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RESEARCH ARTICLE

Molecular characterization of *Pseudomonas fluorescens* inhibiting the chickpea wilt pathogen *in vitro*

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ABSTRACT

Chickpea (*Cicer arietinum* L.) is the most important pulse crop of India. Wilt caused by *Fusarium oxysporum* f.sp. *ciceri* is the major limiting factor of chickpea production. The *Pseudomonas fluorescens* is used as a biocontrol agent against the chickpea wilt pathogen. However, the diversity of such bacteria that can exhibit antagonism against wilt pathogen is poorly exploited. Plant growth-promoting rhizobacterial strains belonging to fluorescent Pseudomonads were isolated from the rhizosphere of chickpea. Ten isolates which exhibited strong antifungal antagonistic activity against *Fusarium oxysporum* f.sp. *ciceri* mainly through the production of antifungal metabolites were characterized by PCR-RAPD analysis. A considerable level of molecular diversity was determined among the rhizobacterial isolates of *Pseudomonas fluorescens* isolated from different chickpea growing areas of Gujarat.

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INTRODUCTION

Pseudomonas fluorescens strains had been reported to control several diseases caused by soil borne pathogens (Vidhyasekharan and Muthemilan, 1995) and is 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 (Emmert and Handelsman, 1999). The mode of action of the antagonistic organisms against various soil borne plant pathogens, include biosynthesis of antibiotics, production of hydrolytic enzymes, production of siderophore and competition for substances (Velzahan *et al.*, 1999). Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. The main objective of this study was the characterization of *P.fluorescens* having a potential for the control of chickpea fungal pathogen, *Fusarium oxysporum* f.sp. *ciceri.*

MATERIAL AND METHODS

Isolation of microbe:

Pseudomonas fluorescens isolates used in the present experiment were isolated based on its capability to produce siderophore, indole acetic acid like compound and salycillic acid by following the method of Vlassak *et al.* (1992) from chickpea rhizosphere of ten different chickpea growing areas of Gujarat(India) using selective Kings B (KB) medium (Simon and Ridge, 1974). Isolation of the pathogenic fungus *Fusarium oxysporum* f.sp. *ciceri* was made by tissue isolation technique using solidified potato dextrose agar (PDA) medium in Petri plates (Subramanian, 1954). The list of RAPD primers used for molecular characterization of microbes has been given in Table A.

Antagonism:

Per cent growth inhibition of *Fusarium oxysporum* f.sp. *ciceri.* by *Pseudomonas fluorescens* was measured using 20 ml of King's B+PDA medium (1:1) by dual culture techniques (Reddy *et al.*, 2008) with some modification and optimization

Table A : List of RAPD primers used for molecular characterization of microbes

Sr. No.	Primer series	Sequence 5'- 3'	GC content (%)	Tm (^oC)
1.	OPA-01	CAGGCCCTTC	70	38.2
2.	OPA-02	TGCCGAGCTG	70	42.4
3.	OPA-07	GAAACGGGTG	60	34.5
4.	OPA-09	GGGTAACGCC	70	38.7
5.	OPA-11	CAATCGCCGT	60	40.8
6.	OPA-19	CAAACGTCGG	60	36.6
7.	OPD-03	GTCGCCGTCA	70	41.4
8.	OPD-05	GATGACCGCC	70	38.8
9.	OPD-15	CATCCGTGCT	60	34.9
10.	OPD-20	ACCCGGTCAC	70	38.2
11.	OPE-01	CCCAAGGTCC	70	37.3
12.	OPE-03	CCAGATGCAC	70	37.4
13.	OPE-18	GGACTGCAGA	70	42.4
14.	OPG-02	GGCACTGAGG	60	28.3
15.	OPG-11	TGCCCGTCGT	60	28.6

of media. All the inoculated plates were incubated at 30 ±1 ^{0}C. Index of antagonism was determined after six days of incubation (DAI) as described by Zarrin *et al.* (2009).

DNA extraction:

Various isolates of *Pseudomonas fluorescens* and pathogen, *Fusarium oxysporum* f.sp. *ciceri* were grown on KB and PDA broth. After 6 days of growth in incubation chambers 30^{0}C, microbes were harvested by centrifugation at 10000 rpm for 10 minutes, pallets were washed successively with chilled sterile water and chilled acetone. The dehydrated microbes obtained were desicated under vacuum to remove acetone vapours for 20 min. The dried sample was stored at -20^{0}C until further use for DNA isolation. Genomic DNA from microbes was isolated as method described by Punekar *et al.* (2006) and Ranganath *et al.* (2002).

