Biological Control of Rice Sheath Blight Disease by *Pseudomonas fluorescens* Isolates

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Abstract

Sheath blight of rice caused by *Rhizoctonia solani* is an economically important disease affecting rice production. Eight *Pseudomonas fluores-cens* strains were isolated from rhizosphere of rice seedlings collected from Andhra Pradesh and Tamilnadu. These strains were characterized with PCR based RAPD technique and tested for their *in-vitro* antago-nistic activity against *R. solani*. Crude metabolites from one particular isolate of *P. fluorescens* (P.f 003) were extracted with organic solvents such as ethyl acetate and petroleum ether and these were tested against *R. solani*. Commonly used fungicides in rice sheath blight disease management such as hexaconazole, carbendazim, copper oxy chloride and mancozeb at 150 ppm were screened against mycelial growth of *R. solani* using poisoned food technique. All the strains tested were exhibited antagonistic activity against *R. solani*. One isolate, P.f 003 gave 78% inhibition compared to control. All the fungicides and crude extracts of P.f.003 inhibited the mycelial growth of *R. solani*. Highest inhibition was recorded with hexaconazole and ethyl acetate crude metabolite extract. The results offer a scope for integrating *P. fluorescens* with chemical fungicides for control of sheath blight of rice.

Introduction

Sheath blight caused by *Rhizoctonia solani* is a major soil-borne disease affecting rice production (Ou, 1985). Currently, the disease is managed mostly by application of systemic fungicides and antibiotics (Dev and Mary, 1986). No genetic resistance has been reported for this disease and all the rice cultivars are susceptible to the pathogen. The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that are used for enhancing crop growth and suppressing plant diseases. Fluorescent pseudomonads are a group of PGPR that can promote growth and suppress plant pathogens. Their applicability as biocontrol agents has drawn wide attention because of production of secondary metabolites such as siderop-hores, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Gupta *et al*., 2001).

The present study was aimed at isolation, characterization, *in-vitro* screening of several rhizosphere isolates of P. *fluorescens* from rice seedlings against *R. solani.* Also studies were conducted to determine the sensitivity of sheath blight pathogen to fungicides as well as to the crude metabolites of *P. fluorescens* in order to devise strategies for rice sheath blight disease management.

Materials and Methods

Isolation and characterization of *Pseudomonas fluorescens*

Eight strains of *P. fluorescens* were isolated from rhizosphere of rice seedlings grown in Andhra Pradesh and Tamilnadu on King's B medium (King *et al*., 1954). Bacterial colonies that have exhibited fluorescence at 365 nm were selected and purified for further studies.

Genotyping of these strains were performed using PCR-RAPD along with the standard strain MTCC 1749. DNA extraction was carried out by the modified method of Byun *et al.* (1986). Two ml of sterile distilled water was added to bacterial culture grown on King's B medium and the extract was taken into eppendorf tubes. To this, 800 µl of extraction buffer was added and the tubes were incubated at 65°C for 30 min. Then, 200 µl of phenol and 200 µl of chloroform were added to the same tubes and were mixed by tapping for 10 min. The supernatant was collected and 5µl RNase was added and later incubated at 37°C for 1 hr. To this, equal volume of chloroform was added and mixed for 10 min and centrifuged at 13,000 rpm for 10 min. After centrifugation, the supernatant was collected in fresh tubes and to that 2/3rd volume of isopropanol was added and mixed gently for 5 min and again centrifuged at 13,000 rpm for 10 min. The pellet was collected and to this 500 µl of 70% ethanol was added, then tapped for 5 min and again centrifuged at 13,000 rpm for 10 min. The pellet was dried in heater for 1 hr and later dissolved in TE buffer for further studies. DNA quantification was done spectrophotometrically at a wavelength of 260 nm and 5 ng/µl were prepared and used for amplification.

PCR reactions were carried out with 20 µl of reaction mixture containing 10X buffer (with 2.5mM $MgCl_2$), 2 µl of 2mM dNTP mixture, 2 µl of 2µM primer, 0.5µl of Taq DNA polymerase 3U; 8µl of H_2O, and 15 ng of template DNA. Samples were amplified on thermal cycler (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 number of cycles was 40, with the final extension time of 10 min. A 10 µl of each reaction was electropho-resed 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.

In-vitro antagonism against Rhizoctonia solani

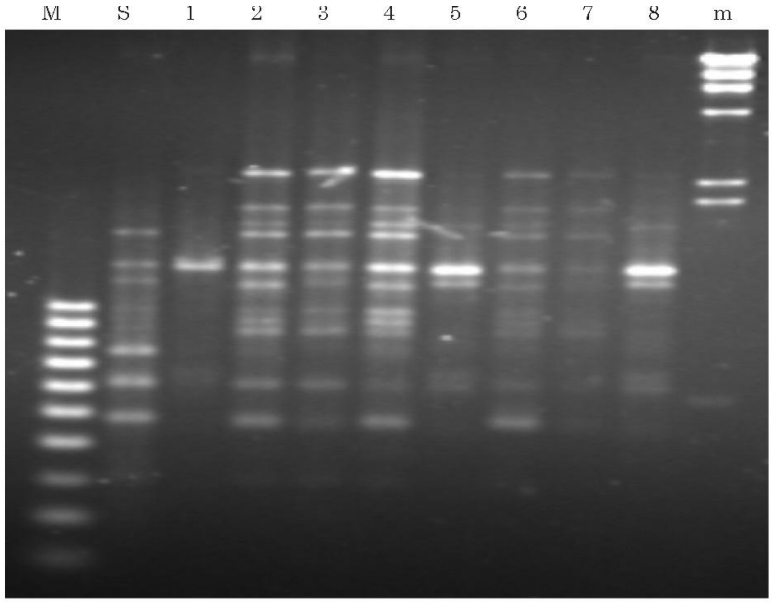
The antagonistic ability of *P. fluorescens* strains against *R. solani* was determined by dual culture technique (Rabindran and Vidyasekaran, 1996). The efficacy of commonly used fungicides against *R. solani* and crude metabolites of *P. fluorescens* (P.f 003) were tested by poisoned food technique (Dhingra and Sinclair, 1985). The fungicides, Bavistin (carben-dazim 50% WP), Blitox (copper oxy chloride 50% WP), ContafPlus (hexaconazole 5% SC) and Indofil M45 (mancozeb 75% WP) were screened in the present study. Crude metabolites were extracted from strain *P. fluorescens* (P.f 003) grown on King's B by partitioning with ethyl acetate and petroleum ether following the procedure of Tripathi and Johri (2002). These fungicides and crude metabolites were amended into PDA at 150 ppm and poured into petri dishes. PDA plates without fungicides or crude metabolites served as control.

