Research Article

Morphological and Molecular Variability in *Fusarium udum isolates* Causing Vascular Wilt of Pigeonpea in Tamil Nadu

Smitha Kodoth Padinhare*, Rajeswari Mohan, Alice Devadason and Thiruvengadam Raguchander

*Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore- 641 003, India

Abstract

The present investigation was carried out to study the variability among *Fusarium udum* isolates causing vascular wilt disease of pigeonpea in Tamil Nadu. Totally 10 isolates of the fungus were recovered from different pigeonpea growing regions of Tamil Nadu. Morphological observation revealed the occurrence of variation with respect to colony type, growth pattern, and size of macroconidia and microconidia. All the 10 isolates were confirmed as *Fusarium udum* by PCR amplification of genomic DNA using species specific primers. Random Amplified Polymorphic DNA studies demonstrated the existence of variability within the isolates of *Fusarium udum* causing pigeonpea wilt. An understanding on the variability among isolates will help a long way in developing better management strategies.

Keywords: Pigeonpea, vascular wilt, *Fusarium udum*, RAPD, variability

***Correspondence**

Author: Smitha Kodoth Padinhare

Email: smitpath@gmail.com

Introduction

Pigeonpea, commonly known as red gram or arhar (*Cajanus cajan* (L) Millsp.), is the one of the most important legume crops of the tropical and subtropical countries. India, being the world's largest producer and consumer of pigeonpea, contributes to almost 80% of the global pigeonpea area and production. The most important biotic factor limiting the production of pigeonpea is the vascular wilt caused by *Fusarium udum* (Butler). Under favourable environmental conditions, wilt may cause up to 50 to 100 per cent yield loss in pigeonpea [1]. The yield loss due to this disease also depends upon the stage at which the plants wilt and it can approach over 50 per cent and even up to100 per cent when wilt occurs at the pre-pod stage [2]. The pathogen *F. udum* is host specific, heterogenous and mainly soil borne. [3] and [4] reported the cultural and the pathogenic variability in the fungus. Different isolates of *F. udum* have been reported from India by [5] which differ in their mycelial color, substrate color, mycelial growth and virulence. Because of the existence of high pathogen variability, management of the disease using resistant cultivars will be a challenging task. For breeding resistant varieties, knowledge of pathogen variability in the particular crop area is essential. Randomly Amplified Polymorphic DNA offers several advantages and provides genetic information on the isolates. [6] Characterized the genetic diversity in pigeonpea wilt pathogen using 14 isolates collected from major pulse growing regions of India and grouped all 14 *F. udum* isolates into three major clusters by cluster analysis of ITS-RFLP. RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens [7].

Materials and Methods

Isolation, Identification and Pathogenicity of Fusarium isolates

The vascular wilt pathogen *Fusarium udum* was isolated from root and collar region of infected plants. Isolation was done as per the standard protocols [8]. All the isolates were purified by single spore isolation described by [9].

The pure cultures on PDA were used for the observation of phenotypic characters such as colony features, growth rate and pigmentation. The conidial characters were studied by microscopic observation following slide culture of the fungus [10]. Publications of [11] were used for the identification of *Fusarium* isolates on a species level based on morphological features.

Pathogenicity was proven in the susceptible pigeonpea cultivar CoRg7by modified root injury method [12] with slight modifications. Earthen pots were filled with sterilized potting mixture. In the middle of the earthen pots, 15 cm length cut pieces of PVC pipes of 5 cm diameter were immersed and the soil inside was scooped out. The surface

sterilized pigeonpea seeds were sown in the pot just at the periphery of the PVC pipe all around. When the seedlings were 10 to 15 days old, the PVC pipes were gently removed which exposed the entire root system and facilitated infection. The gap created by removing the pipes were filled with *Fusarium udum* multiplied in sterilized sand-maize medium at the rate of 20 g kg^{-1} soil [13]. After symptom development, re-isolation was done and compared with the original culture for confirmation of the pathogen identity.

Molecular Characterization of Fusarium isolates

The genomic DNA of all the isolates was extracted from the mycelium of *Fusarium* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by [14]. The total DNA was confirmed by resolving on 0.8% of agarose gel electrophoresis.

