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Analysis of genetic diversity among the isolates of *Pseudomonas fluorescens* isolated from onion rhizosphere region

■ **S. MALATHI**

AUTHOR FOR CORRESPONDENCE :
S. MALATHI
Horticultural Research Station
(T.N.A.U.), OOTY, (T.N.) INDIA
Email : malathi_agri@rediffmail.com

ABSTRACT : *Pseudomonas fluorescens* was isolated from different onion growing areas of Tamil Nadu, India and they were screened against *Fusarium oxysporum* f.sp.*cepae in vitro*. RAPD analysis was carried out using twelve random primers, each of them consisted of 10 base pairs. Analysis of the genetic co-efficient matrix derived from the scores of RAPD profile, showed that minimum and maximum per cent similarities among *Pseudomonas fluorescens* isolates were in the range of 10 to 92 per cent, respectively. Cluster analysis by using the unweighted pair-group method with arithmetic average (UPGMA), clearly separated the isolates into 2 major clusters (A and B) confirming the genetic diversity among the isolates of *Pseudomonas fluorescens* isolated from onion.

KEY WORDS : *Pseudomonas fluorescens*, Onion rhizosphere soil, Genetic diversity, RAPD

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Onion (*Allium cepa* var. *aggregatum* G.Don) is one of the important crops grown in India amongst vegetables and spices. Basal rot is the most destructive disease of onion and causes yield loss in all onion growing areas of the world (Coskuntuna and Ozer, 2008). The basal rot of onion is caused by *Fusarium oxysporum*.f.sp.*cepae*.

Fungicides were used for the management of the disease. The wide spread use of fungicides to control the disease has lead to an increase of health hazards due to their phytotoxic residual and pollution effects therefore, using some other means of disease control instead of agrochemical. Among the various antagonists used for the management of plant diseases, plant growth promoting rhizobacteria (PGPR) play a vital role. These bacteria may mediate the biocontrol by one or more of the several mechanisms of disease suppression. Because of these reasons, biological control is a good alternative method, as compared to chemical control which destroys a range of micro and macro-organisms and has a limited impact on the environment (Sigee, 1993).

Antibiotic compounds produced by fluorescent pseudomonads strains play key a role in the suppression of various soilborne plant pathogens (Thomashow and Weller, 1996). Antibiotics produced by different plant growth promoting rhizobacteria have a broad-spectrum activity. The antibiotic 2,4 diacetylphloroglucinol (2,4 DAPG) produced by several strains of *P. fluorescens*, not only have activity against a wide range of plant pathogenic fungi but also have antibacterial, antihelminthic and phytotoxic properties (Keel *et al.*, 1992 and Thomashow and Weller, 1996). Siderophores produced by fluorescent pseudomonads that inhabit the plant rhizosphere have received much attention over the past decade, largely because of their proposed role in the biological control of soil borne plant pathogens and

in disease suppressiveness (Loper, 1990).

Diverse micro-organisms inhabit in the rhizosphere regions have potential to control the soil borne pathogens which cause root disease in crops. Among the different micro-organisms fluorescent pseudomonads produces secondary metabolites which inhibit the growth of pathogens (Haas and Keel, 2003). To understand the molecular details of fluorescent pseudomonads helps us to overcome existing limitations and to designing of improved strategies for the development of biocontrol consortia (Mark *et al.,* 2006). The diversity and beneficial activity of the plant-bacterial association and its understanding is important to sustain agro-ecosystems for sustainable crop production. Highly sensitive diagnostic assays, based on the polymerase chain reaction (PCR) of target-specific sequences, have been implemented successfully for the identification of important biocontrol agents (Ravi Charan *et al*., 2010). Application of molecular marker techniques has been useful for understanding of genetic variations in the different isolates of *Pseudomonas fluorescens*. Molecular analysis using PCR-based RAPD method is useful to differentiate such strains at the intraspecific level.

The present investigation reveal to what extent the diversity exists in the different isolates of *Pseudomonas fluorescens* isolated from onion rhizosphere region by using RAPD markers.

