Int.J.Curr.Microbiol.App.Sci (2018) *7*(10): 2109-2122

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 10 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.710.243

Cultural, Morphological and Molecular Variability of *Fusarium oxysporum* f. sp. *udum* Isolates by RAPD Method

P.H. Ghante*, K.M. Kanase, S.P. Kale, R.L. Chavan, K.M. Sharma and S.B. Ghuge

Department of Plant Pathology, College of Agriculture, Parbhani, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani 431 402 (M.S), India

**Corresponding author*

Keywords

Pigeonpea wilt, *Fusarium oxysporum* f. sp. udum, *in vivo*, Cultural, morphological and molecular characteristics

Article Info

Accepted:
15 September 2018
Available Online:
10 October 2018

A B S T R A C T

Cultural, morphological and molecular characteristics of *Fusarium oxysporum* f. sp. *udum* were studied where eight isolates indicated a great variability amongst them. However, the isolate FOC-2 (Jalna) exhibited maximum mycelial growth of 90 mm. The isolates *viz*., Jalna (FOC-2) and Beed (FOC-3) produced partially submerged (FOC-2) to submerged (FOC-3) white sparse dense growth with smooth margin and bright white substrate pigmentation, respectively. Maximum micro-conidial, macro-conidial and chlamydospore size (17.20 µm, 30.50 x 7.00 µm and 21.80 x 19.60 µm) were recorded in isolate Jalna (FOC-2). The micro-conidia were more or less oval to cylindrical with no septation. The macro-conidia were typically sickle shaped curved, fusoid varied in the size and number of septation (3-5). The chlamydospores were round to oval in shape. Genetic diversity was analyzed based on data obtained by 10 RAPD primers. Most of the primers were found 91.66 to 100 per cent polymorphic in nature. All primers had amplified total number of 144 bands among which 140 and 4 were found polymorphic and monomorphic, respectively. The cluster I comprised isolates FOC-1 (Aurangabad) and FOC-6 (Nanded) together and showed 57.60 per cent similarity to each other; however, cluster II comprised six isolates [FOC-2 (Jalna), FOC-3 (Beed), FOC-4 (Osmanabad), FOC-5 (Latur), FOC-7 (Parbhani) and FOC-8 (Hingoli)] together showing 53.88 per cent similarity. All of these six isolates of cluster II were from different region showing maximum similarity in the range of 59.00 to 100 per cent.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is known by more than 350 vernacular names, the most popular being arhar, yellow dhal, red gram, tur (India), congo pea, gandul, guandu (Brazil), angola pea (United Kingdom), catjang pea, ambrevade, pois d'angdie (French-speaking West Africa), quinochoncho (Venezuela). Pigeonpea ranks fourth in importance as edible legume in the world. Pigeon pea is extensively grown throughout the tropics, subtropics and warmer equatorial regions of Asia, East Africa and Central America in lower altitude areas between 30^{o} N to 30^{o} S, particularly in the semi-arid and lower humid tropics.

Globally, it is grown on approximately 5 million hectares in about 82 countries of the

world. The major production area is located in India, Myanmar, Kenya, Malawi, Uganda and Tanzania.

The pigeonpea is the first seed legume plant to have its complete genome sequenced. The sequencing was first accomplished by a group of 31 Indian scientists from the Indian Council of Agricultural Research, New Delhi (India). India alone occupies three-fourth of the global harvested area and contributes almost a similar share in production. Pigeonpea occupies a prominent place in Indian rainfed agriculture. It is the second most important pulse crop next to chickpea, covering an area of around 4.42 m ha (occupying about 14.5% of area under pulses), production of 2.86 MT (contributing to 16% of total pulse production) and productivity of about 707 kg/ha. Deep roots improve physical properties of the soil and pulverize the soil. The plants shed large amount of leaves, this biomass adds organic matter to soil. Besides, it also leaves 30-50 kg 'N' to the succeeding crop and also benefiting the inter-cropped cereals through increased 'N' supply. Pigeonpea in some areas is an important crop for green manure, providing up to 90 kg nitrogen per hectare.

The area of pigeonpea in Maharashtra is increased from 10.39 lakh ha to 15.33 lakh ha in 2016-17. Area of pigeonpea was highest in 2016-17 (15.33 lakh ha) while the production and productivity were highest during 2013-14 i.e.10.34 lakh tones and 906 kg/ha, respectively. In 2016-17 estimated production of pigeonpea in Maharashtra is 11.70 lakh tonnes. In Marathwada, area under pigeonpea was 5.95 lakh ha during 2016-17, while production and productivity were highest during 2013-14 i.e. 5.16 lakh tones and 933 kg/ha, respectively (Anonymous 2017). Maharashtra contributes 30.29 % in terms of area with 28.29 % of production at national level (average of last ten years). Percentage of area increase during 2016-17 as compared to previous year (2015-16) is 27.25 %, 32.22 % and 33.64 % in India, Maharashtra and Marathwada, respectively. In general, there is low productivity of pulses including pigeonpea. Because, the crop is grown on marginal lands, low rainfall areas, poor management, poor crop husbandry, high rate of flower and fruit drop, non- uniform maturity, pod shattering and susceptibility to pests and diseases.

Wilt caused by *Fusarium udum* is the most destructive disease of pigeonpea throughout India. The plant mortality up to 50 per cent has been observed with severe infection of wilt. The main symptoms are wilting of seedlings and adult plants. The wilting starts gradually showing yellowing and drying of leaves followed by wilting of whole infected plant. The affected plants can easily be recognized in patches in the field. Wilt appears on the young seedlings but mainly observed during flowering and podding stage. Surveys conducted for the disease by Kannaiyan *et al.,* (1984) have indicated it to be a major problem in the states of Bihar and Maharashtra (Reddy *et al.,* 1990). *Fusarium* wilt characterized by wilting of the affected plants and characteristic internal browning or blackening of the xylem vessels extending from root system to stems. Partial wilting of the plants (Upadhyay and Rai, 1992) and patches of dead plants (Reddy *et al.,* 1993) were reported to be common in the fields during advanced stages of plant growth. Investigation was carried out to study cultural, morphological and molecular variability of test pathogen.

