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Detection of Antibiotic Compounds from Liquid Formulation of *Pseudomonas fluorescens* (Pf1) by TLC and HPLC Analysis

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Antibiotics are generally considered as low molecular weight organic compounds produced by microbes and play an active role in the bio-control of plant diseases. Fluorescent pseudomonad bacterial strains are known to suppress fungal growth under *in vitro* as well *in vivo* by the production of one or more antifungal antibiotics. Production of antibiotic compounds by different age old liquid formulations of *P fluorescens* (Pf1) was assayed by Thin Layer Chromatography (TLC). The presence of the antimicrobial compounds *viz*., Phenazine 1-carboxylic acid (PCA), Pyoluteorin (PLT), Oxychlororaphine, Pyoluteorin (PLT), Pyocyanine were detected with different Rf values. 180 days old Pf1 liquid formulation was verified for the production of diacetylphloroglucinol (DAPG) by using Analytical High Performance Liquid chromatography (HPLC). The result of HPLC analysis revealed that DAPG from Pf1 was recorded the retention time of 4.1 min at 270 nm. Similarly same result was obtained from the DAPG standard with the retention time of 4.5 min. Hence the present study, indicate that different days old formulation of Pf1 up to180 days showed positive result for the production of all these antibiotics. It provides the evidence to Pf1 liquid formulation might act against the plant disease through the mechanism of antibiosis when applied to cropping system. Therefore, antibiotics improve the ecological fitness of these bacteria in the liquid culture which can further influence long-term biocontrol efficacy and supports the use liquid based biocontrol agents for sustainable production of agricultural crops.

Key words: Liquid formulation, DAPG, Crude antibiotics, Rf values, *Pseudomonas fluorescens.*

Fluorescent pseudomonad bacterial strains are known to suppress fungal growth *in vitro* by the production of one or more antifungal antibiotics that may also have activity *in vivo*. Root associated fluorescent pseudomonads produce and excretes secondary metabolites which are inhibitory to plant pathogenic organisms including fungi, bacteria and nematodes[1]. Several strains of *Pseudomonas* spp. have been shown to produce wide array of antibiotics which includes 2, 4-diacetyl phloroglucinol, hydrogen cyanide, kanosamine, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, pyocyanin and viscosinamide as well as several other uncharacterized moieties[2]. Phloroglucinols, phenazines, pyoluteorin, pyrrolnitrin, cyclic lipopeptides all of which are diffusible and hydrogen cyanide is volatile in nature[3]. Among these metabolites the polyketide compound DAPG has received particular attention because of its broad-spectrum antifungal, antibacterial and antihelminthic activity[4]. DAPG is synthesized by condensation of three molecules of acetyl coenzyme A with one molecule of malonyl coenzyme A to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate DAPG by a biosynthetic route utilizing a chalcone synthase

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(CHS) type enzyme[5]. Numerous studies have demonstrated the determinative role of DAPG production in the suppression of a variety of soil borne diseases by fluorescent pseudomonads[1].

DAPG producing pseudomonads are commonly found in the rhizosphere of important crops such as rice, wheat, maize, pea, tobacco, tomato, cucumber and they have been shown to be an important biological component of the natural suppressiveness of certain agricultural soils. Several studies have demonstrated that *Pseudomonas* strains with the ability to produce the antifungal metabolite DAPG can be isolated at high frequencies from suppressive soils[6]. The development of sensitive methods for the *in situ* detection of DAPG has strengthened the link between DAPG producing pseudomonads and suppressive soil. Given the ecological importance of DAPG production, biocontrol efficacy of *Pseudomonas* has been irrefutably linked to this antimicrobial metabolite[7,8]. Strains of *Pseudomonas fluorescens* that produce the antibiotic 2,4 diacetylphloroglucinol (2,4-DAPG) are responsible for the natural suppression of take all diseases of wheat (*Gaeumannomyces graminis* var. *tritici*), known as take-all decline (TAD)[9].

Phenazines are nitrogen containing heterocyclic pigments synthesized by *Brevibacterium*, *Burkholderia*, *Pseudomonas* and *Streptomyces*[10]. Currently, over 50 naturally occurring phenazine compounds have been described. Growth conditions determine the number and type of phenazine synthesized by an individual bacterial strain. *P. fluorescens* 2-79 produces mainly phenazine 1-carboxylic acid (PCA) whereas *P. aureofaciens* 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines. The major PHZ synthesized by *P. aeruginosa* is pyocyanin. Almost all PHZ exhibit broad-spectrum activity against bacteria and fungi[11]. It has been shown that bacterization of wheat seeds by *P. fluorescens* strains 30-84 and 2-79 provides primary protection against *G. graminis tritici* on account of release of PHZ. In addition to inhibiting fungal pathogens, PHZ play an important role in microbial competition in rhizosphere including survival and competence[12]. Use of mutants of strains 30- 84 and 2-79 has confirmed their long-term survival in wheat rhizosphere on account of their ability to produce PHZ[13]. Structural and functional analysis was performed for the synthesis of PCA[14].

