ownley et al, 1992) but in our result we did not observe any significant change medium pH after day 2 and day 5 of growth. On day 2, Pi, Fe^{2+}, citrate with Pi, citrate with Fe^{2+} and citrate with Mo^{2+} have shown negative effect on antifungal activity. On day 5 only glucose with nitrate and citrate with glucose and nitrate have shown negative effect on antifungal activity. Contribution order of factors acting positively to antifungal activity on day 2 is, glucose>citrate>citrate +glucose while on day 5 antifungal activity the order is, citrate> citrate +glucose>Fe^{2+}>glucose>citrate +Pi=citrate+Fe^{2+}.Combination with Fe^{2+} and citrate have shown increased antifungal activity on day 5 and was thought to be mediated by induction of antifungal traits specifically. It is in support of previous observation that iron stimulates bicontrol activity (Slininger and Jackson, 1992) and cyanide production (Keel et al, 1989). However the antifungal activity on day 2 is negatively regulated in following order, citrate +Pi > citrate +glucose + KNO_3>> citrate+Fe^{2+} and on day 5 in the order citrate + glucose +KNO_3> citrate+KNO_3> glucose + KNO_3>Pi=KNO_3. Glucose and citrate individually and in combinations, supported well to growth and antifungal activity. Combination with mineral supplementation of $Fe2^{+}$, Mo^{2+}, Zn^{2+} and Pi showed pigmentation after day 5 of growth and 40% antifungal activity and thought to positive effect of Pi on growth and positive effect of $Fe2^{+}$ on antifungal traits. Combination with glucose, citrate and nitrate and did not have any amended mineral nutrient and

have shown more growth (day 2 OD_{600nm} > 4.0) and antifungal activity on day 2 and less growth (day 5 OD_{600nm} >3) and antifungal activity on day 5 which could be possible due to high C/N ratio which will induce rpoN and also antifungal traits and have not any interference of heavy metals. Combination with Fe^{2+}, nitrate and phosphate showed weak growth and antifungal activity which could be possible due to very low C/N ratio but only low growth because of supplementation of Pi and Fe^{2+}. Effect of factors on antifungal activity showed many variations in effect (+/-) and contribution compare to its effect on growth. Possible reason could be that the growth is result of basic metabolism and cumulative effect of many supportive pathways but antifungal activity is contributed by limited number of pathways so the variation in effect was observed. Citrate and Pi individually have positive effect on the growth but their combination have shown negative effect which show a kind of the shift of bacterial physiology/behavior to nutrients present individually and in combinations.

Combination without any amendments (considered as negative control) showed low growth but higher antifungal activity on day 5 than on day 2, which suggest that in diluted nutrient broth antifungal trait gets induced on day 5. Kumar and Shimizu, (2010) reported that, as C/N ratio increases the transcript level of rpoN get increased, which encodes σ^{54}, and this could be the possible reason for the induced antifungal traits.

Fig.3.34 shows the effect of nutritional factors and their contribution to growth of *Pf* CHA0 at day 2 and day 5. Citrate, glucose, Zn^{2+} individually, citrate along with glucose and triple combination of citrate, glucose and nitrate have shown positive effect on growth. Citrate and Pi, citrate and zinc, Mo^{2+}, citrate and Mo^{2+}, triple combination of citrate, glucose and KNO_3 have shown negative effects on growth. Citrate and Pi individually have shown positive effect and contribution of 9% and 5% respectively on day 2 and 10 % and 1 % respectively on day 5, however the combination of citrate and Pi showed negative effect on growth and contributed to 18 % on day 2 and 19 % on day 5 (Fig. 3.35). Contribution order of the factors acting positively on the day 2 growth is, glucose > Zn^{2+}>citrate +glucose>citrate>Pi>KNO_3, while for the day 5 growth the order is Zn^{2+}> citrate+ glucose > citrate > glucose >Pi=KNO_3. In our result, citrate and glucose individually and in combination, have shown strong positive effect on *Pf* CHA0 growth on day 2 and day 5, which is in accordance to previous finding that an organic acid or a tricarboxylic acid cycle intermediate, not glucose, is usually the preferred carbon source in *Pseudomonas* spp. (Mac Gregor et al, 1992). Of the minerals, zinc was found to be supportive

much to *Pf* CHA0 growth on day 2 and day 5. However, the contribution order of negatively acting factors on day 2 growth is, citrate +Pi = Citrate +glucose + KNO_3 > Mo^{2+} and for day 5 it is, citrate +Pi >citrate +glucose + KNO_3 >citrate+ Zn^{2+}. Ammonium molybdate has been reported to be a strong inhibitor of acid phosphatase activity (Glew et al, 1988) and the process of phosphorylation / dephosphorylation plays a crucial role in many metabolic processes (Hunter, 1995).

Table 3.15 Effect of different nutrient combinations on growth and antifungal activity by *Pf* CHA0

Variables			**Responses**			
Nutrient combinations	*Run no. by DE8*	*Supplemented (mM) C : N : Pi : Minerals (Fe, Mo and Zn) ratio*	*Growth OD λ600nm (AU)*		*Antifungal Activity (%)*	
			2 d	*5 d*	*2 d*	*5 d*
1. Dilute NBY	5	0:0:0:0	0.25	0.24	0	44.3
Dilute NBY supplemented with:						
2. ZnSO4, KNO3, (NH4)6Mo7O24	15	0:10:0:0.35(Zn):0.5(Mo)	0	0	0	0
3. KH2PO4,KNO3, FeSO4	8	0:10:10:0.5(Fe)	0.20	0.59	17	0
4. ZnSO4,KH2PO4,(NH4) 6Mo7O2,FeSO4	10	0:0:10:0.5(Fe):0.35(Zn): 0.5(Mo)	1.67	2.96	14.3	50
5. Citrate,KH2PO4,KNO3 ,(NH4)6Mo7O24	12	300:10:10:0.5(Mo)	0.14	0.29	0	44.3
6. Citrate , FeSO4, (NH4)6Mo7O24	7	300:0:0:0.5(Fe) :0.5(Mo)	0.22	0.14	0	10.7
7. Citrate , KH_2PO_4, $ZnSO_4$	3	300:0:10:0.35(Zn)	0.28	0.29	11	0
8. Citrate ,KNO_3, $FeSO_4$,$ZnSO_4$	9	300:10:0:0.5(Fe) :0.35(Zn)	5.24	2.40	11	70.7
9. Glucose, $ZnSO_4$, $FeSO_4$	4	600:0:0:0.5(Fe):0.35(Zn)	0.18	0.08	0	18.6
10. Glucose, KH_2PO_4, $(NH_4)_6Mo_7O_{24}$	2	600:0:10:0.5(Mo)	0.22	0.18	10.7	10.7
11. Glucose,KNO_3,$(NH_4)_6$ Mo_7O_{24},$FeSO_4$	14	600:10:0:0.5(Fe) :0.5(Mo)	0.24	0.26	18.7	30
12. Glucose, KH2PO4, KNO_3, $ZnSO_4$	6	600:10:10:0.35(Zn)	4.04	3.73	0	0

13. Citrate ,Glucose, KH_2PO_4,$FeSO_4$	1	900:0:10:0.5(Fe)	3.48	3.04	10.7	70.7
14. Citrate ,Glucose, KNO3	13	900:10:0:0	4.53	2.83	61	38.6
15. Citrate,Glucose,$ZnSO_4$, $(NH_4)_6Mo_7O_{24}$	11	900:0:0:0.35(Zn):0.5(Mo)	4.61	2.83	44.3	64.3
16. Citrate,Glucose,$ZnSO_4$, KH_2PO_4,KNO_3,$(NH_4)_6$ Mo_7O_{24}, $FeSO_4$	16	900:10:10:0.5(Fe): 0.35(Zn):0.5(Mo)	4.58	2.76	47	70.7

