



## Characterization of secondary metabolites produced during interaction of *Pseudomonas fluorescens* with *Fusarium oxysporum*

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### ABSTRACT

Characterization of the Secondary Metabolites (SM) produced by *Pseudomonas fluorescens* (NAIMCC-B-00361) while growing in mixed culture conditions with fungal pathogen *Fusarium oxysporum* (NAIMCC-F-00811) was done to better mimic antagonism and interaction in a natural environment. Nutrient media, viz. King's B Broth (KBB), Potato Dextrose Broth (PDB), Pigment Production Medium (PPM), Czapek Dox broth Medium (CDB) and half minimal media (50% KBB and 50% PDB) were assessed and KBB was found to be the best medium for the production of these metabolites. Antifungal assay of crude metabolite extract was done using poison food technique and the results showed that the crude chloroform extract of metabolites (mixed culture of *P. fluorescens* and *F. oxysporum*) grown in KBB medium showed 50.47% inhibition of mycelial growth of *F. oxysporum* followed by the chloroform extract of solo culture of *P. fluorescens* which showed 45.38% inhibition of mycelial growth of *Bipolaris oryzae*. Antibacterial assay of crude metabolite extract was done using Agar well diffusion technique and the results revealed that the crude chloroform extract of mixed culture of *P. fluorescens* and *F. oxysporum* grown in KBB medium showed 25.67 mm zone of inhibition against *Bacillus subtilis*, whereas the extract of solo culture of *P. fluorescens* showed 18.67 mm zone of inhibition against *Klebsella pneumoniae*. The qualitative confirmation of antibiotic production by solo culture of *P. fluorescens* and mixed culture using TLC revealed the presence of antibiotics, i.e. 2, 4-DAPG, pyrrolnitrin and phenazine. HPLC analysis of crude chloroform extract of SM produced by solo culture of *P. fluorescens* and mixed culture showed the presence of 2,4-DAPG at retention time 20.707 and 20.698 respectively.

**Key words:** Secondary metabolites, *Pseudomonas fluorescens*, HPLC, TLC, 2,4-DAPG

The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPR) and primarily *Pseudomonas fluorescens* is identified as an important PGPR with ability for plant growth promotion and effective plant disease management properties (Mazzola *et al.* 1992). Their applicability has drawn wide attention because of the production of secondary metabolites such as siderophores, antibiotics, volatile compounds, hydrogen cyanide (HCN) and phytohormones (Kumar *et al.* 2004). Biocontrol mechanism to suppress fungal pathogens by *Pseudomonas* spp. normally involves the production of antibiotics and *P. fluorescens* has a gene cluster that produces a suite of antibiotics including compounds such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine, pyrrolnitrin (PRN), pyoluteorin (PLT) and also biosurfactant antibiotics (Sharma *et al.* 2016, Angayarkanni *et al.*

2005). Fluorescent *Pseudomonas* is uniquely capable of synthesizing many of these antibiotics, not only to enhance its own fitness but also to help in the maintenance of soil health and biological protection of crops from pathogens (Gaur *et al.* 2004).

Interspecies interactions involve the action of multiple genetic and metabolic pathways which can result in mutualistic or antagonistic bacterial effects. The molecular basis of some ecological interactions have been linked to secondary metabolites (SM) which are organic, biosynthesized compounds such as antibiotics and toxins and are not essential for basic growth and reproduction in organisms. They are reported to have possible role in defense, chemical signaling and host-microbe interactions (Schirmer *et al.* 2005). Many unique and biologically active SMs continue to increase the interest of academic and industrial researchers. However, pure cultures of microbes often fail to yield reliable or consistent biosynthesis of SMs (Muscholl-Silberhorn *et al.* 2008). Study of the root-associated bacteria and their antagonistic potential is important not only for understanding their ecological role in the rhizosphere and their interaction with plants but also for multifarious biotechnological applications. Keeping in view, the importance of *Pseudomonas fluorescens*, the

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present investigation was based on characterization of the Secondary Metabolites (SM) produced by *P. fluorescens* while they grow in mixed culture conditions with *F. oxysporum* to better mimic antagonism and interaction in a natural environment.

## MATERIALS AND METHODS

**Microbial cultures:** Cultures of *Pseudomonas fluorescens* (NAIMCC-B-00361) and *Fusarium oxysporum* (NAIMCC-F-00811) were procured from National Agriculturally Important Microorganisms Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan, UP.

