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Full Length Research Paper

Genetic diversity and pathogenic variability among Indian isolates of *Fusarium udum* infecting pigeonpea (*Cajanus cajan* (L.) millsp.)

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Genetic diversity and pathogenic variability among *Fusarium udum* isolates collected from different geographical locations of India were studied. All the isolates exhibited variable levels of virulence against a susceptible pigeonpea cultivar (T-21). The genetic diversity allelic variations among these isolates were estimated using RAPD molecular markers. All the thirteen RAPD primers were found to be highly reproducible and produced a total of 126 loci of which 69 loci were polymorphic. Primer OPB 17 amplified highest number of polymorphic bands with maximum polymorphic information content (PIC) of 4.00. Percentage of polymorphism revealed by individual primers varied from 33.3 to 76.9% with an average of 53.4%. Further, cluster analysis of OPB-17 and provided a substantially discrimination of all the isolates. Our results showed a high degree of variability in pathogenicity and genetic diversity among the populations. Therefore the present studies indicate that the *F. udum* may have significant impact towards the emergence/evolutionary development.

Keywords: *Fusarium* wilt, pathogenecity, pigeonpea, RAPD.

INTRODUCTION

Pigeonpea (*Cajanus cajan* (L) millsp.) is one of the most extensively grown legume crops in India, accounting for almost 90% of world's area and production (Dhanasekar et al., 2010). Vascular wilt of pigeonpea caused by *Fusarium udum* is the most important disease, causing more economic damage to the crop. The fungus can survive on infected plant debris in the soil for about three years and causes serious yield losses, some times upto 100% in susceptible cultivars (Kiprop et al., 2002). The total production loss due to this disease in India alone was estimated to be approximately 97,000 tones per year (Saxena et al., 2010).

Use of resistant cultivars is the most practical and economical method for any disease management practices. However, in case of vascular wilt caused by *F. udum*, deployment of resistant varieties may become extensive because of the high level of genetic variability in the pathogenic population (Baldev and Amin, 1974). Moreover, *F. udum* isolates from the same site/host have been shown to exhibit high variability in cultural characteristics (Reddy and Chaudhary, 1985; Gaur and Sharma, 1989; Jeswani et al., 1977; Kiprop et al., 2002). Pathogenicity tests are the only means of determining the pathological effect of fungal strains present in diseased plants or in soil samples. However, in some pathosystems, race identification by pathogenicity assays provides very little information about genetic diversity within, or relatedness among races of the pathogen. These assays are cumbersome, time-consu-

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ming, require extensive facilities and are often influenced by the conditions of the experimental system. Therefore, characterization of genetic variation among pathogenic isolates of *F. udum* may be a primary step to understand and correlate strain variation with regard to population structure.

Studies on genetic relationships and phylogeny among *Fusarium* species have been conducted at molecular level on various *Fusarium* sp (Belabid et al., 2004; Kiprop et al., 2005; Bogale et al., 2007; Wang et al., 2010). However, these techniques are proven to be slow, expensive and are not amenable for assessment of genetic variation in large scale population genetic studies.

Several researchers have grouped *Fusarium* sp population from different plant host by using randomly amplified polymorphic DNA (RAPD) analysis and suggested that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium* sp. into their respective pathogenic groups (Jana et al., 2003). RAPD markers have been used for analysis of genetic diversity among different *F. oxysporum* formae specials (Balmas et al., 2005; Bayraktar et al., 2008). However, no comprehensive effort has been made to investigate genetic and pathogenic variability among *F. udum* isolates obtained from various agro-climatic zones of India. The present study was aimed to determine the degree of cultural and pathogenic variability among the F. *udum* isolates collected from different pigeonpea and their alternate hosts (*Crotalaria verrucosa* and *Cicer arietinum*) and to estimate the genetic relatedness by using RAPD markers.

MATERIALS AND METHODS

Fungal isolates and cultural characteristics

Thirty isolates of *F. udum* infecting pigeonpea were isolated from seven states of India. All the isolates were maintained at National Agriculturally Important Microorganisms Culture Collection (NAIMCC), India (Table 1). Cultural variability of each isolate was studied after 7 days of inoculation on potato sucrose agar plates at 25 ºC under dark. Morphological and physiological variability in *F. udum* isolates were recorded at every 24 h for each culture.