Materials and Methods

The experiment was conducted at Department of Plant Pathology, College of Agriculture Parbhani, VNMKV, Parbhani (M.S.). The pathogen was isolated from diseased leaves of Pigeonpea on PDA incubated at 27±2 ºC. Ten

highly virulent, test isolates of *F. udum*, representing four agro-climatic zones were subjected to study for their cultural variability. Quantity of 20 ml autoclaved and cooled PDA medium was dispensed in sterile glass petriplates (90 mm diam.) and allowed to solidify at room temperature. Aseptically, these plates were inoculated separately by putting in the centre a mycelial disc (5 mm) obtained from actively growing a week old pure culture of the test isolates and incubated at 27 + 1 ^{o}C. Three PDA plates / isolate / replication were maintained

Cultural variability

Ten highly virulent, test isolates of *F. udum*, representing four agro-climatic zones were subjected to study for their cultural variability. The experiment was planned in CRD and the ten test isolates were replicated thrice.

Observations on cultural characteristics *viz*., colony diameter, colony colour, colony appearance, colony shape and colony margin, zonation, substrate pigmentation *etc*. were recorded after a week of incubation and sporulation was recorded at 10 days of incubation, sporulating culture of the test isolates in Petri plates was flooded with 10 ml distilled water and was gently scraped with camel hair brush, to obtain spore suspension. Temporary mount on glass slide, of the spore suspension was prepared, mounted under research microscope (10X objective lens), counted the spores under five random microscopic fields and averaged. Based on (Kumar and Choudhary, 2006) scale, the test isolates were categorized.

Morphological variability

Temporary mounts in Lactophenol cotton blue stain on glass slides of the sporulated cultures of 10 test isolates were prepared separately and covered with glass slide. The morphological characteristics *viz*., length and breadth, septation of microconidia and macro-conidia of each test isolate (10 days old pure culture growth on PDA) were recorded by using J image software, TS view and with the help of the compound microscope (make: Labomed Vision 2000 as well as Olympus) at 400X magnification under 10 random microscopic fields.

Molecular variability

Molecular variability among 10 isolates of *F. udum* was analyzed by RAPD molecular markers.

Isolation of genomic DNA

The genomic DNA of the 10 test isolates of *F. udum* was isolated, separately by using standard 2 % cetyl trimethyl ammonium bromide (CTAB) extraction method.

Quantification of DNA

Spectrophotometer was used for quantitative and qualitative analysis of the DNA of the test isolates. Five µl of DNA sample was added in Cuvette carrying 0.995 µl of sterile H_2O and absorbance was measured at 280 nm wave lengths. Similarly, the purity of DNA was checked by measuring the ratio of OD at A260/A280 nm. The quantification of DNA was calculated by using following formula.

$$\text{DNA (µg/µl)} = \frac{\text{OD at 260 nm X dilution factor}}{1000} \text{ X } 50$$

Primer screening

Available RAPD primers were used for screening of *Fusarium udum*. The primers were screened on the basis of reproducible and scorable amplification for analysis of *Fusarium udum.* For example, positively screened primers (OP series A to Z) along with their sequence are mentioned below.

Cultural variability

Grade	Sporulation	No. of spores per microscopic field
-	Absent	Nil
+	Poor	1 -10
++	Fair	11 – 30
+++	Good	31 – 50
++++	Excellent	More than 50

Primer screening

Sr. No.	Primer	Sequence 5'-3'	Sr. No.	Primer	
1	OPA-03	AGTCAGCCAC	10	OPC-05	GATGACCGCC
2	OPA-09	GGGTAACGCC	11	OPC-14	TGCGTGCTTG
3	OPA-17	GACCGCTTGT	12	OPC-19	GTTGCCAGCC
4	OPB-04	GGACTGGAGT	13	OPC-20	ACTTCGCCAC
5	OPB-10	CTGCTGGGAC	14	OPD-02	GGACCCAACC
6	OPB-12	CCTTGACGCA	15	OPD-03	GTCGCCGTCA
7	OPB-15	GGAGGGTGTT	16	OPD-05	TGAGCGGACA
8	OPB-20	GGACCCTTAC	17	OPD-07	TTGGCACGGG
9	OPC-01	TTCGAGCCAG			

RAPD analysis of *F. udum* isolates

The PCR protocol for RAPD reaction was optimized with various PCR components and thermal cycler programme. Master mix (24 µl) containing all of the reactants, except template DNA were dispensed in autoclaved PCR tubes (0.2 ml). Genomic DNA of each isolate of *F. udum* was added to the individual tubes containing the master mix.

The contents of each tube were mixed by tapping with fingers, followed by a brief spun to collect the content at bottom of the tube. These tubes were placed in Thermocycler (Bio Rad, USA) and subjected to PCR according to the standardized protocol.

The amplified RAPD product was separated by electrophoresis in 1.5 % agarose gel with 1 X TAE buffer, stained with ethidium bromide (0.5 µg/ml) at 90 V for 1.0 to 1.5 hrs and photographed using gel documentation system (Alpha Innotech, USA). The sizes of the amplification product were estimated using 100 bp to 1 kb ladder (Fermentas, UK). The polymorphism was detected by comparing RAPD product of the test isolates of *F. udum*

Data scoring and analysis

The amplified products generated from RAPD-PCR reaction were resolved on 1.5 % agarose gel. The RAPD amplicons showing monomorphic and polymorphic pattern were scored and amplicon size was determined by comparison with 1 kb DNA ladder (Fermentas, U.K.). Jaccard's similarity coefficient (J) was used to calculate similarity between pairs of varieties, which was as follows (Jaccard, 1908).

$J = n_{xy} / n_t - n_z$

n_{xy} is the number of bands common to variety x and y

n_t is the total number of bands present in all samples and

n_z is the number of bands absent in x and y but, found in all samples

RAPD fingerprint data was scored in present (1) or absent (0) forms, data matrices were generated and used to plot dendrogram exploited for phylogenetic analysis, by using Jacquards' similarity coefficient, using the software NTSYS pc2.02i and Exerter Software.