Pyoluteorin (PLT) is an aromatic polyketide antibiotics consisting of a resorcinol ring derived through polyketide biosynthesis. PLT is produced by several *Pseudomonas* sp. that suppress plant diseases caused by phytopathogenic fungi[15]. PLT mainly inhibits the oomycetous fungi including *Pythium ultimum* and it is strongly active, when applied to seeds, PLT-producing pseudomonads decrease the severity of *Pythium* damping-off[16]. Biosynthesis of PLT is initiated from proline or a related molecule, which condenses serially with three acetate equivalents coupled to chlorination and oxidation at unidentified stages[14].The main aim of this experiment was to evaluate whether *P. fluorescens* (Pf1) could produce antibiotics under liquid conditions.

MATERIALS AND METHODS

Pf1-Liquid formulation

Pseudomonas fluorescens strain Pf1 was obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, India and used for this study. Liquid based Pf1 formulation was prepared in nutrient broth amended with glycerol as per the procedure described by Manikandan et al[17]and maintained long term for further analysis.

Detection of antibiotics from liquid formulation by TLC

Crude antibiotics

For the detection of crude antibiotics, different days of liquid formulation were extracted with ethyl acetate and the dried sample was dissolved with 1.5 ml methanol. Sample of 5µl was spotted onto silica gel plate (Merck, Silica gel 60 $F_{254,}$ Germany). The plates were developed with Isopropanol: Ammonia: Water (8:1:1) and visualized by short wave length (254 nm). For specific antibiotics the Rf values was calculated.

2, 4-diacetyl phloroglucinol (2, 4-DAPG)

Different age old cultures of *P. fluorescens*, Pf1 were grown separately in 20ml of pigment production broth (peptone, 20 g; glycerol, 20 ml**;** NaCl, 5 g; KNO_3, 1 g; distilled water, 1 l; pH 7.2) for four days on a rotary shaker at 30°C. The fermentation broth was centrifuged at 3500 rpm for five minutes in a tabletop centrifuge and the

supernatant was collected. It was acidified to pH 2.0 with 1N HCl and then extracted with an equal volume of ethyl acetate. The ethyl acetate extract was reduced to dryness *invacuo*. The residues were dissolved in methanol and kept at 4°C until use for TLC.

For the detection of an antibiotic, 2, 4-DAPG, a volume of 5 µl of sample was spotted on to the aluminium coated sheets with silica gel. Separation was performed with acetonitrile/ methanol/water (1:1:1) as a solvent system and visualized by short wave length (245 nm) and sprayed with diazotized sulphanilic acid. *Rf* value for the spot confirming 2,4-DAPG was calculated and compared with migration of synthetic 2,4-diacetyl phloroglucinol and for identical color[18].

Phenazine (Phenazine 1-carboxylic acid)

For Phenazine the extraction was done by acidifying the cultures with an equal volume of benzene (Phenazine in the benzene layer) and then extraction of the benzene phase with 5% $NaHCO_3$. PCA was recovered from the bicarbonate layer. The bicarbonate fraction was extracted once with benzene to recover the phenazine. The pigment was air dried, dissolved in methanol and purified by TLC on silica gel with a 250-µm layer thickness. The solvent system containing isopropanol/ ammonia/ water (8:1:1). Plates were viewed under UV light at 254nm and sprayed with diazotized sulfanilic acid (DSA).

Pyoluteorin

The cultures from different age old *P. fluorescens*, Pf1 were grown on King's B broth at 27ºC for 3 days were centrifuged at 14,000 g for 20 min at 4°C and 20 ml of the supernatants were extracted with an equal volume of ethyl acetate for 2 h by using a rotary shaker. The ethyl acetate extracts were dried in a vacuum at 35°C and were dissolved in 1.5 ml of 65% methanol. A volume of 5 µl was applied to aluminum coated sheets with silica gel . Separation was performed with the solvent solution system containing chloroform-acetone (9:1 v/v). The TLC plates were sprayed with dinitro salicylic acid.

