Investigations on Antifungal Metabolites of *Pseudomonas fluorescens* Isolates and their Antagonism Against Major Fungal Pathogens of Rice

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Abstract

Fluorescent Pseudomonads have been successfully employed in contro-lling major fungal plant diseases due to their antifungal metabolites. The present study focuses on characterization of secondary metabolites produced by *Pseudomonas fluorescens* and their *in-vitro* antagonistic ability against rice blast caused by *Magnaporthe grisea* and sheath blight caused by *Rhizoctonia solani*. Among 20 strains of *P. fluorescens* isolated from rhizosphere of rice seedlings, one particular strain P.f 05 was highly effective in inhibiting mycelial growth of rice pathogens. To further char- acterize the production of antifungal metabolites by strain P.f.05, several growth media were used. Among the media tested, King's B medium at 120 rpm with pH of 7.0 and 40% of dissolved oxygen incubated at 28ºC yielded maximum amount of secondary metabolites. Among them, four secondary metabolites were identified through thin layer chromatogr-aphy with R_f values of 0.22, 0.35, 0.42 and 0.51. Of these four, one partic-ular metabolite was found to inhibit the mycelial growth of two rice pathogens significantly higher compared to other three metabolites. Of interest, this metabolite was further characterized by HPLC, NMR and Mass Spectroscopy and identified as 2, 4-diacetyl-phloroglucinol (DAPG). The melting point of 2, 4 DAPG was between 143-175ºC. The infrared spectroscopy spectrum of 2, 4 DAPG showed carbonyl group at 1636 cm^{-1}, other functional groups at 1639 cm^{-1} and OH group at 3434 cm^{-1}. The molecular weight was estimated at M/z 210 by mass spectroscopy which agreed with the composition of $C_{10}H_{10}O_5$ for 2, 4 DAPG. These results offer a scope of identifying superior strains of *P. fluorescens* with high potential of antifungal metabolite production in rhizosphere of rice ecosy-stem for successful management of rice blast and sheath blight diseases.

Introduction

Research on microbial metabolite production and their applicability in controlling plant diseases is gaining momentum in agriculture. The scope of developing these microbial metabolites for commercial pesticides as an alternative to chemical fungicides is gaining importance due to increased concerns on environmental pollution, pathogen resistance and high plant protection costs. Earlier, several of these products have been developed and used as bactericides, fungicides, insecticides or acaricides in agriculture. The metabolites, (Amino-2-chloro-3-phenyl)-4-pyrrole-2-carboxylic acid, 7-chloroindole 3-acetic acid and 3-chloroanthranilic acid were isolated from *Pseudomonas aureofaciens* at an early stage of fermentation (Salcher *et al.,* 1978). Among different plant growth-promo-ting rhizobacteria (PGPR), *Pseudomonas fluorescens* is a widely known biocontrol agent against many soil borne plant pathogens. Several PGPR produce metabolites, which chelate the environmental iron thus making it unavailable to pathogens (Kloepper *et al*., 1981). Several disease suppr-essive antibiotic compounds have also been characterized which includes phenazines (Thomashow *et al.,* 1990), pyrrole type antibiotics (Imanka *et al*., 1965), pyo-compounds (Hays *et al.,* 1995), and indole derivatives (Wratten *et al*., 1977). However, research on new metabolites and its development as agrochemicals by fermentation technology has not been addressed adequately. Therefore, it is considered worthwhile to generate reliable data on the isolation, production, productivity and bio-efficacy of antifungal metabolites of PGPR isolated from rice ecosystem.