PCR amplification using ITS (Internal Transcribed Spacer) and species specific primers

For molecular detection, genomic DNA of each *Fusarium* isolate was subjected to PCR amplification of complete ITS/5.8s rDNA region [15]. ITS1 F (5'-GTCCTAACAAGGTTTC CGTA-3) and ITS4 R (5'-TTCTCCGCTT ATTGATATGC-3) primers were used for the amplification of conserved region with the expected size of 650 base pair. For molecular confirmation at species level, the genomic DNA of *Fusarium* isolates were amplified using species specific primer HFUSF (5'-TCATCACTAACTTCATCACCAAT-3') and the reverse primer HFUSR1 (3'-TGTC GAATGTTAGTAAGTGTTG-5') to get an amplicon size of 200 base pair [16]. PCR amplification was performed using a Mastercycler. The PCR reaction volume 20 µl, contained 2.0 U of Taq polymerase (Sigma-Aldrich), 2 µl of 10X buffer, 1.5 µl of 2.5 mM $MgCl_2$, 1 µl of 2.5 mM dNTP, 2 µl of 10 µM primer, 4 µl of genomic DNA and sterile distilled water. The PCR was performed with an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 46°C and 1 min extension at 72°C, with final extension of 10 min at 72°C. The PCR products were resolved on 1.2% agarose at 85 V, stained with ethidium bromide (0.5µg/ml) and documented using gel documentation system Alpha lmager 2000 (Alpha Innotech, San Leandro, CA, USA)

Random amplified polymorphic DNA (RAPD) -PCR

All the ten isolates of *Fusarium udum* were used for RAPD analysis. The cocktail for the amplification consisted of 4µl of DNA (25 ng/µl), 4µl of primer, 8 µl of PCR master mix, 4µlof sterile distilled water to obtain a final reaction volume of 20 µl. The OPA primer series one to 10 were used to detect polymorphism among the isolates. The DNA sequences (5'-3') of all primers used for DNA amplification are furnished in **Table 1**. The thermal cycler was programmed as initial denaturation at 94 °C for 10 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 36 °C, and 2 min extension at 72 °C with a final extension of 10 min at 72 °C. The amplification products were visualized with a UV transilluminator and photographed in the gel documentation system.

Table 1 Primer sequences used for RAPD-PCR

Primer	Sequence
OPA01	5' CAGGCCCTTC 3'
OPA02	5' TGCCGAGCTG 3'
OPA03	5' AGTCAGCCAC 3'
OPA04	5' AATCGGGCTG 3'
OPA05	5' AGGGGTCTTG 3'
OPA06	5' GGTCCCTGAC 3'
OPA07	5' GAAACGGGTG 3'
OPA08	5' GTGACGTAGG 3'
OPA09	5' GGGTAACGCC 3'
OPA10	5' GTGATCGCAG 3'

