Morphological and molecular characterization of fluorescent pseudomonads isolates from chickpea and mungbean rhizosphere soil of Gujarat

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Abstract

Nineteen isolates were obtained in the serial dilution range of 10^{-2} to 10^{-5} on King's B medium (KB) designated as Pseudomonas fluorescens Pf-1 to Pf-19 from chickpea and mungbean. All the characteristics were dominant for the taxonomical classification of P. fluorescens. The isolates were characterized by standard cultural, morphological and molecular methods, they were found to be rod shaped, colonies dominantly irregular, undulate, convex, smooth and slimy, transparent and characteristic smell on King's B (KB) plate with typical fluorescence on KB medium under UV light and having antagonistic activity against pathogens viz. Macrophomina phaseolina and Rhizoctonia solani causing dry and wet root rot of chickpea and mungbean. Isolates produced yellow-green diffusible pigment of variable intensities on KB medium. 16s rRNA specific region of the ribosomal operon amplified regions from 900-1000 bp for the precise species-level identification of P. fluorescens.

The isolates were identified as P. fluorescens respectively on the basis of 16s rDNA sequence homology. 16s rRNA sequences were subjected to multiple alignments analysis by Tom Hall's offline tool BioEdit. Both intraspecific and interspecific variations were observed in the 16s rRNA sequences. The phylogenetic tree constructed based on the 16s rDNA sequence showed similarity with closely related strains of P. fluorescens using Multiple Sequence Alignment ClustalW software. Isolate Pf-5 (Kheda district) of P. fluorescens depicted best inhibition against M. phaseolina and R. solani.

Keywords: Characterization, Fluorescent Pseudomonads, Operon, 16s rRNA and Phylogenetic tree.

Introduction

Increasing awareness on the deleterious effects of indiscriminate usage of agrochemicals on the environment, human and animals health has led to a search for alternatives to manage biotic stress in legume crops. Furthermore, the usage of fungicides against plant pathogens produces a negative impact on the nodulation of legumes by nitrogen-fixing beneficial bacteria.[14]

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The use of antagonistic plant growth-promoting bacteria has been realized in the recent past. Such bacterial antagonist offers great potential as alternatives to agrochemicals. The specific inherent qualities of the antagonistic bacteria are competition, antibiosis and plant growth promotion.

Among the plant growth promoting rhizobacteria (PGPR), fluorescent pseudomonads are the most exploited bacteria for biological control of soil-borne and foliar plant pathogens. Fluorescent pseudomonads are non-pathogenic rhizobacteria which suppress the soil-borne pathogens through rhizosphere colonization, antibiosis, iron chelation by siderophore production and induced systemic resistance (ISR).

It is now well recognized that Fluorescent pseudomonads are among the most effective rhizosphere bacteria because in addition to disease control, they exert a beneficial effect on plant growth promotion.[5,20]

Material and Methods

The present studies were carried out during 2017-18 and 2018-19 at Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat.

Isolation of *P. fluorescens* from chickpea and mungbean rhizosphere soil: Soil samples were collected from 12 different districts of Gujarat growing chickpea and mungbean (Table 1) and isolations were done to get isolates of *P. fluorescens*. The serial dilution plate technique was used for isolation of the *P. fluorescens*. One gram of soil was transferred aseptically into a test tube having 9 ml distilled water and contents were mixed properly by shaking for five minutes.

One millilitre of aliquot was drawn and transferred to 9 ml distilled water blank (containing sterilized water). The suspension was shaken for one minute before further dilution. Dilutions of 10^{-2} to 10^{-5} were obtained and used for isolation of *P. fluorescens*. Twenty millilitre of King's B medium (KB) was poured into pre-sterilized Petri plates. After solidification of the medium, 0.5 ml of the suspension from each dilution was transferred aseptically into sterilized Petri plates and spread evenly over the surface of the medium.

The plates were incubated at 28±2°C temperature. Development of bacterial colonies was periodically observed up to the seventh day.