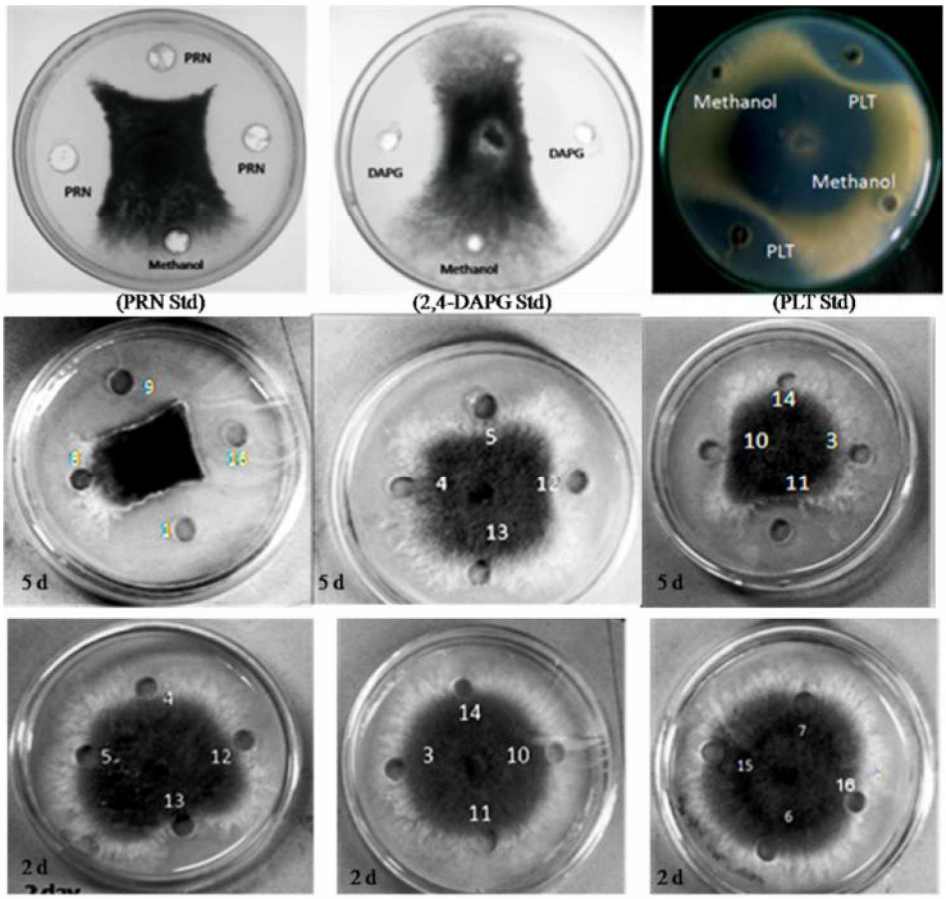


Fig.3.31 Inhibition of *R. bataticola* by pure antibiotic std., ethyl acetate extracts of *Pf* CHA0 grown in various nutritional combinations on 2d and 5d

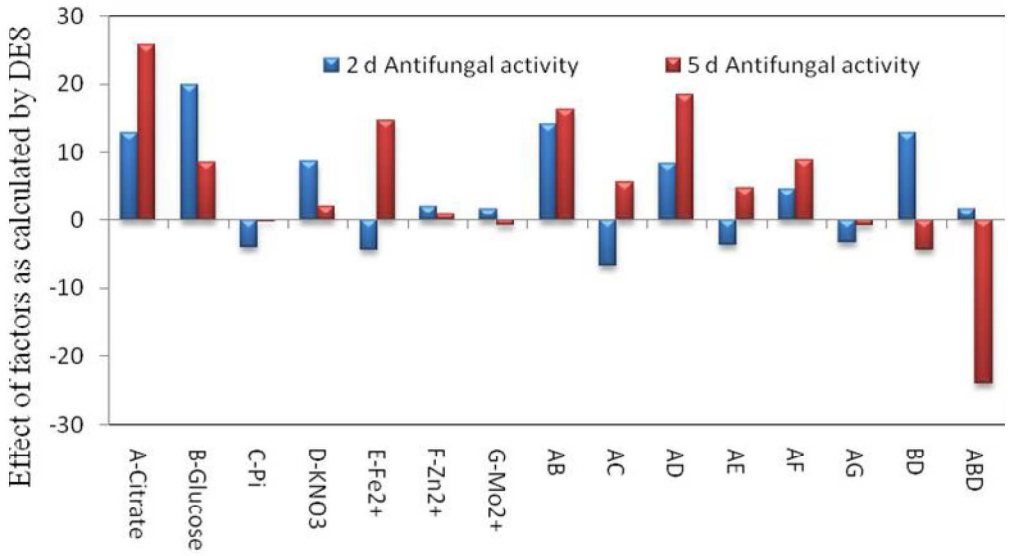


Fig.3.32 Effect of nutritional factors to *Pf* CHA0 antifungal activity at 2 d and 5 d

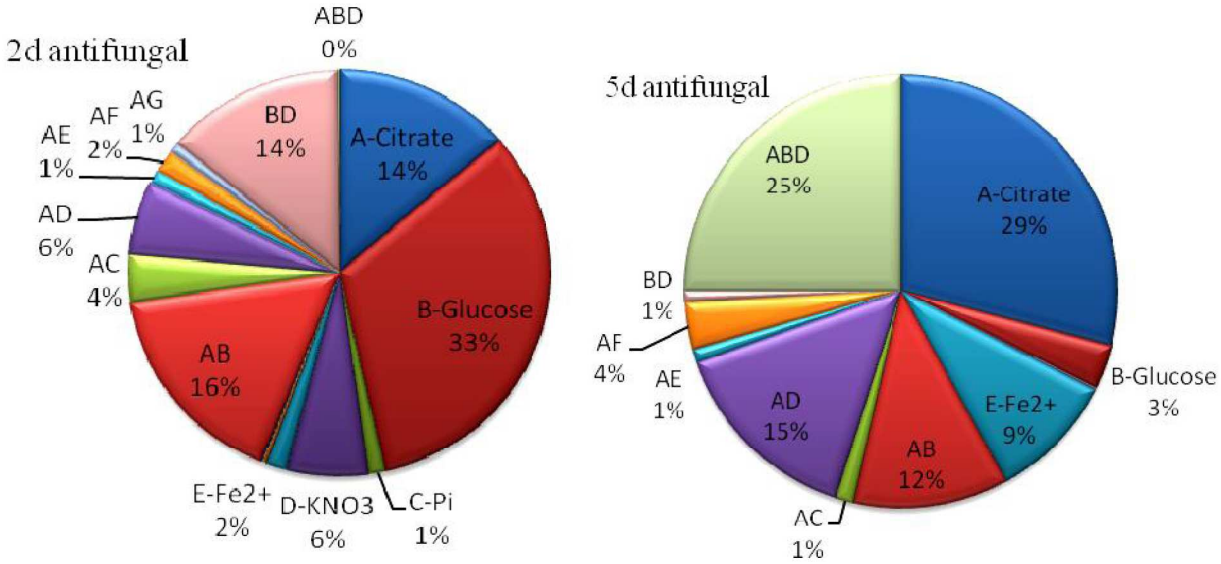


Fig. 3.33 Contribution of nutrients for the effect of nutritional factors on *Pf* CHA0 growth and antifungal activity at 2 d and 5 d