Results and Discussion

Cultural variability among the *F. udum* isolates

The results obtained on cultural characteristics *viz*., mycelial growth (colony diameter), mycelial colour, colony appearance, growth speed, colony shape, margin, sporulation and pigmentation *etc*. in respect of 10 test isolates of *F. udum* grown on PDA (Table 1, 2 and Fig. 1).

Mycelial growth

The results indicated that among the test isolates, mycelial growth was varied from 54.67 mm (FOU 17) to 89 mm (FOU 16). However, it was the highest in isolate FOU 16 (89 mm), followed by the isolates *viz.*, FOU 30 (88.67 mm), FOU 12 (87.67 mm), FOU 6 (84.33 mm), FOU 22 (83.33 mm), FOU 2 (82.67 mm) and these all six were at par with each other.

In rest of the test isolates, mycelial growth was ranged from 81.33 mm to 54.67 mm. significantly; minimum mycelial growth was found in FOU 17 i.e. 54.67 mm.

The maximum (> 80 mm) colony diameter were seen in seven isolates i.e. FOU 2, FOU 6, FOU 12, FOU 16, FOU 19 and FOU 22 with 70 % frequency and medium colony diameter was seen in isolate FOU 3 isolate with 10 % frequency. However, minimum (<60 mm) colony diameter were seen in isolates FOU 13 and FOU 17 with 20 % frequency.

Colony colour

On the basis of colony colour, the test isolates were categorized into four groups. Group I consisted three isolates with white colony (FOU 2, FOU 19 and FOU 30) shared 30 % frequency. Group II contained two isolates with purple colony (FOU 3 and FOU 13) containing 20 % frequency, group III consisted three isolates with pink colony (FOU 6, FOU 12 and FOU 17) had 30 % frequency, group IV consisted two isolates with buff colony (FOU 16 and FOU 22) shared 20 % frequency.

Colony growth rate

On the basis colony growth rate, the test isolates were categorized as fast growing, moderate growing and slow growing. In fast growing category, the five isolates *viz*., FOU 2, FOU 6, FOU 16, FOU 19 and FOU 30 were included with 50 % frequency. In medium / moderate growing category, the two isolates *viz*., FOU 12 and FOU 22 were included with 20 % frequency as well as in slow growing category the three isolates FOU 3, FOU 13 and FOU 17 were included with 30 % frequency.

Colony shape and margin

On the basis of colony shape (circular) and colony margin (non-serrated, smoother serrated), the test isolates were categorized into two groups. The group I included two isolates with circular colony and non-serrated with smooth margin, which were FOU 19 and FOU 30 with 20 % frequency. The group II included the isolates with circular colony and serrated margin, which contained rest of the eight isolates *viz*., FOU 2, FOU 3, FOU 6, FOU 12, FOU 13, FOU 16, FOU 17 and FOU 22 with 80 % frequency.

Sporulation and pigmentation

The sporulation induced by the test isolates varied from fair (++) to excellent (++++). However, it was excellent (++++) in six isolates *viz*., FOU 2, FOU 6, FOU 12, FOU 13, FOU 16 and FOU 17 with 60 % frequency; good (+++) in two isolates *viz*, FOU 3 and FOU 22 with 20 % frequency and fair (++) in two isolates *viz*., FOU 19 and FOU

30 with 20 % frequency.

On the basis of pigmentation, the test isolates were categorized into five groups. The group I included one isolate with dark yellow pigmentation (FOU 2) with 10 % frequency; the group II included four isolates with pink pigmentation (FOU 3, FOU 6, FOU 12 and FOU 13) with 40 % frequency; the group III included two isolates with brown pigmentation (FOU 16 and FOU 22) with 20 % frequency.The group IV included one isolate with yellow to brown pigmentation (FOU 17) with 10 % frequency and the group V included two isolates with light yellow pigmentation (FOU 19 and FOU 30) with 20 % frequency.

Table.1 Cultural variability among the test isolates of *Fusarium udum*

Sr. No.	Parameters	Isolates (District Location) / Characteristics				
		FOU 2 (Ahmednagar)	**FOU 3 (Akola)**	**FOU 6 (Beed)**	**FOU 12 (Jalna-Badnapur)**	**FOU 13 (Jalna-Mantha)**
1	**Colony dia. (mm)**	82.67	63.00	84.33	87.67	58.67
2	**Colour**	White	Purple	Pink	Pink	Purple
3	**Mycelial Appearance**	Luxuriant, appressed, felted and fluffy	Scanty, partially appressed and fibrous	Luxuriant, appressed and fluffy	Luxuriant, partially appressed and fibrous	Scanty, partially appressed and fibrous
4	**Growth speed**	Fast	Slow	Fast	Medium	Slow
5	**Colony shape**	Circular	Circular	Circular	Circular	Circular
6	**Colony margin**	Serrated	Serrated	Serrated	Serrated	Serrated
7	**Sporulation**	++++	+++	++++	++++	++++
8	**Pigmentation**	Dark Yellow	Pink	Pink	Pink	Pink

Continued….

Sr. No.	Parameters	Isolates (District Location) / Characteristics				
		FOU 16 (Latur)	**FOU 17 (Nagpur)**	**FOU 19 (Nashik)**	**FOU 22 (Parbhani)**	**FOU 30 (Satara)**
1	**Colony dia. (mm)**	89.00	54.67	81.33	83.33	88.67
2	**Colour**	Buff	Pink	White	Buff	White
3	**Mycelial Appearance**	Luxuriant, partially appressed and fibrous	Scanty, partially appressed and fibrous	Luxuriant, appressed, felted and fluffy	Luxuriant, partially appressed and fluffy	Luxuriant, appressed, felted and fluffy
4	**Growth speed**	Fast	Slow	Fast	Medium	Fast
5	**Colony shape**	Circular	Circular	Circular	Circular	Circular
6	**Colony margin**	Serrated	Serrated	Non-Serrated, smooth	Serrated	Non-Serrated, smooth
7	**Sporulation**	++++	++++	++	+++	++
8	**Pigmentation**	Brown	Yellow to Brown	Light Yellow	Brown	Light Yellow