Table 1
Isolates of *P. fluorescens*

Isolate No.	Rhizosphere soil	District	Place	Isolate designation
1	Mungbean	Anand	Asodar	Pf-1
2	Mungbean	Anand	Napa	Pf-2
3	Mungbean	Anand	Anklav	Pf-3
4	Mungbean	Anand	Verakhadi	Pf-4
5	Chickpea	Kheda	Umreth	Pf-5
6	Chickpea	Kheda	Lingda	Pf-6
7	Mungbean	Sabarkantha	Moyad	Pf-7
8	Chickpea	Junagadh	Palasva	Pf-8
9	Chickpea	Junagadh	Ambaliya	Pf-9
10	Mungbean	Navsari	Bilimora	Pf-10
11	Mungbean	Banaskantha	Vav	Pf-11
12	Chickpea	Dahod	Jhalod	Pf-12
13	Chickpea	Dahod	Fatepura	Pf-13
14	Mungbean	Mehsana	Badalpura	Pf-14
15	Mungbean	Bhavnagar	Lilivav	Pf-15
16	Mungbean	Vadodara	Padra	Pf-16
17	Chickpea	Ahmedabad	Bavla	Pf-17
18	Chickpea	Kutch	Bharudia	Pf-18
19	Mungbean	Kutch	Amardi	Pf-19

The plates were observed for the appearance of *P. fluorescens* colonies under the microscope and sub-cultured on NA Petri plates for purification. Observations were recorded on morphological characteristics like colony colour, type and growth of colony after seven days of incubation under ultraviolet (UV) light.

DNA isolation: Total genomic DNA of the individual isolate was extracted as described by Nubel et al[16] with certain modifications.

16s rDNA sequence analysis: Genomic DNA was amplified by using PCR Pseudomonas-specific primer set including forward primer, 16s-for (5`-GTGTAGCGGTGAAATGCG-3`) and the reverse primer, 16s-rev (5`-ACGGGCGGTGTGTACAA-3`) for amplification of 900-1000 bp Pseudomonas-specific gene region. For amplification of these primer set, 25 µl of reaction mixture included 2.5 µl (20 ng) of bacterial DNA as template, 2 µl of 10x buffer for Taq DNA polymerase (100 mM of Tris–HCl and 15 mM $MgCl_2$), 0.8 µM of each primer, 2.5 mM of dNTPs and 0.8 µl (3U/ µl) of Taq DNA polymerase (MBI Fermentas AG).

The reaction condition for PCR amplification includes an initial denaturation of 5 min at 95 °C followed by 35 cycles of 1.5 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C with the final extension of 5 min at 72 °C. The amplification was carried out on Biometra thermal cycler. Amplified DNA was electrophoresed in 1.2 % agarose gel at 80 V for 2 h and visualized under Alpha EaseFC4.0.0 UV Gel documentation system.

The sequencing of the PCR product was carried out in automated sequencer ABI 3730 genetic analyzer at Xcelris Genomics Co., Ahmedabad, Gujarat. Sequences were searched using BLAST (Basic Local Alignment Search Tool) program from the GenBank database of NCBI (National Centre for Biotechnology Information), USA.[2] The multiple sequence alignment and pair wise alignment were made using BioEdit version 5.09.[7] The neighbourhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X program version 1.81.[23]

Biocontrol efficacy of *P. fluorescens* isolates against *M. phaseolina* and *R. solani*: Seed and soil-borne pathogens viz. *M. phaseolina* (dry root rot) and *R. solani* (wet root rot) were isolated from respective chickpea and mungbean plants showing typical disease symptoms. *P. fluorescens* isolates were screened against pathogens i.e. *M. phaseolina* and *R. solani* by dual culture method described by Dennis and Webster[4] using PDA medium. PDA medium (20 ml) and were poured in 90 mm sterilized Petri plates and inoculated with the *P. fluorescens* isolates and pathogen *M. phaseolina* and *R. solani* (5 mm disc of 8-10 days old pure culture) opposite to each other near the periphery of Petri plate.

The plates were inoculated with the pathogen alone serving as control. The plates were incubated at 27±1°C temperature. After seven days of incubation, the growth of the pathogen and bioagent was measured and per cent growth inhibition was calculated by the following formula suggested by Vincent[24]. The experiment was carried out for both the pathogens separately.

$$\text{Growth inhibition (\%)} = \frac{\text{DC} - \text{DT}}{\text{DC}} \times 100$$

where DC= Mean diameter of mycelial colony in control treatment (mm) and DT= Mean diameter of mycelial colony in treated set (mm).