PCR-RAPD:

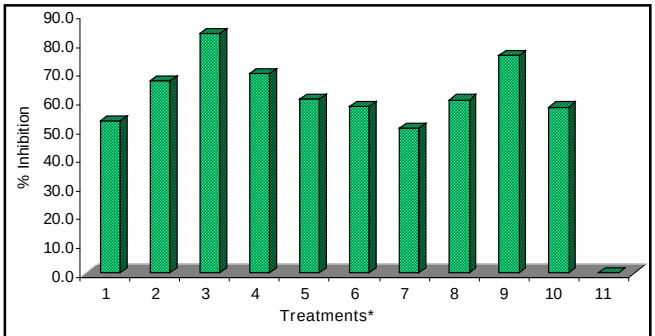
The DNA concentrations were determined spectrophotometrically at a wavelength of 260 nm. Depending on the concentration of DNA determined, a stock solution with a concentration of 1µg/µl was prepared for each isolate. These were subsequently diluted to 5 ng/µl and used in amplification. The RAPD primers used as described in Table 1 were obtained from Bangalore Genei, India.

PCR reactions were carried out in 20µl of reaction containing 10X buffer (with 2.5mM MgCl2), 2µl of 2mM dNTP mixture, 2 µl of 2µM primer, 5µl of Taq DNA polymerase 3U; 8µl of H_2O, and 15 ng of template DNA samples were amplified on DNA thermocycler. (Bio-Rad Thermal Mycycler) using the PCR conditions 94^{0}C for 1 minute, 36^{0}C for 1 minute and 72^{0}C for 2 minutes. The total numbers of cycles were 36, with the final extension time of 10 minutes. A 10 µl of each reaction was electophorosed on 1.5% 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 gel was viewed under UV Trans-illuminator for visualizing amplified DNA bands, and photographed by digital camera, and transferred to computer in JPEG format. Band positions for each isolate and primer combination were scored as either present (1) or absent (0) for phylogenetic analysis using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) system version 2.2 by Exeter Software (Rohlf, 2004). The SIMQUALK programme was used to calculate Jaccard's similarity coefficient and a graphical phenogram (dendrogram) of the genetic relatedness among the microbes was produced by means of the unweighted pair group method with arithmetic average (UPGMA) analysis (Sneath and Sokal, 1973). Size of specific bands of DNA or protein were determined using software *Alphaimager 2200* manufactured by Alpha Ease FC, USA.

RESULTS AND DISCUSSION

In the present investigation, 10 isolates of *P. fluorescens* producing siderophore, IAA like compound and salycillic acid were identified and named as *Pf-1*, *Pf-2* and so on up to *Pf-10*. All the isolates of *P. fluorescens* were found to cause inhibition of the pathogen (Fig. 1). Highest per cent growth inhibition (83.5 %) was recorded in *P. fluorescens*(*Pf-3*) followed by *Pf-9* (75.9 %) at 6 Days after inoculation. Per cent growth inhibition recorded in the present study varied from isolate to isolate. These results are in confirmation with the finding of Vidhyasekharan and Muthemilan (1995). They reported that out of twenty seven isolates, four of *P. fluorescens* were

found to be effective in reducing the radial growth of *F. oxysporum* f.sp. *ciceri*. It was also documented that *P. fluorescens* isolates significantly inhibited the growth of *F. oxysporum* f.sp. *ciceri* in chickpea crop (Inam-ul-Haq *et al*., 2003 ; Kaur *et al.*, 2007).



* Treatments: T_1: *Pf-1* x *F. oxysporum*, T_2: *Pf-2* x *F. oxysporum*, T_3: *Pf-3* x *F. oxysporum*, T_4: *Pf-4* x *F. oxysporum*, T_5: *Pf-5* x *F. oxysporum*, T_6: *Pf-6* x *F. oxysporum*, T_7: *Pf-7* x *F. oxysporum*, T_8: *Pf-8* x *F. oxysporum*, T_9: *Pf-9* x *F. oxysporum*, T_{10}: *Pf-10* x *F. oxysporum*, T_{11}: *F. oxysporum*** alone

Fig. 1 : Growth inhibition of *Fusarium oxysporum* f.sp. *ciceri* during *in vitro* antagonism with *Pseudomonas fluorescens* isolates at 6-day after inoculation (DAI)