Analysis of RAPD-PCR

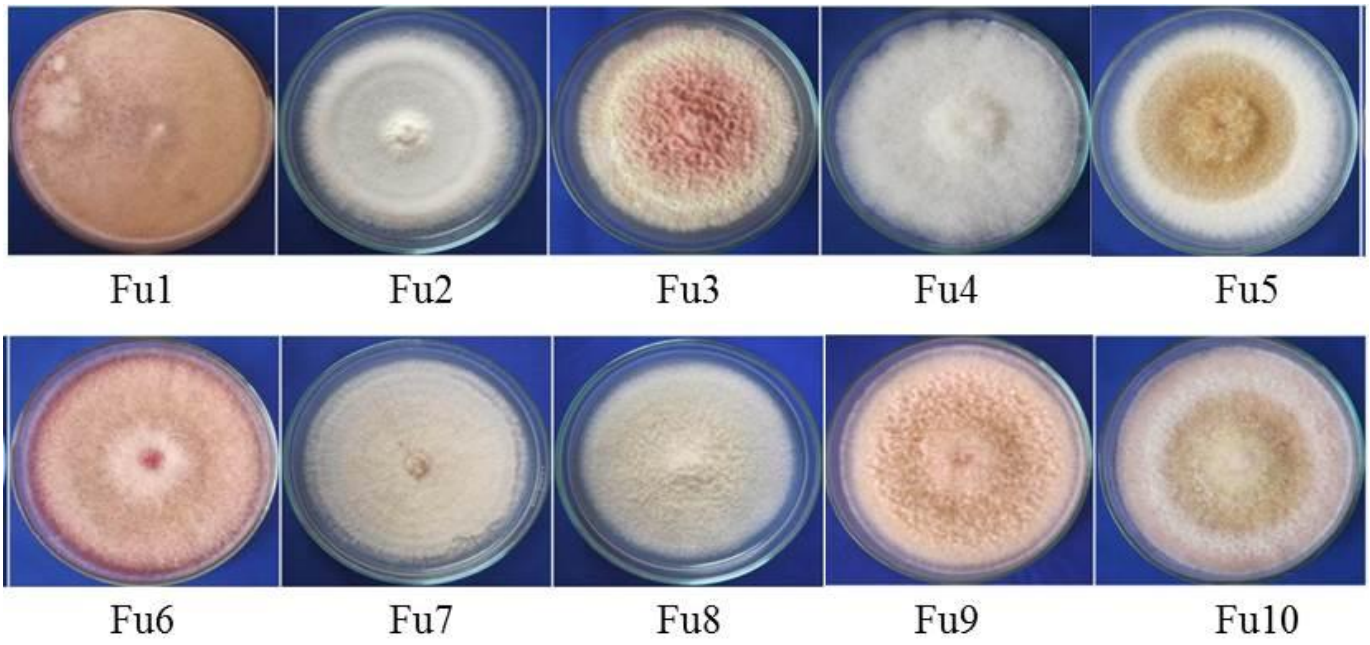
The banding patterns of *Fusarium* isolates were scored for RAPD. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Numerical Taxonomy System Applied Biostatistics, Setauket, New York, USA) described by [17]. Similarity matrix was developed using the Jaccard's coefficient of similarity with the data

matrix. A phenogram was reproduced by the unweighted pair group method for arithmetic average (UPGMA) in the SAHN procedure. Finally, the 10 isolates of *Fusarium udum* were grouped into different clusters using Jaccard's coefficient of similarity [18].

Results and Discussion

Isolation and Identification

The pathogen associated with vascular wilt of pigeonpea was isolated from infected samples collected from different districts of Tamil Nadu *viz*., Coimbatore, Erode, Salem, Dharmapuri, Krishnagiri, Vellore, Thiruvannamalai, Namakkal and Theni. A total of 10 isolates were collected from the above districts. Colonies in culture showed variations in morphological characters (**Figure 1**). The mycelium was initially white which later turned cream, orange or pink.



Fu1 – F. udum Coimbatore isolate
Fu2 – Coimbatore isolate
Fu3 – Erode isolate
Fu4 – Salem isolate
Fu5 – Dharmapuri isolate
Fu6 – Krishnagiri isolate
Fu7 – Vellore isolate
Fu8 – Thiruvannamalai isolate
Fu9 – Namakkal isolate
Fu10 – Theni isolate

Figure 1 *Fusarium udum* isolates from different districts of Tamil Nadu

Coimbatore and Namakkal isolates exhibited salmon pink colonies. The colonies were either appressed or fluffy in nature. Macroconidia was formed in sporodochia, sparse, falcate with hooked apical cell, three to five septate and varied in their size. The size of the macroconidia varied from 11.4-16.3 x 3.0-4.5 µm in the Thiruvannamalai isolate (Fu8) to 27.5-42.7 x 4.5-6.6 µm in Salem isolate (Fu4). The Krishnagiri isolate (Fu6) which formed pink colonies on PDA produced very few spores whereas abundant sporulation was seen in the isolate from Vellore (Fu7) which produced white colonies on PDA. Microconidia was abundant, hyaline, oval, aseptate or with single septa. The size of microconidia ranged from 4.1-12.3 x 4.3-5.8 µm in Thiruvannamalai isolate to 6.7-14 x 2.9-5.3 µm in Vellore isolate (Fu7). Abundant chlamydospore formation was noted in cultures which were terminal or intercalary (**Table 2**).

Molecular characterization of Fusarium isolates

In the present investigation, the genomic DNA amplification of ITS region of all the 10 isolates of *Fusarium* yielded a fragment of approximately 650 bp (**Figure 2**).