Sporulation: ++++ = Excellent, +++ = Good, ++ = Fair, + = Poor, Dia: Diameter

Table.2 Grouping and frequency of *F. udum* test isolates based on cultural variability

Groups	Cultural parameters	No. of isolates	Isolates code	Frequency
1. Colony growth (Range and category)				
Group-I	Maximum (> 80 mm)	07	FOU 2, 6, 12, 16, 19, 22 and 30	70 %
Group-II	Medium (60 to 80 mm)	01	FOU 3	10 %
Group-III	Minimum (< 60 mm)	02	FOU 13 and FOU 17	20 %
2. Colony colour				
Group-I	White	03	FOU 2, FOU 19 & FOU 30	30 %
Group-II	Purple	02	FOU 3 and FOU 13	20 %
Group-III	Pink	03	FOU 6, FOU 12 & FOU 17	30 %
Group-IV	Buff	02	FOU 16 and FOU 22	20 %
3. Mycelium appearance				
Group I	Luxuriant and appressed	04	FOU 2, FOU 6, FOU 19 and FOU 30	40%
Group II	Luxuriant and partially appressed	03	FOU 12, FOU 16 & FOU 22	30%
Group III	Scanty and partially appressed	03	FOU 3, FOU 13 & FOU 17	30 %
4. Growth speed (mm / day)				
Group I	Fast (12 mm / day)	05	FOU 2, 6, 16,19 & FOU 30	50 %
Group II	Medium (10 mm / day)	02	FOU 12 and FOU 22	20 %
Group III	Slow (06 mm / day)	03	FOU 3, FOU 13 and FOU 17	30 %
5. Colony margin				
Group I	Serrated	08	FOU 2, 3, 6, 12, 13, 16, 17 and 22	80 %
Group II	Non-serrated	02	FOU 19 and FOU 30	20 %
6. Sporulation				
Group I	Excellent (++++)	06	FOU 2, 6, 12,13,16 and FOU 17	60 %
Group II	Good (+++)	02	FOU 3 and FOU 22	20 %
Group III	Fair (++)	02	FOU 19 and FOU 30	20 %
7. Pigmentation				
Group I	Dark yellow	01	FOU 2	10 %
Group II	Light yellow	02	FOU 19 and FOU 30	20 %
Group III	Yellow to Brown	01	FOU 17	10 %
Group IV	Pink	04	FOU 3, FOU 6, FOU 12 and FOU 13	40 %
Group V	Brown	02	FOU 16 and FOU 22	20 %

Table.3 Morphological variability among the test isolates of *F. udum*

Sr. No.	Isolates	Micro-conidia		Macro-conidia	
		Av. Size (µm) Length x Breadth	Septation (No.)	Av. Size (µm) Length x Breadth	Septation (No.)
1	**FOU 2**	8.62 x 3.70	0	28.60 x 4.30	1- 4
2	**FOU 3**	7.41 x 3.10	0-1	25.63 x 4.40	2- 4
3	**FOU 6**	6.55 x 2.73	0	23.20 x 3.90	1- 3
4	**FOU 12**	8.74 x 3.92	0	28.29 x 4.10	1- 3
5	**FOU 13**	6.32 x 2.81	0	26.22 x 4.30	2- 4
6	**FOU 16**	9.02 x 4.10	0	31.83 x 3.89	1- 4
7	**FOU 17**	9.34 x 4.15	0- 1	30.86 x 5.52	1- 3
8	**FOU 19**	5.92 x 2.61	0	22.40 x 4.62	2- 3
9	**FOU 22**	8.43 x 3.54	0	27.62 x 4.10	3- 4
10	**FOU 30**	5.33 x 2.62	0	24.80 x 4.28	1- 2

Table.4 Grouping and frequency of *F. udum* test isolates based on morphological variability

Sr. No.	Conidia	Group and size (µm)	No. of Isolates	Code of isolates	Frequency
I. Length x Breadth size					
1	Micro-conidia	Group I: Large (8.1-10 µm x 3-4 µm)	05	FOU 2, 12, 16, 17 and FOU 22	50 %
		Group II: Medium (7.1-8 µm x 3-4 µm)	01	FOU 3	10 %
		Group III: Small (5-7 µm x 2-3 µm)	04	FOU 6, 13, 19 and FOU 30	40 %
2	Macro-conidia	Group I: Large (28.1-32 µm x 4-6 µm)	04	FOU 2, 12, 16 and FOU 17	40 %
		Group II: Medium (26.1-28 µm to 4-5 µm)	02	FOU 13 & FOU 22	20 %
		Group III: Small (22-26 µm x 3-5 µm)	04	FOU 3, 6, 19 and FOU 30	40 %
II. Septation					
1	Micro conidia	Group I: No septation	08	FOU 2,6,12,13,16,19,22 and FOU 30	80 %
		Group II: Single septation	02	FOU 3 & FOU 17	20 %
2	Macro conidia	Group I: Maximum (1-4, 2-4 & 3-4)	05	FOU 2, 3, 13, 16 and FOU 22	50 %
		Group II: Medium (1-3, & 2-3)	04	FOU 6, 12, 17 and FOU 19	40 %
		Group III: Minimum (1-2)	01	FOU 30	10 %

Table.5 Polymorphic amplifications generated by RAPD markers

Sr. No.	Primer	Total No. of amplicons	Average No. of bands / primer	Total No. of Loci	No. of polymorphic Loci	Per cent Polymorphism (%)
1.	OPA 9	10	0.1	3	3	100
2.	OPA 17	7	0.7	4	4	100
3.	OPA 3	9	0.9	4	4	100
4.	OPB 4	19	1.9	5	5	100
5.	OPB 12	12	1.2	4	4	100
6.	OPB 15	15	1.5	9	9	100
7.	OPC 1	9	0.9	2	2	100
8.	OPC 5	6	0.6	3	3	100
9.	OPC 14	18	1.8	7	7	100
10.	OPD 2	19	1.9	7	7	100
11.	OPD 3	23	2.3	6	6	100
12.	OPD 5	16	1.6	8	8	100
13.	OPD 7	14	1.4	5	5	100
14.	OPC 19	21	2.1	8	8	100
15.	OPC 20	16	1.6	7	7	100
16.	OPB 10	38	3.8	10	10	100
17.	OPB 20	22	2.2	6	6	100
Overall		**274**	-	**98**	**98**	**100**
Average		**16.11**	**1.56**	**5.76**	**5.76**	**100**