Results and Discussion

Isolation of *P. fluorescens* isolates: The nineteen isolates were obtained from rhizosphere soil samples of chickpea and mungbean from different villages of twelve districts of Gujarat (Table 1) following the serial dilution plate technique. Samples (approx. 500g) from the rhizosphere of the chickpea and mungbean from the five different locations, one each from the corner and one from the centre of the field were collected and pooled to get a uniform sample. Nineteen isolates were obtained in the serial dilution range of 10^{-2} to 10^{-5} on KB medium designated as *Pseudomonas fluorescens* Pf-1 to Pf-19 (Fig. 1).

Fig. 1: Pure colony of *P. fluorescens*

Cultural and morphological characterization: The colour of the colony of all the isolates was slimish cream and there was slight pigment production. The isolates Pf-1, Pf-18 and Pf-19 formed regular shape, undulate margin, convex elevation, smooth texture, opaque and characteristic water droplet within colonies. The isolates Pf-2, Pf-3, Pf-6, Pf-7, Pf-11, Pf-16 and Pf-17 depicted irregular shape, entire to wrinkle margin, raised to flat elevation, smooth to rough texture, opaque to transparent and characteristic smell to water droplet within colonies whereas Pf-4, Pf-5, Pf-8 and Pf-15 isolates showed round shape, entire margin, smooth texture and characteristic smell while Pf-9, Pf-10, Pf-12 and Pf-13 isolates formed small shape, undulate to entire margin with characteristic smell (Table 2).

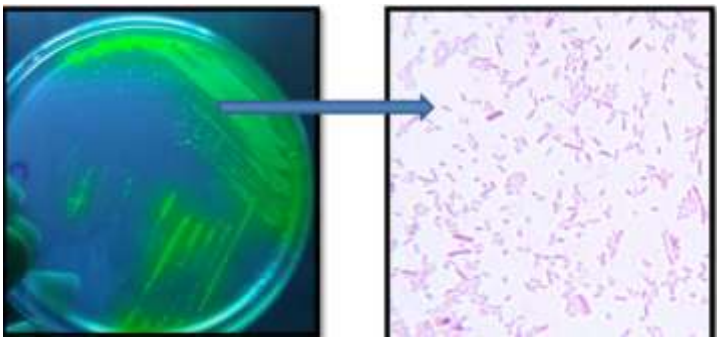
It is a rod-shaped bacterium observed under 100x objective of a light microscope (Fig. 2, ii). The colonies of the isolates were dominantly irregular, undulate, the convex, smooth and slimy, transparent and characteristic smell on KB plate. The colony showed fluorescence on King's B agar medium when observed under UV light (Fig. 2, i). Isolates produced yellow-green diffusible pigment of variable intensities on King's B medium.

These characteristics were regarded as taxonomically useful characteristics for *P. fluorescens*. Colony growth on King's B media was surrounded by fluorescent colour. Manjunatha et al[13] also mentioned that pigmented rhizobacteria that were gram-negative rod shaped belonged to *P. fluorescens* with pigment of colony as the most distinctive cultural characteristics of the microorganism which resulted from yellow to deep purple under different cultural conditions.

Shahzaman et al[21] obtained thirty isolates of *P. fluorescens* were isolated from the rhizosphere of chickpea fields in the Punjab district of Pakistan and observed that all the four isolates viz. Pf 1, Pf 3, Pf 5 and Pf 8 produced round shaped and yellowish colonies and rod shaped cells. Manasa et al[11] isolated fifteen *Pseudomonas* sp. isolates and observed that all the isolates developed small to medium, smooth, glistening colonies; out of the 15 isolates 6 isolates showed yellowish green colour with light green pigmentation and the remaining isolates showed dull white colonies with no pigmentation. These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope.

Pothiraj et al[17] collected ten isolates of *P. fluorescens* on King's B medium from rhizosphere soil of different crops and observed gram-negative rod shaped and fluorescence characteristics of the bacteria.

16s rDNA sequence analysis: All the nineteen isolates belonging to fluorescent pseudomonads from rhizospheric soils of chickpea and mungbean were identified by sequencing of 16s rRNA gene of ribosomal DNA. 16s rRNA gene was amplified in all the nineteen isolates and gave amplicons ranging in size from 900-1000 bp (Fig. 3).