**Extraction of secondary metabolites from *P. fluorescens*:** The effective isolate of *P. fluorescens* was grown in King's B media. The media for secondary metabolite production, viz. King's B Broth (KBB), Potato Dextrose Broth (PDB), Pigment Production Medium (PPM), Czapek Dox broth Medium (CDB) and half minimal media (50% KBB and 50% PDB) were prepared separately and inoculated with pre-grown inoculum of *P. fluorescens* on King's B media and kept in incubator shaker at 28°C and 120 rpm for 2 weeks.

**Extraction of secondary metabolites from co-culture of *P. fluorescens* and *F. oxysporum*:** Nutrient media, viz. King's B Broth (KBB), Potato Dextrose Broth (PDB), Pigment Production Medium, Czapek Dox Medium and Half Minimal Media (50% KBB and 50% PDB) were inoculated with pre-grown inoculum of *P. fluorescens* and 7 day old mycelium of *F. oxysporum*. The inoculated flasks were kept in incubator shaker under fixed parameters of temperature 28°C and rpm-120 for 2 weeks.

**Extraction of crude secondary metabolites with organic solvent:** Crude metabolites were extracted from solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* grown on different media, i.e. KBB, PDB, Half Minimal Media (50% KBB and 50% PDB), PPM and CDB by partitioning with chloroform in the ratio of 1:1. Cell free supernatant was extracted with equal volume of chloroform and was shaken vigorously for 30 min and the procedure was repeated thrice. Organic phase thus obtained was pooled and reduced under vacuum pressure by rotary flash evaporator at 40°C and the crude extract obtained was evaluated for antimicrobial activity.

**Antimicrobial activity of secondary metabolites:** Crude secondary metabolites extracted from solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* were evaluated for antimicrobial activity using poison food technique and agar well diffusion assay.

**Antifungal activity of crude extracted metabolites:** Test pathogenic fungi, viz. *Fusarium oxysporum*, *Rhizoctonia solani*, *Alternaria alternata* and *Bipolaris oryzae* were procured from Division of Plant Pathology, SKUAST-J. Crude secondary metabolite extract of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* were tested for their efficacy against pathogens by poison food technique (Nene and Thapliyal 1971). 20 µl of extracted

crude metabolite (5 mg/ml) was poured on PDA medium before plating. After solidification of medium, 4 mm disc of fungal pathogenic culture was inoculated at the center of each plate and incubated at 28°C. Radial growth of the test fungi was measured after incubation. PDA plates without metabolite served as control. Percentage inhibition radial growth was calculated by using the formula as given below:

$$\text{PIRG} = (R_1 - R_2) / R_1 \times 100$$

where PIRG, Percentage inhibition of radial growth;  $R_1$ , Radial growth of fungal pathogen in absence of secondary metabolites;  $R_2$ , Radial growth of fungal pathogen in presence of secondary metabolites.

**Antibacterial activity of crude extracted metabolites:** Test bacteria *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Micrococcus luteus* were procured from Division of Plant Pathology, SKUAST-J and maintained by routine sub-culturing on Nutrient Agar. Secondary metabolites extracted from *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* were tested for their efficacy against bacterial pathogens by Agar well diffusion method (Valgas *et al.* 2007). The prepared culture plates were inoculated with different test bacteria using plate method. Wells were made on the agar surface with 6mm cork borer. 20 µl of the crude secondary metabolites of concentration 5 mg/ml extracted from culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* were poured into the well using micropipettes. The plates were incubated at 37±2°C for 24 hours for bacterial activity. The plates were observed for the zone of inhibition around the wells. Chloramphenicol (1 mg/ml) served as positive control. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter.

**Identification of metabolites through TLC:** An applicator was used to uniformly spread slurry of silica gel G (25 g of silica gel G powder with 50 ml of water) to a thickness of 250µm on a glass plate (20×20 cm and 0.5cm thickness). The plates were air dried to allow the binder to set. The plates were then activated at 120°C for 1 h just before use. 20 µl samples were applied to thin layer chromatography plates coated with a 250 µm layer of silica gel and developed in chloroform: acetic acid (95:5) solvent system. Spots were visualized under UV light at 254nm. The  $R_f$  value of the band was calculated using the standards formula ( $R_f = \text{Distance traveled by the substance (cm)} / \text{Distance traveled by the mobile phase (cm)}$ ).

