Biochemical and PCR-RAPD Characterization of *Pseudomonas fluorescens* Produced Antifungal Compounds Inhibit the Rice Fungal Pathogens *In vitro*

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Plant growth-promoting rhizobacterial strains belonging to fluorescent pseudomonads were isolated from the rhizosphere of rice. Among 30 strains that were confirmed as *Pseudomonas fluorescens*, these *P. fluorescens* strains was characterized by PCR-RAPD analysis and biochemical methods. Ten exhibited strong antifungal activity against *P. oryzae* and *R. solani* mainly through the production of antifungal metabolites.

Key words: *Pseudomonas fluorescence,* PCR-RAPD, *P.oryzae, R.solani*, Siderophore.

Plant growth-promoting rhizobacteria (PGPR)[1] improve plant growth in two different ways, directly or indirectly. The direct promotion of plant growth by PGPR is through production of plant growth-promoting substances[2], or facilitation of uptake of certain nutrients from the soil[3]. Bacteria found in rice fields produced fluorescent and non-fluorescent pigments on King's b medium were shown to be antagonistic to *R.solani* that causes rice sheath blight[4,5]. Fluorescent pseudomonad strains have been reported to control several diseases caused by soil borne pathogens[6] and are known to survive in the rhizosphere. Biological control of plant diseases using antagonistic microorganisms offers a highly effective, economical and environmental friendly alternative to the use of synthetic pesticides[7]. The mode of action of the antagonistic organisms against various soil-borne plant pathogenic fungi, include biosynthesis of antibiotics, production of hydrolytic enzymes[8], production siderophore and competion for substrates. 2, 4-diacety phloroglucinol, which inhibits growth of pytopathogenic fungi[9]. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. The main objective of this study characterization of *P. fluorescens* for the management of rice fungal pathogens *P.oryzae* and *R.solani*.

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MATERIAL AND METHODS

One hundred soil samples were collected from 50 different locations representing rhizosphere of mainly rice around Andhra Pradesh, using King's B medium[10]. Colonies that showed fluorescence at 365 nm were selected and further purified on the same medium. Pathogens used were *Pyricularia oryzae* and *Rhizoctonia solani* obtained from Directorate of Rice Research, Hyderabad, INDIA.

The strains were tested for antifungal activity against these pathogens by dual culture technique[11]. *P.fluorescens* strains isolates were streaked at one side of Petri dish (1cm away from the edge) containing PDA medium. A 5 mm mycelial dic 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±2°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.

Based on bioefficacy studies the efficient strains were selected for testing to determine whether siderophore or antibiotics are responsible for the antagonistic property of strains[12]. The King's B agar medium was sublimented with 1% of 100mM $FeCl_3$ and inoculated with 3 day old culture of *P. fluorescens* strains in the 4 corners of the petiplate. Similarly it was inoculated in the iron free medium. An actively growing mycelial disc of 9 mm diameter of *R. solani* and *P.oryzae* was inoculated in center of the petriplate. A week after inoculation, inhibition of mycelial growth was observed in both iron amended King's B agar containing antibiotics. We also observed the changes in the color of iron deficient medium, which indicates that siderophore, is responsible for fluorescent pigment production.

The genotyping was performed by PCR-RAPD analysis. The purpose of this study was to apply the RAPD technique for characterization of *P. fluorescens* and evaluate the ability of this technique to differentiate between them. A total of 8 most efficient strains of *P. fluorescens* along with MTCC (1749) strain were used in this study. The DNA was extracted by the modified method of [13]. The supernatant taken in eppendorf tube, added 800 µl of extraction buffer and incubated at 65°C for 30 min, in the same tube added 200 µl of phenol and 200 µl of Chloroform mixed it by tapping for 10 min. Collected supernatant and added 5µl RNase and incubated at 37°C for 1 hr. Added equal volume of chloroform, mixed for 10 min and centrifuged at 13,000 rpm for 10 min. After centrifugation collected supernatant in fresh tube and added 2/3rd volume Isopropanol, mixed gently for 5 min and centrifuged at 13,000 rpm for 10 min. Collected pellet and added 500 µl of 70% ethanol, tapped for 5 min and again centrifuged at 13,000 rpm for 10 min, removed supernatant and the pellet was dried in heater for 1 hr. The pellet was dissolved in TE buffer for further studies.

The DNA concentrations were determined spectrophotometrically at a wavelength of 260 nm. Depending on the concentration of DNA determined, a stock solution with a concentration of 1g/ml was prepared for each isolate. These were subsequently diluted to 5 ng/ml used in amplification. The RAPD primers were used in Table-1.

PCR reactions were carried out in 20µl of reaction containing 10X buffer (with 2.5mM $MgCl_2$), 2µl of 2mM dNTP mixture, 2 µl of 2µM primer, 5µl of Taq DNA polymarase 3U; 8µl of H_2O, and 15 ng of template DNA samples were amplified on DNA thermocycler. (MJ research, USA, India) using the PCR conditions 94°C for 1 min, 36°C for 1min, and 72°C for 2 min. The total numbers of cycles were 40, with the final extension time of 10 min. A 10 ml of each reaction was electophorosed on 1.8% agarose gel run at constant voltage (6v/cm) in 0.5 x TBE and stained with ethidium bromide (10mg/ml). The DNA marker used was 1kb ladder. The gels were photographed under UV light with Polaroid film 667.