(i) **(ii)**

Fig. 2: (i) Fluorescence observation under UV light and (ii) microscopic view of *P. fluorescens*

Table 2
Colony characteristics of isolates of *P. fluorescens*

Isolates	Colony characteristics					
	Shape	**Margin**	**Elevation**	**Texture**	**Opacity**	**Special characteristics**
Pf-1	Regular	Undulate	Convex	Smooth & slimy	Opaque	Water droplet
Pf-2	Irregular	Entire	Slightly raised	Smooth	Opaque	Characteristic smell
Pf-3	Irregular	Entire	Slightly raised	Smooth	Opaque	Characteristic smell
Pf-4	Round	Entire	Slightly raised	Smooth & slimy	Opaque	Characteristic smell
Pf -5	Round	Entire	Convex	Smooth	Transparent	Characteristic smell
Pf-6	Irregular	Wrinkle	Flat	Rough	Opaque	Water droplet
Pf-7	Irregular	Wrinkle	Flat	Rough	Opaque	Characteristic smell
Pf-8	Round	Entire	Slightly raised	Smooth	Transparent	Characteristic smell
Pf-9	Small	Undulate	Convex	Smooth & slimy	Opaque	Characteristic smell
Pf-10	Small	Entire	Slightly raised	Smooth	Opaque	Characteristic smell
Pf-11	Irregular	Entire	Slightly raised	Smooth	Opaque	Characteristic smell
Pf-12	Small	Undulate	Convex	Smooth & slimy	Opaque	Characteristic smell
Pf-13	Small	Undulate	Convex	Smooth & slimy	Opaque	Characteristic smell
Pf-14	Irregular	Undulate	Convex	Smooth & slimy	Transparent	Characteristic smell
Pf-15	Round	Entire	Slightly raised	Smooth	Transparent	Characteristic smell
Pf-16	Irregular	Wrinkle	Flat	Rough	Opaque	Water droplet
Pf-17	Irregular	Wrinkle	Flat	Rough	Opaque	Water droplet
Pf-18	Regular	Undulate	Convex	Smooth & slimy	Opaque	Water droplet
Pf-19	Regular	Undulate	Convex	Smooth & slimy	Opaque	Water droplet

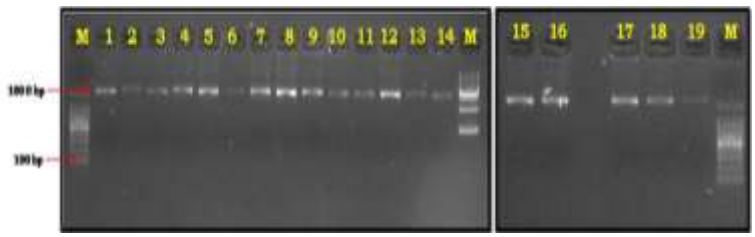


Fig. 3: 16s rRNA gene amplification from isolates of *P. fluorescens*

Amplicons were cloned and sequenced by Automated Sequencer ABI 3730 genetic analyzer at Xcelris Genomics Co., Ahmedabad. Sequences and then subjected for BLAST analysis for its identity and confirmation and subsequently submitted to the National Centre for Biotechnology Information (NCBI) GenBank. All isolates are monomorphic indicating that all are same. The accession number for sequences of 16s rRNA gene for the nineteen isolates of *Pseudomonas* spp. is presented in table 3.

Multiple alignments of nucleotide sequences of 16s rRNA gene of *P. fluorescens* isolates: 16s rRNA sequences were subjected to multiple alignments analysis by Tom Hall's offline tool BioEdit. Both intraspecific and interspecific variations were observed in the 16s rRNA sequences. Among the isolates, isolates Pf-7, Pf-8 and Pf-9 with Pf-17, Pf-18 and Pf-19 showed the highest distance among likelihood and isolate Pf-2 also showed the highest distance with isolate Pf-7, Pf-8 and Pf-9. Isolates Pf-12 with Pf-17, Pf-18 and Pf-19 showed a similar pattern of likelihood. This indicates that isolates are different from each other at the genetic level.

The lowest distance was observed with isolates Pf-7, Pf-8 and Pf-9 with Pf-12. It indicates that these isolates are genetically more related as compared to other isolates under study. Multiple sequence alignment revealed that quite a number of gaps were introduced in the alignment within the 16s rRNA region of rDNA. Position of 16s rRNA sequences showing variation is depicted in fig. 4.

Phylogenetic analysis of 16s rRNA sequences: The phylogenetic tree was constructed by using MEGA 5.10 version software with nucleotide sequences of the sequenced 16s rRNA gene of all the nineteen isolates. *P. fluorescens* was isolated from chickpea and mungbean rhizospheric soil. Nucleotide sequence of all these isolates separated them into fourteen distinct phylogenetic sub-clades (Fig. 5).