**HPLC analysis of secondary metabolites:** Reverse-phase HPLC method was used for determination of secondary metabolites using method of Brodhagen *et al.* (2004) with slight modifications. Each crude sample was dissolved in HPLC grade DMSO (1 ml) before analysis. RP-HPLC (SHIMADZU Corporation) was used to determine the retention times of the compounds present in the chloroform extracts of secondary metabolites from solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum*. The HPLC diode array detector was

programmed to observe and record absorbance at 254 nm. The samples were injected (20 µl) into the HPLC equipped with a C18 reverse phase column (5 µm; 4.6×150 mm) and eluted at 1 ml/min. The initial eluent, 80% acetonitrile/ water (v/v, both acidified with 0.1% acetic acid), was run for 2 min. followed by a linear gradient to 80% acetonitrile (acidified with 0.1% acetic acid) over 32 minute period. The HPLC mobile-phase consisted of Acetonitrile: Water (80:20) at a flow rate of 0.8 ml/min and detected at a wavelength of 254 nm. Resolutions results may vary from column to column.

## RESULTS AND DISCUSSION

**Extraction of secondary metabolites:** Procedures for extracting most of the secondary metabolites are well documented and they are known to produce at higher levels during growth and stationary phases (Roze *et al.* 2011). Reported scientific literature perceives fluorescent *Pseudomonas* to secrete secondary metabolites with high biological activities (Hu *et al.* 2005, Ayyadurai *et al.* 2006). In our results, *P. fluorescens* was found to produce Secondary Metabolites (SM) in different nutrient media, viz. King's B Broth (KBB), Potato Dextrose Broth (PDB), Pigment Production Medium, Czapek Dox Medium and Half Minimal Media (50% KBB and 50% PDB) and King's B broth was found to be the best medium for production of secondary metabolites. Reddy *et al.* (2009) found King's B medium to yield maximum amount of secondary metabolites.

Isolation of already reported compounds empirically becomes more advantageous with choice of extraction protocols. Liquid-liquid extraction processes are widely used with different solvents among which ethyl acetate and chloroform are the most popular solvents (Al-Dhabi *et al.* 2016). In the present investigation, chloroform was found to be the best solvent as most of the secondary metabolites are miscible with it.

**Antifungal activity of crude extracted metabolites:** The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in KBB medium exhibited varying mycelial percent inhibitions of mycelial growth against different test fungi {*Fusarium oxysporum*

(50.47%), *Rhizoctonia solani* (44.99%), *Alternaria alternata* (39.50%) and *Bipolaris oryzae* (46.40%)} while the chloroform extract of solo culture of *P. fluorescens* showed mycelial inhibition of 40.97% against *Fusarium oxysporum*, 32.43% against *A. alternata*, 38.75% *Rhizoctonia solani* and 45.38% against *Bipolaris oryzae*. The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in half minimal media (50% KBB and 50% PDB) exhibited mycelial inhibition of 30.39% against *F. oxysporum*, 33.27% against *A. alternata*, 26.02% *Rhizoctonia solani* and 30.88% against *Bipolaris oryzae*, whereas the chloroform extract of solo culture of *P. fluorescens* showed mycelial inhibition of 27.43% against *F. oxysporum*, 29.84% against *A. alternata*, 25.18% *Rhizoctonia solani* and 25.23% against *Bipolaris oryzae*. However, the crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in PDB medium exhibited mycelial inhibition of 23.79% against *F. oxysporum*, 14.63% against *A. alternata*, 26.14% *Rhizoctonia solani* and 18.86% against *Bipolaris oryzae* whereas the chloroform extract of solo culture of *P. fluorescens* showed mycelial inhibition of 16.80% against *F. oxysporum*, 11.40% against *A. alternata*, 18.38% *Rhizoctonia solani* and 12.97% against *Bipolaris oryzae* (Table 1).