The polymorphisms can be detected by the use of random amplified polymorphic DNA (RAPD) which does not require prior knowledge of the genome. RAPD has been commonly used for finger printing biological control agents (Chapon *et al.*, 2002). In the present investigation, 15 primers were examined and of these, three primer (OPA-02, OPE-18 and OPG-02) failed to give any amplified product of DNA. Thus, 12 out of 15 primers were selected for evaluating the molecular differences existing in 10 isolates of *Pseudomonas fluorescens* which inhibits the growth of fungal pathogen, *F. oxysporum* f.sp. *ciceri*. However, molecular characterization of pathogen, *F. oxysporum* f.sp. *ciceri* was also carried out to identify the genetic variation and diversity and it was compared with various isolates of *P. fluorescens*. A total 160 bands were produced by 12 RAPD primers with an average frequency of 13 bands per primer. Total 148 polymorphic bands were generated out of which 114 were polymorphic and shared between at least two individuals. The 37 bands were polymorphic and unique while 12 were monomorphic (Table 1). The per cent polymorphism furnished by each primer ranged between 63.6 and 100.

The calculated PIC values for RAPD markers ranged from 0.687 to 0.934 (Table 1). The lowest PIC values obtained by OPD-20 and highest was with OPD-03. Thus, on the bases of PIC value, primer OPD-03 (Plate 1) gave best results among the primer used for characterization of *P. fluorescens* isolates studied. The RAPD profile of present investigation showed only with 12 random primers, hence screening of more number of primers is recommended to evaluate the present set of *P. fluorescens* isolates, effectively.

The Jaccards similarity coefficient showed homomorphism between some isolates of *P. fluorescens*. The single fungal pathogen (*F. oxysporum* f.sp. *ciceri*) showed the minimum similarity with the group of *P. fluorescens* isolates (Table 2). The dendrogram obtained indicates that there was a major cluster consisting of 10 isolates of *Pseudomonas fluorescens* of total 11 microbes, whereas one

Table 1 : Polymorphism obtained with different RAPD primers generated from ten *Pseudomonas fluorescens* isolates and *Fusarium oxysporum* f.sp. *ciceri*

Sr. No.	Name of primer	Polymorphic bands			Mono morph. bands	Total bands	Poly-mor. (%)	PIC
		S	U	T				
1.	OPA-01	10	3	13	2	15	86.7	0.912
2.	OPA-07	11	2	13	1	14	92.9	0.897
3.	OPA-09	8	1	9	1	10	90.0	0.864
4.	OPA-11	8	3	11	1	12	91.7	0.882
5.	OPA-19	11	5	16	0	16	100.0	0.915
6.	OPD-03	16	4	20	0	20	100.0	0.934
7.	OPD-05	11	8	19	1	20	95.0	0.925
8.	OPD-15	5	2	7	4	11	63.6	0.882
9.	OPD-20	4	0	4	0	4	100.0	0.687
10.	OPE-01	9	4	13	0	13	100.0	0.891
11.	OPE-03	7	2	9	2	11	81.8	0.887
12.	OPG-11	12	2	14	0	14	100.0	0.915
	Total	111	37	148	12	160		

S = Shared; U = Unique;
T = Total polymorphic bands;
PIC = Polymorphism information content;

Table 2 : Jaccard's similarity coefficient between the *Pseudomonas fluorescens* isolates based on the RAPD data

	Pf-1	*Pf-2*	*Pf-3*	*Pf-4*	*Pf-5*	*Pf-6*	*Pf-7*	*Pf-8*	*Pf-9*	*Pf-10*	*Foc*.*
Pf-1	1.000										
Pf-2	0.667	1.000									
Pf-3	0.288	0.377	1.000								
Pf-4	0.740	0.725	0.327	1.000							
Pf-5	0.625	0.693	0.422	0.683	1.000						
Pf-6	0.359	0.425	0.588	0.410	0.495	1.000					
Pf-7	0.707	0.693	0.318	0.750	0.619	0.368	1.000				
Pf-8	0.717	0.755	0.315	0.853	0.613	0.353	0.800	1.000			
Pf-9	0.730	0.716	0.355	0.827	0.657	0.402	0.706	0.862	1.000		
Pf-10	0.405	0.375	0.363	0.397	0.414	0.351	0.414	0.410	0.400	1.000	
*Foc.**	0.310	0.304	0.188	0.305	0.233	0.184	0.282	0.304	0.308	0.260	1.000

* Foc.= *Fusarium oxysporum* f.sp. *ciceri*

fungal pathogen (*F. oxysporum* f.sp. *ciceri*) was found to be different from the rest of *P. fluorescens* isolates (Fig. 2). Out of ten *Pseudomonas fluorescens* isolates, *Pf-1*, *Pf-9*, *Pf-8*, *Pf-4* and *Pf-7* showed more than 70 % similarity. This confirms that bacteria isolated from different geographical areas can also share some genetic relatedness. Sub cluster A_2 consisted of 2 isolates, and shared 58% similarity. These two isolates *Pf-3* and *Pf-6* gave 83.5% and 58.2 % inhibition against test fungus, *F. oxysporum* f.sp. *ciceri*, respectively.