Table.6 Similarity index in DNA fingerprinting of *F. udum* isolates

	FOU 2	FOU 3	FOU 6	FOU 12	FOU 13	FOU 16	FOU 17	FOU 19	FOU 22	FOU 30
FOU 2	1.0000000									
FOU 3	0.0000000	1.0000000								
FOU 6	0.0000000	0.1525424	1.0000000							
FOU 12	0.0000000	0.1363636	0.3846154	1.0000000						
FOU 13	0.0000000	0.0882353	0.2553191	0.3666667	1.0000000					
FOU 16	0.1666667	0.0800000	0.0208333	0.0689655	0.0588235	1.0000000				
FOU 17	0.0000000	0.2894737	0.1451613	0.1041667	0.1111111	0.0714286	1.0000000			
FOU 19	0.0000000	0.1600000	0.3793103	0.2916667	0.2250000	0.0540541	0.2200000	1.0000000		
FOU 22	0.0217391	0.1964286	0.2714286	0.2241379	0.1372549	0.0909091	0.2500000	0.4629630	1.0000000	
FOU 30	0.0000000	0.2881356	0.3424658	0.2500000	0.1166667	0.0178571	0.2539683	0.3134328	0.2597403	1.0000000

Table.7 Dis-similarity index in DNA fingerprinting of *F. udum* isolates

	FOU 2	FOU 3	FOU 6	FOU 12	FOU 13	FOU 16	FOU 17	FOU 19	FOU 22	FOU 30
FOU 2	0.0000000									
FOU 3	1.0000000	0.0000000								
FOU 6	1.0000000	0.8474576	0.0000000							
FOU 12	1.0000000	0.8636364	0.6153846	0.0000000						
FOU 13	1.0000000	0.9117647	0.7446809	0.6333333	0.0000000					
FOU 16	0.8333333	0.9200000	0.9791667	0.9310345	0.9411765	0.0000000				
FOU 17	1.0000000	0.7105263	0.8548387	0.8958333	0.8888889	0.9285714	0.0000000			
FOU 19	1.0000000	0.8400000	0.6206897	0.7083333	0.7750000	0.9459459	0.7800000	0.0000000		
FOU 22	0.9782609	0.8035714	0.7285714	0.7758621	0.8627451	0.9090909	0.7500000	0.5370370	0.0000000	
FOU 30	1.0000000	0.7118644	0.6575342	0.7500000	0.8833333	0.9821429	0.7460317	0.6865672	0.7402597	0.0000000

Fig.1 Cultural (Colony growth) variability among the test isolates of *F. udum*

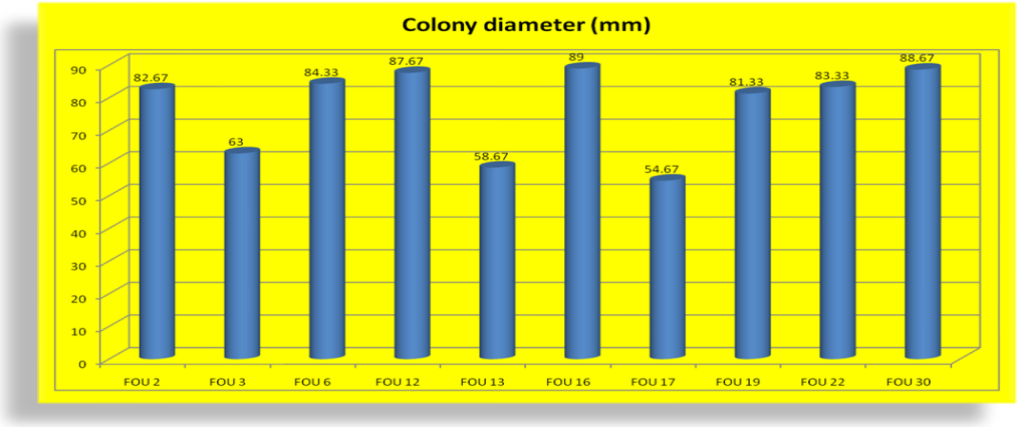
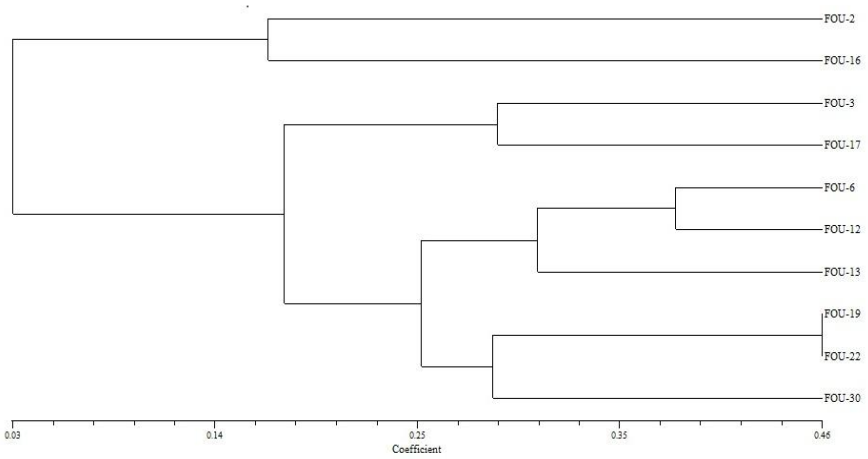


Fig.2 Dendrogram based on RAPD analysis depicting relationship between 10 test isolates of *F. udum*