The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in PPM medium exhibited varying mycelial percent inhibitions against different test fungi {*Fusarium oxysporum* (22.69%), *Rhizoctonia solani* (15.85%), *Alternaria alternata* (18.81%) and *Bipolaris oryzae* (26.80%)} of mycelial growth of *F. oxysporum* while the chloroform extract of solo culture of *P. fluorescens* showed mycelial inhibition of 16.89% against *Fusarium oxysporum*, 15.01% against *A. alternata*, 19.78% *Rhizoctonia solani* and 14.44% against *Bipolaris oryzae*. The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in CDB medium exhibited mycelial inhibition of 20.75% against *F. oxysporum*, 15.04% against *A. alternata*, 15.42% *Rhizoctonia solani* and 15.33% against *Bipolaris oryzae* whereas the chloroform extract of solo culture of *P. fluorescens* showed

Table 1. Antifungal activity of crude chloroform extract of secondary metabolites of solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum*

| Crude Secondary Metabolites Extract (5 mg/ml) | Nutrient media | Percent inhibition of mycelial growth |                             |                           |                         |
|-----------------------------------------------|----------------|---------------------------------------|-----------------------------|---------------------------|-------------------------|
|                                               |                | <i>Fusarium oxysporum</i>             | <i>Alternaria alternata</i> | <i>Rhizoctonia solani</i> | <i>Bipolaris oryzae</i> |
| <i>P. fluorescens</i>                         | KBB            | 40.97                                 | 32.43                       | 38.75                     | 45.38                   |
|                                               | PDB            | 16.80                                 | 11.40                       | 18.38                     | 12.97                   |
|                                               | KBB+PDB        | 27.43                                 | 29.84                       | 25.18                     | 25.23                   |
|                                               | PPM            | 16.89                                 | 15.01                       | 19.78                     | 14.44                   |
|                                               | CDB            | 16.58                                 | 11.78                       | 17.76                     | 14.84                   |
| <i>P. fluorescens</i> and <i>F. oxysporum</i> | KBB            | 50.47                                 | 39.50                       | 44.99                     | 46.40                   |
|                                               | PDB            | 23.79                                 | 14.63                       | 26.14                     | 18.86                   |
|                                               | KBB+PDB        | 30.39                                 | 33.27                       | 26.02                     | 30.88                   |
|                                               | PPM            | 22.69                                 | 18.81                       | 15.85                     | 26.80                   |
|                                               | CDB            | 20.75                                 | 15.04                       | 15.42                     | 15.33                   |

mycelial inhibition of 16.58% against *F. oxysporum*, 11.78% against *A. alternata*, 17.76% *Rhizoctonia solani* and 14.84% against *Bipolaris oryzae* (Table 1). The possible high antifungal and antibacterial activity of chloroform extract may be attributed to polar nature of the solvent in which maximum number of secondary metabolites are extracted which may be causing synergistic effect. Earlier, antifungal metabolites, i.e. Phenazine and 2,4 DAPG extracted from *Pseudomonas fluorescens* recorded 67.03 and 77.34 per cent inhibition of mycelial growth of *Fusarium oxysporum* f.sp. *cepae* causing basal rot of onion respectively (Malathi 2015). *P. putida* WCS 358r produced phenazine-3-carboxylic acid (PCA) and 2, 4 DAPG which has antifungal and antibacterial action (Bakker *et al.* 2002). *P. chlororaphis* isolate PA 24 and *B. subtilis* isolate CBE 4 produced 2,4 DAPG and phenazine which were inhibitory to the growth of the *P. aphanidermatum* in turmeric and to the other soil borne pathogens, viz. *M. phaseolina*, *F. oxysporum* f. sp. *cubense* and *Sclerotium rolsii* (Kavitha 2004). Phenazine and 2,4-DAPG antibiotics were inhibitory to mycelial growth of *M. phaseolina* and *R. solani* under *in vitro* condition (Kamalakkannan 2004).

**Antibacterial activity of crude extracted metabolites:** Antibacterial activity of crude chloroform extract of SM secreted during co-culture of *P. fluorescens* and *F. oxysporum* using agar well diffusion assay displayed significant activity against bacterial pathogens (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Micrococcus luteus*) at a concentration of 5 mg/ml. The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in KBB medium showed maximum zone of inhibition, i.e. 25.67 mm against *B. subtilis*, 24.67 mm against *Escherichia coli*, 22.67 mm against *Klebsiella pneumoniae*, 16.33 mm against *Staphylococcus aureus* and 21 mm against *Micrococcus luteus* while the chloroform extract of SM extracted from solo culture of *P. fluorescens* showed 12.33 mm zone of inhibition against *Bacillus subtilis*, 16.33 mm against *Escherichia coli*, 18.67 mm against *Klebsiella pneumoniae*, 11.33 mm against *Staphylococcus aureus* and 16.00 mm against *Micrococcus luteus* (Table 2).