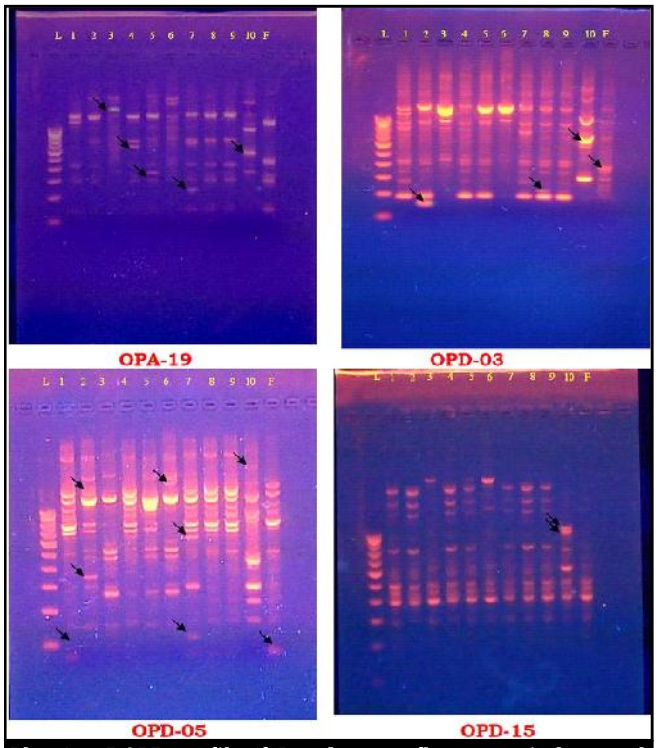
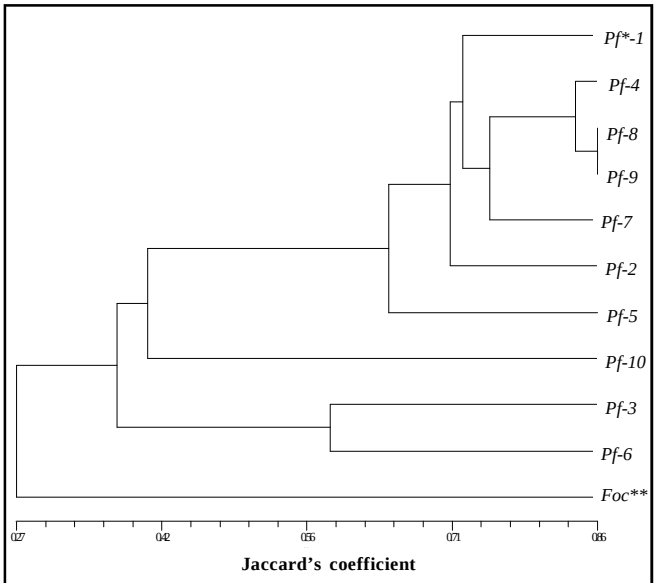


Plate 1: RAPD profile of *Pseudomonas fluorescens* isolates and *Fusarium oxysporum* f.sp. *ciceri* obtained with different primers. (L=0.1 to 1.0 kbp, 1 to 10 = *Pseudomonas fluorescens* isolates Pf-1 to Pf-10, F= *Fusarium oxysporum* f.sp. *ciceri)*

In view of the growth inhibition of fungal pathogen, these two isolates significantly differed; however, they fell in the same group (Fig. 2). The highest growth inhibition of pathogen, *Fusarium oxysporum* f.sp. *ciceri* was 83.5% and 75.9% by *Pf-3* and *Pf-9*, respectively, during *in vitro* study which were placed in different cluster. However, dendrogram showed no clear-cut grouping of the *Pseudomonas fluorescens* isolates by the level of antagonism.