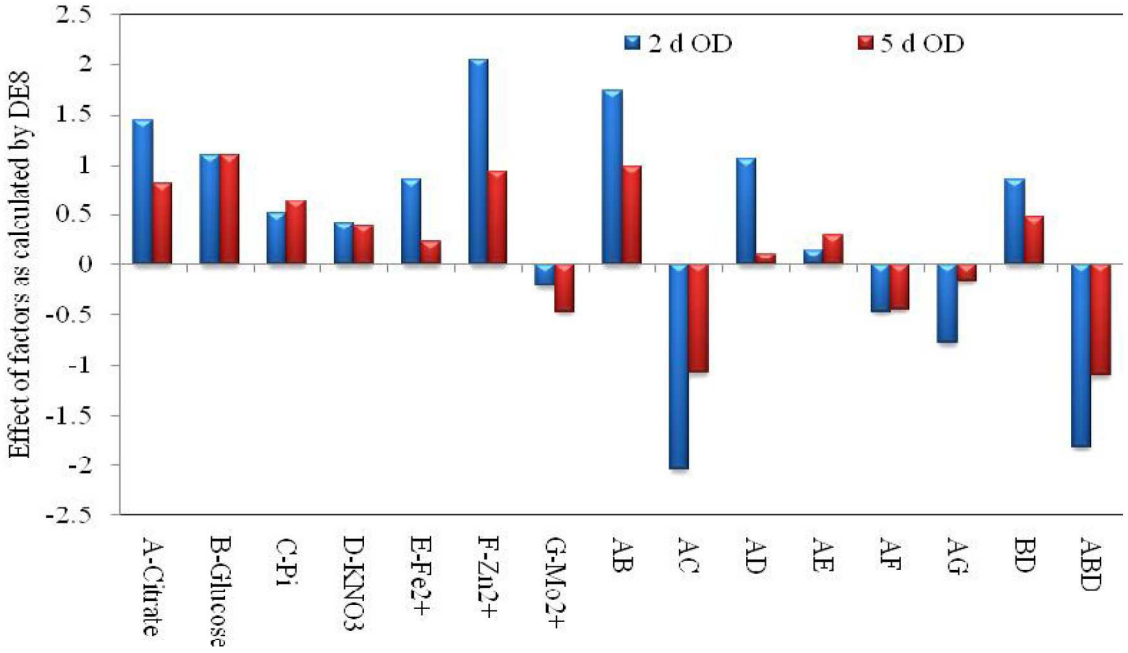


Fig. 3.34 Effect of nutritional factors to *Pf* CHA0 growth (OD 600nm) at 2d and 5d

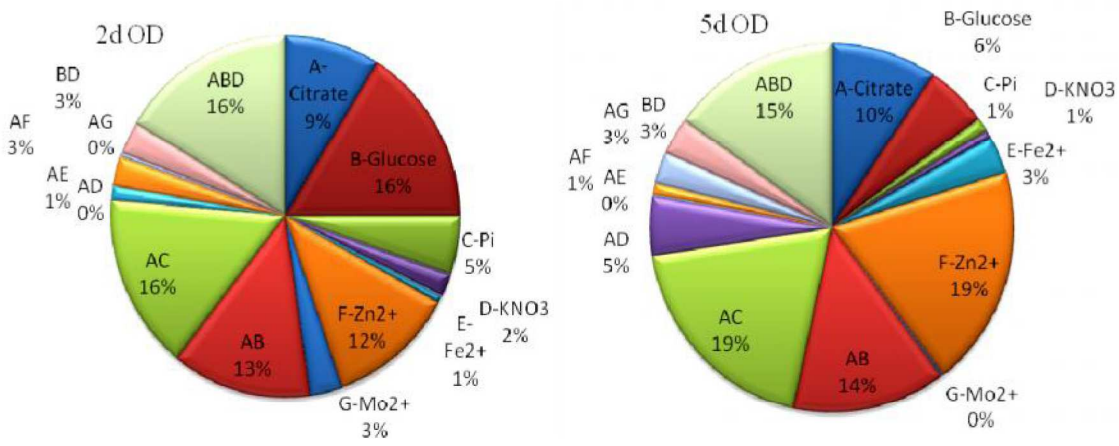


Fig .3.35 Contribution of factors to the effect of factors on OD600nm at 2d and 5d

3.3.6.2 Effect on nutritional factors on antibiotic biosynthesis:

PRN biosynthesis under different nutrient combinations is depicted in Table 3.16. Significant variation in biosynthesis level of PRN was observed across all combinations on day 2 (p=0.0016) and day 5 (p=0.0045). Nutrient combination amended with $FeSO_4$, KH_2PO_4 and KNO_3, combination amended with KH_2PO_4, $(NH_4)_6Mo_7O_2$, $FeSO_4$ and $ZnSO_4$, combination amended with $FeSO_4$, Glucose, KNO_3 and $(NH_4)_6Mo_7O_{24}$, and combination amended with citrate, $FeSO_4$,

glucose, KH_2PO_4, KNO_3, $(NH_4)_6Mo_7O_{24}$ and ZnSO4 supported PRN biosynthesis on day 2 while certain other combinations contain Mo supported PRN biosynthesis on day 5. It supports the previous observation by Duffy and Defago (1999), that the production of PRN by *Pf* CHA0 was stimulated by Mo^{2+}. It suggests that direct improvement in the bio control effectiveness by NH_4-Mo could be indirectly mediated by altered enzymatic activity. Effect of nutritional factors and their contributions to PRN biosynthesis is depicted in Fig.3.36 and Fig.3.37. Fe^{2+}, Pi, citrate with glucose and triple combination of nitrate, glucose with nitrate showed positive effect on PRN biosynthesis on day 2. On day 5, PRN biosynthesis is primarily influenced by citrate. Fe^{2+} showed strong positive effect on PRN biosynthesis with contribution 29% on day 2 and 13 % on day 5 PRN biosynthesis (Fig. 3.37). It is in support of previous observation that iron stimulates biosynthesis of a variety of antifungal metabolites (e.g., zwittermycin A (Milner et al, 1995), kanosamine (Milner et al, 1996), phenazine (Slininger and Jackson, 1992).

Inorganic phosphate has been reported to repress the production of antibiotics including diacetylphloroglucinol and pyrrolnitrin by *Pf* CHA0 (Duffy and Defago 1999). Phosphate repression has been reported for other polyketide antibiotics (e.g. anthracycline and tetracycline) and phenazines in *Pseudomonas* spp. (Turner and Messenger 1986; Martın et al. 1994), and for zwittermycin A and kanosamine in Bacillus (Milner et al. 1995, 1996) and may be a common phenomenon in soil bacteria. Contribution order of factors acting positively to PRN biosynthesis on day 2 is, Fe^{2+} > citrate+glucose >glucose+KNO_3 > Pi> KNO_3>citrate +Pi while on day 5 PRN biosynthesis the order is, citrate>Fe^{2+}>citrate+KNO_3> citrate+Fe^{2+} > Zn^{2+}> KNO_3=citrate +Pi.(Fig. 3.37) The negative effect of factors on PRN biosynthesis on day 2 is in the order, Citrate +glucose + KNO_3 >Citrate +Fe^{2+}>citrate >Zn^{2+} and on day 5 in the order, glucose + KNO_3> citrate+glucose +KNO_3>Pi>Citrate +glucose (Fig 3.37).