Pyocyanine

Cultures of different age old Pf1 were grown in 5 ml of PPB and then incubated on a rotary shaker at 30ºC for 4 days. The extraction was done using above procedure. The benzene phase was removed and the pyocyanine which remained in the aqueous fraction was extracted with an equal volume of chloroform. The pigment was air dried, dissolved in methanol and remaining procedure was same as like oxycholroraphne.

Oxychlororaphine

The extraction was similar as in the case of Phenazine. Here bicarbonate layer was removed. The oxychlororaphine fraction was recovered from the benzene layer. The pigment was air dried and dissolved in methanol. The pigment was spotted on TLC on silica gel with a 250-µm layer thickness. Spots were detected in the solvent system containing ethyl acetate/chloroform (9:1). Plates were viewed under UV light at 254nm and sprayed with diazotized sulfanilic acid (DSA).

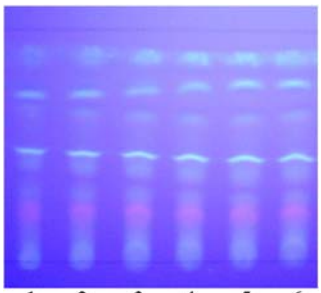
Detection of an antibiotic DAPG by HPLC

First, 180 days old culture of *P. fluorescens*, Pf1 was inoculated into 50 ml of pigment production broth and incubated for 24 h at 27°C. The bacterial culture was centrifuged at 14,000 g for 20 min at 4°C and the supernatant was extracted with an equal volume of ethyl acetate for 2 h by using a rotary shaker. The ethyl acetate extract was dried in vacuum at 35°C and were dissolved in 1.5 ml of 65% methanol. Aliquots were filtered (pore size, 0.2 mm; Millipore; Sigma) and analyzed with a shimadzu LC – 20 AT model HPLC equipped with SPD – M20A prominence Diode array detector (DAD) fitted with RP-18e Chromolith column. It was thermostatically controlled at 40°C. The column was washed for 10 min with a pure methanol before each analysis. The flow rate was 0.5ml/min. DAPG was detected at a wavelength of 270 nm and their retention time was identified**.**

RESULTS AND DISCUSSIONS

The antibiotics such as crude antibiotics, 2,4-DAPG, phenazine, pyoluteorin, oxychlororaphine, and pyocyanine production by different age old cultures of Pf1 was tested. In the crude antibiotics assay, Pf1 culture significantly produced the various antibiotics in different storage periods (Fig. 1).

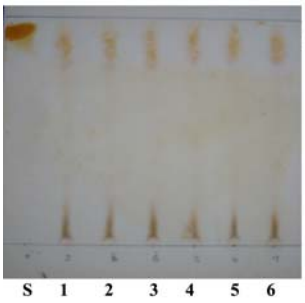
The presence of DAPG was detected by spraying dinitrosalicylicacid (DNS) on the TLC plate with an R_f value of 0.88 which was coincided with standards (Fig. 2 a). For phenazine and oxychlororaphine, the TLC plates after



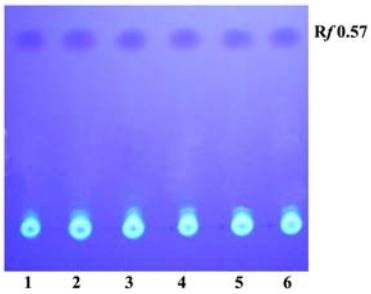
1	2nd day	4	90th day
2	30th day	5	180th day
3	60th day	6	Talc formulation

Fig 1. Detection of Crude antibiotics production by Pf1 liquid formulation at different days intervals

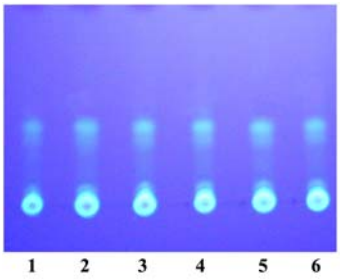
development with DNS showed distinct spots of bright orange colour with R_f value of 0.57. Pyoluteorin was detected for the different age old cultures of Pf1 with R_f value 0.05. Pyocyanine was detected with the R_f value of 0.53 for the different age old cultures of Pf1 (Fig. 2 b-e). HPLC analysis of DAPG from Pf1 at 260 nm recorded the retention time of 4.56 min for the standard DAPG and 4.45 min for DAPG from Pf1 of liquid formulation (Fig. 3). The results of current study showed that, primary mechanism of biocontrol by fluorescent pseudomonads involves production of antibiotics such as 2, 4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyoluteorin (PLT), oxychlororaphine and pyocyanin.



