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Molecular characterization and Antagonistic Potential of phenazine-1-carboxylic acid producing Pseudomonas fluorescens isolates from economically important crops in South India

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Abstract

In plant disease management, Fluorescent Pseudomonads are among the widely used Plant Growth-Promoting Rhizobacteria (PGPR). So, the present study is focused on the isolation of *Pseudomonas fluorescens* from rhizosphere soil samples of different locations in Andhra Pradesh and Tamil Nadu, and its antagonistic activity against *Sclerotium rolfsii* through dual culture technique. Molecular characterization of effective isolates in dual culture technique for phenazine-1-carboxylic acid (PCA) producing *Pseudomonas fluorescens* isolates and genetic diversity was performed among PCA producing isolates and other effective isolates in dual culture of *Pseudomonas fluorescens* through RAPD analysis.

Keywords: *Pseudomonasfluorescens, PCA, Sclerotiumrolfsii, polymorphism.*

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Introduction

In present days Fluorescent pseudomonads are one of the biocontrol agents which reduce the usage of fungicides, pesticides in concern to soil pollution in agriculture land. *Pseudomonas fluorescens* is a plant growth promoting rhizobacteria which stimulate growth to the plant bacteria is mainly because of the production of indole-3- acetic acid (IAA) [1, 2] and it has a capability to suppress soil borne pathogens in various crops [3] with respect to production of secondary metabolites such as Hydrogen cyanide (HCN), Phytohormones, Siderophores, Antibiotics like 2,4-Diacetylphloroglucinol, Phenazine-1-Carboxylic Acid, Pyocyanin, Pyrrolnitrin, Pyoluteorin [4,5,6,7,8]. *P. fluorescens* also produce antifungal metabolites which suppresses the soil borne fungal pathogens [9,10,11]. Of these, Phenazine-1-Carboxylic Acid (PCA) is an important anti fungal metabolite from pseudomonads which plays a major role in biocontrol [12]. Phenazines play a role in biocontrol by undergoing oxidation–reduction transformations in the target cells and accumulate the toxic superoxide radicals in the cells[13,14]. Earlier studies showed that the antibiotic phenazine-1-carboxylic acid of *Pseudomonas fluorescens* 2-79 has high potential against *Gaeumannomyces graminis var. tritici* which causes root disease in wheat [15]. Stem rot is a dreadful disease caused by soil borne fungal pathogen *Sclerotium rolfsii* affects the production of groundnut and also it leads to significant yield losses [16]. The present study discusses the antagonistic activity against stem rot

pathogen, identification of Phenazine-1-Carboxylic Acid producing *P. fluorescens* and genetic variability of collected *P. fluorescens* in groundnut based cropping systems.

Materials and methods

Isolation and identification of Pseudomonas fluorescens.

Rhizosphere soil samples (n= 35) were collected from groundnut, cowpea, pigeon pea and sunflower crops in and around Chittoor and YSR Kadapa districts of Andhra Pradesh and Coimbatore district of Tamil Nadu, India. By following serial dilution the rhizosphere samples were plated on King's B medium and the plates were incubated at 37 ± 2^0C for 24hrs [17]. Fluorescent colonies were selected under Ultra violet light at wavelength 365nm. These isolates were further characterized by morphological and biochemical methods by using standard procedures for confirmation of *P. fluorescens*. Identified isolates were maintained as glycerol stocks and frozen in vials at 80°C for long-term storage.

Dual Culture Studies

The stem rot disease caused by *Scleotium rolfsii* was isolated from an experimental groundnut fields of Regional Agricultural Research Station (RARS), Tirupati,Andhra Pradesh, India. The culture was maintained on potato dextrose agar (PDA) for further studies. *In vitro* screening of *P. fluorescens* strains for antifungal activity against mycelial growth of *S. rolfsii* on PDA medium through dual culture technique [18]. Each treatment was replicated for five times. A mycelium disc (4mm diameter) taken from an actively growing fungal culture of *S. rolfsii* was placed on the surface of the PDA medium. Simultaneously, *P. fluorescens* that were identified and confirmed through biochemical tests were streaked away from the mycelium disc at sides towards the edge of petriplates. Plates inoculated with fungal mycelium disc alone was used as control and incubated at 25^0C until fungal mycelia completely covered the agar surface in control plates in the dark. Strains that inhibited mycelial growth of *S. rolfsii* were identified. Five days after incubation, the inhibition of the mycelial growth of the pathogen was measured.

