ISSN 0974-4169 www.ajrconline.org

RESEARCH ARTICLE

Isolation of secondary metabolites from *Pseudomonas fluorescens* and its Characterization

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ABSTRACT

Twenty *Pseudomonas fluorescens* strains were isolated from rice growing soil samples and characterized. One of the *P.fluorescens* isolated and identified from the dual culture test. It was fermented for secondary metabolite in a small scale and extracted with ethyl acetate. The isolated metabolite tested against rice fungal pathogens. The structure of the compound was elucidated by high-resolution NMR spectroscopy.

KEY WORDS *Pseudomonas fluorescens*, antimicrobial metabolites, rice fungal pathogens

INTRODUCTION:

The genus pseudomonas is one of the most diverse Gram-negative bacterial genera, isolated from sources ranging from plants to soils and water of this genus are straight or slightly curved rods, motile by means of polar flagella. *Pseudomonas* is characterized by their ability to grow in simple media at the expense of a great variety of simple organic compounds, without needing organic growth factors. King's B media is an optimal for most species of pseudomonas isolation[1], 1954) developed the first selective media (King's A and King's B) for the isolation of fluorescent pseudomonads.

In recent years there has been much success in obtaining biological control of plant pathogens using bacterization techniques[2-6]. Bacteria used, as inoculants are mostly *Pseudomonas fluorescens-Putida* types obtained from soils and plant surfaces. Some of these bacteria produce metabolites, which chelate the environmental iron thus making it unavailable to pathogens[7]. To date a number of these diseases suppressive antibiotic compounds have been characterized chemically and include N-containing heterocycles such as phenazines[8-10] , pyrrole type antibiotics[11-12] , pyo-compounds[13], and indole derivatives[14]. A small number of antibiotics like compounds that do not contain nitrogen have also been isolate from fluorescent pseudomonads[15], one of these metabolites 2, 4-diacetylphloroglucinol (DAPG), is a major factor control of a range of plant pathogens[16-17].

Received on 05.02.2009 Modified on 13.02.2009
Accepted on 22.02.2009
Asian J. Research Chem. 2(1): Jan.-March, 2009;Page 26-29

The antibiotic DAPG is produced by Pseudomonads of worldwide origin, and its biosynthetic locus is conserved in Pseudomonads obtained from diverse geographic locations[18-19]. Bacteria that produce DAPG play a key role in agricultural environments, and their potential for use in sustainable agriculture is promising.

MATERIALS AND METHODS:

Isolation of *Pseudomonas fluorescens*: The *P.fluorescens* strains were isolated from 100 soil samples collected from rhizosphere region of rice in Andhrapradesh and Tamilnadu using King's B medium. Colonies that showed fluorescence at 365 nm were selected and further purified.Rice fungal pathogens *P.Oryzae* and *R.Solani* were procured from Directorate of Rice Research, Hyderabad, India. The strains were tested for antifungal activity against these pathogens by dual culture technique[20]. *P.fluorescens* isolates were streaked at one side of Petri dish (1 cm away from the edge) containing PDA medium. A. 5 mm mycelial disc from seven days old PDA culture of pathogens were placed on the opposite side in the Petri dish perpendicular to the bacterial streak and plates were incubated at room temperature (28 $\pm 2^0$C) for 3-7 days. At the end of incubation period, the zone of inhibition was recorded by measuring the distance between the edges of the fungal mycelium and the antagonistic bacterium. Plates inoculated with fungus only served as control. Three replications were maintained for each isolate.

Development of fermentation technology for production of metabolites from *P. fluorescens*

Developed the fermentation technology for the production of effective secondary metabolites from *P. fluorescens*. In

this the most effective strain (P.f 05) were used for fermentation in different media.

Preparation of Inoculum:
The effective strain (P.f 05) was grown in 100ml of King's B media in 250 ml conical flask in orbital shaker at 28^0C, 120 rpm, for 24 hours.

The selected medias (King's A, King's B, Nutrient broth and Nutrient broth with glucose) were prepared individually. Each media was pored into fermenter, then the fermenter sterilized at 121^0C, 15 lbs pressure, for 20 min. After cooling the media was inoculated with pregrown inoculum of *P. fluorescens* (P.f 05). The fermentor was performed under fixed parameters, temp 28^0C, rpm-120, DO_2-40%, pH-7.0 for 96 hours. The turbidity of the culture was measured by estimating the optical density through spectrophotometer at hourly intervals. The culture was centrifuged at 10,000 rpm for 15min to get the cell free filtrate. These culture filtrates were used to study the efficacy against rice fungal pathogens.

Extraction of crude metabolites with selected organic solvents
Crude metabolites were extracted from the effective growth medium (King's B) by partitioning with organic solvents *viz.,* Ethyl acetate, Petroleum Ether, Chloroform, Acetone, Hexane[21] the five solvents were being tried for extraction of secondary metabolites. The extracted metabolites without concentration were tested for their efficacy against pathogens by poison food technique[22]. The different concentrations of extracted crude metabolite (0.2%, 0.5%, 1.0%, 3.0% and 5.0%) were prepared and poured on PDA medium, after a 4 mm disc of *P.oryzae* and *R.solani* culture was inoculated at the center of each plate, three replications were maintained for each treatment and the petridishes were incubated at 28^0C. The radial growth of the fungus was measured 5 days after incubation. PDA plates without metabolite served as control.