PCR was performed for the characterization of *F. udum* using species specific primers HFUSF (5'-ATCATCACTAACTTCATCACCAAT-3') and the reverse primer HFUSR1 (3'TGTCGAATGTTAGTAAGTGTTG-5'). DNA of all the isolates of *Fusarium* were amplified with the species specific primers to yield amplicon size of 200 base pair confirming all the isolates to be *F. udum* (**Figure 3**).

Table 2 Morphological features of *Fusarium udum* isolates from pigeonpea

Place of collection	Isolate	Colony diameter in 7 days	Colony morphology	Mycelial colour	Pigmentation	Sporula-tion*	Size of macroconidia (µm)**	Size of microconidia (µm)**
Coimbatore	Fu1	90 mm	Circular margin with fluffy growth	Salmon pink	Buff	++	16.5-26.8 x 3.2-4.7	6.0-14.1 x 4.1-5.0
Coimbatore	Fu2	70 mm	Circular margin, circular zones	White	Cream	++	16.3-23.9 x 2.0-4.2	6.2-11.8 x 2.1-3.8
Erode	Fu3	64 mm	Wavy margin, slightly appressed	Creamy Pink	Cream	++	17.1-25.8 x 3.5-4.9	5.8-12.5 x 2.5-4.3
Salem	Fu4	78 mm	Wavy margin, fluffy growth	White	Cream	++	27.5-42.7 x 4.5-6.6	6.0-15.7 x 3.1-3.7
Dharmapuri	Fu5	80 mm	Circular margin, fluffy growth	Buff	Straw	+++	21.4-33.8 x 2.5-3.0	5.1-15.7 x 2.9-3.5
Krishnagiri	Fu6	85 mm	Circular margin, fluffy growth	Pink	Dark pink	+	23.7-41.5 x 2.5-3.8	6.2-13.1 x 2.6-3.9
Vellore	Fu7	62 mm	Circular margin, concentric, appressed growth	White	Light yellow	++++	19.7-38.6 x 3.2-4.6	6.7-14 x 2.9-5.3
Thiruvanna-malai	Fu8	73 mm	Circular margin, concentric, appressed growth	Cream	Cream	++	11.4-16.3 x 3.0-4.5	4.1-12.3 x 4.3-5.8
Namakkal	Fu9	85 mm	Circular margin with fluffy growth	Salmon pink	Straw	++	22.7-34.2 x 3.2-5.1	6.7-13.2 x 3.3-4.3
Theni	Fu10	90 mm	Circular margin with fluffy growth	Light pink	Cream	++	17.7-40.8 x 4.6-6.3	5.8-11.9 x 2.7-3.5

* (+) – Scanty, (++) – Moderate, (+++) – Good, (++++) – Abundant ** Mean of 100 observations

