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Morphogenetic variations among the isolates of Pigeon pea wilt pathogen isolated from different regions of Bundelkhand

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Abstract

Vascular wilt of pigeon pea [*Cajanus cajan* (L.) Millsp.] caused by *Fusarium udum* Butler is a significant threat to pigeon pea growers worldwide. This pathogen invades the xylem tissues of pigeon pea plants, leading to considerable losses for growers. Variability in the virulence of the pathogen is observed across different geographical regions growing the same crop. Pathogens isolated from selected areas of Bundelkhand exhibited morphological, cultural, and genetic differences, with distinct strains of *F. udum* identified. These variations were labeled as strains FUA, FUB, FUC, FUD, and FUE, and their pathogenicity was assessed. Under cultural conditions, these strains produced cottony colonies ranging from white to yellow, with abundant fluffy aerial mycelium and light yellow to deep red pigmentation on the underside of the plate. Genetic variability among these strains was evaluated using five Random Amplification of Polymorphic DNA (RAPD) primers: OPA2, OPA3, OPE02, OPB1, and A16. Primer OPA3 yielded the maximum number of bands across all five strains of *F. udum*, while primer OPE02 produced the fewest amplifications.

Key words : Wilt Disease, Pathogenesis, Cultural Variation, Genetic Variation, RAPD-PCR.

Fusarium oxysporum is a soil-borne fungus found in diverse soil types, causing various plant diseases including cortical rots, head blights, leaf spots, root rots, fruit rots, cankers, dieback, and vascular wilt. Among these diseases, vascular wilt of crops is particularly significant and is caused by different formae speciales of *Fusarium oxysporum*[15]. *F. oxysporum* is a host-specific pathogen, typically infecting one or a few species of plants and often specific cultivars of those plants. Approximately 120 formae speciales and races have been reported[23]. Vascular wilt of pigeon pea is caused by

F. oxysporum f. sp. *udum* (*F. udum*).

Variations in cultural and morphological characteristics are observed in cultures of *F. udum* isolates, whether from the same site or different geographical locations. These variations also affect pathogenicity on pigeon pea genotypes[10]. Molecular tools, such as Random Amplification of Polymorphic DNA (RAPD) analysis, are useful for identifying correct isolates. Molecular techniques provide accurate differentiation at the DNA sequence level. Nowadays, numerous methods are being adopted to assess variability in Fusarium species, with the majority being DNA-based[7,21]. This study focuses on cultural and genetic variations among strains of *F. udum* collected from pigeon pea fields in several districts of Bundelkhand, Uttar Pradesh.

Isolation of Fusarium udum strains :

Strains of *Fusarium udum* were collected from pigeon pea fields affected by *Fusarium* wilt in three districts of Bundelkhand: Jhansi, Jalaun, and Mahoba (U.P.). The isolated pathogens exhibited cultural and morphological variability upon isolation. Plates with similar characteristics were designated as *F. udum* A (FUA), *F. udum* B (FUB), *F. udum* C (FUC), *F. udum* D (FUD), and *F. udum* E (FUE). Each designated strain was mass multiplied on PDA and incubated in a BOD incubator at 28±2°C. The experiment was conducted in the Department of Botany, Bundelkhand University, Jhansi. Growth patterns, colony color, and reverse plate characteristics were recorded at regular intervals using the Munsell color chart. The color of the reverse plate on PDA was determined. These isolated strains were further characterized by RAPD analysis of genomic DNA preparations with random primers. Polymorphism was detected by recording the presence or absence of bands, revealing similarities or dissimilarities at the DNA level among the isolates. Details of the RAPD analysis is provided under the analysis of *F. udum* isolates provided by Biokart India Pvt. Ltd.

Pathogenesis :

Pathogenesis of isolated pathogenic strains was conducted using Koch's postulate. Cultures of pathogenic strains were mass multiplied on PDA medium, and 10% of the inoculum was thoroughly mixed with autoclaved soil in sterilized polybags at the time of seed sowing, with five replicates of each strain and control maintained. Observations were made for up to six weeks, and the results on pathogenicity were recorded and tabulated based on the visibility of wilt symptoms on the aerial parts of the seedlings. The pathogen was then re-isolated and confirmed by preparing temporary slides.