Group-1 included isolate of Pf-15 and Pf-14, group-2: Pf-16; group-3: Pf-1; group-4 Pf-3; group-5 Pf-13; group-6: Pf-11 and Pf-10; group-7: Pf-6; group-8: Pf-5 and Pf-4; group-9: Pf-12; group-10: Pf-9; group-11: Pf-8 and Pf-7; group-12: Pf-19; group-13: Pf-18 and group-14: Pf-17 and Pf-2. Joint phylogenetic analysis of all the nucleotide sequences of the 16s rRNA region exhibited significant phylogenetic distance of about 100 per cent. Phylogenetic analysis showed that isolates Pf-1, Pf-10, Pf-11, Pf-14, Pf-15, Pf-16 and Pf-13 were grouped together and they have been isolated from mungbean and chickpea irrespective of the area of collection. Isolates Pf-4, Pf-5 and Pf-6 were collected from one region.

However, their source of the collection was different (isolate Pf-4 from mungbean from Anand, isolate Pf-5 and Pf-6 from chickpea from Kheda). These indicated that isolate Pf-5 and Pf-6 had similarity at the genetic level that matches with geographical location. A similar pattern was observed for isolate Pf-8, Pf-9 (chickpea from Junagadh) and Pf-12 was isolated from chickpea (Dahod) while isolate Pf-7 was isolated from mungbean (Sabarkantha). Isolates Pf-17, Pf-18 and Pf-19 were collected from Ahmedabad and Kutch region. In these isolates, isolate Pf-18 and Pf-19 were isolated from the Kutch region, while Pf-2 and Pf-17 were isolated from Anand and Ahmedabad region respectively.

All details of likelihood similarity among the total isolates are shown in the similarity index (Table 4). Multiple sequence alignment revealed that quite a number of gaps were introduced in the alignment within the 16s rRNA gene of ribosomal DNA.

The results of the present investigation based on 16s rRNA region corroborate with the results achieved by Issar et al[8], Rameshkumar et al[19], Manjunatha and Naik[12], Rajwar and Sahgal[18] and Garrido-Sanz et al.[6] They characterized strains of *Pseudomonas* spp. based on sequencing of 16s rRNA gene and applied multigene approaches especially the 16s rRNA specific gene of the ribosomal operon for the identification of *Pseudomonas* species as well as phylogenomic analysis validating the results based on the PCR-based system for species-level classification of *P. fluorescens.*

Biocontrol efficacy of isolates of *P. fluorescens* against *M. phaseolina*: The results presented in table 5 revealed that all the isolates of *P. fluorescens* and native *T. asperellum* significantly inhibited the mycelial growth of *M. phaseolina***.** Significantly minimum mycelial growth of *M. phaseolina i.e.*13.18 mm was observed with Pf-5 isolate with maximum growth inhibition of 85.36 per cent followed by isolate Pf-1 with 21.15 mm of mycelial growth with 76.50 per cent growth inhibition, Pf-6 with 21.65 mm of mycelial growth with 75.94 per cent growth inhibition, Pf-12 with 23.55 mm of mycelial growth with 73.83 per cent growth inhibition and isolate Pf-13 with 24.06 mm of mycelial growth with 73.27 per cent growth inhibition after 7 days of inoculation.

Similarly, native isolate of *T. asperellum* significantly inhibited the mycelial growth of *M. phaseolina* i.e. 86.21 per cent (Fig. 6, i). Therefore, the isolate Pf-5 of *P. fluorescens* and native isolate of *T. asperellum* showing best inhibition against *M. phaseolina* were selected for further studies against biotic and abiotic stresses.