The crude chloroform extract of SM obtained from co-culture of *P. fluorescens* and *F. oxysporum* grown in PDB medium showed zone of inhibition zone of inhibition, i.e. 12.33 mm against *B. subtilis*, 8.67 mm against *Escherichia coli*, 10.67 mm against *Klebsiella pneumoniae*, 6.67 mm against *Staphylococcus aureus* and 9.00 mm against *Micrococcus luteus*, whereas the chloroform extract of SM extracted from solo culture of *P. fluorescens* showed zone of inhibition of 6.67 mm against *Bacillus subtilis*, 5.67 mm against *Escherichia coli*, 8.00 mm against *Klebsiella pneumoniae*, 4.67 mm against *Staphylococcus aureus* and 7.33 mm against *Micrococcus luteus* (Table 2). The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in half minimal media (50% KBB and 50% PDB) media showed zone of inhibition i.e. 13.00 mm against *B. subtilis*, 6.00 mm against

*Escherichia coli*, 11.33 mm against *Klebsiella pneumoniae*, 6.00 mm against *Staphylococcus aureus* and 7.67 mm against *Micrococcus luteus* while the chloroform extract of SM extracted from solo culture of *P. fluorescens* showed zone of inhibition of 9.00 mm against *Bacillus subtilis*, 6.67 mm against *Escherichia coli*, 11.00 mm against *Klebsiella pneumoniae*, 4.33 mm against *Staphylococcus aureus* and 8.33 mm against *Micrococcus luteus* (Table 2).

The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in PPM medium showed zone of inhibition i.e. 8.67 mm against *B. subtilis*, 4.33 mm against *Escherichia coli*, 10.33 mm against *Klebsiella pneumoniae*, 4.33 mm against *Staphylococcus aureus* and 11.00 mm against *Micrococcus luteus*, whereas the chloroform extract of SM extracted from solo culture of *P. fluorescens* showed zone of inhibition of 9.67 mm against *Bacillus subtilis*, 5.33 mm against *Escherichia coli*, 8.67 mm against *Klebsiella pneumoniae*, 6.67 mm against *Staphylococcus aureus* and 5.33 mm against *Micrococcus luteus* (Table 2). The crude chloroform extract of SM extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in CDB medium showed zone of inhibition, i.e. 8.33 mm against *B. subtilis*, 8.67 mm against *Escherichia coli*, 6.67 mm against *Klebsiella pneumoniae*, 9.33 mm against *Staphylococcus aureus* and 6.67 mm against *Micrococcus luteus* while the chloroform extract of SM extracted from solo culture of *P. fluorescens* showed zone of inhibition of 4.67 mm against *Bacillus subtilis*, 5.00 mm against *Escherichia coli*, 7.00 mm against *Klebsiella pneumoniae*, 6.67 mm against *Staphylococcus aureus* and 9.00 mm against *Micrococcus luteus* (Table 2).

Earlier, the crude bacterial extracts was found to inhibit the growth of *Bacillus subtilis*, *Paracoccus paratrophus*, *Pseudomonas diminutus* and *Micrococcus luteus* with zones of inhibition of 15 mm, 25 mm, 8 mm and 3 mm respectively (Mezaache-Aichour *et al.* 2013). The endophytic *Pseudomonas aeruginosa* showed significant antibacterial activity against *Staphylococcus aureus* followed by *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhi* (Sunkar and Nachiyar 2013). The antibacterial activity of 2,4-diacetylphloroglucinol (2,4-DAPG) extracted from *Pseudomonas fluorescens* was evaluated against 23 vancomycin resistant *Staphylococcus aureus* (VRSA) strains and against vancomycin resistant *Enterococcus* spp. (Isnansetyo *et al.* 2003).