Identification of metabolites by TLC
Thin layer chromatography (TLC) was carried out with the crude extract on silica gel (TLC silica gel. 60, 20 x 20, 0.5 mm, Merck and Co, Inc) with benzene: acetic acid (95:5) solvent system. The crude extract (30 µl) was spotted, and the solvent front was allowed to run for approximately 16 cm. The running lane was then dried thoroughly; elution of compound was detected at 365 nm. After cut into portions (1 by 2.5 cm), these portions were scraped into micro centrifuge tubes and extracted with 100% acetone. The silica residue was removed by centrifugation and the supernatant was transferred to a second set of micro centrifuge tubes. The individual metabolites were again spotted on TLC plate along with 2, 4- DAPG standard (Sigma Co., U.S.A) for confirmation of metabolites. Each fraction was concentrated by evaporating off the acetone and tested for antifungal activity of individual metabolites by Poisoned food technique (Nene and Thapliyal 1971) at 0.2%, 0.4%, 0.6%, 0.8% and 1.0% concentrations.

The effective antifungal metabolite (R_f 0.35) was purified by column chromatography, in the glass column (50 x 2 cm) packed with slurry of silica gel (60-120 mesh) preactivated at 120^0C for 4 hours. After the column was successively eluted with hexane-benzene (1:3) and collected 25 ml fractions with a flow rate of 1 ml/min. These fractions were distilled on water bath and monitored by TLC. The fractions of similar compositions were mixed together for further studies.

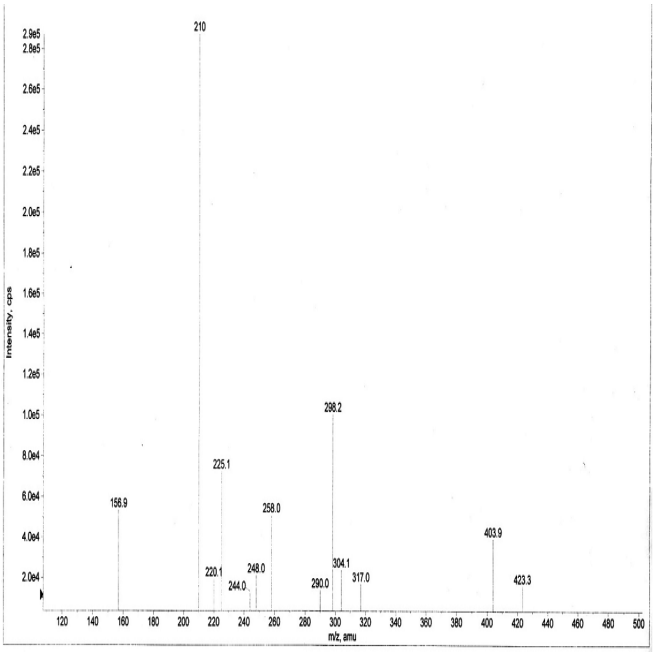

Fig-1: Mass Spectra of 2, 4-Diacetylphluoroglucinol

Characterization of Antifungal metabolite by HPLC, NMR, IR and Mass spectroscopy
HPLC analysis:
The HPLC system consisted of a Waters pump (model 510), a Rheodyne injection valve with a 2 µl loop, and an analytical column (150 mm x 4.5 mm i.d) by 6 mm [outer diameter]) packed with octadecyl silica (Hypersit; particle size 5µm). The spectrophotometric detector (model 440) was from Waters Associates and was linked to a Phillips 8251 strip chart recorder, which plotted the detector outputs. The reagents for mobile phase preparation were of HPLC grade, and all mobile-phases used were filtered and degassed on a Millipore HPLC filtration system with 0.45 µm pore size membrane filters. Unless otherwise stated the HPLC mobile-phase consisted of Acetonitrile: water (50:50) at a flow rate of 1.0 ml/min and detected at a wavelength of 254 nm.

NMR, IR and Mass spectroscopy:
The dried purified antifungal metabolite was crystallized by dissolving in ethyl acetate (6 ml) and filtered to remove insoluble impurities. The filter was washed with ethyl acetate (2 ml) and the wash was added to ethyl acetate solution. After leaving this solution over night at -20^0C, the

crystals were collected. The melting point of crystallized metabolite was determined by subjecting a capillary tube filled at one end with dried powder to heat at 180^0C. The crystallized antifungal metabolite was re-suspended in a minimum quantity of acetone and analyzed by nuclear magnetic resonance spectroscopy (NMR- 400 MHz), Fourier transform infrared spectroscopy (FTIR, model 460 and Jasco) and mass spectroscopy (EIMS) to identify the chemical structure.

