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Original Research Article

Analysis of Genetic Diversity of *Fursarium udum* Causing Wilt of Pigeon Pea

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A B S T R A C T

Pigeonpea is an important pulse crop in arid and semiarid region of India. Several biotic and abiotic constrain hamper its production. *Fusarium* wilt caused by *Fusarium udum* Butler is one of the most important biotic factors causing considerable amount of losses in grain yield. Pathogenic variability among the isolates of *F. udum* causing pigeonpea wilt has been studied based on morphological analysis and genetic marker. In the present investigation eighteen isolates of *Fusarium* species were analyzed. RAPD marker is used to study pathogenic diversity in the fields of pigeonpea among the various regions of Maharashtra and Karnataka. Morphological characterization of eighteen isolates of *Fusarium* spp. grown on artificial PDA media was observed. *In planta* pathogenecity test of eighteen samples was carried out by inoculation of suspension in the soil plastic pots containing pigeonpea plantlets. The infected plants were observed and the wilting symptoms like falling of leaves, yellowing of leaves were recorded. A total of 20 RAPD primers were screened with genomic DNA of *Fusarium spp.* Among these 8 primers showed polymorphism and these primers were selected for estimation of genetic diversity and genetic relationship among all species of isolates. The molecular analyses showed isolates memberFU-1 and FU-2 are most distant from origin. The isolates FU-2 and FU-18 are closely related and FU-18 is close to the origin. Based on morphological and pathogenicity analysis the identity of all isolates was confirmed.

Introduction

Pigeonpea (*Cajanus cajan* (L) Millsp.) is a perennial legume crop. This crop belongs to the Leguminoseae family. The word Cajanus comes from Malay word 'Katschang' or 'Katjang' which means pod or bean. Pigeonpea has many vernacular names like arhar and tur in India, red-gram in Australia, tropical green gram or Gungo pea in Jamaica, Guando in Brazil and Anglo pea in United Kingdom. This crop is originated in Nile valley and costal region of Africa (which coast—Africa is too big). In India it is domesticated about at least 3500 year ago. Pigeonpea is a diploid (2n = 22) species having a genome size of about 833.1 Mbp (Varshney *et al.,* 2012). Pigeonpea is the second most important pulse crop of India, after chickpea. India ranks first in its production and contributes to 90% of global pigeonpea (Mespogu *et al.,* 2012).

It is cultivated over an area of about 3.62 million hectares (occupying about 14.5% of area under pulses). The overall production is estimated of 3.07 million tones (contributing to 1.6% of total pulse production) and productivity is about 650.004 kg/ha (Singh *et al.,* 2015).

In India it is grown in states like Maharashtra, Uttar Pradesh, Madhya Pradesh, Karnataka, Gujarat, and Andhra Pradesh. In Maharashtra it is grown in kharip season and cultivated on 1.21 million hectares (Mha) of land and having production of 29.92 Mha tones and yield is 0.85 kg/ha in Maharashtra (Source: Agricultural Statistic at a Glance 2012). Pigeonpea is not only cultivated as food but also as a forage crop depending upon cultivating region.

Many agricultural inventions and development of high yielding cultivars or hybrids of pigeonpea have lead to improved productivity and yield of pulses. However, pigeonpea cultivation is mainly pegged by some biotic and abiotic factors.

Abiotic factors generally can be addressed using crop management strategies, whereas biotic factors are hard to control. Biotic factors involve various disease causing microbes and pests. Pigeonpea is attacked by more than hundreds of pathogens (Nene *et al.,* 1989) including fungi, bacteria, viruses, nematodes, and mycoplasma like organisms (MLO).

The significant diseases of the pigeonpea are *fusurium wilt* (*Fusarium udum*), sterility mosaic diseases (SMD), leaf spot (*Mycorellasiella cajani*) and to lesser extent powdery mildew (*Leuiellula taurica*). These diseases are of economic concern because of the losses incurred in field conditions. Among to above stated disease *Fusarium wilt* is most prevalent in India and East Africa. This disease is also reported in several other countries such as Bangladesh, Indonesia, Mauritius, Myanmar, Nepal, Nevis, Zambia, Ghana, Kenya, Venezuela, Trinidad and Tobago (Kannaiyan *et al.*, 1984; Reddy *et al.,* 1993; Marley and Hillocks *et al.*, 1996).