Table 3
NCBI GenBank accession numbers of isolates of *P. fluorescens*

Isolates	Identified as	Gene Accession No.	E Value	Identity (%)	Reference Gene Accession No.
Pf-1	*P. fluorescens*	MN099290.1	0.0	100	KX385199.1
Pf-2	*P. fluorescens*	Submitted in NCBI	0.0	100	LT623958.1
Pf-3	*P. fluorescens*	Submitted in NCBI	0.0	100	AJ583090.1
Pf-4	*P. fluorescens*	MN099291.1	0.0	100	MK875666.1
Pf-5	*P. fluorescens*	MN099292.1	0.0	100	HF913573.1
Pf-6	*P. fluorescens*	MN099293.1	0.0	99	KT767782.1
Pf-7	*P. fluorescens*	MN099294.1	0.0	100	MK578898.1
Pf-8	*P. fluorescens*	MN099295.1	0.0	100	AJ310393.1
Pf-9	*P. fluorescens*	MN099296.1	0.0	99	MK883103.1
Pf-10	*P. fluorescens*	MN099297.1	0.0	100	U63901.1
Pf-11	*P. fluorescens*	MN099298.1	0.0	99	KF675203.1
Pf-12	*P. fluorescens*	MN099299.1	0.0	99	KU877638.1
Pf-13	*P. fluorescens*	MN099300.1	0.0	100	U63901.1
Pf-14	*P. fluorescens*	MN099301.1	0.0	99	AB621835.1
Pf-15	*P. fluorescens*	MN099302.1	0.0	100	HQ914783.1
Pf-16	*P. fluorescens*	MN099303.1	0.0	100	MK157087.1
Pf-17	*P. fluorescens*	Submitted in NCBI	0.0	99	LT623958.1
Pf-18	*P. fluorescens*	,,	0.0	100	MK248116.1
Pf-19	*P. fluorescens*	,,	0.0	100	MH773394.1

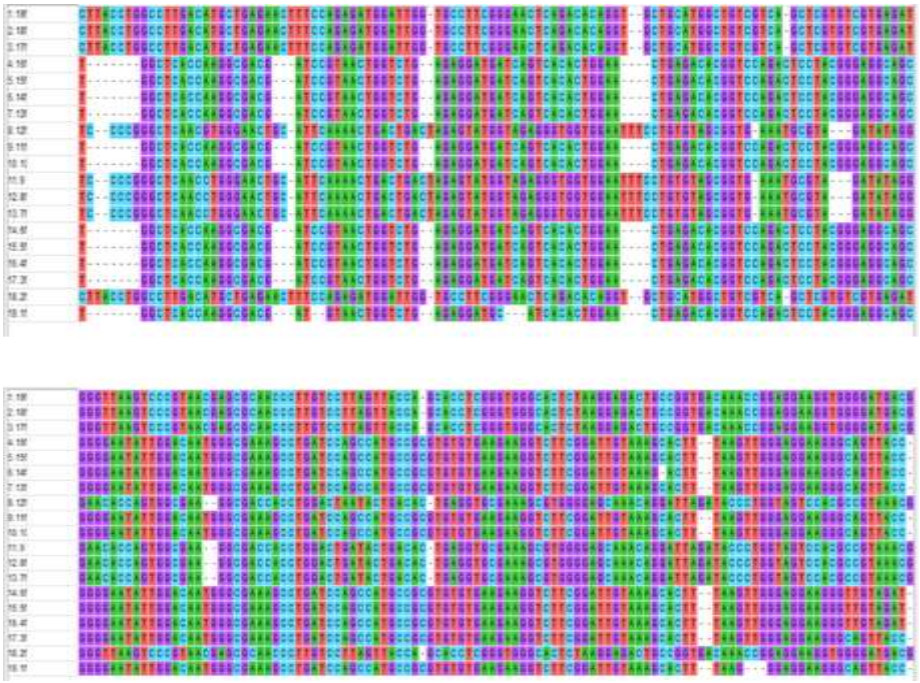


Fig. 4.: Multiple alignment of nucleotide sequences of 16s rRNA sequences of the nineteen *P. fluorescens* isolates

Table 4
Similarity coefficient among the isolates of *P. fluorescens*

	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
19	100																		
18	100	100																	
17	100	100	100																
16	97.844	97.844	97.844	100															
15	97.844	97.844	97.844	100	100														
14	97.844	97.844	97.844	100	100	100													
13	97.921	97.921	97.921	99.964	99.964	99.964	100												
12	96.603	96.603	96.603	98.544	98.544	98.544	98.512	100											
11	97.921	97.921	97.921	99.964	99.964	99.964	100	98.512	100										
10	97.921	97.921	97.921	99.964	99.964	99.964	100	98.512	100	100									
9	96.577	96.577	96.577	98.517	98.517	98.517	98.485	99.991	98.485	98.485	100								
8	96.577	96.577	96.577	98.517	98.517	98.517	98.485	99.991	98.485	98.485	100	100							
7	96.577	96.577	96.577	98.517	98.517	98.517	98.485	99.991	98.485	98.485	100	100	100						
6	97.858	97.858	97.858	99.902	99.902	99.902	99.94	98.522	99.94	99.94	98.495	98.495	98.495	100					
5	97.858	97.858	97.858	99.902	99.902	99.902	99.94	98.522	99.94	99.94	98.495	98.495	98.495	100	100				
4	97.858	97.858	97.858	99.902	99.902	99.902	99.94	98.522	99.94	99.94	98.495	98.495	98.495	100	100	100			
3	97.886	97.886	97.886	99.95	99.95	99.95	99.987	98.561	99.987	99.987	98.534	98.534	98.534	99.94	99.94	99.94	100		
2	100	100	100	97.844	97.844	97.844	97.921	96.603	97.921	97.921	96.577	96.577	96.577	97.858	97.858	97.858	97.886	100	
1	97.844	97.844	97.844	99.987	99.987	99.987	99.959	98.534	99.959	99.959	98.506	98.506	98.506	99.897	99.897	99.897	99.946	97.844	100