RESULTS AND DISCUSSION

Thirty strains of *P.fluorescns* were isolated from one hundred rhizosphere soil samples. The fluorescent pseudomonads were identified according to[14]. 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 dihydrolase positive and were identified as *P.fluoresces*. Among the 30 isolates, 10 isolates P.fluorescens were found to effectively inhibit (55-85%) the mycelial growth of both fungal pathogens.In dual cultures with rhizospheric bacteria, soil borne pathogens[15] were reported to be inhibited The antimicrobial activity of *P.fluorescens* had reported against numerous fungi[16,17] . *P. fluorescens* was shown to effectively inhibit *P.oryzae* and *R.solani* by agar plate method[18].

Iron is a fundamental element for respiration of several aerobic and facultative microorganisms and therefore, its availability in soil is essential[19]. On the other hand, siderophores are low weight compounds with high affinity for Fe^{3+} [20]. The most effective isolates (P.f 001, 003, 005 and 007) were tested in the presence and absence of $FeCl_3$. Among these P.f 003 strain completely inhibited the mycelial growth of the rice pathogens both in presence and absence of $FeCl_3$ (Table 2). Which indicated the siderophore mediation along with antifungal metabolites. Siedrophore production was observed reverse side of petri plates, as green dots and also the change of color of the medium to fluorescent green. However, three strains P.f 001, 005 and 007 partially inhibited fungal growth in the presence and absence of $FeCl_3$ ranging from 25-85 percent. Similarly[21] reported that the *Pseudomonas fluorescens* produced siderophores and antifungal metabolites, which are involved in the control of phytopathogenic fungi. This suggested that these three isolates produced antifungal metabolites were different from the other strains.

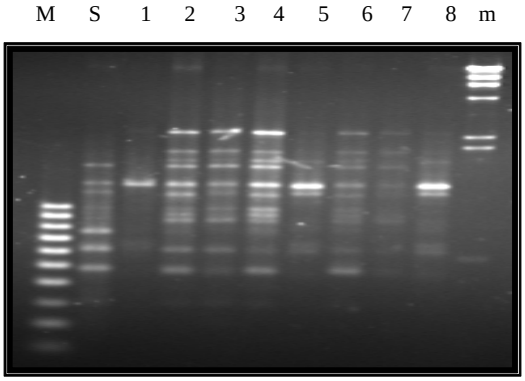
The most efficient eight strains of *Pseudomonas fluorescens* were characterized genotypically by RAPD analysis by comparing with the standard strain of MTCC 1749. As the PCR profiles (Fig 1) revealed that the standard lane shows 6 prominent bands. These 6 bands with molecular weights of 490bp, 520bp, 660bp, 1.3kb, 1.4kb and 1.7kb show sharing with the selected 8 most efficient strains namely P.f 001, 003, 005, 006, 007, 008, 011and 012 (Lane No.1-8). The fragment with molecular weight of 1.4 kb is most common and prominent in all the strains including standard except in P. f 011. Three common bands with molecular weight 490bp,

Table 1. RAPD Primers and their sequence

RAPD Primer number	Primer sequence
OPA 10	GTGATCGCAG
OPA 11	CAATCGCCGT
OPA 12	TCGGCGATAG
OPA 13	CAGCACCCAC
OPA 14	TCTGTGCTGG
OPA 15	TTCCGAACCC
OPA 16	AGCCAGCGAA
OPA 17	GACCGCTTGT
OPA 18	AGGTGACCGT
OPA 19	CAAACGTCGG
OPA 20	GTTGCGATCC

Table 2. Antifungal activity of *P. fluorescens* strains on fungal pathogens (% inhibition) from rice in the presence and absence of FeCl-$_3$

Strain	*Rhizoctonia solani*		*Pyricularia grisea*	
	$FeCl_3$	No $FeCl_3$	$FeCl_3$	No $FeCl_3$
P.f 001	85	26	66	25
P.f 003	100	100	100	100
P.f 005	75	36	68	27
P.f 007	82	30	70	34



M – 1kbp, S-Standard culture of *P. fluorescens*, 1-P.f 001, 2-P.f 003, 3- P.f 005, 4- P.f 006, 5- P.f 007, 6- P.f 008, 7- P.f 011, 8- P.f 012 and m-ë DNA /Hind III digest.

Fig. 1. Agarose gel exhibiting the PCR profile of the *P. fluorescens* DNA amplified with the operon primer (OPA 11)

1.3kb and 1.7kb are observed in strains of P.f 003, 005, 006 and 008 (Lane No.2, 3, 4 and 6), Pair-wise co-efficient similarity based on presence and absence of bands revealed that among 8 strains, 4 strains *viz.*, P.f 003, 005, 006 and 008 exhibited 83% similarity, whereas, three strains (P.f 001,007 and 012) showed 16.67, 50, 66.6% similarity, one strain P.f 011 showed 0% similarity with the standard strain. This confirms that bacteria isolated from entirely different geographical areas can also share some genetic relatedness.

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