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Biochemical studies on genotypic characterization of *Pseudomonas fluorescens* isolates by PCR-RAPD analysis

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Abstract
Plant growth promoting rhizobacterial strains belonging to fluorescent *Pseudomonads* were isolated from the rhizosphere of citrus in Vidarbha region of Maharashtra, India. On the basis of morphological and biochemical studies, they were categorized as *Pseudomonas fluorescens*, *P. putida* and *P. aurigonosa.* Genotyping of these *P. fluorescens* strains was made by PCR–RAPD analysis since differentiation by biochemical methods was limited. Among the 16 primers screened, six primers (OPA-16, OPB-18, OPC-15, OPC-19, OPG-05 and OPG-10) were polymorphic showed better amplification of DNA while other were recorded monomorphic. Similarity co-efficient between each pair of accessions was used to construct a dendrogram using the unweighted paired group method with arithemetic average (UPGMA).

Keywords: *Pseudomonas fluorescens*, PCR-RAPD, citrus, rhizosphere

Introduction
The diversity of *Pseudomonads* present in the soil also depends on the crop in the field and the type of metabolites that produce to arrest of the pathogens. Further differentiation within the same class of *Pseudomonads* based on the chemical component can be done with the help of molecular markers. Many molecular methods are used to detect the presence of soil-borne pathogens and also to assess the genetic variability among the different isolates (Kumar *et al*. 2002) [5]. Biological control of plant diseases using antagonistic microorganisms offers a highly effective, economical and environmental friendly alternative to the use of synthetic pesticides (Emmert, 1999) [1]. The mode of action of the antagonistic organisms against various soil-borne plant pathogenic fungi, include biosynthesis of antibiotics, production of hydrolytic enzymes (Velzahan *et al*. 1999) [15], production siderophore and competition for substrates 2, 4-diacety phloroglucinol, which inhibits growth of pytopathogenic fungi (Thompson and Gould, 1994) [7]. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. In the present investigation, 8 isolates from different location having citrus crop of research interest were genetically characterized using PCR based methods i.e. RAPD. The various isolates were grouped based on the polymorphic pattern obtained with these techniques.

Materials and Methods
Thirty soil samples collected from rhizosphere region of citrus in Vidarbha region using King's B medium (Kings *et al*. 1954) [4]. Colonies obtained on King'B medium were selected and further purified. They were subjected for differentiation and identification through different biochemical test viz., oxidase, catalase, arginine dihydrolysis, nitrate reduction, gelatine liquefaction and urease test. Based on bioefficacy studies the efficient strains were selected for 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 eight most efficient strains of *P. fluorescens* were used in this study. The DNA was extracted by the method of Kumar *et al*. (2002) [5]. 25 ml actively grown *P. fluorescens* in nutrient broth culture was centrifuged in 30 ml centrifuge tube at 6000 rpm for 5 min at 4 ºC. The cell pellet was suspended in 1 ml TE buffer and 0.5 ml N butanol. The suspension was centrifuged at 6000 rpm for 5 min at 4 ºC and discard the supernatant. The pellet was resuspended in 2 ml of TE buffer and centrifuged at 6000rpm for 5 min at 4 ºC to

remove the traces of N butanol. The pellet was resuspunded in 1 ml of TE buffer. 100 µl of 10% SDS and 25 µl of 100 µg/ml proteinase K were added, it was mixed well and incubated at 37 ºC for 1 h. 200 µl of 5 M NaCl and 150 µl of CTAB (10% stock) were added and mixed well and incubated at 4 ºC for 10 min. The cell lysate were deprotinized with 1 ml Phenol: Chloroform mixture, mixed well and centrifuged at 6000 rpm for 10 min at 4 ºC. The aqueous layer was carefully transferred to new 1.5-2 ml microfuge tube and noted the volume. Ice cold isopropanol to the extent of 0.6% volume was added and incubated at -20 ºC for overnight. The suspension was centrifuged at 12000 rpm for 15 min at 4 ºC. The supernatant was discarded. The pellet was dried and resuspended in 50 µl of TE buffer and stored at -20 ºC for further use. The DNA concentration were determined spectrophotometrically at a wavelength of 260 nm. Depending on the concentration of DNA determined, a stock solution with a concentration of 1µg/µl was prepared for each isolate. PCR reactions were carried out in 20 µl of reaction containing 10 X PCR buffer (2 µl); 2.5 mM MgCl2 (1.5 µl); 2 mM of dNTPs(2 µl); 2 µM primer (5 µl); 1 µl of Taq DNA polymerase 5U ; template DNA (2 µl) and sterile water(6.5 µl). DNA samples were aplified on DNA thermocycler (Eppendorf, Germany) using the PCR conditions 94ºC for 4 min, 94 ºC for 30 sec, 35 ºC for 30 sec, 72 ºC for 1 min. The total numbers of cycles were 40, with the final extension time of 10 min at 72 ºC. The PCR products was electophorosed on 1.5% agarose gel run at constant voltage 70 V of 2 hrs in 1xTBE buffer and stained with ethidium bromide solution (0.5 µm/ ml). The DNA marker used was 10 kbp ladder. The gels were photographed and analyzed using gel documentation system (Alpha Innotech Crop, USA). The bands which appeared consistently, were evaluated. The pair wise coefficient similarity based on the presence and absence of bands was calculated.

Results and Discussion

Thirty strains of *P. fluorescens* were isolated from soil samples of citrus collected from different region of Vidarbha, Maharashtra, India. All the fluorescent bacterial antagonists were gram negative, rod shaped and all produced yellowish green pigment on King's B medium. All were Oxidase and Arginine dihydrolase positive and were identified as *P. fluorescens*. Among the 30 isolates, 8 isolates of *P. fluorescens* were found to inhibit (13.55 - 38.88%) the mycelial growth of *Phytophthora* (Table 1).