The levels of PLT under different nutrient combinations shown in Table 3.16. PLT levels vary significantly in all 15 combinations on the day 2 (p=0.02) and day 5 (p=0.0098). PLT biosynthesis level was high in combination in $FeSO_4$, KH_2PO_4, $(NH_4)_6Mo_7O_2$, and $ZnSO_4$, combination amended with citrate ,glucose, $(NH_4)_6Mo_7O_{24}$ and $ZnSO_4$, combination amended with citrate ,glucose, and KNO_3 and combination amended with citrate, $FeSO_4$,glucose, KH_2PO_4, KNO_3, $(NH_4)_6Mo_7O_{24}$ and ZnSO4 on day 2. High PLT biosynthesis on day 5 was observed in the combination amended with citrate, $FeSO_4$, glucose and KH_2PO4 , combination

amended with $ZnSO_4$, KH_2PO_4, $(NH_4)_6Mo_7O_2$,$FeSO_4$ and combination amended with citrate, $FeSO_4$, glucose, KH_2PO_4, KNO_3, $(NH_4)_6Mo_7O_{24}$ and $ZnSO_4$ (Table.3.16). Fig. 3.38 shows the significant effect of nutritional factors and their combinations on PLT biosynthesis ($p<0.05$). Zinc increased PLT biosynthesis confirming the previous finding of Duffy and Defago, 1999, that zinc increased PLT biosynthesis in all fluorescent *Pseudomonas* strains able to produce this antibiotic, but the level of stimulation varied. Positive contribution of factors on PLT biosynthesis on day 2 showed following order, citrate+ glucose> glucose=citrate> glucose+ KNO_3> citrate+KNO_3 while on day 5 PLT biosynthesis the order is, citrate +glucose> citrate+KNO_3 > glucose+KNO_3(Fig.3.39) The negatively acting factors on PLT biosynthesis on day 2 showed the order, citrate +Fe^{2+} =Citrate +Pi >>citrate+glucose +KNO_3 and on day 5 in the order citrate+ glucose +KNO_3> KNO_3>> glucose>citrate. PLT biosynthesis is maximally repressed by combination of citrate and Fe^{2+}, citrate and phosphate on day 2 (Fig 3.39). However on day 5, PLT biosynthesis is maximum repressed by triple combination of citrate, glucose and nitrate followed by nitrate confirming the finding that PLT is repressed by glucose.

The 2, 4- DAPG biosynthesis levels under different nutrient combinations are shown in Table 3.16. 2, 4- DAPG biosynthesis varies significantly in all 15 combinations on the day 2 ($p=0.009$) but none significantly on day 5 ($p=0.10$). Glucose, citrate with Fe^{2+}, citrate with Zn^{2+} and triple combination of citrate, glucose and nitrate have shown significant positive effect on 2,4- DAPG biosynthesis at day 2 ($p<0.05$). The effect of nutrients on the 2, 4- DAPG biosynthesis on day 2 and day 5 is depicted in Fig. 3.40. Positive contribution of factors acting on 2, 4- DAPG biosynthesis on day 2 follows the order, glucose>citrate+ Zn^{2+}. 2, 4- DAPG biosynthesis on day 2 is stimulated by glucose followed by combination of citrate and Zn^{2+} confirming the previous observation that increased 2, 4- DAPG biosynthesis by glucose (Duffy and Defago, 1999). However the negatively acting factors on 2, 4- DAPG biosynthesis on day 2 follow the order, citrate + Fe^{2+} > citrate+ glucose +KNO_3 (Fig 3.41). On day 5, 2, 4- DAPG is repressed by Pi which supports previous observation that 2, 4- DAPG production by *Pf* CHA0 was almost abolished by 10 mM phosphate. Growth was increased 5- to 10-fold by 100 mM phosphate amendment (Duffy and Defago, 1999).

Table 3.16 shows the summary of effect of factors on antifungal activity, PRN, DAPG and PLT production. Combination of citrate and glucose has shown positive effect on day 2 and day 5 antifungal activities and well correlated by its positive effect on PRN and PLT production.

Glucose showed the positive effect on day 2 antifungal activity and was correlated with its positive effect on 2, 4- DAPG production. Combination of citrate and Fe^{2+} has shown negative effect on day 2 antifungal activity and it was supported well by negative effect of this combination on day 2 PRN, 2, 4- DAPG and PLT production. Citrate has shown positive effect on day 5 antifungal activity and correlated with its positive effect on day 5 PRN production. Triple combination of citrate, glucose and nitrate has negative effect on antifungal activity and correlated also with its negative effect on PRN and PLT production. The importance of nutrient status to pyoluteorin production is corroborated by the observation that pyrrolquinoline quinone, a cofactor required by glucose and alcohol dehydrogenases, represses pyoluteorin production confirm that pyoluteorin production is linked to the physiological status of the cell (Whistler et al, 2000).

Table 3.16 Biosynthesis of PRN, 2, 4- DAPG and PLT under different nutrient combinations

	Variables	**Responses**					
	Supplemented (mM)- C : N : Pi : Minerals (Fe, Mo and Zn) ratio	*PRN production (ng/ml)*		*DAPG production (ng/ml)*		*PLT production (ng/ml)*	
		2 d	*5 d*	*2 d*	*5 d*	*2 d*	*5 d*
1	*0:0:0:0*	411	29.5	26	33.3	60	6590
Dilute NBY supplemented with:							
2	*0:10:0:0.35(Zn):0.5(Mo)*	0.00	0.00	0.0	0.00	0.00	0.00
3	*0:10:10:0.5(Fe)*	1375	4	26.5	22.65	695	90
4	*0:0:10:0.5(Fe):0.35(Zn):0.5(Mo)*	1710	70	82	20	1488	7285
5	*300:10:10:0.5(Mo)*	87	1046	25	80.45	49.35	238
6	*300:0:0:0.5(Fe) :0.5(Mo)*	28.5	574	21	56.95	25	57
7	*300:0:10:0.35(Zn)*	105	5	83.5	29.9	23	142.5
8	*300:10:0:0.5(Fe) :0.35(Zn)*	565	2012.5	17.5	153.5	117	1121
9	*600:0:0:0.5(Fe):0.35(Zn)*	29.5	609	54.5	0	21	203.5
10	*600:0:10:0.5(Mo)*	0.00	0.00	32	26	0	43.5
11	*600:10:0:0.5(Fe) :0.5(Mo)*	1045	120	317.5	1241.5	117	794.5
12	*600:10:10:0.35(Zn)*	402	4	85.5	0	32	44.5
13	*900:0:10:0.5(Fe)*	555	1370.5	8.5	126.25	66	1991
14	*900:10:0:0*	675	306	13	321.5	2193	2493
15	*900:0:0:0.35(Zn):0.5(Mo)*	87.5	930	107.5	17	1875	3388
16	*900:10:10:0.5(Fe):0.35(Zn):0.5(Mo)*	870	1014	99.5	23.5	1229	2893

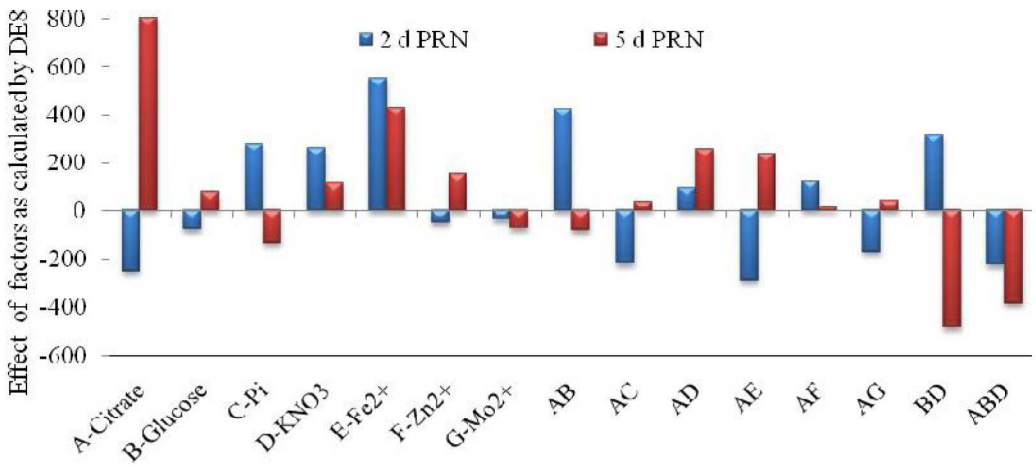


Fig.3.36 Effect of nutritional factors and its combinations on PRN production at 2d and 5d