Isolation of DNA and PCR analysis:

The bacterial colonies from the selected plate were suspended in 100µl of lysis solution (0.05M NaOH, 0.25% SDS) and incubated for 15min at 100ºC. The suspension was centrifuged for 1min at 12,000 rpm and diluted with 50 fold sterile distilled water. Identification of PCA producing *Pseudomonas fluorescens* from other isolates was done by using PCA primers (PCA-F-5'-TTGCCAAGCCTCGCTCCAAC-3' and PCA-R-5'-CCGCGTTGTTCCTC GTTCAT-3') which is specific for PCA antibiotics [19]. PCR amplification was carried out in a 25µl reaction mixture which contained five microlitres of the diluted suspension, 2.5mM of $MgCl_2$, 10mM of each dNTPs, 10 pmols of each primer, 2.5 U/µl of Taq DNA polymerase (Fermentas Canada). The PCR amplification was performed in PCR machine (Eppendorf Pro S, Germany). The PCR

program consisted of an initial denaturation at 94°C for 10min followed by 35 cycles of 94°C for 30 seconds, annealing at 58°C for 45seconds, extension at 72°C for 45seconds and final extension at 72°C for 10 min. The PCR amplified products were separated on 1% agarose gel electrophoresis.

Random Amplified Polymorphic DNA (RAPD) analysis of Pseudomonas fluorescens isolates

In this study the bacterial isolates were screened with seven different random primers OPC-9, OPD-1, OPD-2, OPD-3, OPE-01, OPF-06, and OPM-10 (Operon technologies Inc.) and all these primers were found to be useful in generating polymorphism among them. These *P. fluorescens* isolates were identified based on their higher antagonistic activity under in dual culture studies, of which phenazine producing *P. fluorescens* isolates are also present. For further studies these strains were considered as the best *P. fluorescens* isolates in the fields. The sequences of RAPD primers are given in the Table 2.

Each primer was used to study polymorphism between the isolates by RAPD assay with genomic DNA extracts from all the isolates as template DNA. Master mix was prepared freshly using 2.5µl of 10X assay buffer, 2.0 µl of 2.0mM $Mgcl_2$, 0.5 µl of 10mM dNTPs, 0.2 µl of 0.5 units of Taq DNA polymerase, 2 µl of 0.6 um primer and sterile double distilled water (17.8µl) for all the isolates to avoid band handling errors. The master mix was distributed to microfuge tubes of 0.2ml capacity at 23 µl per tube and 2 µl of 25-100 ng templates DNA from the respective isolates was added to make the total reaction volume to 25 µl. PCR amplification was carried out by 4 min of initial denaturation at 94^0 C followed by 40 cycles of denaturation at 94^0 C for 1 min, annealing at 37^0 C for 2 min, extension at 72^0 C for 2 min and final extension at 72^0C for 10 min. Amplified PCR products were subjected to 1% agarose gel electrophoresis with 1X TBE buffer as running buffer. The banding patterns were visualized under UV transilluminator with ethidium bromide (10mg/ml) staining. The DNA banding profiles were documented in the gel documentation system and compared with 1 Kb DNA ladder (Fermentas, USA). RAPD banding profiles were scored to presence and absence of bands.

For each isolate RAPD pattern was evaluated by assigning character state ‘1’ to the reproducible bands and ‘0’ for the absence of bands in the gel. From the generated data matrix Jaccard similarity coefficient was calculated for each pair wise comparison [20]. SPSS package was used for the preparation of cluster analysis and subsequent dendrogram.