RESEARCH METHODS

Isolation of *Pseudomonas fluorescens* from the rhizosphere region :

Pseudomonas fluorescens was isolated from the rhizosphere soil collected from onion growing areas of Tamil Nadu, India. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. Ten gram of rhizosphere soil was transferred to 250 ml Erlenmeyer flask containing 100ml of sterile distilled water. After thorough shaking, the antagonist in the suspension was isolated by serial dilution plate method. From the final dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium (King *et al.,* 1954) and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature (28±2^{0}C) for 24 hours. Colonies with characteristics of *Pseudomonas* sp. were isolated individually and purified by streak plate method (Rangaswami, 1993) on King's B medium. The pure cultures were maintained on respective agar slants at 4^{0}C.

Screening of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp.*cepae* under *in vitro* conditions :

The *Pseudomonas fluorescens* isolates were tested for their inhibitory effect on growth of *Fusarium oxysporum* f.sp.*cepae* by following the dual culture technique (Dennis and Webster, 1971). The bacterial isolates were streaked on one side of the Petri dish (1 cm away from the edge of the plate) on PDA medium and a mycelial disc (8 mm diameter) of five day old *Fusarium oxysporum* f.sp.*cepae* culture was placed on the opposite side of the Petri dish perpendicular to the bacterial streak. The plates were incubated at room temperature (28 $\pm$ 2^{o}C) for 4 days. After four days of incubation, the pathogen growth and inhibition zone were measured and expressed in mm.

DNA extraction of *Pseudomonas fluorescens* isolates:

Total DNA was isolated from bacterial strains using standard protocols (Sambrook *et al*., 1989). Bacterial cultures were grown on KB broth for 48 h at 25^{0}C. Bacterial colonies (2 mm diameter) were suspended in 100 µl of lysis solution (0.05 M NaOH), 0.25 per cent sodium dodecyl sulfate (SDS) and incubated for 15 min at 100ºC. The suspension was centrifuged for one min at 12,000 rpm and diluted 50-fold with sterile distilled water. Five microlitres of the diluted suspension was used for PCR.

RAPD-PCR analysis of *Pseudomonas fluorescens* isolates :

Genotypic characterization of the *Pseudomonas* isolates was done by using a PCR-based fingerprinting method with randomly amplified polymorphic DNA (RAPD) markers (Williams *et al.,* 1990). The heat-lysed suspension of rhizobacteria was used as DNA source. Totally 12 random ten mer primers were used for RAPD analysis. PCR-RAPD amplification were carried out in a 20 µl reaction containing 10 X *Taq* buffer (with 2.5 mM $MgCl_2$), 2 µl; 2 mM dNTP mixture, 2 µl; 2 mM primer, 5 µl; *Taq* DNA polymerase, 3 U; H_2O, 8 µl and 50 ng of template DNA samples were amplified with DNA thermo cycler (Eppendorf Master Cycler Gradient,

Westbury, New York) using the PCR conditions 92ºC for 4 min, 28ºC for 1 min and 72ºC for 2 min. The total number of cycles was 40 with the final extension time of 10 min. In each amplification reaction, a control sample without DNA was included. The amplification products were separated on a 2 per cent agarose in 1 X TBE buffer (Tris-borate, 90 mM; EDTA, 2 mM; pH 8.3) containing 0.5 µg of ethidium bromide per ml at 60 V for 3 h. The amplification products were visualized with a UV transilluminator and photographed in the gel documentation system (Alpha Innotech Corporation). All PCR - RAPD analysis were repeated at least three times and only the RAPD bands which appeared consistently were evaluated.

Analysis of RAPD-PCR results :

The banding patterns were scored for RAPD primers in each fluorescent pseudomonad isolate starting from the small size fragment to large sized one. Presence and absence of each band in each isolate was coded as 1 and 0, respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc programme version 2.02 (Exeter Software, New York, USA) described by Rohlf (1993). A dendrogram was constructed based on Jaccard's similarity co-efficient using the marker data from fluorescent Pseudomonads isolates with UPGMA (Jaccard, 1998).