Fusarium wilt of pigeonpea is a soil borne disease and it is known to cause heavy losses every year in India. The fungal pathogen causing wilt can survive on infected plant debris in soil for about 2-3 years and result in high yield losses sometimes up to 100% in susceptible cultivars (Kipro *et al.,* 2005). The crop losses caused by this disease in Indian subcontinent ranged from 16% to 47% (Prasad *et al.,* 2003). *Fusarium* species are known to cause disease in pigeonpea are *Fusarium udum, F. semitectum, F. monilyformii* and *Fusarium solani*. Among these *Fusarium udum* is the most significant pathogen due to its severe pathogenicity and high penetration value.

Molecular biology has played a major role in genetic understanding of the pathogen analysis at molecular level. Availability of genetic markers such as random amplification of polymorphic DNA (RAPD) has helped in analysis of genetic diversity in pigeonpea (Yadav *et al.,* 2012). In RAPD analysis random primers are used to amplify the DNA of interest. This technique involves use of random decamers in a PCR reaction resulting in amplification of many discrete DNA. Using this technique we can identify different species of *Fusarium* causing disease in pigeonpea. In this study we employed RAPD primers to test genetic relationship and molecular diversity among isolates of *Fusarium* wilt. The molecular marker analysis was used to determine genetic distance between the isolates of *F. udum* and other species of *Fusarium*. Such molecular analysis can be used to identify the traits responsible for disease causing ability of the *Fusarium* species in pigeonpea. *Fusarium* wilt is a dangerous disease in legumionous crops. We need to find certain varieties that can be resistant against the disease using marker-assisted selection.

Materials and Methods

Agarose, SDS, Tris–HCl, EDTA-disodium chloride (HiMedia), dNTPs and Taq DNA polymerase (MB Fermentas). RAPD primers were obtained from Operon Technologies (Almeda ® California). All other chemicals and reagents used were of analytical grade.

Collection, isolation, purification and maintenance of *Fusarium* species isolates

Survey was conducted among pigeonpea growing regions of Maharashtra and Karnataka during year 2016-17. Several samples of pigeonpea plants affected with *Fusarium* wilt were collected. From this collection eighteen (18) root samples infected with *Fusarium udum* were isolated. These samples will be referred as isolates in hereafter in this study. The list of root samples collected from the farmers' field that was infected with *Fusarium* sp. is described in Table 1.

Isolation and purification of pathogen

The collected samples of infected roots and stems were washed under running tap water to remove adhered soil. Further, these samples are cut in small sections with the help of sterilized scalpel and were treated with 0.01% $HgCl_2$ for 5 min followed by washing with 70% ethanol for 1 minute and then washed thoroughly with sterilized water for 3 times each wash consisted of 5 min.

Pathogenicity test

Pathogenic potential of isolates of *Fusarium* was tested by proving Koch's postulates (Nene *et al.,* 1981). Mycelial disk of eighteen *Fusarium* species isolates were inoculated with sterilized potato dextrose broth separately and incubated at 28 ± 2º c for 7-8 days. These cultures are now poured into potting mixture @40-50 ml broth culture/pot (Fig.2). For the growth of pathogen isolates necessary conditions were provided and observations of symptoms and plant mortality were recorded.

Morphological variability

Artificial growth media i.e. potato dextrose agar (PDA) media is poured in the glass petriplates (5 mm) and were inoculated with sterilized root sections. Such inoculated plates were incubated at 28±2ºC incubator. The growth of fungal cultures was observed for 5 days and observations were recorded.

For the visualization of mycelium under microscope we have used Lactophenol blue dye. Further pare morphology and mycelium of *Fusarium* isolates were observed under compound microscope. The pure culture of *Fusarium* isolates were further utilized in pathogenicity test.

Genomic DNA extraction

The genomic DNA of fungal pathogen was extracted using the protocol developed and standardized by Chavhan (2004).