Pathogenicity assay

Pathogenicity assay for each *F. udum* isolate was performed on susceptible cultivar of pigeonpea cv. T-21 as per the standard pot-culture inoculation method as described by Nene and Haware (1980). In brief the inoculum was prepared by supplementing 5 agar plugs (1 mm) of *F. udum* isolate grown on PSA at 25 ± 2 ºC for 10 days in to the conical flasks (250 ml) containing 50 gm of sterile substrate (45 g sand + 5 g pigeonpea meal). After 10 days of incubation, inoculum was mixed with 1 kg of sterile soil in pots (25 cm diameter) containing surface sterilized (0.1% $HgCl_2$, 2 min) pigeonpea seeds (6 seeds/pot) for growth upto 8 weeks at 25 ± 1 ºC with relative humidity 30–50% and 14 h photoperiod (light intensity, 297^{-1} moles/(sec.m^2). Plants growing on uninoculated sterile soil served as control. The wilt incidence on host plant was recorded up to 8 weeks and percentage of disease incidence (*I* %) was calculated.

Wilt incidence (*I* %) = Number of wilted plants/ Total number of plants × 100

Grading of wilt incidence (*I %)* = 0–20% - avirulent, >20–50% - moderately virulent; >50% - highly virulent.

Genomic DNA extraction and RAPD fingerprinting

The genomic DNA extracted from all the *F. udum* isolates (Table 1), as described by Babu (2007). Thirteen random primers were analyzed to obtain specific fingerprinting patterns in *F. udum*. PCR was prepared in a total reaction volume of 25 µl containing 2.5 µl of 10X PCR buffer (100 mM, Tris-HCL, pH 8.3, 250 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTPs, 50 pmol primer, 1U of *Taq* DNA polymerase (Bangalore Genei, India) and 20-50 ng genomic DNA. The PCR was programmed for 5 min of initial denaturation at 94ºC followed by 40 cycles at 95ºC for 1 min, 35ºC for 1 min, 72ºC for 2 min and 72 ºC for 8 min. The PCR products were resolved by 1.6% agarose gel containing ethidium bromide (0.25 mg/ml) and visualized under UV transilluminator.

Statistical data analysis

All bands were scored for presence (1) or absence (0) at positions and the scores were assembled in a rectangular data matrix. The data was analyzed by Jaccard's coefficient using NTSYS-PC numerical taxonomy package Version 2.0; (Exeter Biological Software, Setauket, NY). The dendrograms were constructed using UPGMA clustering program (Rohlf, 1998). The fingerprints generated by different primers were compared for their relatedness among isolates.

RESULTS

Cultural characteristics

Mycelial growth pattern of isolates varied from sparsed to abundant, but mycelia in culture remained fluffy to suppressed or scanty fibrous. Up on ageing (2 to 7 days after inoculation) the colour of mycelia varied from yellow/white to grayish purple, while extracellular pig-

Table 1. Cultural and virulence characteristics of *Fusarium udum* isolates from different geographical locations of India