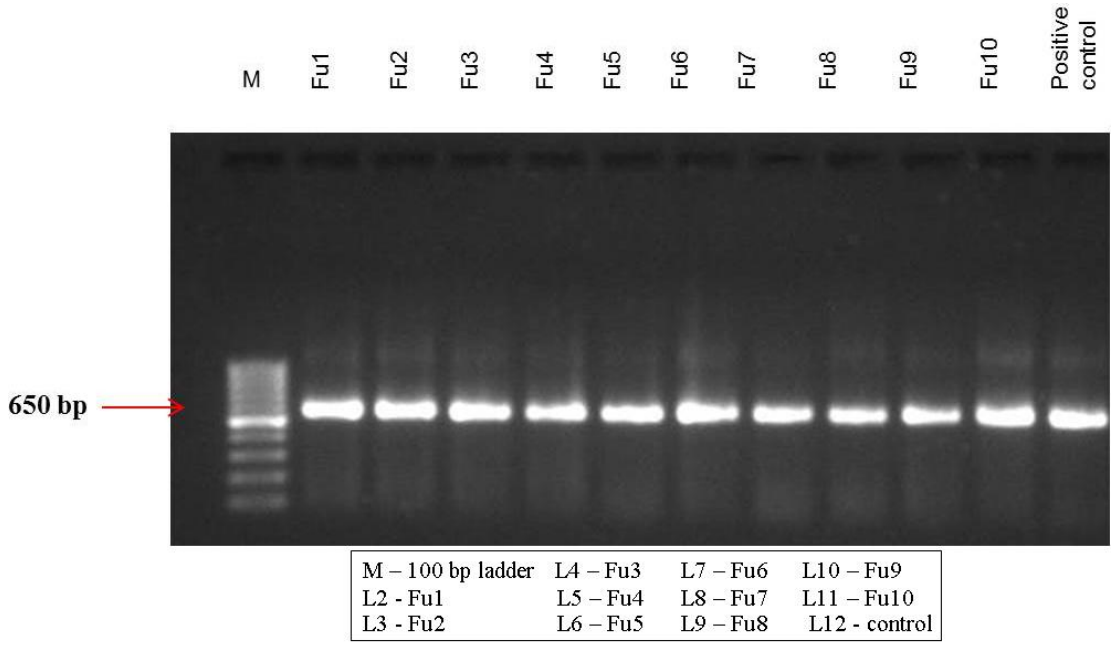


Figure 2 PCR amplification of ITS region of *Fusarium* isolates