Molecular diversity analysis

All the reactants required for PCR were added together to form reaction mixture. The reaction mixture consisted of 2.5 µL of 10X PCR buffer, 1.7 µL of 1.5 mM $MgCl_2$, 2.0 µL of dNTP mix (2.5mM each), 0.3 µL of Taq DNA polymerase (1.25 U/µl), 1.5 µL of primers, 1.0 µL of template DNA. The final volume of reaction 25 µl was maintained in sterile PCR tubes (0.2 mL). After that isolated genomic DNA of each sample of *Fusarium species* was added to individual prepared tubes containing master mixture. The tubes were subjected to

tapping with figures followed by brief spin for mixing the contents thoroughly and the subjected to PCR standardized protocol for amplification of DNAs.

The PCR amplification was carried out using Master cycler epgradient S (Eppendrof) with pre-denaturation at 94ºc for 4 minute. Then 35 cycles of 94ºC for 1 minute. Primer annealing at 37ºC and extension at 72ºC for 1 minute. Eventually the reaction was completed using a final extension of 72ºC for 10 minute.

The test for presence of DNA is done by using 0.8% agarose gel electrophoresis procedure. The results of PCR amplification were separated on 1.2% agarose gel in 1X TAE using gel-electrophoresis at 100V for 40-45 minutes. After this by using ethidium bromide staining, the bands were detected. Standard DNA of 1kb molecular weight is used as marker (Fig. 1) scoring was done visually on the basis of clearly resolved bands for presence or absence. Jaccard's similarity co-efficient was estimated from NTSYS-pc software.

Data analysis

Qualitative analysis of RAPD bands were scored as present (1) or absent (0). Data matrices were generated and these were used to plot the Dendrogram by software NTSYS-pc.

Results and Discussion

Diversity of *Fusarium species* causing wilt of pigeonpea is studied using morphological analysis and genetic markers in the present investigation. The application of RAPD marker is described to study pathogenic diversity in different regions of pigeonpea growing states of Maharashtra and Karnataka.

In planta pathogenicity test

Selected eighteen different isolates of *Fusarium* sp. were inoculated in the soil plastic pots carrying pigeonpea plantlets. The isolates resulted in appearance of wilting symptoms like drooping of leaves, yellowing of leaves starting from apical shoots, progressing downwards and eventually wilting of whole plants. The pigeonpea plantlets died after a month from the date of inoculation. The fungal isolates were taken from these infected plants and were compared with fungal cultures for morphology and pathogenic penetrance levels. Control plants of this experiment remained healthy. This helps to establish pathogenicity using the Koch's postulates.

Morphological characterization of isolates of *Fusarium* species

Morphological characters of eighteen isolates of *Fusarium* species were observed on artificial media i.e. Potato Dextrose Agar (PDA) media. The inoculum obtained from isolates was allowed to grow on PDA for five days.

Morphological characteristics of the cultures are presented in Table 2. Among the 18 isolates FU2, FU4, FU6, FU10, FU15, and FU17 showed slow growth and produced pigmentation viz., white, white, light, light pink, light, dark yellow, and yellowish white respectively. FU3, FU7, FU8, FU9, FU12, FU13, FU14, and FU18 showed moderate growth and produced pigmentation viz. creamy white, pale yellow, creamy white, light orange, white pale yellow, creamy white, orange white and white respectively.

While, FU1, FU5, FU11, and FU16 showed fast growth and produced pigmentation viz., light yellow, yellow orange, orange yellowish, and white respectively.