Materials and Methods

Isolation and *in-vitro* screening of *Pseudomonas fluorescens* against rice pathogens

Rhizosphere soil samples were collected from rice seedlings grown from Andhra Pradesh and Tamilnadu and isolation of *P. fluorescens* strains studies were carried out on King's B medium. Colonies that have shown fluorescence at 365 nm were selected, purified and used for further studies. These strains were screened for their *in-vitro* antagonistic ability against rice blast pathogen *Magnaporthe oryzae* and sheath blight pathogen *Rhizoctonia solani* by dual culture technique (Rabindran and Vidyasekaran, 1996). Bacterial isolate was streaked at one side of petri dish (1cm away from the edge) containing PDA. Five mm mycelia plug from seven-day-old PDA cultures of rice pathogens were placed at the opposite side of petri dishes perpendicular to the bacterial streak. Petri dishes were then incubated at 28 ±2°C for 5 days. Petri dishes inoculated with fungal discs alone were served as control. Three replications were maintained for each isolate. Observations on width of inhibition zone and mycelia growth of test pathogens were recorded and percent inhibition of pathogen growth were calculated.

Standardization of fermentation technology for production of anti-metabolites

Strain p.f.05 of *P. fluorescens* was selected because of its antagonistic ability superior than other strain was used to optimize anti-metabolite production. Various media such as on King's A, King's B, nutrient broth and nutrient broth plus glucose were used to growth strain p.f.05 in fermentor. The fermentor was operated at 28 C with 120 rpm and aerated with 40% diluted oxygen and maintained at pH 7.0 pH for 96 h. The turbidity of the bacterial growth was measured by estimating the optical density through spectrophotometer at hourly intervals. Active cells of strain p.f.05 were centrifuged at 10,000 rpm for 15 min to get cell free anti-metabolites. These metabolites were tested or *in-vitro* against rice pathogens as described above.

Identification of metabolites by Thin Layer Chromatography

Thin layer chromatography (TLC) was used with the crude extract of *P. fluorescens* strain p.f.05 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 and the elution of compound was detected at 365 nm. The cut portions (1 cm x 2.5 cm) were scraped into micro centrifuge tubes and were 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 at 1.0% concentrations.

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

Characterization of antifungal metabolites

HPLC analysis

To develop a rapid method for detection of DAPG production by *P. fluoresecens* P.f 05, a modified HPLC based method was developed by incorporating a sample pre-treatment step which eliminates non-C_{18}-retained material.

NMR, IR and Mass Spectroscopy

The dried purified antifungal metabolite collected above 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^{0}C, 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°C. 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) was used to identify the chemical structure of the metabolite.

Results and Discussion

Twenty bacterial strains were isolated from rhizosphere soil samples collected from rice seedlings grown from Andhra Pradesh and Tamilnadu. All the strains were gram negative, rod shaped and 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 20 strains, *P. fluorescens* P.f 05 was found significantly inhibit the mycelial growth of rice pathogens. Studies on standardization of fermentation technology for *P. fluorescens* showed that King's B medium was the best for production of anti-metabolites.

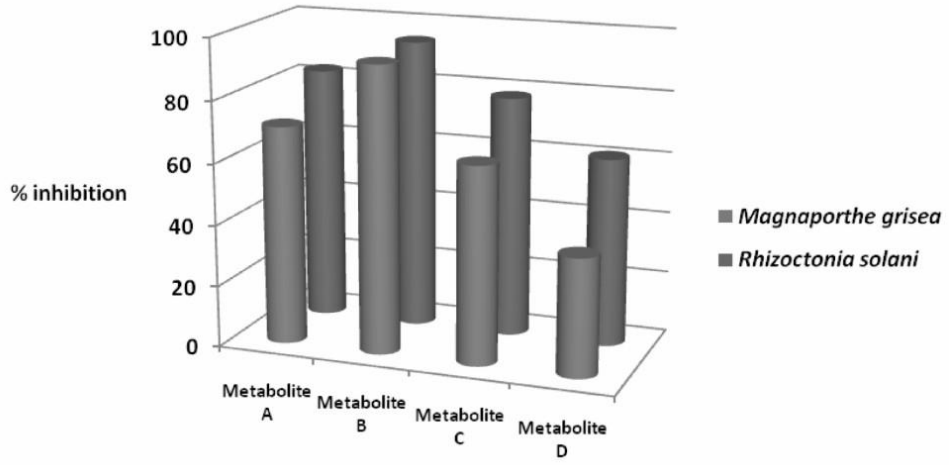

Fig. 1. *In-vitro* efficacy of metabolites of *Pseudomonas fluorescens* strain p.f.05 on mycelial growth of rice pathogens.