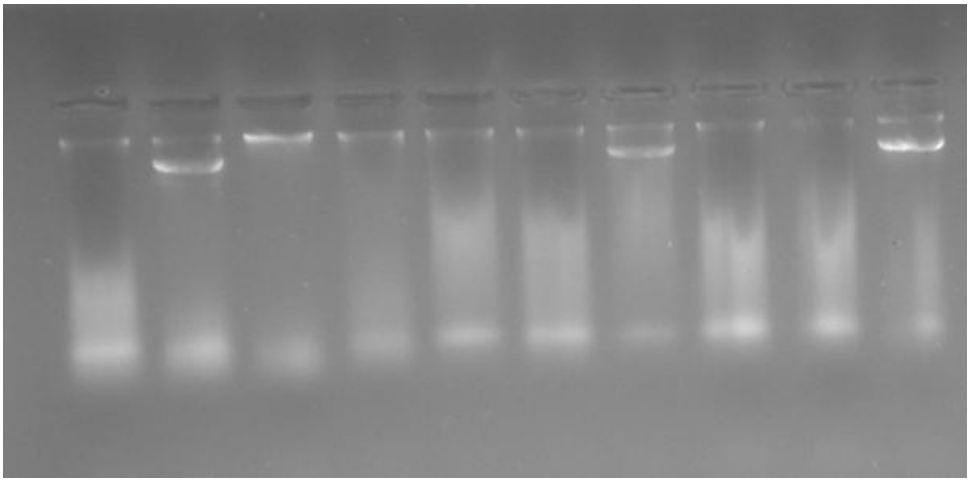


Fig.3 RAPD fingerprient profile of 10 isolates of *F. udum* (DNA)

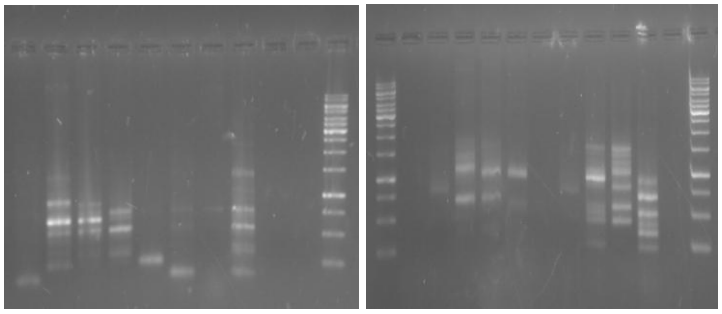


Fig.4 RAPD fingerprient profile of 10 isolates of *F. udum* by using primer OPC 19 & OPC 20 Lane M- marker (1 kb DNA ladder); Lanes 1-10 isolates

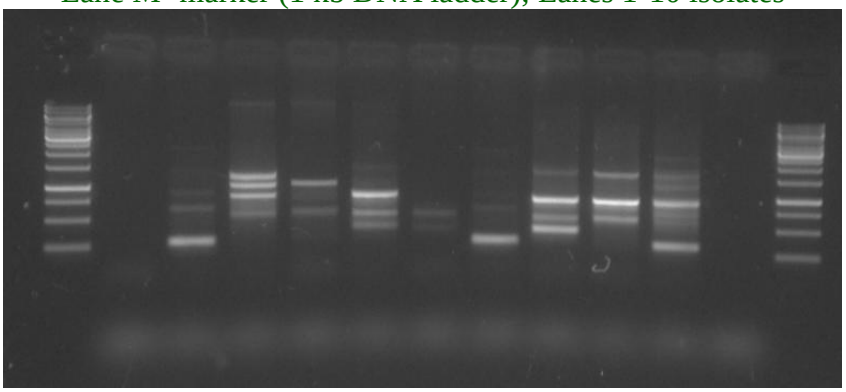


Fig. 5 RAPD fingerprient profile of 10 isolates of *F. udum* by using primer OPB 10 Lane M- marker (1 kb DNA ladder); Lanes 1-10 isolates

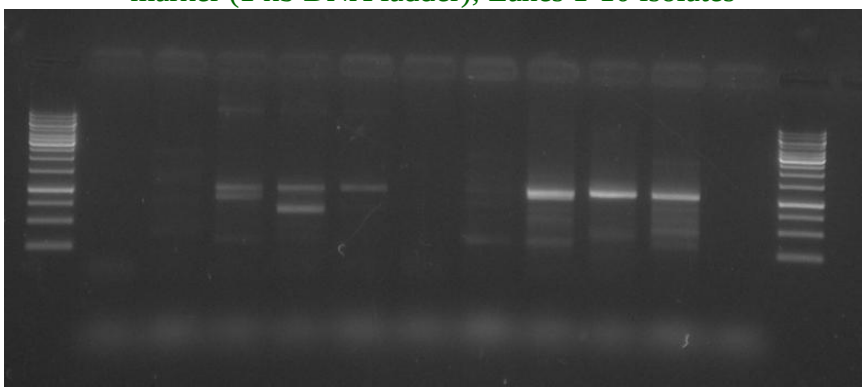


Fig.6 RAPD fingerprient profile of 10 isolates of *F. udum* by using primer OPB 20 Lane M- marker (1 kb DNA ladder); Lanes 1-10 isolates

Morphological variability among *F. udum* isolates

Conidial size

The results revealed that all the ten isolates exhibited a wide range of variability in respect of size of microconidia and macro-conidia. Average size of micro-conidia of the test isolates was ranged from 5.33 µm x 2.62 µm (FOU 30) to 9.34 µm x 4.15 µm (FOU 17). However, maximum micro-conidial size (9.34 x 4.15) was recorded in isolate FOU 17. This was followed by the isolates *viz.*, FOU 16 (9.02 x 4.10), FOU 12 (8.74 x 3.92), FOU 2 (8.62 x 3.70), FOU 22 (8.43 x 3.54), FOU 3 (7.41 x 3.10), FOU 6 (6.55 x 2.73), FOU 13 (6.32 x 2.81), FOU 19 (5.92 x 2.61) and FOU 30 (5.33 µm x 2.62 µm).

Average size of macro-conidia of the test isolates was ranged from 22.40 µm x 4.62 µm (FOU 19) to 31.83 µm x 3.89 µm (FOU 16). However, maximum macro-conidial size (31.83 x 3.89) was recorded in isolate FOU 16.