fluorescens based on the 16s rRNA gene sequences

The present findings are in harmony with earlier workers. The effectiveness of *P. fluorescens* against *M. phaseolina* has been recorded by Kumar et al[10] and Alice and Sundravadana[1].

Biocontrol efficacy of isolates of *P. fluorescens* against *R. solani*: All the isolates significantly inhibited the mycelial growth of *R. solani* over control and also restricted the sclerotial production. Significantly minimum mycelial growth of 14.50 mm was observed with isolate Pf-5 with maximum growth inhibition of 83.89 per cent followed by isolate Pf-6 which recorded 21.11 mm of mycelial growth and 76.54 per cent of growth inhibition. Pf-2 showed 22.38 mm of mycelial growth and 75.13 per cent of growth inhibition, Pf-1 recorded 23.13 mm of mycelial growth (74.30%) and Pf-13 recorded 25.60 mm of mycelial growth (71.56%) after 7 days of inoculation.

Similarly, a native isolate of *T. asperellum* recorded the mycelial growth of 11.73 mm and growth inhibition of 86.97 per cent **(**Table 5, Fig. 6, ii**)**. Therefore, the isolates Pf-5 of *P. fluorescens* and native isolates of *T. asperellum* showing maximum inhibition against *R. solani* were selected for further studies against biotic and abiotic stresses. The potentant antagonistic effect of *P. fluorescens* against *R. solani* has been reported by Bautista et al[3], Nabrdalik and Grata[15] and Sivakamasundari and Usharani.[22]

Conclusion

The 16s rRNA specific region of the ribosomal operon amplified regions from 900-1000 bp for the precise identification of *Pseudomonas* species as well as phylogenomic analysis validated the results based on the PCR-based system for species-level classification of *P. fluorescens*. Phylogenetic analysis using MEGA 7.0.26 showed that isolates Pf-1, Pf-10, Pf-11, Pf-13, Pf-14, Pf-15 and Pf-16 were grouped together and they have been isolated from mungbean irrespective of the area of collection. Isolates Pf-4, Pf-5 and Pf-6 were collected from one region.

Table 5
In vitro* efficacy of *P. fluorescens* isolates against *M. phaseolina* and *R. solani

Trt. No.	Isolates	*M. phaseolina*		*R. solani*	
		Mycelial growth (mm)	Growth inhibition (%)	Mycelial growth (mm)	Growth inhibition (%)
T_1	Pf-1	21.15 f	76.50	23.13 ghi	74.30
T_2	Pf-2	24.21 ef	73.10	22.38 hi	75.13
T_3	Pf-3	27.96 de	68.93	30.18 bcdef	66.47
T_4	Pf-4	30.30 bcd	66.33	27.31 defg	69.66
T_5	Pf-5	13.18 g	85.36	14.50 j	83.89
T_6	Pf-6	21.65 f	75.94	21.11 i	76.54
T_7	Pf-7	28.61 de	68.21	27.50 cdefg	69.44
T_8	Pf-8	31.71 bcd	64.77	31.75 bcd	64.72
T_9	Pf-9	32.73 bcd	63.63	34.75 b	61.39
T_{10}	Pf-10	35.76 b	60.27	31.50 bcd	65.00
T_{11}	Pf-11	35.55 bc	60.50	30.83 bcde	65.74
T_{12}	Pf-12	23.55 ef	73.83	27.58 cdefg	69.36
T_{13}	Pf-13	24.06 ef	73.27	25.60 fghi	71.56
T_{14}	Pf-14	30.00 cd	66.67	31.88 bcd	64.58
T_{15}	Pf-15	33.11 bcd	63.21	29.75 bcdef	66.94
T_{16}	Pf-16	34.30 bc	61.89	32.40 bc	64.00
T_{17}	Pf-17	30.65 bcd	65.94	33.20 b	63.11
T_{18}	Pf-18	28.28 de	68.58	29.90 bcdef	66.78
T_{19}	Pf-19	28.28 de	68.58	26.50 efgh	70.56
T_{20}	Control	90.00 a	0.00	90.00 a	0.00
SEm. ±.		1.63	----	1.46	----
F-Test		Sig.	---	Sig.	---
CV %		10.31	----	9.28	----