Table.1 List of *Fusarium* species isolates utilized in the study

Sr.no	Isolates Code No.	Species	Host	Origin
1	*FU-01*	*F.udum*	*Cajanus cajan*	Ahmednagar, M.S
2	*FU-02*	*F.udum*	*Cajanus cajan*	Nashik M.S
3	*FU-03*	*F.udum*	*Cajanus cajan*	Pune, M.S
4	*FU-04*	*F.udum*	*Cajanus cajan*	Aurangabad, M.S
5	*FU-05*	*F.udum*	*Cajanus cajan*	Jalna, M.S
6	*FU-06*	*F.udum*	*Cajanus cajan*	Parbhani, M.S
7	*FU-07*	*F.udum*	*Cajanus cajan*	Latur, M.S
8	*FU-08*	*F.udum*	*Cajanus cajan*	Akola, M.S
9	*FU-09*	*F.udum*	*Cajanus cajan*	Nagpur, M.S
10	*FU-10*	*F.udum*	*Cajanus cajan*	Jalna (Mantha),M.S
11	*FU-11*	*F.monilyformy*	*Cajanus cajan*	VDCOAB, Latur (Pure culture)
12	*FU-12*	*F.udum*	*Cajanus cajan*	Bidar, Karnatka
13	*FU-13*	*F.udum*	*Cajanus cajan*	Jalgaon, M.S
14	*FU-14*	*F.udum*	*Cajanus cajan*	Deoni, M.S
15	*FU-15*	*F.udum*	*Cajanus cajan*	Dhanegaon, M.S
16	*FU-16*	*F.udum*	*Cajanus cajan*	Nilanga, M.S
17	*FU-17*	*F.udum*	*Cajanus cajan*	Badnapur (BDN), M.S
18	*FU-18*	*F.semitectum*	*Cajanus cajan*	VDCOAB, Latur (Pure culture)

Table.2 Description of morphological characters of pigmentation mycelia Growth pattern and diameter

Sr.no	Isolates Code No.	Pigmentation	Mycelia Growth Pattern	Diameter in cm
1	*FU-01*	Light yellow	Fast growth	9.2 cm
2	*FU-02*	white	Slow growth	4 cm
3	*FU-03*	Creamy white	Moderate growth	6.3 cm
4	*FU-04*	white	Slow growth	4.7 cm
5	*FU-05*	Yellow orange	Fast growth	9 cm
6	*FU-06*	white	Slow growth	4.3 cm
7	*FU-07*	Pale yellow	Moderate growth	5.9 cm
8	*FU-08*	Cream white	Moderate growth	5 cm
9	*FU-09*	Light orange-white	Moderate growth	7.3 cm
10	*FU-10*	Light pink	Slow growth	4.8 cm
11	*FU-11*	Orange yellowish	Fast growth	9 cm
12	*FU-12*	Pale yellow	Moderate growth	6.3 cm
13	*FU-13*	Creamy white	Moderate growth	7.2 cm
14	*FU-14*	Orange white	Moderate growth	5.2 cm
15	*FU-15*	Light-dark yellow	Slow growth	3 cm
16	*FU-16*	White	Fast growth	9.3 cm
17	*FU-17*	Yellowish white	Slow growth	3.8 cm
18	*FU-18*	white	Moderate growth	5.7 cm

Table.3 RAPD DNA fingerprint analysis

Sr.no	Primer	Sequence	Number of Nucleotides (Bases)
1	OPB-7	GGTGACGCAG	10
2	OPD-12	CACCGTATCC	10
3	OPD-13	GGGGTGACGA	10
4	OPD-14	GTTCCCCAAG	10
5	OPD-15	CATCCGTGCT	10
6	OPD-16	AGGGCGTAAG	10
7	OPD-18	GAGAGCCAAC	10
8	OPD-20	ACCCGGTCAC	10