**Identification of metabolites through TLC:** Thin layer chromatography (TLC) of the crude secondary metabolites produced by *P. fluorescens* grown in KBB using chloroform as an extraction solvent showed four spots at  $R_f$  0.78, 0.73, 0.69 and 0.45. However, chloroform extract produced by co-culture of *P. fluorescens* and *F. oxysporum* showed three distinct spots at  $R_f$  0.79, 0.78 and 0.49. TLC of the chloroform extract of crude metabolites extracted from co-culture of *P. fluorescens* and *F. oxysporum* grown in PDB showed one spot at  $R_f$  0.45 whereas the crude chloroform extract of the metabolites extracted from *P. fluorescens* showed two spots at  $R_f$  0.94 and 0.64 (Table 3).

Table 2 Antibacterial activity of chloroform extract of crude secondary metabolites of solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum*

| Crude Secondary Metabolites Extract (5 mg/ml) | Nutrient media | Zone of inhibition (mm)   |                          |                         |                              |                              |                           |
|-----------------------------------------------|----------------|---------------------------|--------------------------|-------------------------|------------------------------|------------------------------|---------------------------|
|                                               |                | Chloramphenicol (1 mg/ml) | <i>Bacillus subtilis</i> | <i>Escherichia coli</i> | <i>Klebsiella pneumoniae</i> | <i>Staphylococcus aureus</i> | <i>Micrococcus luteus</i> |
| <i>P. fluorescens</i>                         | KBB            | 27                        | 12.33                    | 16.33                   | 18.67                        | 11.33                        | 16.00                     |
|                                               | PDB            | 23                        | 6.67                     | 5.67                    | 8.00                         | 4.67                         | 7.33                      |
|                                               | KBB+PDB        | 23                        | 9.00                     | 6.67                    | 11.00                        | 4.33                         | 8.33                      |
|                                               | PPM            | 16                        | 9.67                     | 5.33                    | 8.67                         | 6.67                         | 5.33                      |
|                                               | CDB            | 32                        | 4.67                     | 5.00                    | 7.00                         | 6.67                         | 4.00                      |
| <i>P. fluorescens</i> and <i>F. oxysporum</i> | KBB            | 26                        | 25.67                    | 24.67                   | 22.67                        | 16.33                        | 21.00                     |
|                                               | PDB            | 22                        | 12.33                    | 8.67                    | 10.67                        | 6.67                         | 9.00                      |
|                                               | KBB+PDB        | 24                        | 13.00                    | 6.00                    | 11.33                        | 6.00                         | 7.67                      |
|                                               | PPM            | 28                        | 8.67                     | 4.33                    | 10.33                        | 4.33                         | 11.00                     |
|                                               | CDB            | 20                        | 8.33                     | 8.67                    | 6.67                         | 9.33                         | 6.67                      |
| CD (P=0.05)                                   |                |                           | 2.51                     | 2.44                    | 2.2                          | 2.18                         | 1.98                      |

The crude chloroform extract of the metabolites extracted from co-culture of *P. fluorescens* and *F. oxysporum* using Half minimal media (50% PDB + 50% KBB) showed two different spots at  $R_f$  0.97 and 0.67, whereas the chloroform extract of solo culture of *P. fluorescens* also showed three distinct spots at  $R_f$  0.69, 0.78 and 0.87. Crude chloroform extract of the metabolites extracted from co-culture of *P. fluorescens* and *F. oxysporum* using Pigment production media (PPM) showed three different spots at  $R_f$  0.69, 0.73 and 0.77, whereas the chloroform extract produced by solo culture of *P. fluorescens* showed three distinct spots at  $R_f$  0.69, 0.78 and 0.87.

The results of conformation of antibiotic production by solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* by using thin layer chromatography revealed the presence of antibiotics, i.e. 2, 4-DAPG,