Treatment means with the letter/letters in common are not significant by DNMRT at 5 % level of significance.

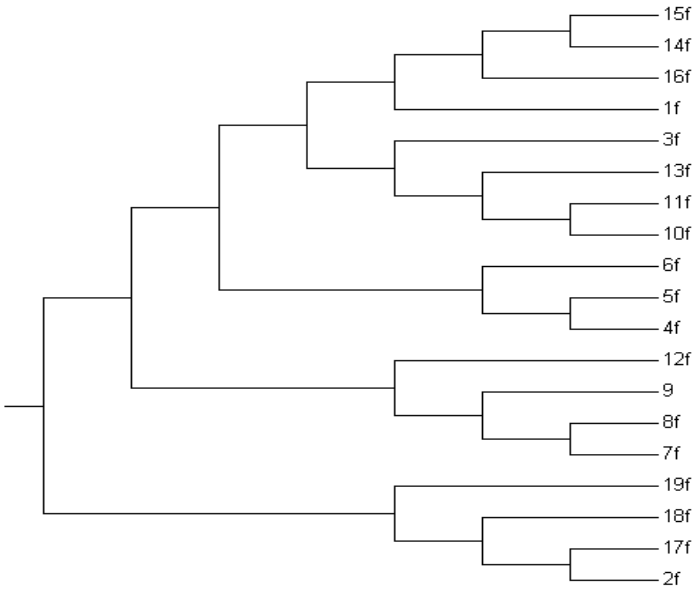


Fig. 5: Phylogenetic tree of nineteen isolates of *P. fluorescens* through analysis of 16s rRNA gene sequences

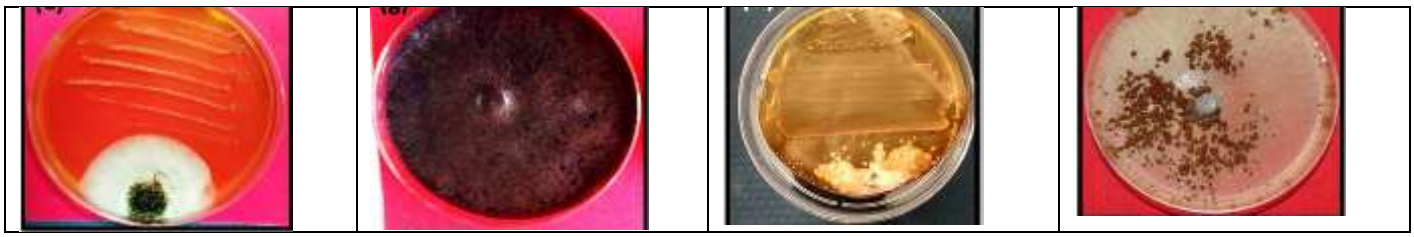


Fig. 6: Effectiveness of *P. fluorescens* in inhibiting mycelial growth of (i) *M. phaseolina* and (ii) *R. solani*

However, their sources of the collection were different (isolate Pf-4 from mungbean, isolate Pf-5 and Pf-6 from chickpea). These indicated that isolate Pf-5 and Pf-6 had similarity at the genetic level that matched with geographical location. *In vitro* antagonism testing with isolate Pf-5 exhibited minimum mycelial growth of 13.18 mm with *M. phaseolina* with maximum growth inhibition of 85.36 per cent followed by isolate Pf-1 with 21.15 mm of mycelial growth with 76.50 per cent growth inhibition. A similar trend was observed in *R. solani* with minimum mycelial growth of 14.50 mm with isolate Pf-5 with maximum growth inhibition of 83.89 per cent followed by isolate Pf-6 which recorded 21.11 mm of mycelial growth and 76.54 per cent of growth inhibition.

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