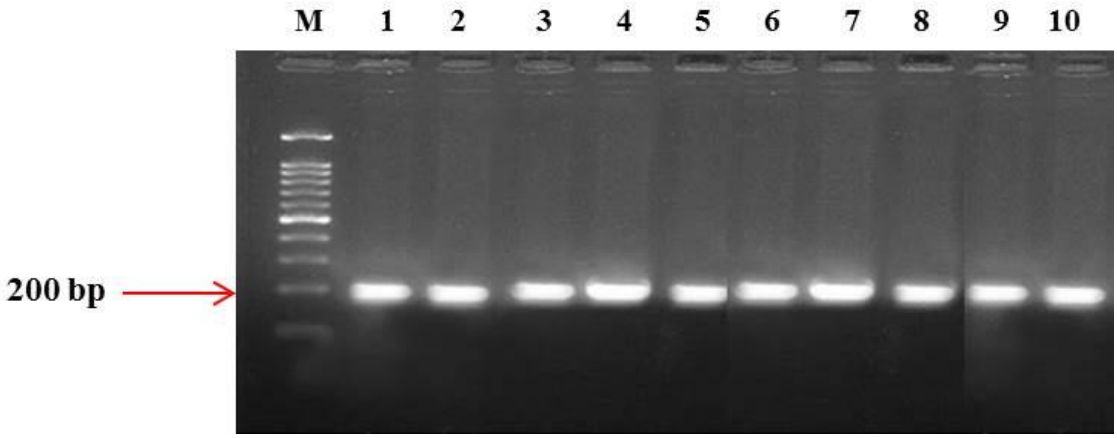
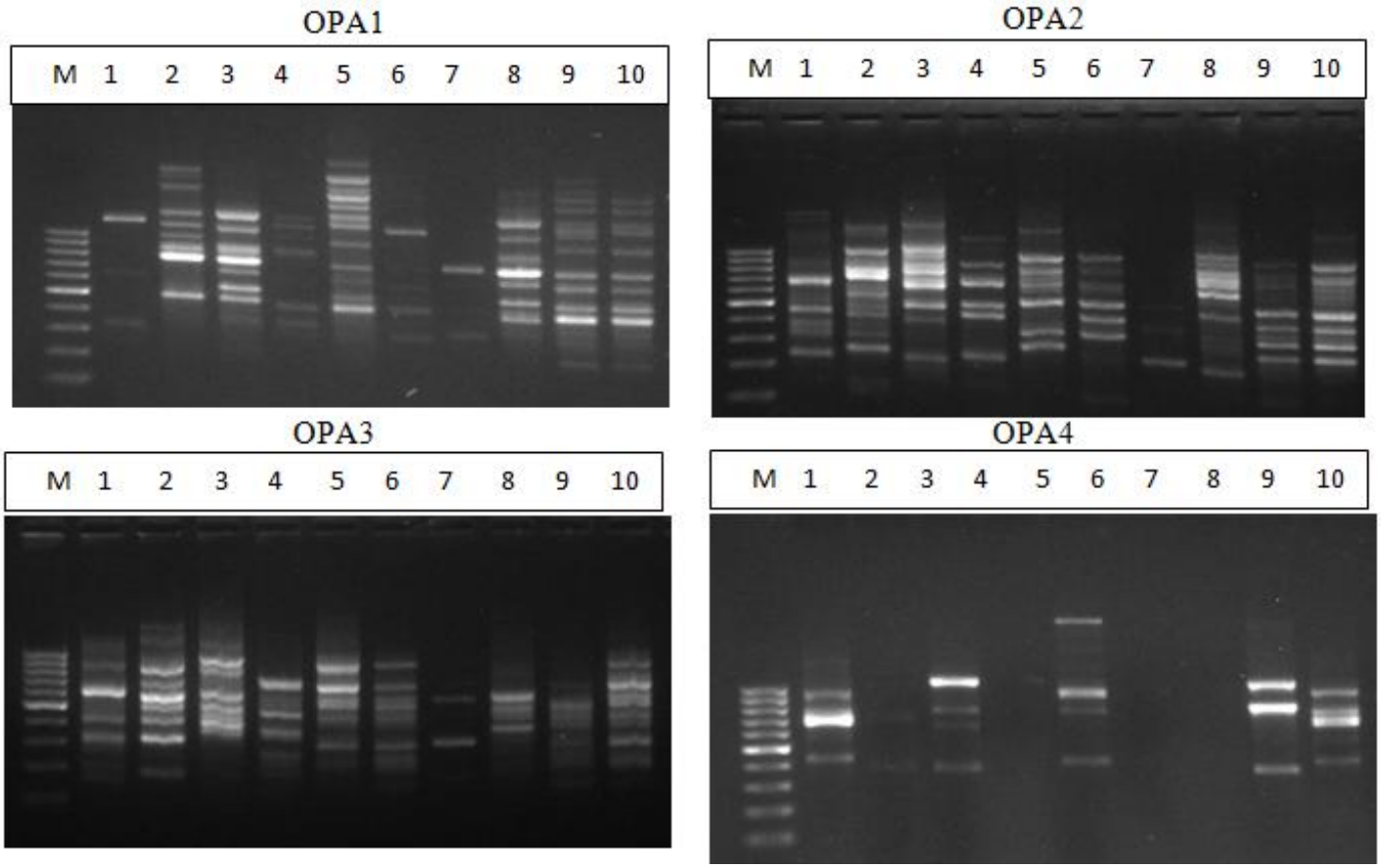