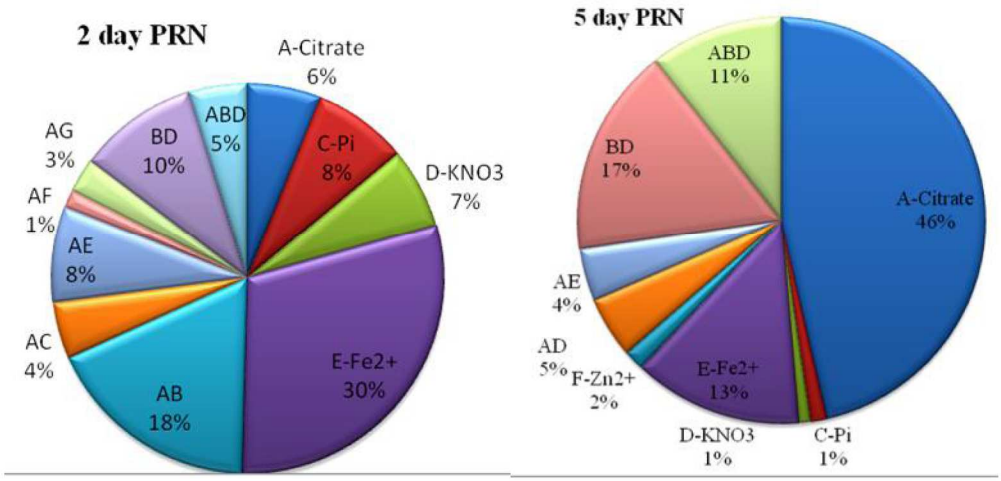


Fig.3.37 Contribution of factors to the effect of factors on PRN production at 2d and 5d

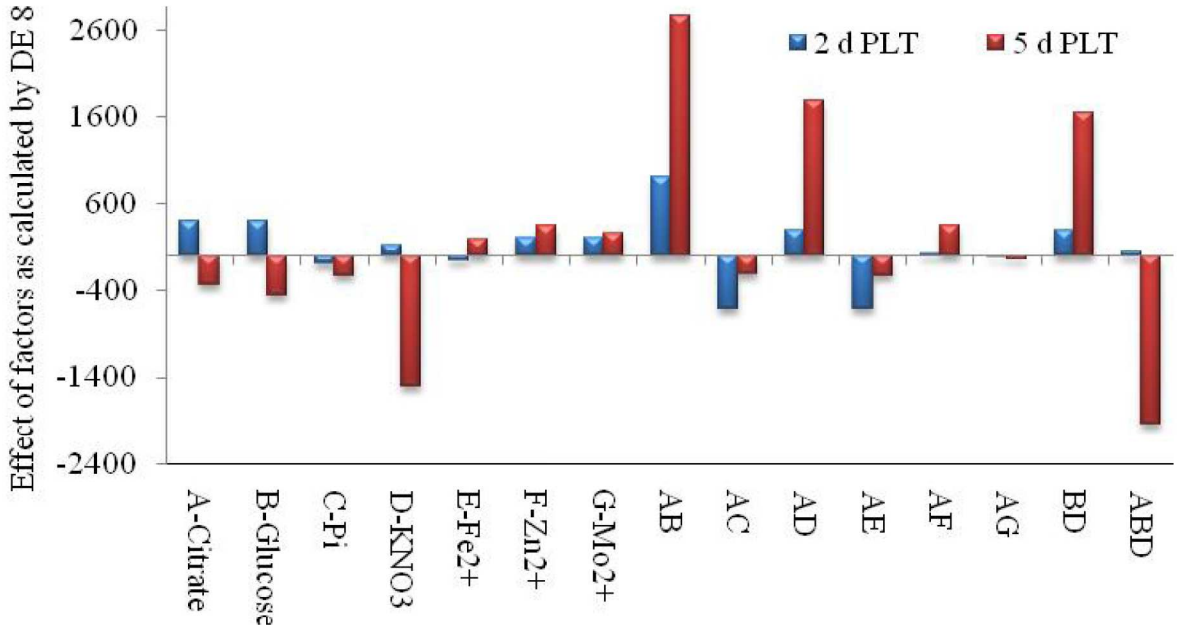


Fig.3.38 Effect of nutritional factors and its combinations on PLT production at 2d and 5d

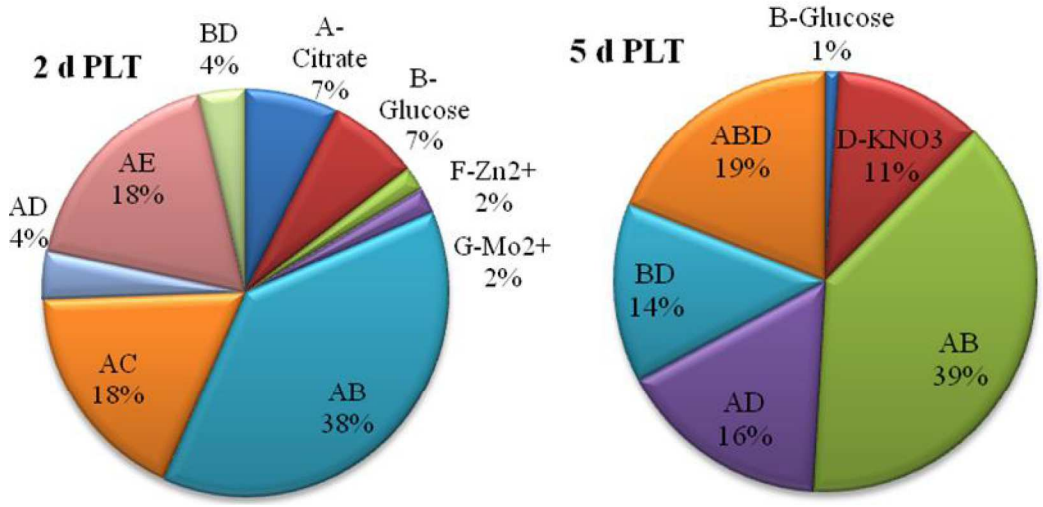


Fig.3.39 Contribution of factors to the effect of factors on PLT production at 2d and 5d