Results of TLC indicated that four antifungal metabolites designated as A, B, C and D were produced by strain P.f 05 corresponding by their R_f values of 0.22, 0.35, 0.42 and 0.51. An identical Rf value of 0.35 for DAPG extracted from strain p.f. 05 and for the DAPG standard was comparable. Antagonistic studies showed with the metabolite B significantly inhibited both the pathogens better than the other three metabolites (Fig. 1).

The melting point of the 2,4 DAPG identified from metabolite B was shown to be between 143-175 C. HPLC spectrum has shown a 2, 4 DAPG peak at 14.635 min. The H^1NMR (200 MHz $CDCl_3$) spectrum of 2, 4 DAPG had shown 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}. The molecular weight was estimated at M/z 210 by mass spectroscopy (Fig. 2), which agreed with the composition of $C_{10}H_{10}O_5$ for 2, 4 DAPG.

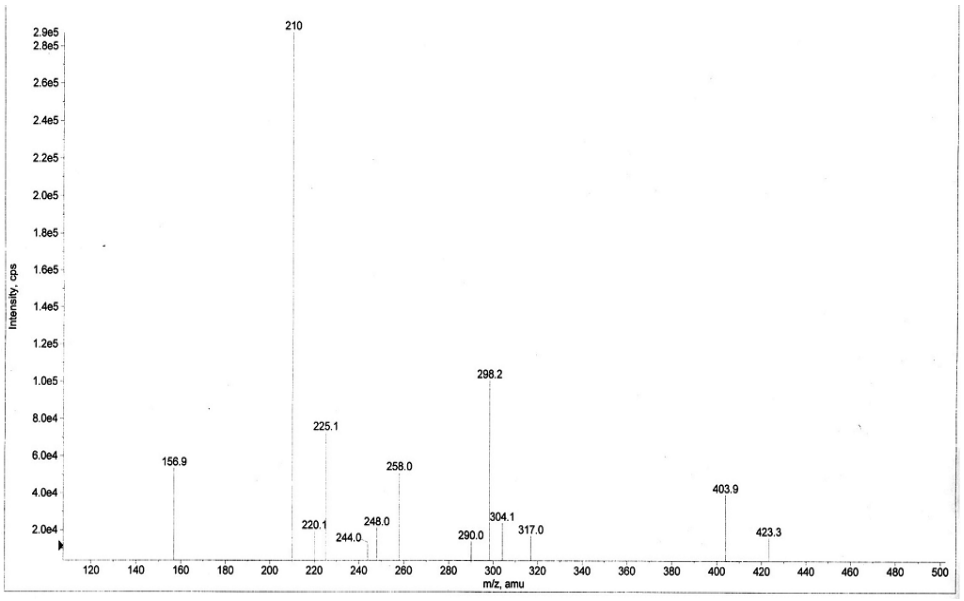

Fig. 2. Mass Spectra of 2, 4-Diacetylphluoroglucinol produced by *Pseudomonas fluorescens strain* p.f.05.

The metabolite, 2,4- DAPG produced by *P. fluorescens* is a major factor in controlling a range of plant pathogens. DAPG produced by Pseudomonads of world-wide origin, and its biosynthetic locus is conserved in Pseudomonads obtained from diverse geographic locations (Keel *et al*., 1996). Bacteria that produce DAPG play a key role in agricultural environments, and their potential for use in sustainable agriculture is promising. To conclude, antibiotic production by *P. fluorescens* is now recognized as an important feature in plant disease suppression. Our results suggest that DAPG production in crop rhizosphere is an important factor contributing to reduction in disease severity in rice from soil borne plant pathogens. Further, identification of superior strains of *P. fluorescens* with rapid and abundant production potentiality of 2, 4- DAPG is essential for effective management of diseases in rice production.

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