The experiments were repeated three times and the data was analyzed by ANOVA and the treatment means wee compared by Duncan's Multiple Range Test ($p<0.05$).

Results and Discussion

Eight bacterial strains were isolated from rice rhizosphere soil samples from Andhra Pradesh and Tamilnadu. These strains were characterized genotypically through RAPD analysis by comparing with the standard strain of MTCC 1749. The PCR profiles revealed that the size standard has six prominent bands (Fig. 1). These six bands with molecular weights of 490bp, 520bp, 660bp, 1.3kb, 1.4kb and 1.7kb show sharing with these *Pseudomonas* strains 001, 003, 005, 006, 007, 008, 011 and 012 (Lane 1-8). The fragment with molecular weight of 1.4kb is most common and prominent in all the strains including the standard strain except in P.f 011. Three common bands with molecular weight of 490bp, 1.3kb and 1.7kb are observed in strains of *P. fluorescens* 003, 005, 006 and 008 respectively (lane no. 2, 3, 4 and 6). Pair-wise coefficient similarity matrix revealed that four strains viz., P.f 003, 005, 006 and 008 exhibited 83% similarity whereas three strains (P.f 001, 007 and 012) have shown 16.67, 50 and 66.6%

similarity with the standard strain. However, the strain P.f 011 has exhibited 100% dissimilarity with the standard strain. The results suggest that these *P. fluorescens* isolated from different geographical locations can also exhibit genetic relatedness to a certain extent.



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-λ DNA /Hind III digest.

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

Dual culture studies revealed that the mycelial growth of *R. solani* in PGPR challenged plates ranged from 2.0 to 8.1 cm as against control (9.0 cm). Among the bacterial strains, the P. f 003 effectively controlled the pathogen with a mycelial growth of 2.0 cm and an inhibition of 77.8% (Table 1). The sensitivity of *R. solani* to commonly used fungicides and also to the crude metabolites of *P. fluorescens* (P.f 003) indicated that the test pathogen was significantly inhibited by all the fungicides and crude metabolites (Fig. 2).

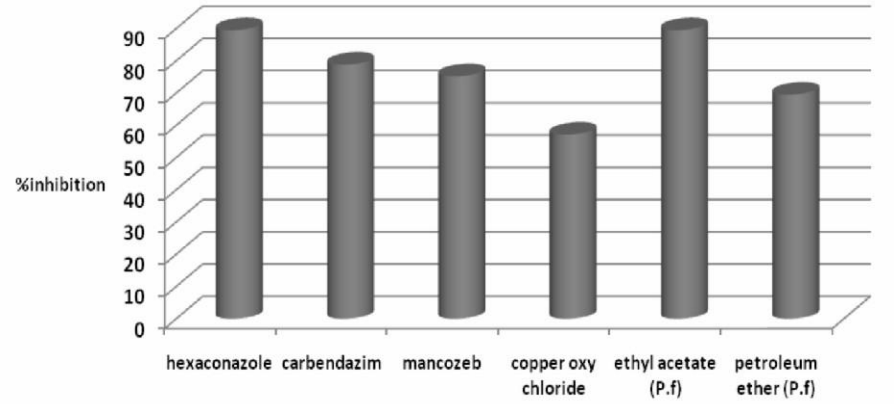


Fig. 2. Sensitivity of *Rhizoctonia solani* to commonly used fungicides and certain crude metabolites of *Pseudomonas fluorescens* at 150 ppm concentration.

Table 1. *In-vitro* antagonism of different *Pseudomonas fluorescens* strains on mycelia growth of *Rhizoctonia solani.*

P. fluorescens strain	Mycelial growth (cm)	Growth inhibition(%)
P.f 001	7.8	13.33[c]
P.f 003	2.0	77.78[a]
P.f 005	8.0	11.11[c]
P.f 006	7.9	12.22[c]
P.f 007	8.0	11.11[c]
P.f 008	7.2	20.00[b]
P.f 011	8.1	10.00[c]
P.f 012	8.0	11.11[c]
Control	9.0	0

Means of three replications.

Means followed by a common letter are not significantly different at 5% level by DMRT.

The results of the present study indicated the prevalence of antagonistic *P. fluorescens* strains in the rhizosphere of rice. Further, the strains from different locations have genetic relatedness to a certain extent. Identification of potential antagonistic PGPR would be useful in managing sheath blight disease effectively under field conditions as an alternative or supplement to chemical fungicides.

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