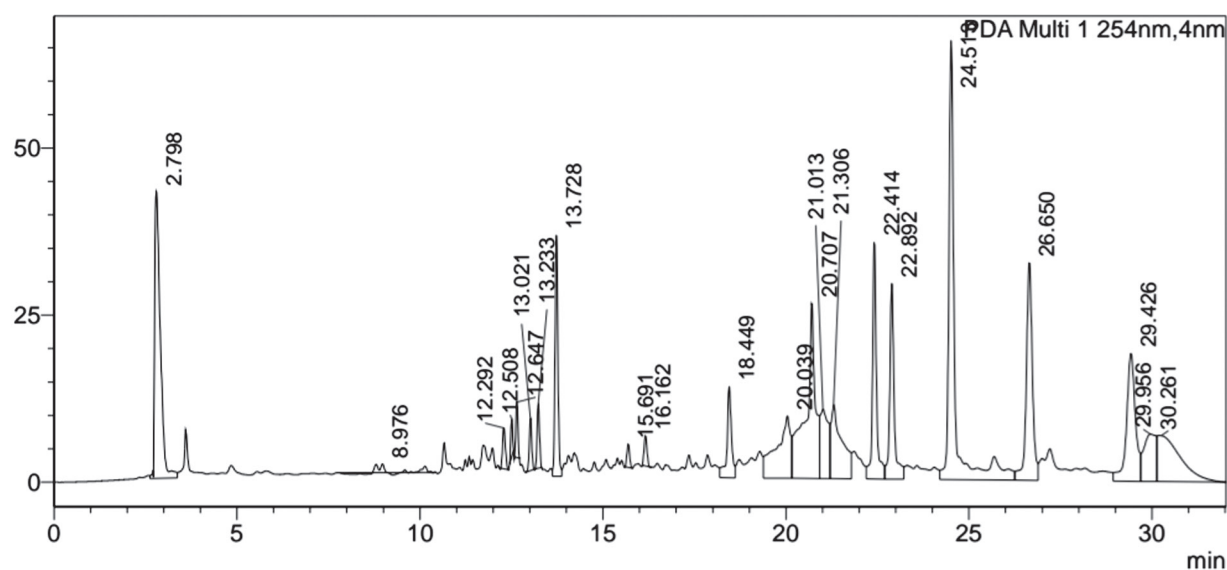
Pyrrolnitrin and Phenazine. Spots having  $R_f$  in the range of 0.81 to 0.89 which is close to the  $R_f$  value of synthetic 2,4-diacetyl phloroglucinol (0.81-0.89), spots having  $R_f$  value in the range of 0.5 to 0.80 which is close to the  $R_f$  value of synthetic phenazine (0.25, 0.47, 0.55, 0.63) and spots having  $R_f$  value in the range of 0.71 to 0.77 which is similar to the  $R_f$  value of synthetic pyrrolnitrin (0.73, 0.75). Earlier, three strains (PF1, FP7 and PB2) were reported to produce 2,4-DAPG which had the same  $R_f$  value as synthetic phloroglucinol (0.76) (Nandakumar *et al.* 2002). Distinct spots of 2,4-DAPG were reported to have  $R_f$  of 0.88 that appeared on the TLC plate after spraying diazotized sulphanilic acid (Rosales *et al.* 1995). Secondary metabolite secreted by *Pseudomonas brassicacearum* J12 with  $R_f$  value of 0.7 was characterized as 2,4-DAPG which inhibited the growth of phytopathogenic bacteria *Ralstonia solanacearum* (Zhou *et al.* 2012).

**High-Performance Liquid Chromatography (HPLC) analysis of secondary metabolites:** As the crude chloroform extract of SM of co-culture of *P. fluorescens* and *F. oxysporum* and solo culture of *P. fluorescens* showed maximum activity against the fungal and bacterial pathogens, so these extracts were further characterized by High Performance Liquid Chromatography (HPLC) and Liquid Chromatography and Mass Spectrometry (LC-MS). High Performance liquid chromatography (HPLC) analysis of crude chloroform extract of SM produced by solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum* revealed the presence of 2,4-DAPG at retention time ( $t_R$ ) 20.707 and 20.698 (Fig 1 and Fig 2). 2,4-DAPG from *Pseudomonas fluorescens* was characterized by TLC and HPLC. The DAPG extracts of soil inoculated with *P. fluorescens* and challenge inoculated with *F. oxysporum* f. sp. *cubense* eluted at retention time ranging from 20.00 min to 21.30 min in HPLC and  $R_f$  value 0.88 in TLC developed by acetonitrile : methanol : water (1:1:1) solvent system (Saravanan and Muthusamy 2006).