Results and discussion

Nearly 35 bacterial colonies were isolated from the collected rhizosphere soil samples of different plant species in different places. Of these, only 21 bacterial isolates were tested positive for *P. fluorescens* based on morphological and biochemical characterization studies. For these *P. fluorescens* isolates specific identification numbers were given as mentioned in Table -1. Dual culture studies between bacterial isolates and stem rot pathogen in In vitro conditions indicated that inhibition of test pathogen ranged from 0 to 67.5% (Table- 1). Of them, maximum inhibition was obtained with CBI1 (67.5%), followed by CTR3 (59.3%) and YPD2 (45.6%). Comparative analysis on mean antagonistic abilities of *P. fluorescens* isolates collected from different regions indicated that Coimbatore isolates exhibited highest inhibitory per cent of 67.5% and 59.3% chittoor, followed by isolates from Yerpedu (45.6%), Madanapalli (35.6%), Kodur (31.3%), Piler (30.1%) regions (Table-1). The inhibition of PCA isolates ranged from 45.6% to 67.5%, whereas for other isolates, it ranged from 13.6% to 59.3% (Table-1).

Only two (CBI 1, and YPD 2) out of 8 isolates were confirmed to be producing phenazine through PCR analysis with PCA specific primers. The PCR amplification of DNA for these isolates is predicted at 1100bp (Fig-1). It was noticed that there was no amplification in case of remaining isolates. In the present study for the amplification of DNA from *Pseudomonas fluorescens* isolates the primers which were used gave a reproducible and scorable band with high percentage of polymorphism (Fig- 2 & Table- 2). The obtained amplified products were specific to each primer and they ranged from 6 to 21. A total of 85 amplified products were obtained from the seven selected primers. Maximum polymorphism (100%) was observed with OPC9, OPD1, OPD2, OPD3, OPE1, OPF6 and OPM10 primers and the bands obtained with these primers were polymorphic with size ranging from 100bp to 3000bp (Fig 3). The maximum genetic variation observed between isolates CBI1 and PLR1 (100%) closely followed by YPD2 and KDR5 (97.7%). CBI1 and MNP2 were genetically closer with 16.7% similarity followed by KDP3 and PLR1 (14.8%).The similarity co-efficients subjected to SPSS package to produce a dendrogram, produced two major clusters (Fig-3) having CBI1, MNP2 and TPT1, KDR5 is one cluster and remaining isolates KDP3, PLR1 and YPD2, CTR3 in another cluster which in turn were grouped separately in two sub-clusters.

Table 1: *In vitro* antagonistic potential of various *Pseudomonas fluorescens* isolates collected from various crop rhizospheres of groundnut based cropping system against groundnut stem rot pathogen, *Sclerotiumrolfsii*.

Identification number of *P. fluorescens* isolates	Place of collection	Crop	% Inhibition of Mycelial growth of *S. rolfsii*[1*]
CBI1	Coimbatore	Cowpea	67.5[a]
CBI2	Coimbatore	Groundnut	0[g]
YPD1	Yerpedu	Sunflower	0[g]
YPD2	Yerpedu	Groundnut	45.6[c]
KDP1	Kadapa	Pigeon pea	0[g]
KDP2	Kadapa	Cowpea	0[g]
KDP3	Kadapa	Groundnut	16.7[ef]
TPT1	Tirupati	Pigeon pea	13.6[f]
TPT2	Tirupati	Groundnut	0[g]
CTR1	Chittoor	Pigeon pea	0[g]
CTR2	Chittoor	Cowpea	0[g]
CTR3	Chittoor	Sunflower	59.3[b]
MNP1	Madanapalli	Groundnut	0[g]
MNP2	Madanapalli	Cowpea	35.6[d]
PLR1	Piler	Sunflower	30.1[def]
PLR2	Piler	Groundnut	0[g]
KDR1	Kodur	Sunflower	0[g]
KDR2	Kodur	Cowpea	0[g]
KDR3	Kodur	Groundnut	0[g]
KDR4	Kodur	Pigeon pea	0[g]
KDR5	Kodur	Groundnut	31.3[de]

Means followed by a common letter in the columns are not significantly different at 31.3[de] $p<0.05$
[1]Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control
* Values are means of five replications