S.No	NAIMCC Accession no	Host	Geographical origin	Mycelium Colour	Substrate Colour	Mycelial Growth	Virulence *
1	CABI 193652	*Cajanus indicus*	Hyderabad, Andhra Pradesh	White	White cream	Fluffy	M
2	CABI 170431	*Crotalaria verrucosa*	Hyderabad, Andhra Pradesh	Grayish purple	Mulbur purple	Scanty fibrous	M
3	CABI 241426	*Cicer arietinum*	Hyderabad, Andhra Pradesh	Yellowish	Gold brown	Fluffy	L
4	NAIMCC-F-1708	*Cajanus cajan*	Kanpur, Dehat, Uttar Pradesh	White	Gold brown	Scanty fibrous	H
5	NAIMCC-F-1703	*Cajanus cajan*	Fatehpur, Uttar Pradesh	Pinkish	Mulbur purple	Suppressed	H
6	NAIMCC-F-1702	*Cajanus cajan*	IIPR farm, Uttar Pradesh	Yellowish	White cream	Scanty fibrous	H
7	NAIMCC-F-1710	*Cajanus cajan*	Fatehpur Uttar Pradesh	Yellowish	Gold brown	Fluffy	M
8	NAIMCC-F-1714	*Cajanus cajan*	IIPR farm, Kanpur,U. P	White	Mulbur purple	Fluffy	H
9	NAIMCC-F-1688	*Cajanus cajan*	Banda, Uttar Pradesh	Yellowish	White cream	Suppressed	M
10	NAIMCC-F-1686	*Cajanus cajan*	Banda, Uttar Pradesh	White	Mulbur purple	Scanty fibrous	H
11	NAIMCC-F-1709	*Cajanus cajan*	Kanpur Dehat,Uttar Pradesh	Yellowish	White cream	Fluffy	M
12	NAIMCC-F-1685	*Cajanus cajan*	Banda, Uttar Pradesh	Yellowish	White cream	Scanty fibrous	M
13	NAIMCC-F-1713	*Cajanus cajan*	BHU, Varansi, Uttar Pradesh	Yellowish	Mulbur purple	Suppressed	H
14	NAIMCC-F-1674	*Cajanus cajan*	Kanpur, Dehat, Uttar Pradesh	Yellowish	White cream	Fluffy	M
15	NAIMCC-F-1211	*Cajanus cajan*	Tandawa, Jharkhand	White	Gold brown	Fluffy	M
16	NAIMCC-F-1214	*Cajanus cajan*	Sangbaria, Jharkhand	Grayish purple	Mulbur purple	Suppressed	M
17	NAIMCC-F-1212	*Cajanus cajan*	Lakhiah, Jharkhand	Yellowish	Mulbur purple	Scanty fibrous	M
18	MTCC 2755	*Lasperysi leucostoma*	Not known	Grayish purple	Mulbur purple	Suppressed	M
19	NAIMCC-F-1215	*Cajanus cajan*	Maurshidabad, West Bengal	White	White cream	Fluffy	H
20	NAIMCC-F-571	*Cajanus cajan*	Faridabad, Haryana	Yellowish	Gold brown	Scanty fibrous	M
21	NAIMCC-F-563	*Cajanus cajan*	Rohtak, Haryana	Yellowish	White cream	Fluffy	M
22	NAIMCC-F-562	*Cajanus cajan*	Rohtak, Haryana	Yellowish	Mulbur purple	Fluffy	H
23	NAIMCC-F-570	*Cajanus cajan*	Rohtak, Haryana	White	White cream	Fluffy	M
24	NAIMCC-F-561	*Cajanus cajan*	Sonipat, Eastern Haryana	Yellowish	Gold brown	Fluffy	M
25	NAIMCC-F-234	*Cajanus cajan*	Rangareddy, Andhra Pradesh	Grayish purple	Mulbur purple	Suppressed	H
26	NAIMCC-F-567	*Cajanus cajan*	Bhiwani, Central Haryana	White	Mulbur purple	Fluffy	H
27	NAIMCC-F-559	*Cajanus cajan*	Faridkat, Central Punjab	White	Gold brown	Fluffy	M
28	NAIMCC-F-557	*Cajanus cajan*	Baran, South Eastern Rajasthan	Grayish purple	Mulbur purple	Suppressed	H
29	NAIMCC-F-556	*Cajanus cajan*	Dholpur, Rajasthan	Yellowish	White cream	Fluffy	H
30	NAIMCC-F-569	*Cajanus cajan*	Sonipat, Haryana	White	White cream	Fluffy	M

*Moderately susceptible (>20-50% wilt), H-Highly susceptible (>50% wilt), *F. udum* against pigeonpea susceptible cultivar (cv. T-21)
NAIMCC - National Agriculturally Important Microbial Culture Collection, India, **MTCC**- Microbial Type Culture Collection, India.
CABI-U.K - Centre for Agriculture and Biosciences International, U.K