Molecular marker :

In this study, five primers from the Operon series were used. This series relies on previously synthesized primer sequences from Operon technology. The names, sequences, temperatures, and percent content of the primers are given in Table-1.

Isolation and purification of genomic DNA of Fusarium udum strain :

The culture was retrieved and placed in a mortar, then homogenized with 1 ml of extraction buffer. The homogenate was

Table-1. Details of primers used in the RAPD-PCR analysis

S.no.	Oligo name	Sequence (5'→3')	Tm (°C)	GC-Content
1	OPA2	TGCCGAGCTG	34	70%
2	OPA3	GGGTCCAAAG	32	60%
3	OPE02	GGTGCGGGAA	27	70%
4	OPB1	GTTTCGCTCC	25	60%
5	A16	AGCCAGCGAA	32	60%

transferred to a 2 ml microcentrifuge tube. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the tubes and mixed gently by shaking. The tubes were centrifuged at room temperature for 15 minutes at 14,000 rpm. The upper aqueous phase was collected in a new tube, and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 minutes at 14,000 rpm was transferred to a new tube.

The DNA was precipitated from the solution by adding 0.1 volumes of 3 M Sodium Acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 minutes of incubation at room temperature, the tubes were centrifuged at 4°C for 15 minutes at 14,000 rpm. The DNA pellet was washed twice with 70% ethanol and then briefly with 100% Ethanol and air dried. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA, 5 µl of DNase-free RNase A (10 mg/ml) was added to the DNA.

Table-2. DNA quantification of different strains of *F. udum*

S.no.	Sample ID	DNA (µg/ml)
1	A	61.7
2	B	62.8
3	C	60.3
4	D	59.7
5	E	60.8

The thermal cycling conditions of template DNA, primers and reactant to repeated cycles for this analysis was given below.

Table-3. RAPD-PCR conditions

S. no.	Reaction Mixture (20 µl)		Cycling Conditions		
1	Template DNA	25 ng	Initial Denaturation	2 minutes at 95°C	
2	Primer	1 µM	Denaturation	1 minute at 95°C	40 Cycles
3	dNTPs	0.5mM	Annealing	1 minute at 34°C	
4	Mgcl2	2.5mM	Extension	2 minutes at 72°C	
5			Final Extension	5 minutes at 72°C	

From the pathogenesis findings, it was noted that seeds infested by the pathogen exhibited a decrease in germination percentage compared to the control (Table-4). There was approximately a thirty percent reduction in seed germination, which could be attributed to the pathogen or potentially to variability in the seeds themselves. Table-5 displays the results of the analysis of variance (ANOVA) test for germination percentage. The p-value for the "Between Groups" variation is 0.008127, indicating a statistically significant difference in germination percentage between at least two of the groups being compared. The F-value, which exceeds the critical F-value for a significance level of 0.05, further supports this significance. Panwar *et al.*[17], Sukumar *et al.*[22], and Rangaswamy *et al.*[18] conducted similar studies on the pathogenicity of *F. udum* isolates, observing virulence with minor variations in incidence and symptoms. Table-6 presents p-values indicating significant differences in infection rates of pigeon pea leaves among different pathogen isolates.

Variations in isolates may stem from the genetic origin of the host or pathogen. Higher diversity levels are crucial for the adaptability of the pathogen across a wider range of environments. *F. udum* has been classified into different groups based on colony characteristics, mycelial growth, mycelial width, spore size, and colony pigmentation by various researchers[12,16,20]. A culture-dependent approach was utilized to assess the cultural variability of *F. udum* from different pigeon pea fields. The pathogen produced cottony, profuse, fluffy colonies with white to yellow colony color and light yellow to deep red pigmentation (Fig. 1). The mycelium was septate and hyaline, producing long, slender, septate micro and macroconidia with slightly curved or long curved spores with pointed or blunt ends (Fig. 2). Five different strains (A, B, C, D, and E) of *F. udum* were obtained from twelve villages (Table-7). Mahesh *et al.*[14] collected 41 isolates of *F. udum* from different states of India, all showing a wide range of variations in mycelial color and pigmentation. Several studies support these findings[1,8,13,19].