RESEARCH FINDINGS AND DISCUSSION

Fifteen native isolates of *Pseudomonas fluorescens* were tested for their antagonistic activity against *Fusarium oxysporum* f.sp.*cepae* by dual culture technique. Among the isolates Pf 12 significantly exerted highest (74.68) per cent reduction of mycelial growth with 8.21 mm inhibition zone followed by Pf 27 recorded 66.28 per cent reduction of mycelial growth with 6.84 mm inhibition zone (Table 1). *Pseudomonas* isolates were the major potential biocontrol agents against foliar and soil borne pathogens. In this study, Pf 12 and Pf 27 of the *Pseudomonas* isolates were found to be the most effective in inhibiting the growth of *Fusarium oxysporum* f.sp.*cepae*. It might be due to the production of antibiotics, volatile compounds and lytic enzymes. Many strains of *Pseudomonas* have been found to produce broad spectrum antibiotics *viz*., phenazine, pyrrolnitrin, pyoverdine, 2,4-diacetylphloroglucinol (Gardener *et al*., 2000), lytic enzymes such as chitinases and b-1,3-glucanases which degrade fungal chitin (Velazhahan *et al*., 1999), production of siderophore (Loper, 1988), production of HCN (Ahl *et al*., 1986) and induced systemic resistance (Van Peer *et al*., 1991).

The RAPD analysis of fifteen *Pseudomonas* isolates with twelve random ten mer primers were

Table 1 : Efficacy of different isolates *Pseudomonas fluorescens* against *Fusarium oxysporum*.f.sp.*cepae in vitro*

Isolates	Mycelial growth (cm)*	Per cent reduction over control	Inhibition zone (mm)*
Pf5	5.02	43.01	3.54
Pf12	2.23	74.68	8.21
Pf17	7.50	14.86	0.00
Pf23	5.84	33.71	2.27
Pf27	2.97	66.28	6.84
Pf30	4.70	46.65	4.21
Pf31	6.42	27.12	1.92
Pf33	6.52	25.99	0.96
Pf39	6.42	27.12	1.21
Pf40	7.65	13.16	1.88
Pf45	7.88	10.55	0.93
Pf48	6.24	29.17	0.79
Pf49	6.80	22.81	0.65
Pf51	4.70	42.65	3.11
Pf56	6.80	22.81	0.81
Pf59	7.05	19.97	0.00
Control	8.81	-	-
C.D. (P=0.05)	0.58	-	-

*mean of three replications

amplified the DNA fragments with different molecular weight. Each RAPD pattern was compared with other patterns and Euclidean distance matrix was calculated. Of these, 4 random primers *viz*., R1, Pgs3, Pgs2 and AP12 produced easily scorable and reliable banding patterns, which were used for RAPD analysis of test isolates (Fig. 1). Analysis of the genetic coefficient matrix, derived from the scores of RAPD profile, showed