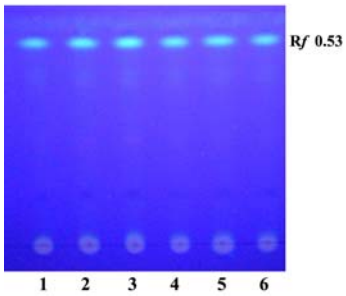
a). 2, 4-diacetyl phloroglucinol (2, 4-DAPG)



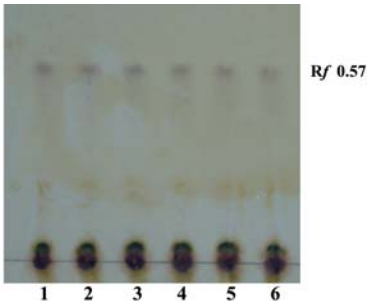
b). Phenazine



C). Pyoluterin

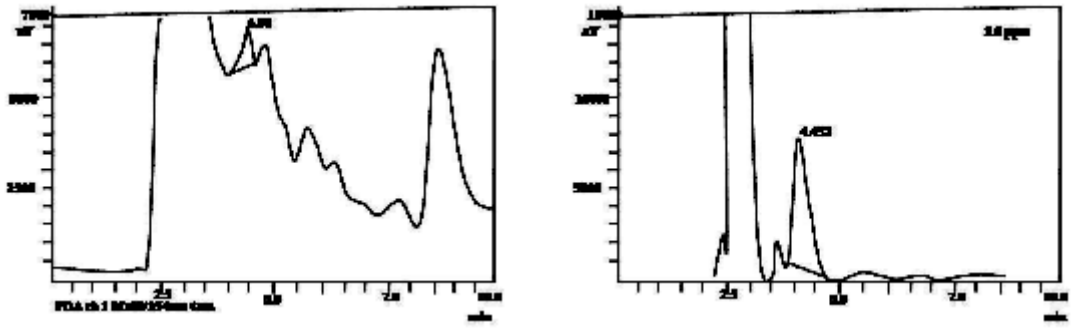


D). Oxychloraphine



E). Pyocyanine

Fig 2. Detection of different antibiotics from Pf1 liquid at different days old formulations



i). Pf1 sample

ii). DAPG standard

Peak Report

Name	Retention time
Pf1 - DAPG	4.66
Standard DAPG @ 1ppm	4.44
Standard DAPG @ 2ppm	4.55
Standard DAPG @ 5ppm	4.50

Fig 3. HPLC chromatogram of *Pseudomonas fluorescens* (Pf1) for the production of antibiotic DAPG

The results of this study has been supported with the following reports such as[19], *P. fluorescens* produced antibiotics to protect plants against a range of soil-borne fungal pathogens is well documented. The mechanisms of action of phenazines are assumed that they diffuse across or insert into the membrane and act as reducing agent, resulting in the uncoupling of oxidative phosphorylation and the generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to the organisms. Phenazine plays a vital role in the management of soil-borne pathogens. However, Thomashow et al[20] have obtained evidence that bacterial phenazine antibiotic production contributes to the longterm survival of *P. fluorescens* strain 2-79 and 30-84 in soil habitats. The compound 2, 4-DAPG, which is produced by certain plant-associated fluorescent *Pseudomonas* species of worldwide origin, is of particular significance to agriculture because of its activity *in situ* against a variety of root and seedling pathogens. Furthermore, DAPG acts as a signaling compound inducing the expression of its own biosynthetic genes[15].

Under certain conditions, antibiotics improve the ecological fitness of these bacteria in the rhizosphere which can further influence longterm biocontrol efficacy[21]. PLT was involved in protection of cress from damping-off by CHA0, but did not contribute to the strain's ability to suppress damping-off of cucumber. *Pseudomonas* strain CHA0 produces several antifungal metabolites in addition to DAPG, noticeably siderophores, HCN and PLT[4]. DAPG is involved in disease suppression by *P. fluorescens* strain CHA0. Mutants that were defective for the production of phenazine, PLT, PRN and HCN showed less activity in biological control[3].

In the present study, different days old cultures of Pf1 up to180 days showed positive result to all these antibiotics *viz.*, 2, 4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyoluteorin (PLT), oxychlororaphine and pyocyanin. The presence of certain antibiotics in liquid formulation might have an inhibitory effect against plant pathogens. Therefore by analyzing the antibiotic compounds, added advantage to liquid formulation of Pf1 for successful usage in disease management practices.

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