Table 1: Per cent growth inhibition of *Phytophthora* by *P. fluorescens*

Sr. No.	Isolates	% growth inhibition	Location
1	Pf_1	22.44	Akola
2	Pf_6	13.55	Akot
3	Pf_9	26.00	Akoli Jahagir
4	Pf_{14}	24.33	Warud
5	Pf_{15}	29.44	Warud
6	Pf_{18}	15.77	Amravati
7	Pf_{19}	26.22	Katol
8	Pf_{20}	38.88	Katol

In the present work a total 16 primers were used in RAPDs and 6 were found to be polymorphic (Table2). The amplified DNA fragments ranged from 9.00-0.30 kbp. The number of polymorphic loci amplified varied with different primers and isolates.

Table 2: List of primers screened against *Pseudomonas fluorescens* isolates

Sr. No.	Primer Screened	Primers sequences
1	OPA10	GTGATCGCAG
2	OPA12	TCGGCGATAG
3	OPA13	CAGCACCCAC
4	OPA14	TCTGTGCTGG
5	OPA15	TTCCGAACCC
6	OPA16	AGCCAGCGAA
7	OPB10	CTGCTGGGAC
8	OPB11	GTAGACCCGT
9	OPB14	TCCGCTCTGG
10	OPB15	GGAGGGTGTT
11	OPB18	CCACAGCAGT
12	OPC15	GACGGATCAG
13	OPC19	GTTGCCAGCC
14	OPC20	ACTTCGCCAC
15	OPG05	CTGAGACGGA
16	OPG10	AGGGCCGTCT

All the bands were scored for their presence and absence in the 8 isolates and a similarity matrix was constructed using UPGMA program. Cluster analysis carried out based on the similarity data generated from the 8 isolates using 6 primers accounted for a total of 296 polymorphic DNA bands. The various *P. fluorescens* isolates were divided into two major classes (Fig. 1). Two of the isolates i.e. Pf_{15} and Pf_{14} formed one groups. Second class consisted of different subgroups with Pf_9, Pf_6 and Pf_{19}, Pf_{18} and Pf_{20} and Pf_1. The similarity among these isolates ranged from 0.158 to 0.044. Genetic variability exists among the various isolates of *Pseudomonads* depending on the geographical locations from where these were collected, crops grown in a specific region and the agricultural practices employed in the location (Picard and Bosco, 2008) [9]. DNA-based (genotypic) approaches (Saharan and Naef 2008; Schutte *et al.* 2008) [13, 14] have increasingly been applied to microbial identification and classification. In fact, these molecular approaches have resulted in the birth of a new ecology subspecialty. Generally, these methods tend to be dependent on bacterial growth variables, more stable, less time-consuming and are very useful for determining phylogenetic relationship among microbial isolates and for assigning strains into specific groups. The similarity data obtained with RAPD primers identified 2 major groups by cluster analysis. The grouping does not appear to be based on geographic origin as Pf_{14} and Pf_{15} were from the same location but not from the same field. The genotypic and phenotypic diversity of *P. fluorescens* could be based on the geographic origin or functional differences in the genome of the strains (Naik *et al.* 2008) [6].

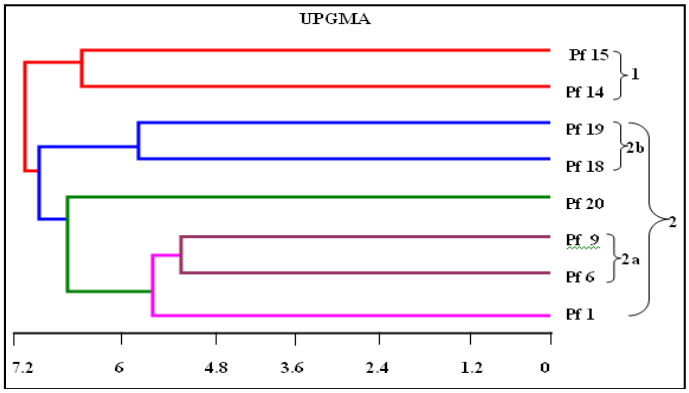

Fig 1: Phylogenic tree of 8 *Pseudomonas fluorescens* isolates obtained by UPGMA, distance coefficient on the basis of RAPD

RAPDs are able to differentiate a large number of isolates unique bands that can be used for fingerprinting and protecting commercial *P. fluorescens* isolates. It is important to combine molecular diversity with the antibiotic-producing property of the isolates to properly identify the different strains prevalent in crop fields. Sabir *et al.* (2013) [11] worked on the electrophoretic analysis of RAPD band profiles of *Bacillus thuringiensis* and observed the presence of polymorphism among the studied isolates. Pethannan *et al.* (2018) [8] isolated *P. aeruginosa* from various clinical samples and evaluated for their variability and genetic relationship using PCR based Randomly Amplified Polymorphic DNA (RAPD) technique. Further genetic relationship was determined using RAPD technique. A total of 7 were isolated and characterized biochemically and identified belonging to *P. aeruginosa*. Cluster analysis and phylogenetic tree reveal close relatedness between *P. aeruginosa* strain P1, P3, P4, P5 and P6 but distantly related to the P2 and P7. Molecular methods help to identify novel strains of *Pseudomonads* which will be more effective as biocontrol agents in a particular region. It is important to correlate molecular diversity with functional diversity to assess the utility of these isolates as biocontrol agents for a particular crop in local fields which can then be extrapolated to the same crop in other fields or even other crop.

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