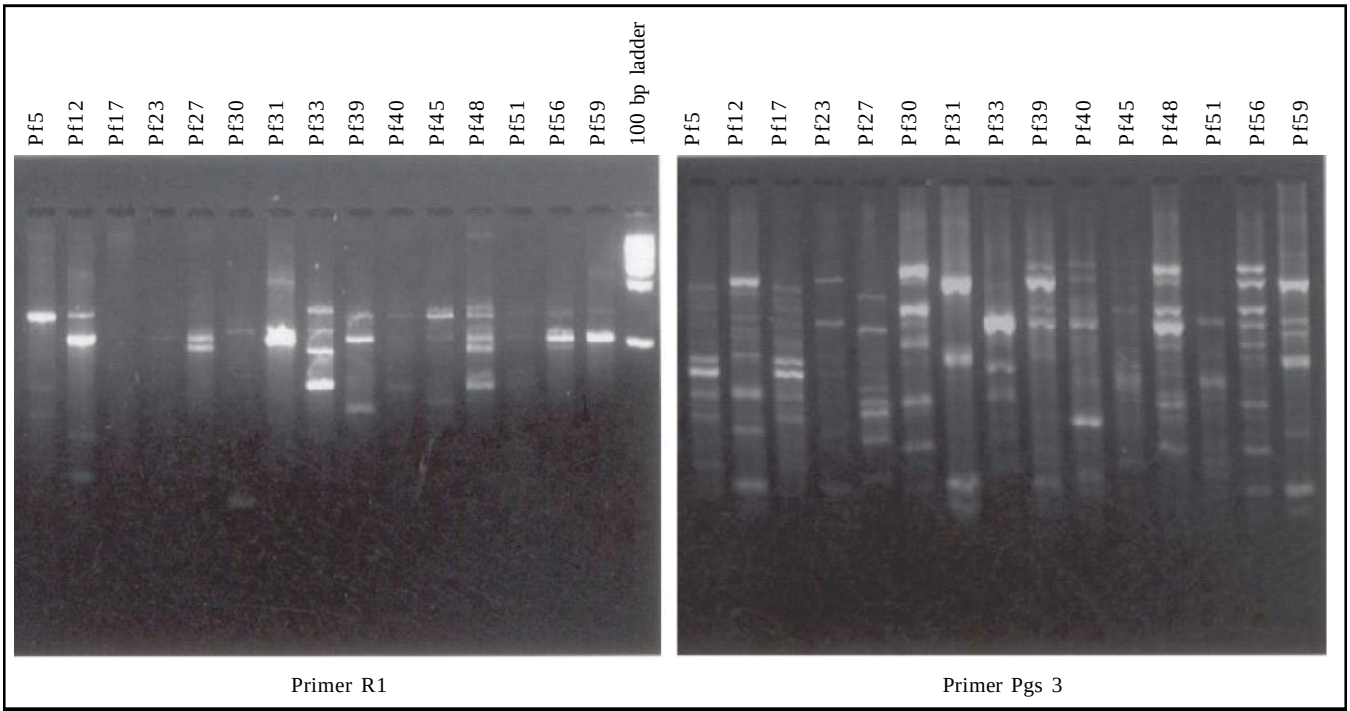

Fig. 1 : RAPD analysis of primers amplified DNA fragment of *Pseudomonas fluorescens*

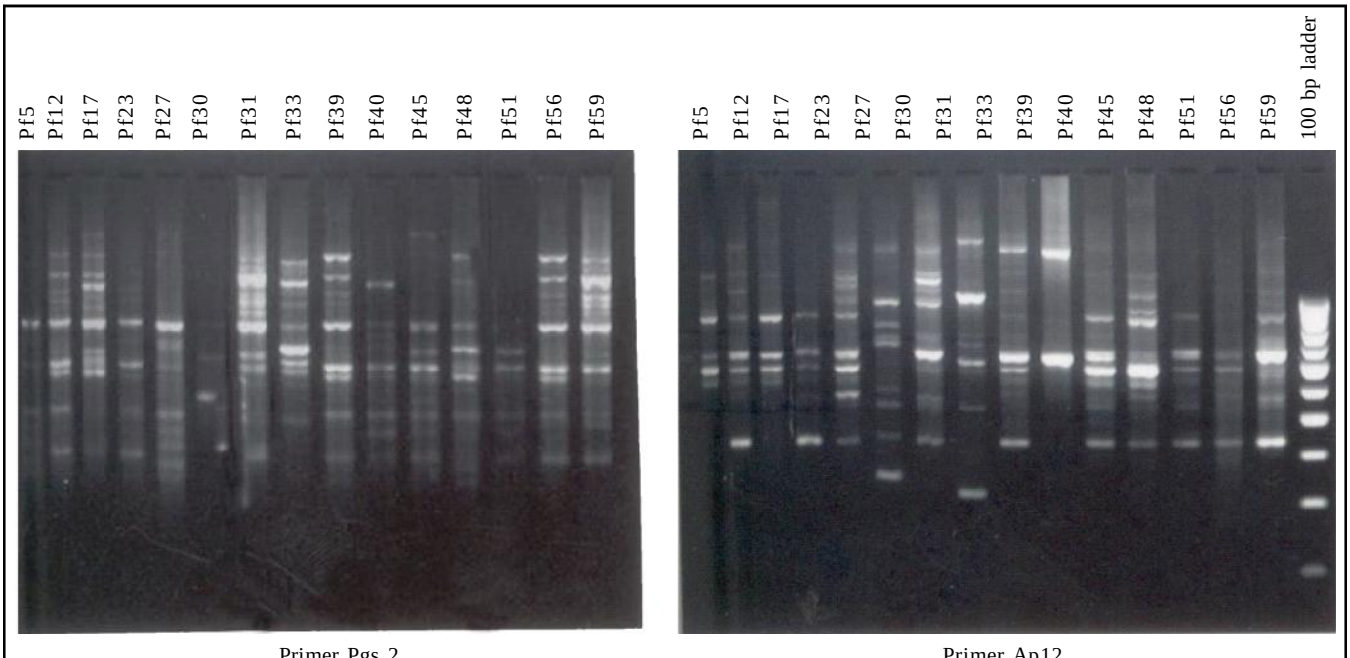

Fig. 2 : Cluster analysis of RAPD patterns of *Pseudomonas fluorescens* isolates collected from different areas of Tamil Nadu

that minimum and maximum per cent similarities among the *Pseudomonas* isolates were in the range of 10 to 92 per cent, respectively (Table 2). Cluster analysis, using UPGMA, clearly separated the isolates into 2 clusters