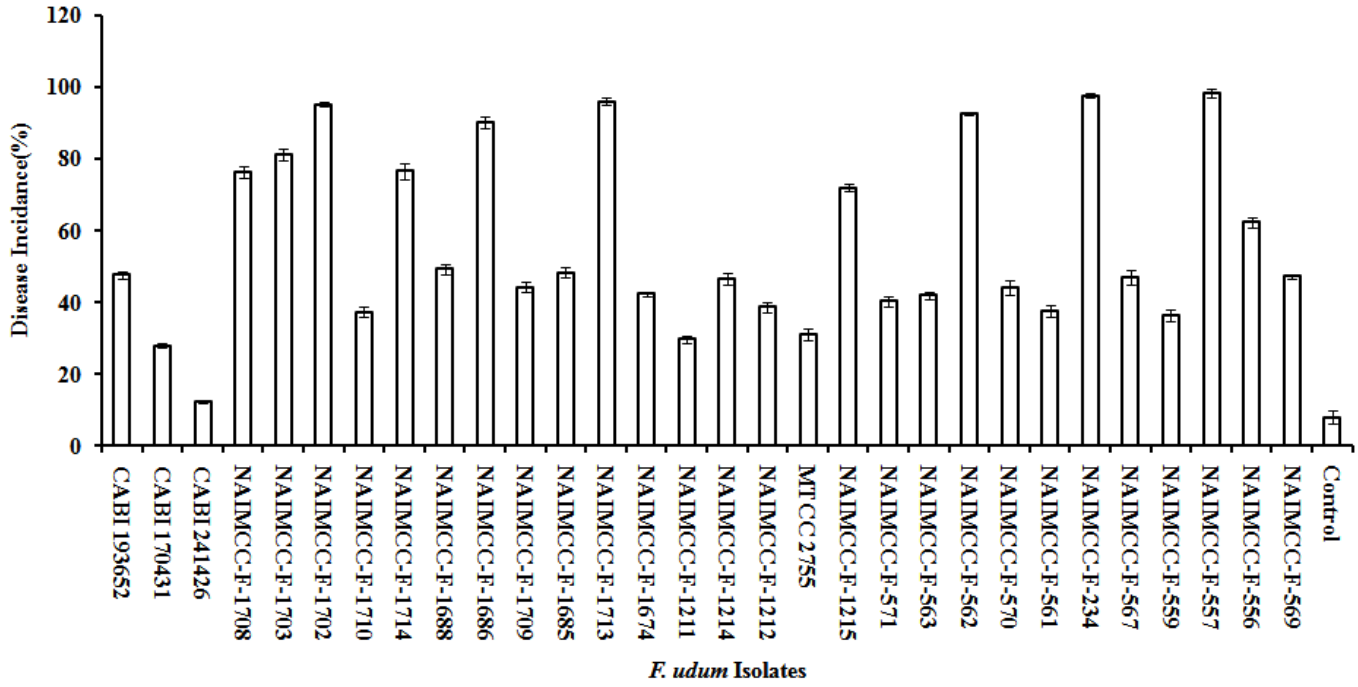

Figure 1. Disease severity of *Fusarium* wilt on pigeonpea (T-21) susceptible variety. Bars indicate standard errors of the mean values, different letters indicate significant difference at the 5% level between disease severity after pathogen inoculation

ments of different colours (golden brown, white cream and mulbur purple) were secreted in to the medium. Majority of the isolates showed fluffy mycelial growth with yellow colour and golden brown to mulbur purple substrate pigmentation in the medium (Table 1).

Pathogenicity assay

The pathogenicity assays revealed a highly variable interaction of various isolates on the pigeonpea plant (cv. T-21). Typical symptoms such as epinasty, interveinal yellowing of lower leaves, followed by drooping of the leaves and discolouration of vascular tissue appeared on pigeonpea plants following which leaves dried and detached from the branches. Percentage of plants exhibiting wilt symptoms ranged from 12 to 98%, with an average disease incidence of 56.31%. Necrosis generally began at 4 to 6 weeks of post-inoculation and was near completion after 8 weeks. Based on the reaction on host and disease index, all the isolates were grouped into avirulent (1), moderately virulent (17) and highly virulent (12) categories (Table 1 and Figure 1).

RAPD-PCR analysis

All the thirteen arbitrary primers used to characterize the genetic diversity of thirty different isolates of *F. udum* were successfully amplified a total of 126 DNA fragments with an average of 9.69 amplicons per primer. Out of 13 decamers, the OPB-17 produced consistently reproducible band pattern with maximum polymorphism (76.9%). The primer OPB-17 produced 13 bands with maximum polymorphic information content (PIC) of 4.00 followed by primers OPB-8 and OPV-10 having 12 bands each with 3.5 and 3.5 PIC values, respectively. The lowest number of bands (6) was observed in the primer OPA-4. Further diversity analysis was carried out for the data obtained from OPB17. The UPGMA dendrogram analysis separated 30 different *F. udum* isolates into three major genotypes and ten sub groups at 50% and 75% arbitrary level of similarities respectively. The major genotypic group-I included 22 isolates whereas group II and III consisted of 3 and 5 isolates, respectively (Figure 3). Individual primers were also informative in providing specific RAPD polymorphism for strainal differentiation (Table 2).