Figure 3 PCR amplification of *Fusarium* isolates using species specific primers

Random Amplified Polymorphic DNA Profiling

Genetic variation among the isolates of *Fusarium udum* from wilt infected pigeonpea plants was studied by RAPD analysis using 10 OPA primers. The primer OPA6 produced 11 polymorphic bands. Ten polymorphic PCR products were obtained with the primer OPA1. The primers OPA2, OPA8 and OPA9 produced eight polymorphic bands. Seven polymorphic bands were produced by OPA7. Primers OPA3 and OPA5 produced six polymorphic bands. OPA4 and OPA10 produced five and four polymorphic bands respectively. The PCR products size ranged from 0.1 to 3kb (**Figure 4**).

Comparative analysis of the PCR profile for each primer was done on the basis of presence or absence of bands at the same level. Presence of a band was scored as one and absence as zero. The diversity among the isolates using RAPD markers was calculated by UPGMA method and the genetic distance between each isolate is represented in the form of dendrogram (**Figure 5**) and similarity matrix (**Table 3**).



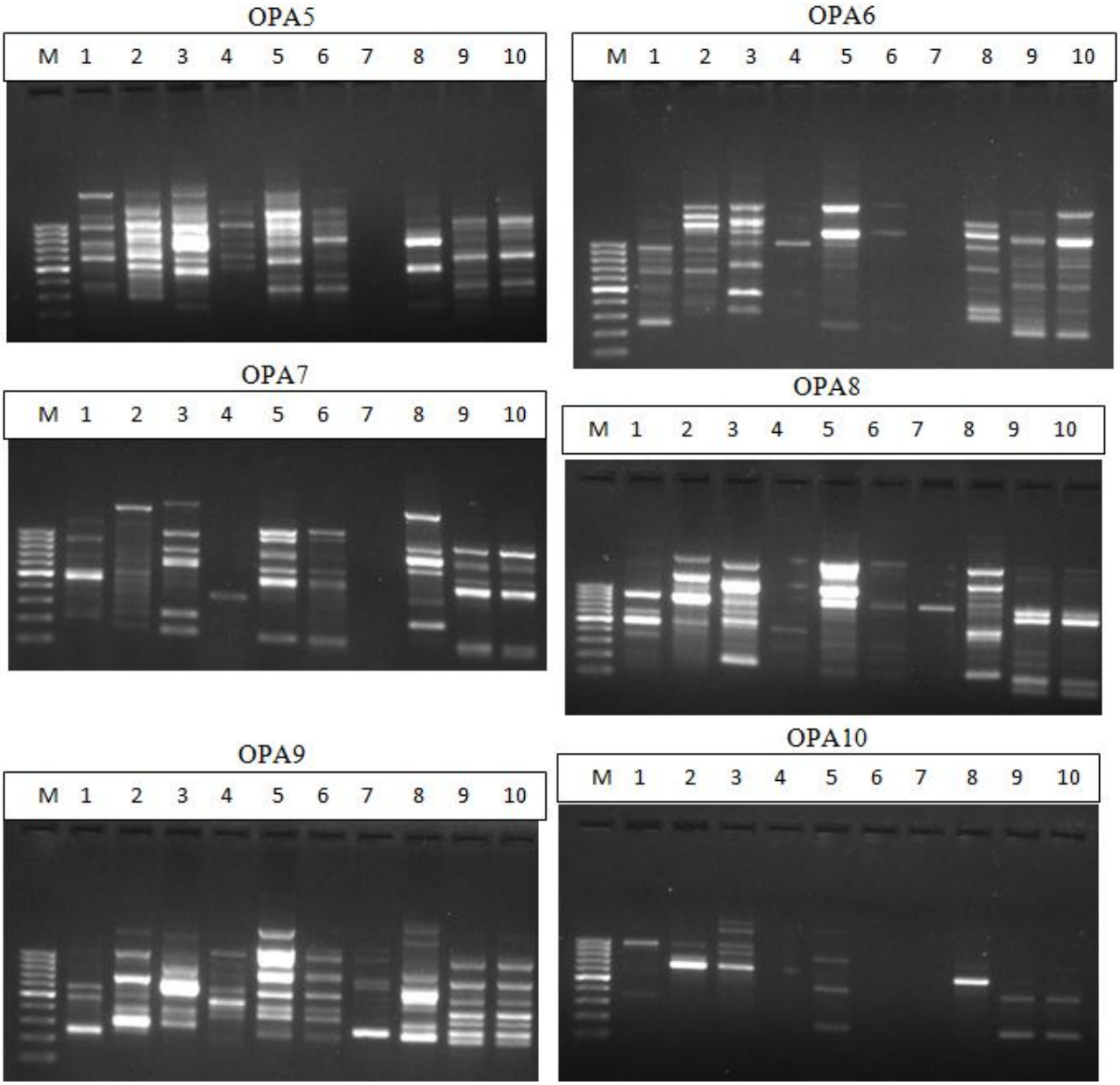


Figure 4 RAPD profile of *Fusarium udum* using RAPD primers

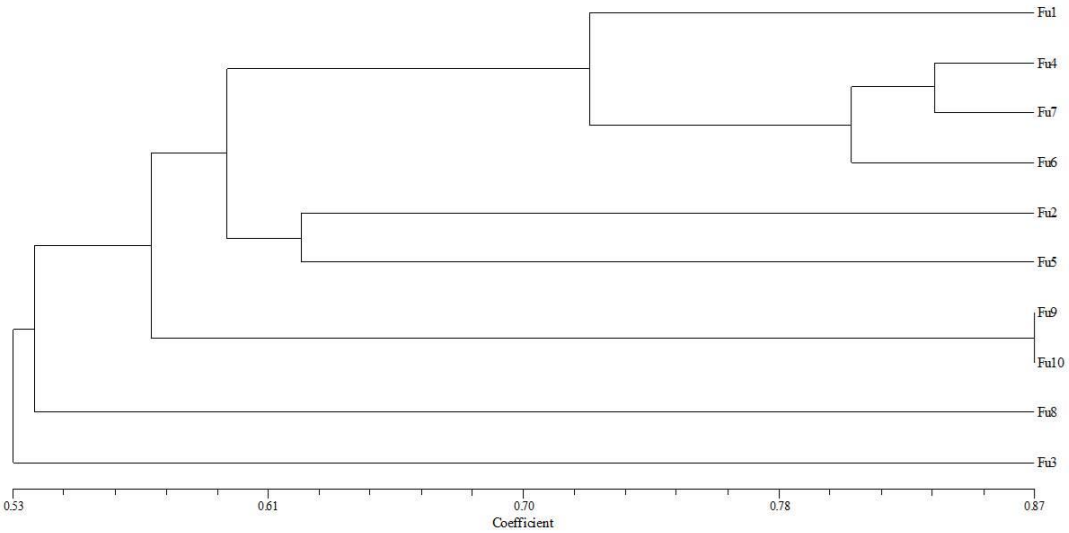


Figure 5 Dendrogram of the isolates constructed from RAPD data using UPGMA method

Table 3 RAPD similarity matrix of pigeonpea *Fusarium udum* isolates

S.No	*F. udum* isolates	Fu1	Fu2	Fu3	Fu4	Fu5	Fu6	Fu7	Fu8	Fu9	Fu10
1	Fu1	1									
2	Fu2	0.61	1								
3	Fu3	0.56	0.61	1							
4	Fu4	0.72	0.58	0.54	1						
5	Fu5	0.57	0.62	0.54	0.56	1					
6	Fu6	0.70	0.62	0.57	0.80	0.69	1				
7	Fu7	0.73	0.59	0.53	0.83	0.57	0.81	1			
8	Fu8	0.51	0.54	0.51	0.57	0.50	0.54	0.58	1		
9	Fu9	0.53	0.43	0.40	0.63	0.59	0.61	0.60	0.51	1	
10	Fu10	0.51	0.48	0.47	0.66	0.61	0.63	0.58	0.51	0.87	1

Fu – *Fusarium udum*

Cluster analysis of the RAPD products revealed high degree of genetic polymorphism. The ten isolates were resolved into five main groups. Group I was divided into two clusters, A and B at a coefficient value of 0.73. Clutster A contained the single isolate from Coimbatore *viz.,* Fu1. Cluster B was against sub-clustered into two at a similarity coefficient of 0.8 as C and D. Subcluster C contained two isolates Fu4 (Salem) and Fu7 (Vellore) which merged at a similarity coefficient of 0.85. Subcluster D contained the isolate Fu6 (Krishnagiri). Group II contained two isolates, Fu2 (Coimbatore) and Fu5 (Dharmapuri) with a similarity coefficient of 0.62. Group I and II merged at a similarity coefficient value of 0.60. Group III contained two isolates, Fu9 (Namakkal) and Fu10 (Theni) with a similarity coefficient of 0.87. Group IV was represented by the isolate Fu8 (Thiruvannamalai) and Group V by Fu3 (Erode).The similarity coefficient between all the 10 isolates ranged from 0.53 to 0.87. Maximum similarity of 87 per cent was noted between the isolates from Namakkal and Theni *viz.,* Fu9 and Fu10 respectively.

Genetic variability studies in plant pathogen populations are an essential pre-requisite for analysing the co-evolution in the plant pathosystem. RAPD-PCR has been successfully employed to characterize strains and races in phytopathogenic fungi. In recent years, numerous DNA based methods have been increasingly used to study variability in pathogenic *Fusarium* population [19] and [20]. DNA fingerprinting has been successfully used for characterization of individual *Fusarium* isolates and grouping them into standard racial classes. The genetic diversity and pathogenic variability among *F. udum* isolates collected from different geographical locations of India were estimated using RAPD molecular markers [5]. [6] characterized 14 isolates of *F. udum* collected from major pulse growing regions of India using 24 RAPD primers and grouped all the isolates into three major clusters.

Conclusion

The present study clearly indicates the existence of genetic variability within species level among the vascular wilt pathogen. An in depth study of this will help in evolving better varieties which can cope with the evolving pathogen variability.

Acknowledgement

The authors gratefully acknowledge the funding from Department of Science and Technology, SERB, Government of India for carrying out this investigation and Tamil Nadu Agricultural University for providing the facilities for carrying out the research work.

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Publication History
Received 06th Oct 2017
Revised 20th Oct 2017
Accepted 20th Oct 2017
Online 30th Oct 2017