RAPD analysis is a reliable method for differentiating Fusarium isolates and studying species variation[9,2,3]. After morphological variability, diversity analysis of F. udum strains was conducted with five RAPD primers at different configuration levels, generating a total of 40 bands with an average of 8 bands per primer. Primer OPA3 yielded the highest amplification (15 bands), followed by OPA2 and A16 (Figure 3). Primer OPE02 produced the fewest amplifications, with a single band position in fungal strain B. Different climatic conditions significantly influence isolates of the same species collected from different environmental conditions, as observed by Panwar *et al.*[17]. Birajdar *et al.*[5] screened a total of 20 RAPD primers, with 8 primers yielding polymorphic patterns, consistent with findings by Wang *et al.*[24], Bogale *et al.*[6] Kiprop *et al.*[11], and Belabid *et al.*[4] at the molecular level in *Fusarium*.

Table-4. Pathogenicity test for different isolates of *Fusarium udum*

S. No	Treat-ments	Germi-nation %	After 30 Days			After 45 Days		
			Plant Height (cm)	No. of Leaves	Infected Leaves	Plant Height (cm)	No. of Leaves	Infected Leaves
1	FU A	69.99	14.8	43.2	22.8	18.6	9.6	5.4
2	FU B	66.66	13.6	42.8	22	20.6	10.4	6
3	FUC	64.66	13.2	40.6	23.4	20	10.8	6.8
4	FUD	70.66	13.6	44	20.6	20.8	10.4	7.4
5	FUE	66.66	14	42.6	22.4	19	11.6	6.4
6	Control	96.66	15	65.4	-	23.2	87.2	-

Table-5. ANOVA Test for germination percent

Source of variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3616.055	5	723.211	4.068837	0.008127*	2.620654148
Within Groups	4265.854	24	177.7439			
Total	7881.908	29				

$P<0.05$

Table-6. F test for infected leaves of pigeon pea in different isolates of *Fusarium udum* strain

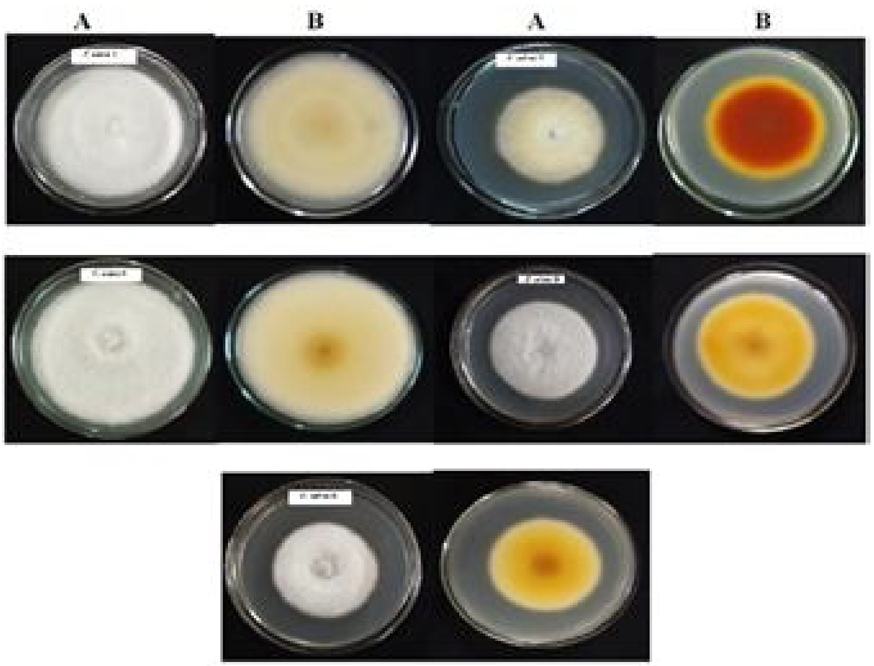
Mean	47.08	26.08
Variance	106.372	1167.632
Observations	5	5
df	4	4
F	0.091101	
P(F<=f) one-tail	0.01975*	
F Critical one-tail	0.156538	

$P<0.05$

Table-7. Morphological characters of different isolates of *Fusarium udum*

S. No.	Isola-tes	Isolated Place	Colony Characters	Growth	Mycelium	Pigmen-tation (Reverse)	Spore morphology
1	A	Chirgaon, Bara, Bagi, Saidnagar	White to cream	Fast	Septate, Hyaline	Light Yellow	Micro and macroconidia observed. The microconidia were minute, slightly curved with blunt ends.