Table 2: Primer survey for determination of polymorphism in different isolates Of *Pseudomonas fluorescens*

RAPD 10-mer		Total No. of bands	Polymorphic bands
OPC-9	5’ CTCACCGTCC 3’	08	08
OPD-1	5’ ACCGCGAAGG 3’	21	21
OPD-2	5’ GGACCCAACC 3’	18	18
OPD-3	5’ GTCGCCGTCA 3’	13	13
OPE-1	5’CCCAAGGTCC3’	06	06
OPF-6	5’GGGAATTCGG3’	09	09
OPM-10	’TCTGGCGCAC3’	10	10
	Total	85	85

Total number of bands = 85; Polymorphic bands = 85; Percent of polymorphism = 100%

Figure 1: Amplification profile of the PCA primers of *Pseudomonas fluorescens* isolates. Lane M-1Kb Ladder; Lane 1-5: (CBI 1, and YPD 2)

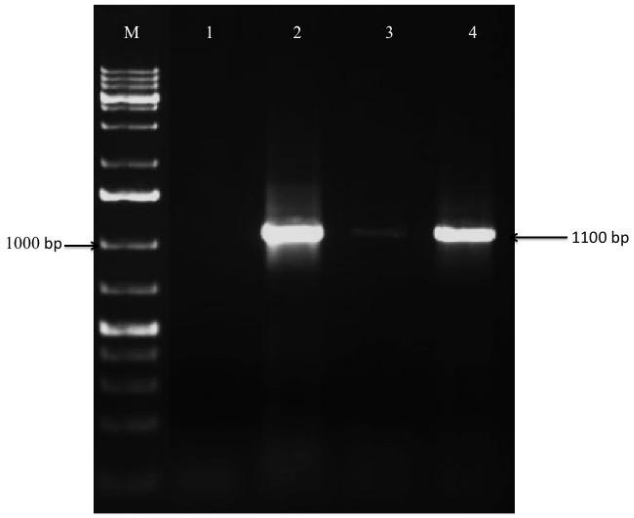


Figure 2: Amplification profile of the RAPD primers (OPD1 and OPD2) of *Pseudomonas fluorescens* isolates. Lane M-1Kb Ladder; Lane 1-8: (CBI 1, YPD 2 MNP 2, TPT 1, KDR 5, KDP 3, PLR 1, YPD 2, CTR 3)

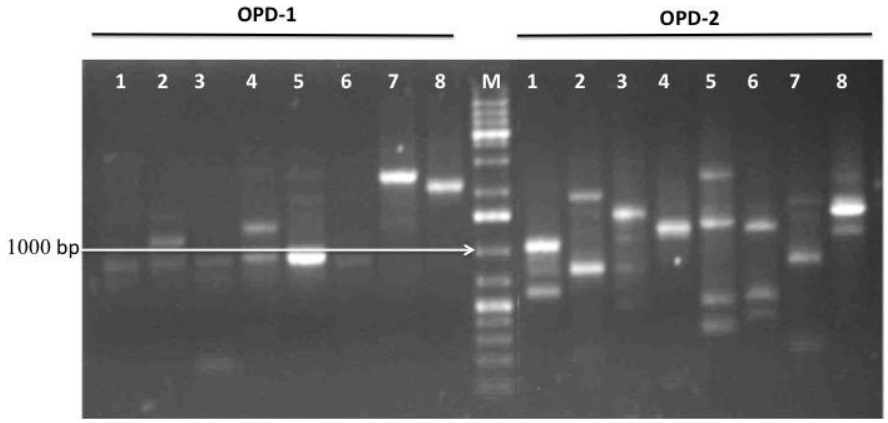
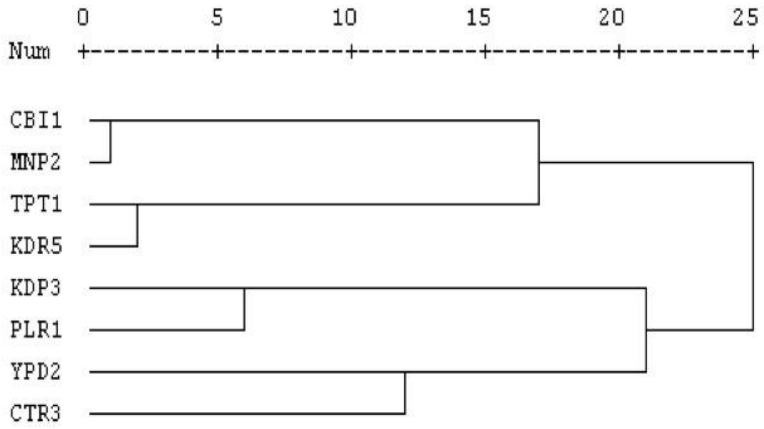