RESULTS AND DISCUSSION:

Twenty strains of *P.fluorescens* were isolated from 150 rhizosphere soil samples. The fluorescent pseudomonads were identified. All the fluorescent bacterial antagonists were gram negative, rod shaped and all produced yellowish green pigment on King's B medium. All were gelatin liquifiers and oxidase and arginine dihydrogenase positive and were identified as *P.fluorescens*. Among the 20 isolates, one isolate *P.fluorescens* 05 was found to effectively inhibit (50-85%) the mycelial growth of all fungal pathogens tested. In dual cultures with rhizospheric bacteria, soil borne pathogens P.oryzae and R.solani[23] were reported to be inhibited. The antimicrobial activity of *P. fluorescens* had reported against numerous fungi[24] (Khan MS and Zaidi A. 2002), *P.fluoresens* was shown to effectively inhibit *P.oryzae* and *R. solani* by agar plate method[25] reported that *P.fluorescens* inhibit a maximum of 69.8%growth of *R.solani* inciting seedling disease of okra when compared to control. Investigation on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new pesticide products. Several such products have been developed and used as bactericide, fungicide, acaricide, insecticide or acaricide in agriculture. However, research on new metabolites and its development, as agrochemical by fermentation technology has not been addressed adequately in India. Therefore, it is considered worthwhile to generate reliable data on the isolation, production, productivity and bioefficacy of antifungal metabolites from some native *P. fluorescens*. In this investigation developed the fermentation technology for the production of effective secondary metabolites from *P. fluorescens*. We found that the King's B medium was best for production of effective metabolites through fermentation technology with parameters of rpm 120, p^H 7.0, temperature 28^0C and dissolved oxygen 40%. A new metabolite (Amino-2-chloro-3-phenyl)-4-pyrrole-2-Carboxylic acid was isolated from *P. aureofaciens* at an early stage of fermentation, and also isolated 7-Chloroindole 3-acetic acid and 3-chloroanthranilic same organism[26].

The metabolites were extracted from *P. fluorescens* (P.f 05) culture filtrate (1:1 ratio) using different organic solvents (Ethyl acetate, petroleum ether, chloroform, acetone and hexane) and studied their bioefficacy of each solvent against rice fungal pathogens at different concentrations .Among the metabolites tested against the rice fungal pathogens, the metabolite extracted with ethyl acetate was effectively inhibited (89-90%) both the pathogens tested at 5 per cent concentration. The next best was petroleum ether (66-70%) at 5% per cent. he TLC showed four antifungal metabolite compounds were produced by strain P.f 05 on TLC plate as indicated by their R_f values (0.22, 0.35, 0.42 and 0.51), TLC showed an identical Rf value of 0.35 for DAPG extracted from *P. fluorescens* (05 strain) and for the DAPG standard.

The ethyl acetate extract obtained from cell free nutrient broth culture of P.f 05 isolate was subjected to coloumn chromatography for the purification of the metabolites present in the extract. The column was successively eluted with hexane: benzene (25:75). The individual metabolites was purified in column chromatography were tested for their bioefficacy against fungal pathogens of rice. Out of four individual metabolites tested against rice fungal pathogens the metabolite showed R_f 0.35 was effectively inhibited both the pathogens (93-94%) at 1.0 per cent concentration. The order of performance was R_f 0.22 (71-83%), R_f 0.42 (64-78%) and R_f 0.51 (38-61%) at 1.0 per cent concentration on both pathogens tested.

To develop a rapid method for detection of DAPG production in Vivo, a simple HPLC based method was developed. This assay shows the presence of DAPG in culture supernatants. Phloroglucinols have previously been investigated chromatographically by using HPLC[27]. However in this study, we modified the HPLC procedure for the detection of a particular phloroglucinol (DAPG), incorporating a sample pretreatment step that eliminates non-C_{18}-retained material. However, in this study, we identified DAPG production in P.f 05 strain *Pseudomonas fluorescens.* Also observed DAPG production in HPLC[28]. The melting point of the 2, 4 diacetyl phloroglucinol showed $143\text{-}175^0$C. HPLC spectrum showed 2,4 diacetyl phloroglucinol peak at 14.635 min. The H^1NMR (200 MH_Z $CDCl_3$) spectrum of 2,4 diacetyl phloroglucinol showed peaks at δ 2.3, δ 5.0 and δ 5.9. The C^{13} NMR spectrum showed peaks at δ 199.8, δ 167.9, δ 163.1, δ104.3, δ 96 and δ 29.9. The FTIR (KBr) spectrum showed carbonyl group at 1636 cm^{-1} and other functional groups at 1639 cm^{-1} and OH group at 3434 cm^{-1}, other functional groups at 2923, 2851 cm^{-1} and the molecular weight was estimated at M/z 210 by mass spectroscopy (Fig 1), which agreed with the composition $C_{10}H_{10}O_5$ for 2,4 diacetyl phloroglucinol.

CONCLUSION:

In conclusion, antibiotic production by fluorescent pseudomonas spp. is now recognized as an important feature in plant disease suppression by some strains. However due to the scarcity of nutrients in most soils, antibiotic production is generally restricted. Thus the results suggest that DAPG production in crop rhizosphere is yet another important that reduces the severity of this important rice diseases. This could be further exploited or accelerated by developing superior 2, 4- DAPG producing strains as microbial inoculants for rice.

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