RAPD markers linked to antagonism of 10 isolates of *Pseudomonas fluorescens* with pathogen, *Fusarium oxysporum* f.sp. *ciceri* have been tabulated in Table 3. The highest (83.5%) fungal growth inhibition was achieved by



*Pf = *Pseudomonas fluorescens*
** Foc=*Fusarium oxysporum* f.sp. ciceri

Fig. 2 : Dendrogram depicting the genetic relationship among the ten isolates of *Pseudomonas fluorescens* isolates based on the RAPD data

Pf-3 during *in vitro* antagonism followed by *Pf-9* (75.9 %). RAPD markers linked to this trait were noticed with some primers used in the present investigation. Total three primer out of twelve, primer OPA-01, OPA-11 and OPA-19 gave three specific amplicon (709, 363 and 1805 bp, respectively) for *Pf-3* which were not yielded by any other *Pseudomonas fluorescens* isolates. Similarly, OPA-07 and OPA-11 detected 2018 and 339 bp markers associated with *Pf-9*. However, OPA-11 generated common RAPD markers of 363 and 339 bp for *Pf-3* and *Pf-9* (first two highest inhibitory acting antagonists). Primer OPA-7 generated unique band of DNA for pathogen, *Fusarium oxysporum* f.sp. *ciceri* only and produced 443, 1011 bp markers which may be useful to identify pathogen from *Pseudomonas fluorescens* isolates. However, number of other primer also generated unique marker band from DNA of *Fusarium oxysporum* f.sp. *ciceri* (Table 3). The maximum 8 numbers of unique marker bands of varying base pairs were generated by primer OPD- 05 for different isolates of *Pseudomonas fluorescens* as well as fungal pathogen, *Fusarium oxysporum* f.sp. *ciceri*. Similarly, for *Pf-10* and *Fusarium oxysporum* f.sp. *ciceri*, maximum primers used in the present study generated unique marker bands of varying base pairs.

Literature showed efficiency of RAPD to identify various *Pseudomonas fluorescens* isolates and also fungal pathogen through molecular characterization. Davide *et al.* (2007) used twelve arbitrary decamer primers to generate RAPD marker patterns from 46 bacterial strains. The sizes of the amplified DNA fragments obtained were 390 bp to 1100 bp for different markers. The other 10 markers either did not generate any *Pf153*-specific amplicon or did not show any consistent amplifications for all the bacterial strains tested. Reddy and Rao (2009) studied 30 isolates of *Pseudomonas fluorescens*. They found genetic variation with set of 11 RAPD primers of OPA series exhibiting 16.66 to 83 % similarity, except *PF-011* which showed 0 % similarity with standard strain. Kumar *et al.* (2002) reported from RAPD analysis of *Pseudomonas fluorescens* that bacteria isolated from entirely different geographical areas can also share some genetic relatedness. Singh *et al.* (2006) studied 30 isolates of pathogen, *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolated from rhizosphere soil of chickpea from different locations in Northern India. They found genetic variation with set of 40 RAPD primers.

Thus, the RAPD analysis of *Pseudomonas fluorescens* isolates using random primers, showed presence of high level of polymorphism. The calculated PIC values ranged from 0.687 to 0.934**.** On the bases of PIC value, primer OPD-03

Table 3 : RAPD markers associated with characterization of *Pseudomonas fluorescens* isolates and *Fusarium oxysporum* f.sp. *ciceri*

Sr. No.	Name of primer	Molecular markers (bp)										
		Pseudomonas fluorescens										*Foc*
		1	2	3	4	5	6	7	8	9	10	13
1.	OPA-01	–	–	709	–	–	–	–	–	–	259	173
											1609	
2.	OPA-07	–	–	–	–	–	–	–	–	2018	–	443
												1011
3.	OPA-09	–	–	–	–	–	1071	–	–	–	–	–
4.	OPA-11	–	–	363	–	–	–	–	–	339	–	417
5.	OPA-19	–	–	1805	681	360	–	223	–	–	592	-
6.	OPD-03	–	149	–	–	–	–	–	201	–	949	325
7.	OPD-05	124	345	–	–	–	1695	194	–	–	2267	109
			1560					680				
8.	OPD-15	–	–	–	–	–	–	–	–	–	1092	–
											1245	
9.	OPD-20	–	–	–	–	–	–	–	–	–	–	–
10.	OPE-01	337	291	–	–	–	–	–	–	–	–	478
												1020
11.	OPE-03	–	–	–	–	–	–	–	–	–	266	–
											675	
12.	OPG-11	–	–	–	–	–	2935	–	–	–	–	–
							3451					
	Total	2	3	3	1	1	4	3	1	2	9	8

gave best results for characterization of *Pf*. Three RAPD primer OPA-01, OPA-11 and OPA-19 gave three specific amplicons (709, 363 and 1805 bp, respectively) for *Pf-3*, the best performing isolates.

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