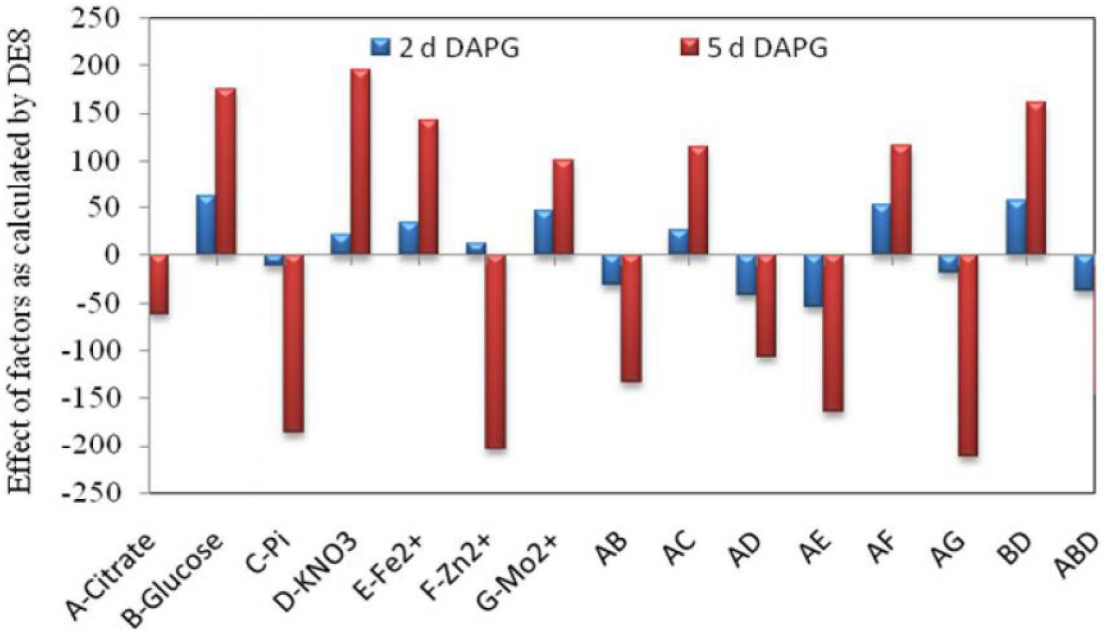


Fig.3.40 Effect of nutritional factors and its combinations on DAPG production at 2d and 5d

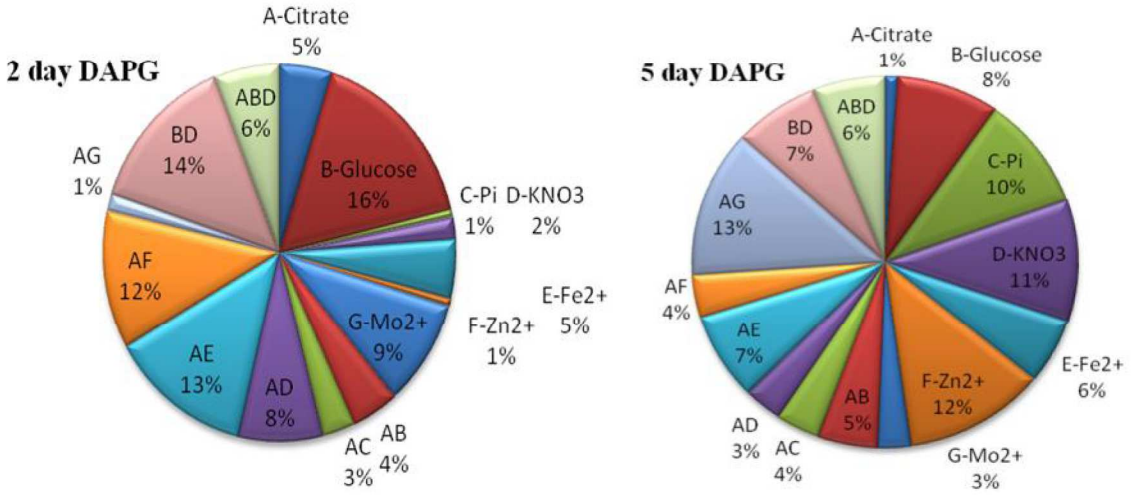


Fig. 3.41 Contribution of factors to the effect of factors on DAPG production at 2d and 5d

Table3.17 Effect of factors and its combinations on the growth, antifungal activity and antifungal metabolite production

		Citrate	Glucose	Zn^{2+}	Pi	KNO3	Citrate+ Glucose	Citrate +Pi	Citrate + Fe	Citrate +Glucose +KNO3	Fe2+	Glucose + KNO3	Citrate+ KNO3	Citrate +Zinc
Growth (OD 600nm)	2 day	+(9%)	+(16%)	+(12%)	+(5%)	+(2%)	+(13%)	-(16%)	NE	-(16%)	NE	NE	NE	NE
	5 day	+(10%)	+(5%)	+(20%)	+(1%)	+(1%)	+(14%)	-(20%)	NE	-(15%)	NE	NE	NE	NE
Antifungal effect	2 day	+(14%)	+(33%)	NE	-(1%)	-(6%)	+(16%)	-(4%)	-(1%)	-(<1%)	-(2%)	-(14%)	-(6%)	NE
	5 day	+(29%)	+(3%)	NE	-(<1%)	-(<1%)	+(12%)	+(1%)	+(1%)	-(25%)	+(9%)	-(1%)	-(15%)	NE
PRN	2 day	-(6%)	NE	-(<1%)	+(8%)	+(7%)	+(18%)	+(4%)	-(8%)	-(11%)	+(30%)	+(10%)	+(<1%)	NE
	5 day	+(46%)	NE	+(2%)	-(1%)	+(<1%)	-(<1%)	+(<1%)	+(4%)	-(5%)	+(13%)	-(17%)	+(5%)	NE
PLT	2 day	+(7%)	+(7%)	NE	NE	+(<1%)	+(38%)	-(18%)	-(18%)	-(<1%)	NE	+(4%)	+(4%)	NE
	5 day	-(<1%)	-(1%)	NE	NE	-(11%)	+(39%)	-(<1%)	-(<1%)	-(19%)	NE	+(14%)	+(16%)	NE
DAPG	2 day	NS	+(16%)	NS	-(1%)	NS	NS	NS	-(13%)	-(6%)	NS	NS	NS	+(12%)
	5 day	NS		NS	-(10%)	NS	NS	NS	NS		NS	NS	NS	NS

(NE-No effect,NS-Non significant)

3.3.6.3 Contribution of PRN, 2,4- DAPG and PLT to antifungal activity:

Based on antifungal activity under all combinations and the biosynthesis level of antifungal metabolites, the percentage contribution of PRN, 2,4- DAPG and PLT to day 2 and day 5 antifungal activity by *Pf* CHA0 depicted in Fig. 3.42. For the day 2 antifungal activity, PLT has contributed 59%, PRN has contributed 26 % and remaining 15% by 2,4- DAPG. For the day 5 antifungal activity PRN has contributed 59%, PLT 37% and 2,4- DAPG only 4%. Regulation of 2,4- DAPG and PLT production in *Pf* CHA0 involves a molecular balance in which each antibiotic induces the expression of its own biosynthetic genes while repressing the expression of the biosynthetic genes of the other antibiotic (Baehler et al, 2005, Shanahan et al, 1992). This could be possible reason of not obtaining significant variations in 2, 4- DAPG production under different nutrient combinations on day 2 and day 5. Another possible explanation for these observations is that other, yet unknown effectors could interfere with the fine-tuned regulation of the 2, 4- DAPG–PLT balance. Similar deviation in 2, 4- DAPG production was obtained by Baehler et al, 2005, as they have used GFP-based reporters to study the antibiotic gene expression at the transcriptional level. The possibility of involvement of GacS/GacA two-component system (Haas and Keel, 2003) may be involved in the modulation of the observed effects as well. Day 2 antifungal found to have maximum positive correlation with PLT

followed by PRN however both PLT and PRN had shown strong positive correlation with OD_{600nm}. Up to day 2, DAPG is found to be not dependent much on OD_{600nm} and PLT. Day 5 antifungal activity was found to have maximum positive correlation with PRN followed by PLT however both PLT and PRN itself had shown a positive correlation with OD_{600nm}. Day 5 PLT levels have shown negative correlation with 2, 4- DAPG production. Day 5 DAPG levels have shown negative correlation with OD_{600nm}, PRN and PLT which proves the earlier observation by Baehler et al, 2005.