Table 2 : Similarity matrix of *Pseudomonas fluorescens* isolates using RAPD primers

Isolates	Pf5	Pf12	Pf17	Pf23	Pf27	Pf30	Pf31	Pf33	Pf39	Pf40	Pf45	Pf48	Pf51	Pf56	Pf59
Pf5	1.000														
Pf12	0.384	1.000													
Pf17	0.266	0.320	1.000												
Pf23	0.642	0.423	0.428	1.000											
Pf27	0.347	0.730	0.333	0.391	1.000										
Pf30	0.100	0.241	0.266	0.277	0.192	1.000									
Pf31	0.304	0.517	0.227	0.409	0.392	0.428	1.000								
Pf33	0.380	0.535	0.181	0.500	0.407	0.380	0.608	1.000							
Pf39	0.400	0.500	0.250	0.450	0.480	0.333	0.384	0.400	1.000						
Pf40	0.181	0.428	0.277	0.350	0.346	0.625	0.478	0.500	0.454	1.000					
Pf45	0.470	0.444	0.294	0.625	0.416	0.315	0.434	0.523	0.550	0.450	1.000				
Pf48	0.315	0.392	0.294	0.444	0.360	0.388	0.375	0.454	0.409	0.318	0.555	1.000			
Pf51	0.437	0.370	0.333	0.600	0.391	0.352	0.409	0.500	0.318	0.500	0.625	0.529	1.000		
Pf56	0.352	0.370	0.250	0.500	0.391	0.352	0.476	0.578	0.380	0.500	0.529	0.529	0.846	1.000	
Pf59	0.411	0.407	0.312	0.562	0.434	0.333	0.454	0.550	0.363	0.473	0.588	0.588	0.923	0.923	1.000