This was followed by the isolates *viz.,* FOU 17 (30.86 µm x 5.52 µm), FOU 2 (28.60 x 4.30), FOU 12 (28.29 x 4.10), FOU 22 (27.62 x 4.10), FOU 13 (26.22 x 4.30), FOU 3 (25.63 x 4.40), FOU 30 (24.80 x 4.28), FOU 6 (23.20 x 3.90) and FOU 19 (22.40 µm x 4.62 µm).

In case of micro-conidial size three groups *viz*., Group I (large), II (medium) and III (small) showed 50 %, 10 % and 40 % frequency respectively where as in case of macro-conidial size groups *viz*., Group I (large), II (medium) and III (small) showed 40 %, 20 % and 40 % frequency, respectively.

Septation

Results revealed least variability was observed among the test isolates in respect of septa on the micro-conidia. Among the test isolates, septation was ranged from 0 to 1. Isolates FOU 3 and FOU 17 were recorded 0-1 septation, whereas isolates FOU 2, FOU 6, FOU 12, FOU 13, FOU 16, FOU 19, FOU 22 and FOU 30 were more or less oval without septation.

Results revealed marked variability among the test isolates in respect of septa on the macro-conidia. Among the test isolates, septation was ranged from 1-2 to 3-4. Of the test isolates, FOU 22 recorded maximum (3-4) septation, followed by the isolates *viz*., FOU 3 and FOU 13 (2-4), FOU 19 (2-3), FOU 2 (1-4), FOU 6 as well as FOU 17 (1-3) and FOU 30 (1-2 septation).

In case of micro-conidial septation two groups *viz*., Group I (No septation) and II (single septation) showed 80 % and 20 % frequency, respectively. Whereas, in case of macro-conidial septation, three groups *viz*., Group I (maximum), II (medium) and III (minimum) showed 50 %, 40 % and 10 % frequency, respectively. (Table 3 and 4)

The pathogenic, cultural and morphological variability of *F. udum* found in present study are in consonance with the earlier reports (Madhukeshwara and Seshadri, 2001; Kiprop *et al.,* 2002; Reddy 2006; Mahesh *et al.,* 2010; Tiwari and Dhar, 2011; Rangaswamy *et al.,* 2012; Kumar and Upadhyay, 2014; Shinde *et al.,* 2014, Rashmi and Chattannavar, 2016). These results indicated that there is existence of pathogenic, cultural and morphological variability in *Fusarium oxysporum* f. sp. *udum* which might be due to environmental variation or struggle of existence or such several causes.

Molecular variability

DNA fingerprinting profile

The RAPD-PCR protocol described by Chavan, (2004) was used with some modifications to produce DNA fingerprinting profile of 10 fungal isolates of *F. udum* species. The PCR amplification reaction was optimized by varying concentration of PCR components. Amplification reaction was carried out in 25 µl reaction mixtures containing 30 ng of fungal genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 10 pmol primers and 1.50 U of *Taq* DNA polymerase. PCR amplification was performed in master cycler gradient, Eppendorf PCR thermocycler.

The program consisted of an initial denaturing at 94 ^{o}C for 4 min, followed by 39 cycles comprising denaturation at 94 ^{o}C, 1 min, annealing at 37 ^{o}C and extension of 2 min. at 72 ^{o}C. The final extension was set at 72 ^{o}C for 10 min. PCR amplified product was separated by electrophoresis on 1.5 % agarose gel in 1X

TAE buffer, stained with ethidium bromide and visualized under gel documentation system.

Diversity analysis using RAPD marker

The genomic DNA of 10 isolates of *F. udum* isolated from pigeonpea crop was subjected for PCR amplification by using RAPD primers. Initially 17 random primers *viz*., OPA to OPD series were screened (random primer kit A, Operon Tech., USA). These 17 primers were found more polymorphic and generated significant data for discrimination of the test 10 isolates.

The average size of amplicons generated by the test primers was ranged between 100 bp to 10 kb. The RAPD-PCR amplification results showed that about 17 RAPD primers generated a total of 274 bands, which were found polymorphic with an average of 16.11 bands per primer. The primers OPB-10, OPB-15, OPC-19, OPC-5, OPC-14, OPD-02 and OPC-20 were found more informative, as they generated maximum number of bands i.e. 10, 9, 8, 8, 7, 7 and 6 bands, respectively.

OPC-1, OPC-5 and OPA-9 generated low number of 2, 3 and 3 bands, respectively. All amplicons were found polymorphic with 100 % polymorphism. The similarity matrix based on Jaccard's coefficient was prepared by using the scored data of banding pattern with the help of NTSys pc software. The highest similarity (0.462) was found between the isolates FOU 19 and FOU 22 followed by 0.384 between FOU 6 and FOU 12. The lowest similarity (0.00) was found between the isolates FOU 2 and FOU 30.

Dendrogram generated by UPGMA cluster analysis based on Jaccard's similarity coefficient obtained from RAPD markers through NTSys pc software revealed two major clusters. The first cluster consisted of two isolates namely FOU 2 and FOU 16 while second cluster contained eight isolates *viz.,* FOU 3, FOU 17, FOU 6, FOU 12, FOU 13, FOU 19, FOU 22 and FOU 30. The isolates FOU 19 and FOU 22 were found to be most similar with 46% similarity. Exactly reciprocal / opposite results were obtained in dissimilarity index.

Molecular variability (Genetic diversity) among *Fusarium udum* was demonstrated earlier by many scientists. (Kumar *et al.,* 2007; Datta *et al.,* 2009; Kiprop *et al.,* 2005; Prasad *et al.,* 2012; Mesapogu *et al.,* 2012 and Shinde *et al.*, 2015)

Thus, in present study pathological, cultural, morphological and molecular variability observed among the isolates of *F. udum* may be attributed to their distribution in different Agro-climatic zones of the Maharashtra state, long term *F. udum* pathogen at a particular location and ability of the pathogens to adopt themselves in different cultivars (Table 5, 6, 7 and Fig. 2).