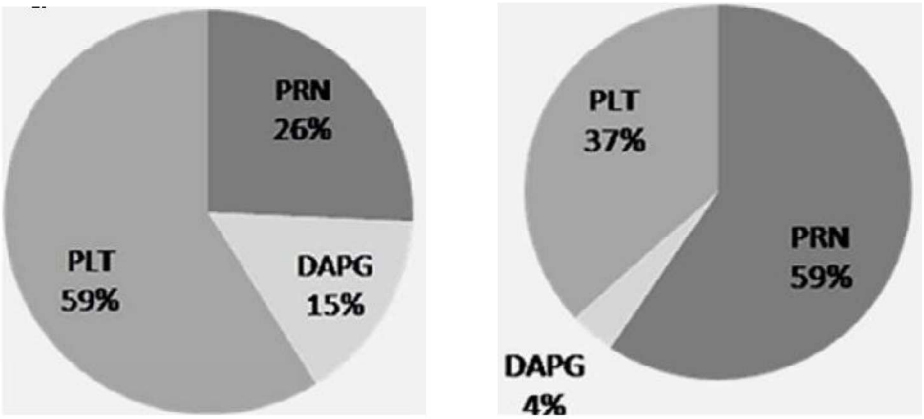


Fig.3.42 Contributions of PRN, PHL and PLT to the 2 day and 5 day antifungal activity

3.3.6.4 Principal Component Aanalysis for day 2 and day 5 antifungal activity:

For the day 2 the biplot graph (Fig. 3.43) shows that on this day of sampling antifungal, OD_{600nm} and PLT biosynthesis showed strong correlation and fall in same zone (-x, +y).While day 2 DAPG and PRN fall in other zone (+x, +y). Based on PCA analysis for the five variables with sixteen different combinations, Pearson correlation and Eigen values were obtained (Fig. 3.43) which shows the degree to which the variables are related with each other. Only the variables with the Pearson value (n) $\geq$ 0.35 have been considered with significant relatedness between them. Day 2 antifungal is positively correlated with PLT maximally (n=0.878) followed by OD_{600nm} (n=0.544), PRN and DAPG. Day 2 PRN has shown positive correlation with OD_{600nm} (n=0.44), antifungal activity (n=0.38). DAPG is nearly independent of OD_{600nm} and PLT (n<0.1). Day 2 PLT has shown high positive correlation with OD_{600nm} (n=0.694) and antifungal activity. Day 5 antifungal activity have shown positive correlation with OD_{600nm} (n=0.522), PRN (n=0.768) and PLT (n=0.475). Day 5 PLT has shown positive correlation with OD (n=0.33) and antifungal activity (n=0.475) but negative correlation with 2,4- DAPG (n= -0.16). 2,4- DAPG

has shown negative correlation with OD_{600nm} (n= -0.187), PRN (n=-0.131) and PLT. For day 2, the results showed that of the first three components, the first component accounted for about 54.52%, the second component about 22.36% and the third component about 13.88% of the total variance in the data set. Three components together accounted for about 90.71% of the total variance and the rest of the components only accounted for about 9.29%. For day 5 biplot graph (Fig. 3.43) shows that PLT and OD_{600nm} shows correlation and falls in same zone (+x, +y) while for day 5, antifungal and PRN falls in other zone (+x,-y). For the day 5, PCA analysis for first three components, the first component accounted for about 45.72%, the second component about 22.72% and the third component about 19.44% of the total variance in the data set. These three components together accounted for about 87.89 % of the total variance and the rest of the components only accounted for about 12.11% (Fig.3.43).

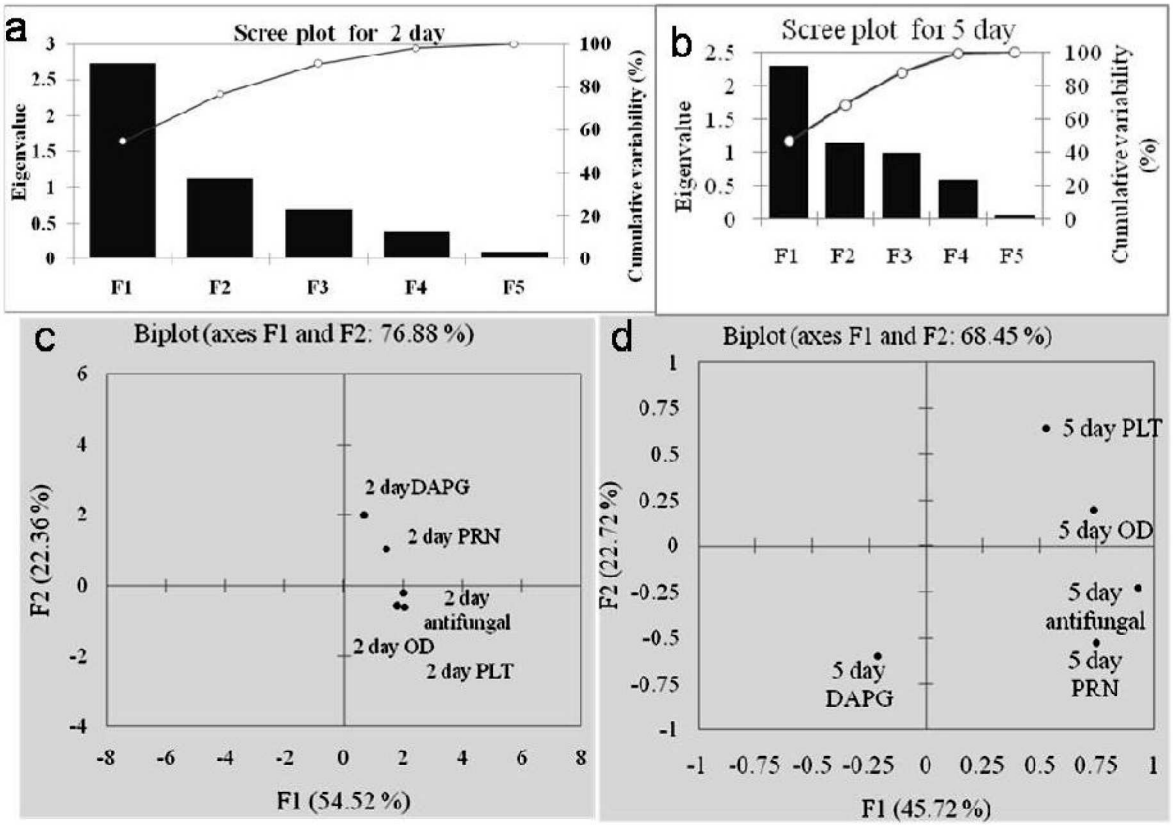


Fig.3.43 Principal Component Analysis (PCA) for the effect of nutritional factors on antibiotic production and antifungal activity of *Pf* CHA0 (a, b) Scree plots for 2d and 5d, (c, d) PCA biplot for 2d and 5d.