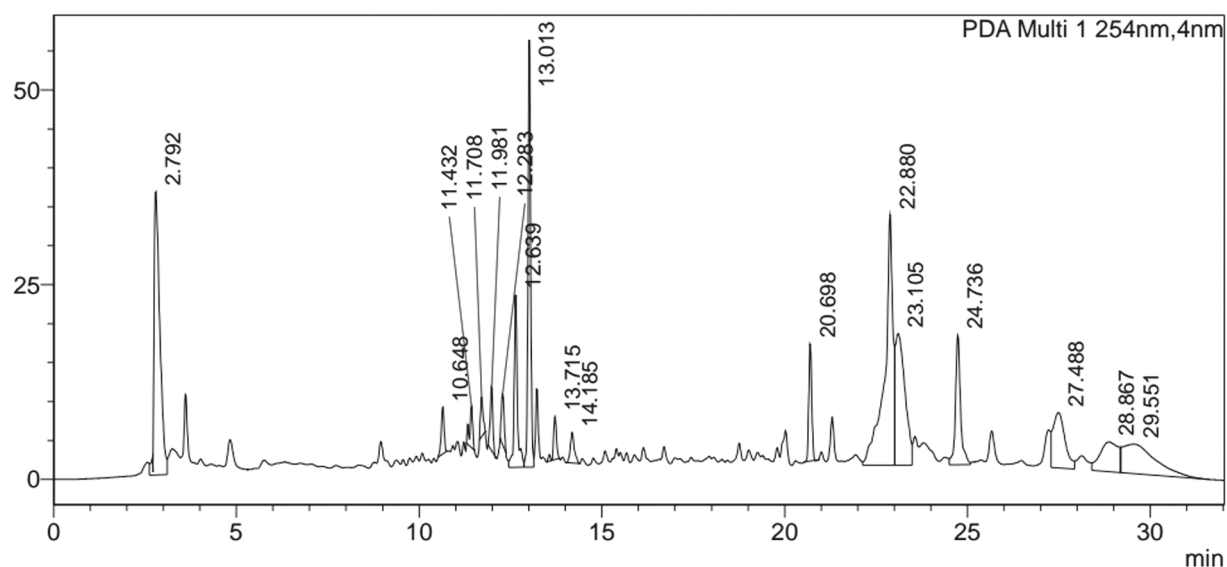
Table 3  $R_f$  values of chloroform extracts of crude secondary metabolites of solo culture of *P. fluorescens* and co-culture of *P. fluorescens* and *F. oxysporum*

| Nutrient media | <i>P. fluorescens</i> | <i>P. fluorescens</i> and <i>F. oxysporum</i> |
|----------------|-----------------------|-----------------------------------------------|
| KBB            | 0.78                  | 0.79                                          |
|                | 0.73                  | 0.78                                          |
|                | 0.69                  | 0.49                                          |
|                | 0.45                  |                                               |
| PDB            | 0.82                  | 0.45                                          |
|                | 0.45                  |                                               |
| KBB+PDB        | 0.94                  | 0.97                                          |
|                | 0.64                  | 0.67                                          |
| CDB            | 0.68                  | 0.64                                          |
|                | 0.77                  | 0.67                                          |
| PPM            | 0.69                  | 0.69                                          |
|                | 0.78                  | 0.73                                          |
|                | 0.87                  | 0.77                                          |

mAU

Fig 1 HPLC chromatogram of chloroform extract of crude secondary metabolites of *P. fluorescens*.

mAU

Fig 2 HPLC chromatogram of chloroform extract of crude secondary metabolites extracted from co-culture of *P. fluorescens* and *F. oxysporum*.

In order to tackle global problems, effective alternatives to chemical control are being investigated and the use of antagonistic microbes as biocontrol agents seems to be one of the promising approaches. With the advent of biocontrol as a potential approach to Integrated Pest Management (IPM) in the area of bacteria-mediated plant disease control, the genus *Pseudomonas* gained considerable importance. It was evident from the study that under *in vitro* conditions, in which we confronted the bacterium and fungus, lacked the complexities and dynamics of natural environments. However, the production of SM by *Pseudomonas* spp. clearly offered insight into the property of antibiosis which further helps it to survive in the natural habitats. Co-cultivation or

mixed fermentation has been recognized as a remarkable successful approach for the discovery of biologically active natural products. Antibiosis occurs during interaction with other microorganisms involving low molecular diffusible volatile as well as non-volatile toxic metabolites or antibiotics involving in suppression of pathogen growth. Finally finding specific molecules or signals that control unique secondary metabolite pathways and their genes may have wider applications for natural products research, microbial ecology and the pharmaceutical industry.

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