References

Anonymous (2017). Annual Report for 2017. Chief Statistician, Pune, Maharashtra, India.

Chavan, R. L. (2004). Study of genetic variability among isolates of *Alternaria* species infecting sunflower: cultural, morphological, pathological, biochemical and molecular investigations. M. Sc. (Agri.) Thesis, M.A.U. Parbhani., Pp. 1-72.

Dutta, S., Rita, R., Dhar, V., Chaudhary, R. G. and Gurha, S. N. (2009). RAPD based diagnosis and diversity analysis of *Fusarium* wilt pathogen of pulse crops. *J. Food Legumes*, 22 (2): 77-81.

Jaccard, P. (1908). Nawelle recherché surla distribution Florale. Bulletin de la Societc Vaucloise des Sciences Naturelles., 44: 223-270.

Kannaiyan, J., Nene, Y. L., Reddy, M. V., Rayan, J. G. and Raju, T. N. (1984). Prevalence of pigeonpea diseases and associated crop losses

in Asia and Amirica. *Trop. J. Pest Management*, 30: 62-71.

Kiprop, E. K., Baudoin, J. P., Mwang'ombe, A. W., Kimani, P. M. and Mergeai, G. (2002). Characterization of Kenyan Isolates of *Fusarium udum* from Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Cultural Characteristics, Aggressiveness and AFLP Analysis., *J. Phytopathol*. 150 (10): 517–525.

Kiprop, E. K., Mwang'ombe, A. W., Baudoin, J. P., Kimani, P. M. and Mergeai, G. (2005). Genetic Variability among *Fusarium udum* isolates from pigeonpea. *African J. Crop Sci*., 13 (3): 163-172.

Kumar, D. and Choudhary, U. (2006). Influence of temperature on mycelial growth and sporulation of *A. brassicae* and *A. brassicicola* causing blight. *J. Res. SKUAST-J*. 5 (1): 48-51.

Kumar, S. and Upadhyay, J. P. (2014). Studies on cultural morphological and pathogenic variability in isolates of *Fusarium udum* causing wilt in pigeonpea. *Indian Phytopathol*, 67 (1): 55-58.

Kumar, V., Chavan, V. B. and Shrivastva, J. P. (2007). Pathogenic and biochemical variability in *Fusarium udum* causing pigeonpea wilt. *Indian Phytopathol.* 60 (3): 281-288.

Madhukeshwara, S. S. and Sesadri, V. S. (2001). Variation and management of *Fusarium udum* of pigeonpea (*Cajanus cajan* (L.) Millsp.) *Trop. Agril. Res*. 13: 380-394.

Mahesh, M., Saifulla, M., Prasad, P. S. and Sreenivasa, S. (2010). Studies on cultural variability of *Fusarium udum* isolates in India. *Inter. J. Sci. Nature* 1 (2): 219- 225.

Mesapogu, S., Bakshi, Achala, Babu, B. K., Reddy, S. S., Sexsena, S. and Arora, D. K. (2012). Genetic diversity and pathogenic variability among Indian isolates of *Fusarium udum* infecting pigeonpea (*Cajanus cajan* (L.) Millsp.). *Inter. Research J. Agril. Sci. Soil Sci*. 2 (1): 51-57.

Prasad, P. S., Saifulla, M., Mallikarjuna, N., Thimmegowda, P. R. and Lakshmipathy, R. N. (2012). Integrated disease management of Pigeonpea wilt *Fusarium udum* (Butler). *Madras Agric. J.*, 99 (10): 811-814.

Rangaswany, E., Pushpavati, B., Mallikarjuna, M. G. and Reddy, P. N. (2012). Morphological and cultural characters of *Fusarium udum*. *Bioinfolet*, 9 (4): 572-575.

Rashmi, U. S. and Chattannavar, S. N. (2016). Cultural and morphological diversity among the isolates of *Fusarium udum* in Karnataka. *J. Fa. M. Sci*., 29 (23): 365-369.

Reddy, B. A. and Saifulla, M. (2006). Variation in growth and morphology of *Fusarium udum* isolates. *Karnataka J. Agril. Sci.*, 19 (2): 318-322.

Reddy, M. V., Nene, Y. L., Kannaiyan, J., Raju, T. N., Saka, V. N., Davor, A. T., Songa, W. P. and Omanga, P. (1990). Pigeonpea lines resistant to wilt in Kenya and Malawi. *Inter. Pigeonpea Newsl*, 6: 34.

Reddy, M. V., Raju, T. N, Sharma, S. B., Nene, Y. L. and McDonald, D. (1993). Hand book of pigeonpea diseases Information Bulletin. 42: 8-10.

Shinde, A. S., Kalaskar, S. R., Rathod, A. H., Sheikh, W. A. and Acharya, S. (2015). RAPD based molecular diversity analysis of different *Fusarium udum* Butler isolates of pigeonpea wilt. *Inter.J. Pl. Protec*., 8 (1): 81-85.

Shinde, V. S., Zagade, S. N. and Chavan, A. A. (2014). Cultural and morphological variation in *Fusarium udum*. *J.Pl. Dis. Sci.*, 9 (2): 237-244.

Tiwari, S. and Dhar, V. (2011). Prevalence of new variants of *Fusarium udum* in India. *Indian Phytopath*, 64: 243-246.

Upadhyay, R. S. and Rai, B., (1992). Wilt of pigeonpea. In: Plant Disease of International Importance (Eds. Singh, U.S., Mukhopadhyaya, A., Kumar, J. and Chaube, H.S.), Prentice Hall, Englewood Cliffs New Jersey, pp. 388-414.

How to cite this article:

Ghante, P.H., K.M. Kanase, S.P. Kale, R.L. Chavan, K.M. Sharma and Ghuge, S.B. 2018. Cultural, Morphological and Molecular Variability of *Fusarium oxysporum* f. sp. *udum* Isolates by RAPD Method. *Int.J.Curr.Microbiol.App.Sci.* 7(10): 2109-2122.
doi: https://doi.org/10.20546/ijcmas.2018.710.243