DISCUSSION

In each virulent group the isolates spread among various RAPD groups and subgroups of *F. udum* had no relationship with cultural or virulence characteristics, nor they had relationship between RAPD and geographical origin of the isolates (Figure 1 and 3). Single spore iso-

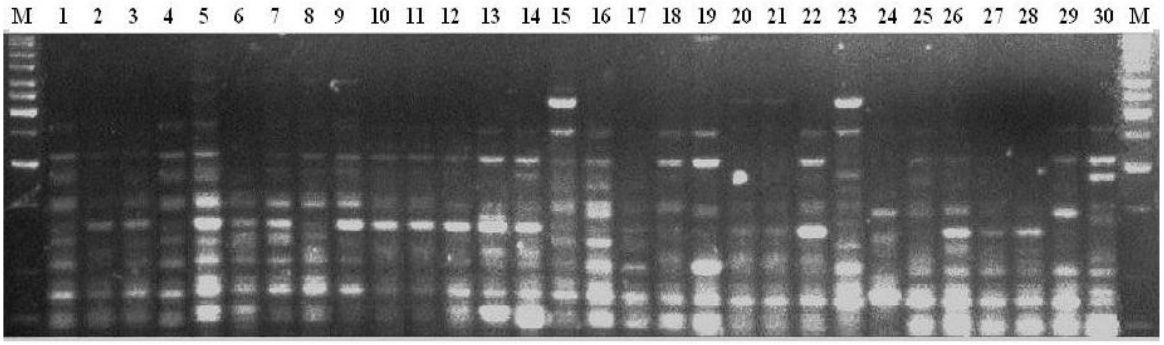

Figure 2. RAPD-PCR fingerprinting: RAPD profiles of 30 isolates of *F. udum* collected from different geographical regions of India were subjected to PCR amplification by using 10-mer RAPD primer OPB-17. Lane 1 to 30 indicating isolates of *F. udum* listed in the Table 1. M- Represents 1Kb ladder.

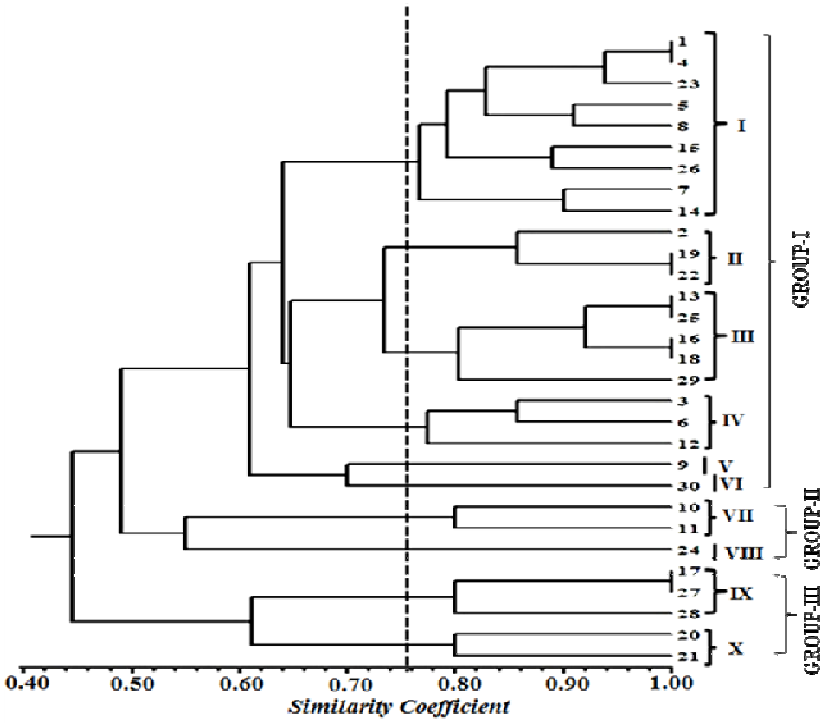

Figure 3. UPGMA-SAHN clustering dendrogram: Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 30 *F. udum* isolates listed in Table 1. Genetic similarity was obtained by RAPD OPB-17, using the Jaccard similarity coefficient.

lates of *F. udum* varied in cultural characteristics based on which all the thirty isolates were classified into four groups by mycelium colour, three groups by aerial mycelium growth and three groups by substrate colour. Our results supported the findings of Okiror, 1986; Shit and Sen Gupta, 1980; Gupta et al., 1988; Gaur and Sharma, 1989 who reported cultural variation in mycelial growth, pigmentation and colony diameter among the isolates of *F. udum*.