2	B	Aata, Okuruaa	White to Yellow	Slow	Septate, Profuse, Hyaline	Reddish brown with yellowring	Long, slender, septate and oval microconidia observed.
3	C	Kulpahad, Ratauli	White to light yellow	Fast	Profuse, Cottony,	Light Yellow with centric dark brown	Micro and macroconidia along with cylindrical or slightly curved ends
4	D	Babina, Lamsar	White	Mode-rate	profuse, fluffy	Yellowish brown	The microconidia were minute, elliptical, curved and the macroconidia are long, curved, pointed at the tips.
5	E	Etora, Jolhopur	White	Slow	extensive and cottony	Yellow to centric brown	Macroconidia are slightly curved or bent with pointed ends.



A- *Colony colour*
B- *Reverse plate colour*

Figure 1. Different strains of *F. udum* showing morphological variability

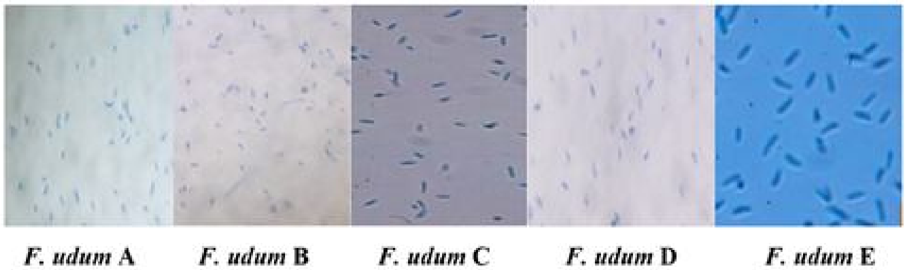
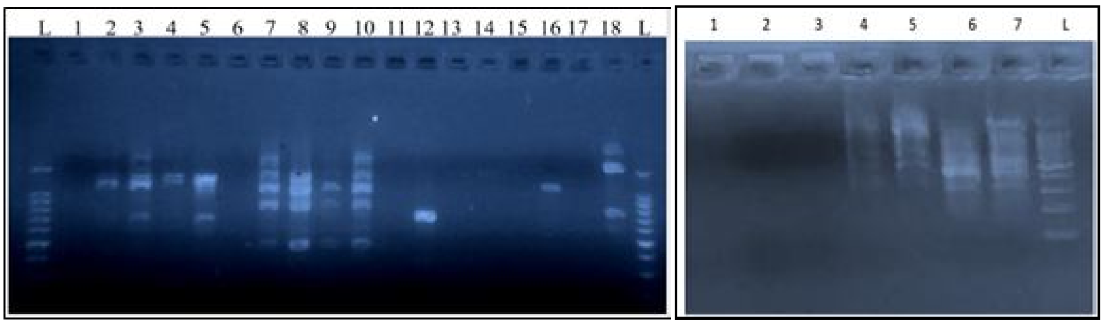


Figure 2. Micrograph of different strains of *F. udum*



Gel Description:
L-Ladder
1-A-OPA2; 2-B-OPA2 3-C-OPA2 4-D-OPA2; 5-E-OPA2;
6-A-OPA3; 7-B-OPA3; 8-C-OPA3; 9-D-OPA3; 10-E-OPA3;
11-A-OPE02; 12-B-OPE02; 13-C-OPE02; 14-D-OPE02; 15-E-OPE02
16-A-OPB1; 17-B-OPB1; 18-C-OPB1
L-Ladder
1- D-OPB1; 2-E-OPB1;
3-A-A16; 4-B-A16; 5-C-A16; 6-D-A16; 7-E-A16
L-Ladder

Fig 3: Band pattern of RAPD fingerprint generated with primer OPA2, OPA3, OPE02, OPBI,A16

In conclusion, *Fusarium oxysporum* poses significant challenges to various crops, including pigeon pea, due to its host-specific pathogenicity. Variability in cultural and genetic characteristics among *F. udum* isolates impacts pathogenicity, with molecular techniques like RAPD analysis aiding in accurate identification. The decrease in seed germination percentage due to pathogen infestation underscores the importance of understanding these variations. Further research on the genetic diversity of *F. udum* strains is crucial for effective management strategies against vascular wilt in pigeon pea cultivation.

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