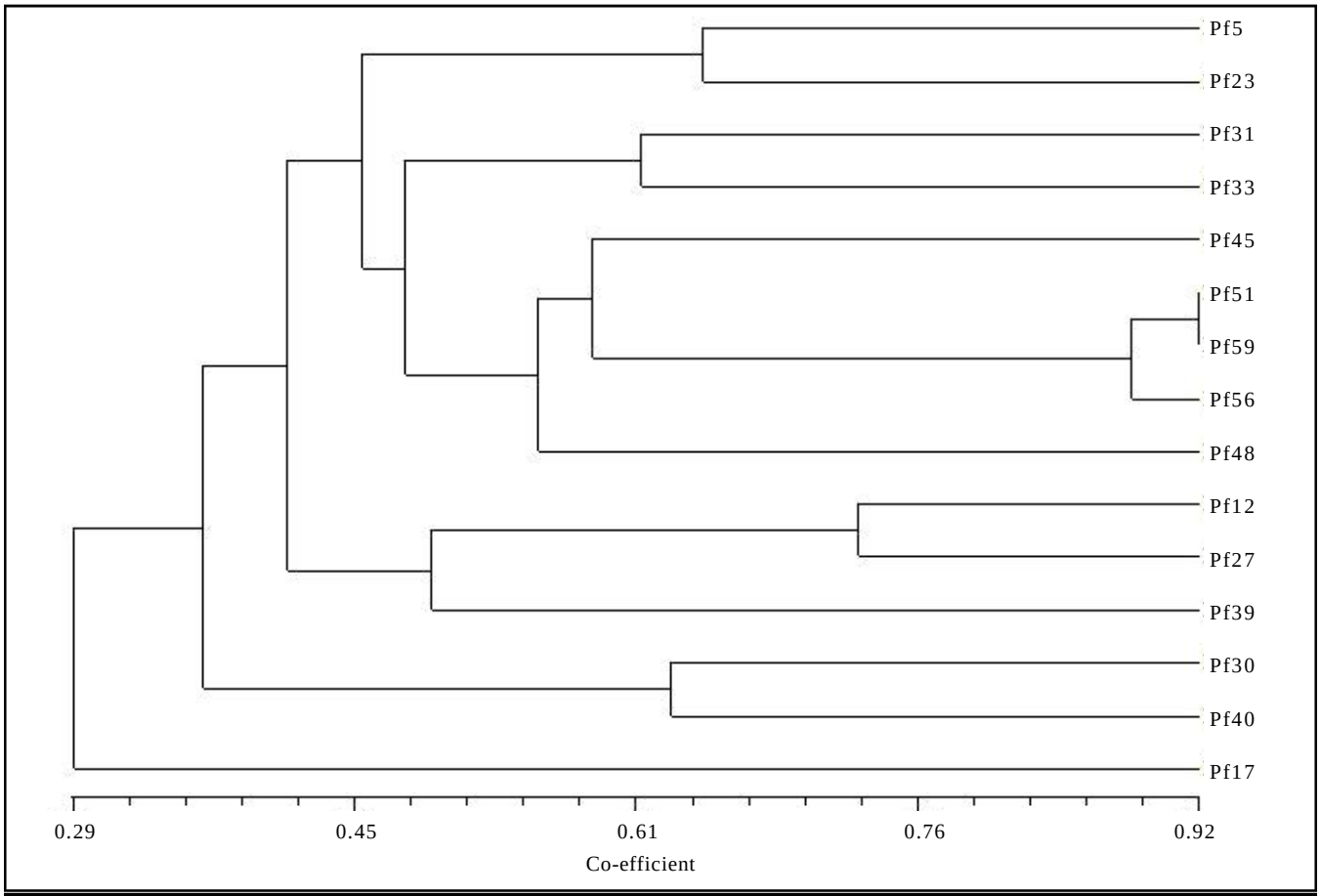

Fig. 3 : The pair wise co-efficient of similarity (Jaccard) was clustered by using the UPGMA algorithm of the NTSYS –Pc programme version 2.0

(A and B) confirming high level of genetic diversity among the isolates of *Pseudomonas*. Cluster A contains one isolate and cluster B contains 14 isolates of *Pseudomonas* (Fig. 2). The isolates Pf 56 and Pf59 showed greater similarity (92%). While the minimum similarity index was recorded at 10 per cent between Pf5 and Pf30 even they were from same cluster group, thus confirming that the *Pseudomonas* isolates collected from entirely different geographical areas can also share some genetic relatedness.

Ellis *et al*. (2000) used a collection of 29 fluorescent pseudomonads involved in the suppression of *P. ultimum* to fatty acid profiling and RFLP analysis of the ribosomal DNA operon (ribotyping) to determine the genetic diversity of the organism possessing similar functions. A small group of genetically related *Pseudomonas* sp. was identified. Each isolate was confirmed to produce diffusible bioactive product, therefore, organisms having similar properties may share some genetic relationship among themselves irrespective of geographical locations. Rameshkumar *et al*. (2002) reported that among 40 strains of fluorescent pseudomonads isolated from the rhizosphere of rice and sugarcane, 18 strains exhibited strong antifungal activity against *R. bataticola* and *F. oxysporum*. Among the isolates the strain JA 16 and MKU 3 showed similar RAPD pattern.

Biological control assumes special significance in ecology conscious and cost-effective alternative strategy for disease management. Rhizobacteria such as *P. fluorescens* and *Bacillus* strains could provide significant levels of disease suppression and substantially enhance plant growth and grain yield. Antagonistic bacteria are considered as ideal biological control agents owing to their rapid growth, easy handling and aggressive colonization of rhizosphere (Gnanamanickam *et al*., 2002).

Pseudomonas fluorescens diversity plays a major role in the success of disease management strategies. In the present study, most of the isolates from the different ecological niche were found to be strongly antagonistic to pathogens, but shared genetic homology with other isolates from entirely different region. Each isolate was confirmed to produce diffusible bioactive product, therefore, organisms having similar properties shared some genetic relationship among themselves irrespective of geographical locations. The results of the present study demonstrate that there is a high level of genetic diversity among isolates of *Pseudomonas fluorescens* in Tamil Nadu. This great genetic deviation detected at the molecular level indicates the aptitude of a biocontrol agent to adapt the different environmental conditions.

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