The cultural characteristics of *F. udum* were found not to be associated with a particular region or district, although the isolates 234 from Ranga Reddy district of Andhra Pradesh, 557 from Baran, South Eastern Rajasthan, 170431 from Hyderabad, Andhra Pradesh

Table 2. List of primers used for RAPD – PCR amplification

S.No.	Name	Sequence (5`- 3`)	%GC	Total number of bands	Monomorphic bands	Polymorphic bands	% of Polymorphism
1.	OPA-01	CAGGCCCTCC	80.0	8	4	4	50.0
2.	OPA-04	AATCGGGCTG	60.0	6	4	2	33.3
3.	OPB-01	GTTTCGCTCC	60.0	10	6	4	40.0
4.	OPB-02	TGATCCCTGG	60.0	8	3	5	62.5
5.	OPB-06	TGCTCTGCCC	70.0	9	4	5	55.5
6.	OPB-07	GGTGACGCAG	70.0	10	4	6	60.0
7.	OPB-08	GTCCACACGG	70.0	12	6	6	50.0
8.	OPB-10	CTGCTGGGAC	70.0	12	5	8	66.6
9.	OPB-11	GTAGACCCGT	60.0	11	5	6	54.5
10.	OPB-14	TCCGCTCTGG	70.0	9	4	5	55.5
11.	OPB-15	GGAGGGTGTT	60.0	10	6	4	40.0
12.	OPB-16	TTTGCCCGGA	60.0	8	4	4	50.0
13.	OPB-17	AGGGAACGAG	60.0	13	3	10	76.9
Total				**126**	**58**	**69**	**694.8**
Average				**9.7**	**4.5**	**5.3**	**53.4**

and 2755 from unknown) produced the purple group of pigments with radial scanty fibrous to suppressed mycelial growth. Cultural characteristics of the isolates were highly dissimilar with their molecular patterns. The phenotypic characteristics of the isolates varied from site to site and plant to plant (Kiprop et al., 2005). Therefore existed reports that single spore isolates of *F. udum* strains vary in their growth pattern, segmentation and capacity to secrete metabolic products (Upadhyay and Rai, 1992).

The virulence of *F. udum* isolates appeared to be independent of cultural characteristics (Okiror, 1986; Gaur and Sharma, 1989). The isolates 234, 557 showed high degree of virulence with 97 and 98 % of disease incidence while the isolates 170431 and 2755 were moderately virulent with 28 and 31 % of disease incidence. Most of the *F. udum* isolates producing fluffy mycelial growth were found to be moderately pathogenic and supported (Shit and Sen Gupta, 1978). OPB-17 was also similar to moderately virulent isolates 170431 and 2755 with similar cultural characteristics were placed in genotype-II and III.

Statistical analysis of RAPD data enabled the classification of Indian *F. udum* isolates into 10 genotypes with 3 RAPD groups. OPB 17 had revealed polymorphisms within reference strains of *F. udum* on alternate hosts and established that DNA fingerprints were useful for genetic characterization and specific identification. Exact correlation could not be attained between virulence, host-related grouping and phylogenetics. The isolates 1708 and 193652 infecting *C. cajan* and *C. indicus* respectively varied in disease incidence by 76% and 48%, respectively but shared 100% genotypic similarity and placed in the same genotype-I, since the F. *udum* isolates were collected from the synonymous cultivars of pigeonpea. The isolate 170431 and 241426 infecting on *C. verrucosa* and *C. arietinum,* respectively showed insignificant disease incidence and were placed in genotype-II and genotype-IV at 86% of similarity coefficient. This suggested that phylogenetic groups do not necessarily correlate with virulence groups (Baayen et al., 2000; Bao et al., 2002) and there was no correlation between RAPD and geographical origin of the isolates. The high variability among *F. udum* strains may be being the deuteromycete, the natural populations which may consist of clonal lineages produced by asexual reproduction (Kiprop et al., 2002).

Overall, the OPB-17 has been shown to be a potential marker for studying the diversity and evolution of *F. udum* to delineate them into host-related grouping and phylogenetics to certain extent. As evident from pathogenicity results of reference isolates *F. udum* isolates emerged as complex group due to host specificity leading to formae specials.

This study adds on *F. udum*, very incomplete information of which is available with respect to the diversity of species that are present in agricultural soils. It also highlights the phylogenetic relationships among different *F. udum* strains. The study may attract the crop breeders to construct genetic linkage maps for molecular tagging of various pathogenic genes of *F. udum* in India.

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