Figure 3: UPGMA-based dendrogram showing relationship among *Pseudomonas fluorescens* isolates through RAPD analysis



P. fluorescens has antagonistic activity on plant pathogens due to the production of several antibiotics [21]. The antibiotics produced by *P. fluorescens* strains include Phenazine-1-carboxylic acid, 2, 4-DAPG, Pyrrolnitrin, and Pyoluteorin [22, 23, 24]. Earlier reports on *P. fluorescens* indicated that it has a capacity to control of a wide range of plant pathogens such as Pythium damping off of cucumber, black root of tobacco, root rot of pea, crown and root rot of tomato, soft rot of potato and take-all of wheat [25, 26, 27, 7, 28, 29,30,23]. Other fungal pathogens that were inhibited under in vitro conditions by *P. fluorescens* include leaf spot (*Alternaria helianthi*); collar rot (*Sclerotium rolfsii*); seedling blight (*Rhizoctonia solani*) and charcoal rot (*Macrophomina phaseolina*) pathogens [31]. *P. fluorescens* had capacity to control the nematode *M. incognita* population which causes the root-knot in tomato [32]. At filed levels suppression of soil borne diseases by *P. fluorescens* isolates is directly correlated to the quantity of antibiotics produced either in the crop spermosphere or rhizosphere [19]. In Invitro conditions antibiotic phenazine-1-carboxylic acid produced from *Pseudomonas fluorescens* strain 2-79 is active against *G.graminis var. tritici* which causes take-all, a major root disease of wheat and other fungal root pathogens [33].

In the present study, isolates of fluorescent Pseudomonads producing PCA were identified through PCR analysis by using specific primers. The isolation studies indicated that the rhizosphere soil samples of different crop species from the areas of Coimbatore (CBI 1) and yerpedu (YPD 2) have PCA producing *P. fluorescens*. In area wise further these PCA producing *P. fluorescens* isolates were examined against soil borne diseases in the fields to enumerate the population status in different crop rhizospheres. The present study gives an idea about the

antagonistic potential of phenazine producing *Pseudomonas fluorescens* and other effective isolates of *Pseudomonas fluorescens* of different crops of rhizospheres soils from Chittoor ,YSR Kadapa and Coimbatore districts against plant pathogen *S. rolfsii* in groundnut (up to 67.5%). In laboratory conditions these isolates effectively reduced the radial growth of stem rot pathogen. There are several reports in the management of stem rot disease in groundnut by the use of plant growth promoting rhizobacteria [34]. Genetic variability studies indicated close association of phenazine producing and other effective isolates of *Pseudomonas fluorescens* of CBI1, PLR1, YPD2 and KDR5 regions. In future, these findings aimed to isolate more potential *P. fluorescens* isolates with PCA production through In vitro studies and evaluation of effective isolates against plant pathogens at field level.

Conclusion

Our findings an indicated the incidence of isolates of *P. fluorescens* from different rhizosphere soil samples in Andhra Pradesh and Tamil Nadu. Some of these isolates have an effective antagonistic activity against *Sclerotium rolfsii* and some are produced antibiotic (PCA). Further studies are aimed for the potential isolates of *P. fluorescens* with the production of PCA through in vitro and their evaluation at greenhouse and field level against fungal pathogens.

Conflict of Interest

We declare that we have no conflict of interest

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