3.3.7 Effect of biocontrol supportive nutritional factors on the antifungal activity by Isolates

3.3.7.1 Effect of biocontrol supportive nutritional factors on the antifungal activity by isolated biocontrol strains:

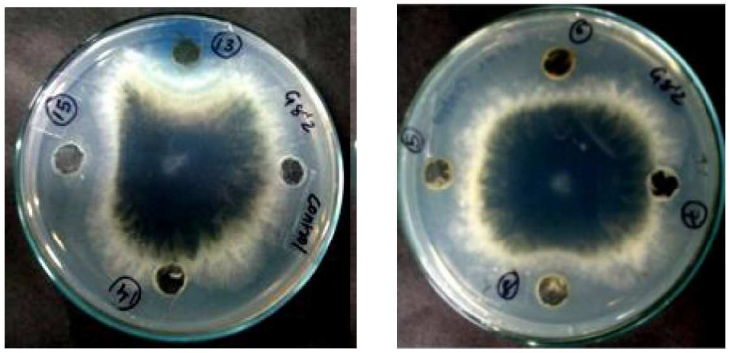
Nutrient combinations which have worked with well with *Pf* CHA0 viz. nutrient combinations number 5, 6 and 8 were selected and found that nutrient combinations number 5 and 8 did not support the antifungal activity of strains whereas nutrient combinations 6 has supported to the antifungal activity (Table 3.18).This indicated an important finding about the strain to strain differences in the nutritional requirements for demonstrating optimal antifungal activity. Strain G20 was selected for performing the experiment same as that with *Pf* CHA0 as G20 showed better antifungal activity than *Pf* CHA0 and 16 nutrient combinations experiment was carried out with it.

Table 3.18: Antifungal activity of 5th day ethyl acetate extracts of different isolates grown in media no- 6

Strain	Antifungal activity (%)		Strain	Antifungal activity (%)
G 16	40.9		G31	45.5
G 18	43.2		G35	34.1
G19	43.2		G36	40.9
G20	45.5		G38	38.6
G22	40.9		G44	43.2
G25	45.5		G 45	45.5
G26	59.1		G46	43.2
G28	38.6		CHA0	55.7
G29	43.2		G31	45.5

3.3.7.2 Effect of combination of nutritional factors on the antifungal activity by G20:

Isolate G20 has shown higher antifungal activity on 2nd day than that of 5th day by ethyl acetate extract (Figs.3.44 and 3.45). Nutrient combination number 6 has shown percentage inhibition above 50% (Table 3.18).



(Control-Methanol, refer table 3.4 for media number (5, 6,13,14,15)

Fig. 3.44 Antifungal activity by G20 extract under different nutrient compositions

Extracts from nutrient combinations number 5, 6 and 8 (table 3.4) of 2nd day have shown moderate inhibition. Iron and citrate are two factors common in all these flasks so it could be concluded that these factors probably have positive effect on the biocontrol physiology of the isolate G20.

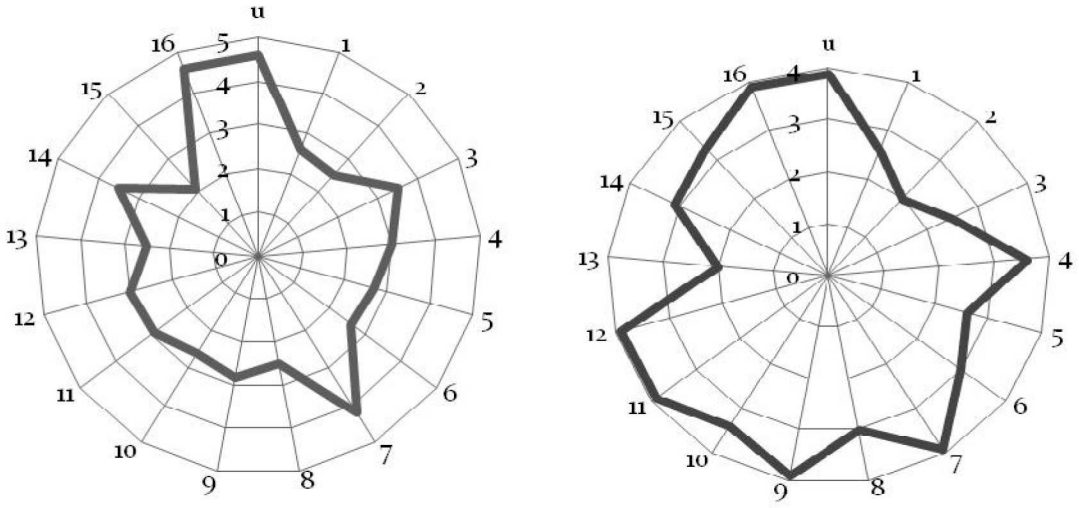


Fig.3.45 Radar graph for antifungal activity by G20 at 2 d and 5 d under different nutrient combinations

3.4 CONCLUSION

Out of total 27 ITS positive, fluorescent *Pseudomonas* strains obtained by repeated plant enrichment, 18 have shown positive amplification for pltC, 15 for phlD, 10 hcnBC positive and 5 prnC positive. Strains 45, G46 and G14 have shown the presence of all antibiotic synthesis genes similar to standard strain *Pf* CHA0. Strains H4, H9, G5 and G22 are similar to standard strains Q287 and Pf-5 in being positive for *phlD, pltC* and *hcnBC* genes. Out of total 61 isolates, 27 were showing antagonistic activity against fungus, out of which only 17 were found to be 2,4-DAPG producers. Among these, some isolates whose antifungal activity was very good but their 2, 4- DAPG production was less for example, isolate G4 and C5. So it can be concluded that the antifungal activity of these isolates may be due to other metabolites like phenazines, pyoluteorin etc. But we also obtained some isolates whose 2, 4- DAPG production was better than *pf* CHA0.

Many nutritional/environmental factors are known to influence the 2,4- DAPG production out of which the effects of two major factors were checked viz. C source and P availability, and their influence on the 2,4- DAPG production of isolates was monitored. The 2, 4- DAPG production of model strain *Pf* CHA0 decreased with increase in inorganic phosphate concentrations. Isolate G8 also showed decrease in 2, 4- DAPG production with increase in phosphate levels, but, the concentration of 2, 4- DAPG produced at each phosphate level was more than that of *Pf* CHA0. Isolate G1 did not show prominent decline in 2, 4- DAPG production in presence of high phosphate. Isolate G8 showed very good 2, 4- DAPG production in presence of sucrose, while 2, 4- DAPG production of *Pf* CHA0 is very less in presence of sucrose. Isolate G2 showed 2, 4-DAPG production pattern similar to *Pf* CHA0 in presence of various carbon sources and better than *Pf* CHA0 in presence of arabinose. Thus isolates G1, G2, G8 can be considered as better 2, 4- DAPG producers than *Pf* CHA0 when influence of carbon sources and phosphate levels are taken into considerations. Production of PRN was studied in *Pf* CHA0 and other isolates. By TLC and antifungal plate assay, production was found in *Pf* CHA0 and G25 as spot was detected at reported Rf value (0.82).

Effect of nutritional factors, like C source and other minerals, on biocontrol physiology of *Pf* CHA0 was studied by statististical approach using Fractional Factorial Design experiment. By software analysis, citrate and nitrate were found to have positive effect on biocontrol trait. Citrate, glucose and nitrate in combination showed positive effect on antifungal activity, whereas, molybdate has shown significantly negative effect. Further combinations of nutritional

factors which have been proved to be good for biocontrol physiology of *Pf* CHA0 viz. nutrient combinations number 5, 6 and 8 were used for checking performance of the isolates. Nutrient combinations number 5 and 8 did not contribute much to the antifungal activity of the while nutrient combinations number 6 showed moderate effect on antifungal activity of the fluorescent pseudomonad isolates locally obtained. Isolate G20 which has shown best antifungal activity was selected for the study of effect of nutritional factors using similar statistical approach as used for *Pf* CHA0. Antifungal activity of 2^{nd} day extract was higher than 5^{th} day extract. Nutrient combinations number 15 had shown positive effect on antifungal activity till 2^{nd} day.

Present study based on the several line of invitro studies, provides a roadmap for the factors and combinations of factors that can be manipulated to improve bacterial inoculants and further it effective combinations should be checked by invivo study at lab scale and field study.These results also expand the application prospect of the biocontrol strains based on the rhizosphere and soil type for better bio-control potential.

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