Fig.1 DNA isolates of *Fusarium udum* as per Table 1

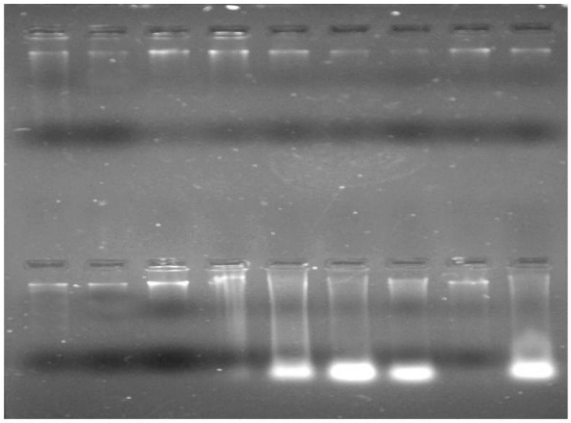


Fig.2a Culture of *Fusarium udum* for pathogenicity test

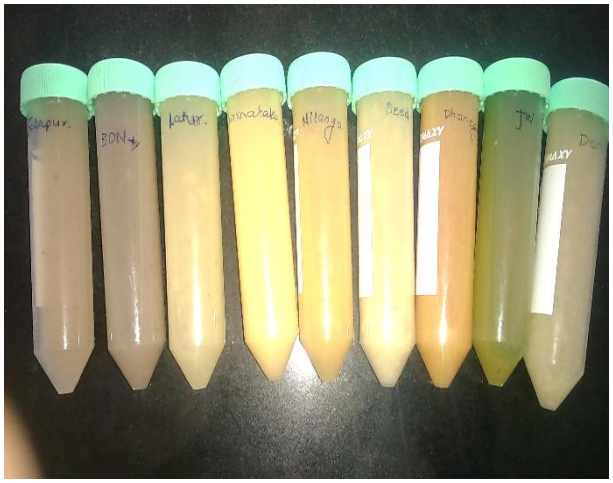


Fig.2b Pathogenesity test of 18 isolates of *F. udum*

Fig.3 Dendrogram analysis of 18 isolates of *Fusarium* species

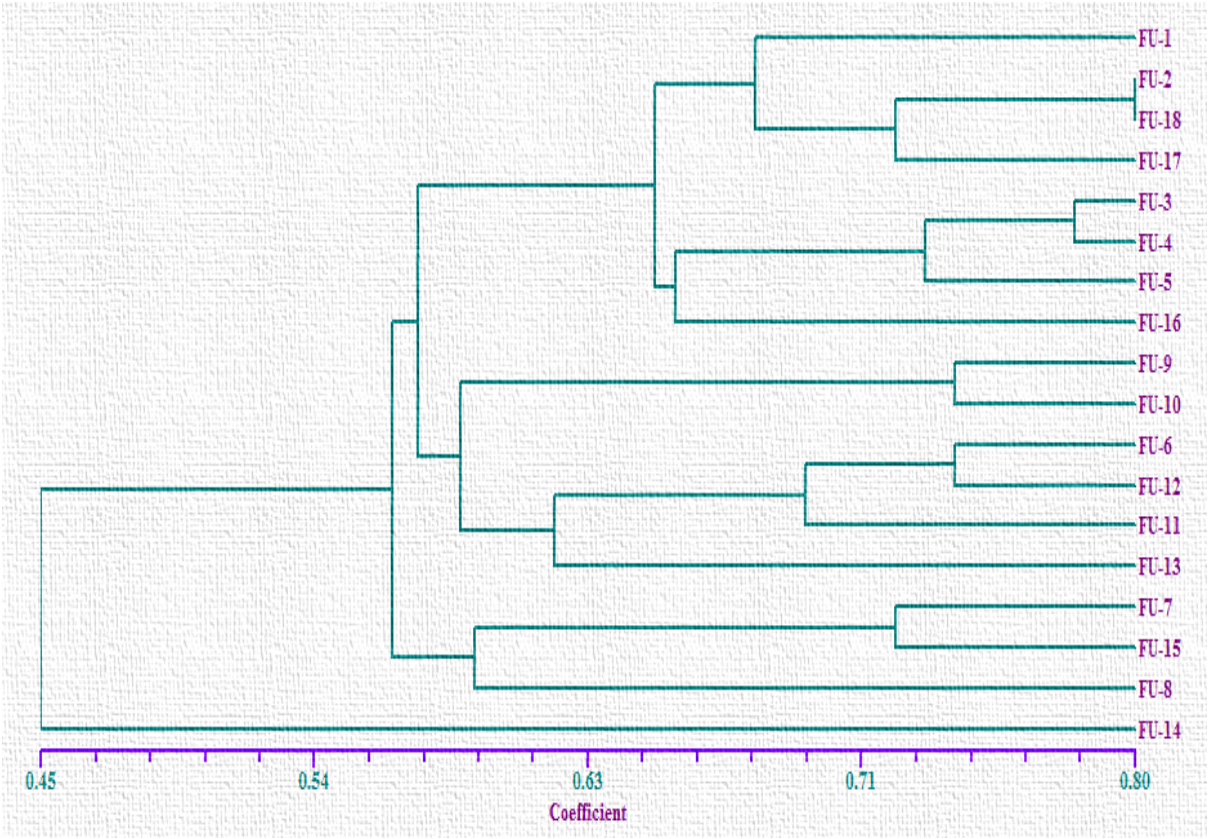
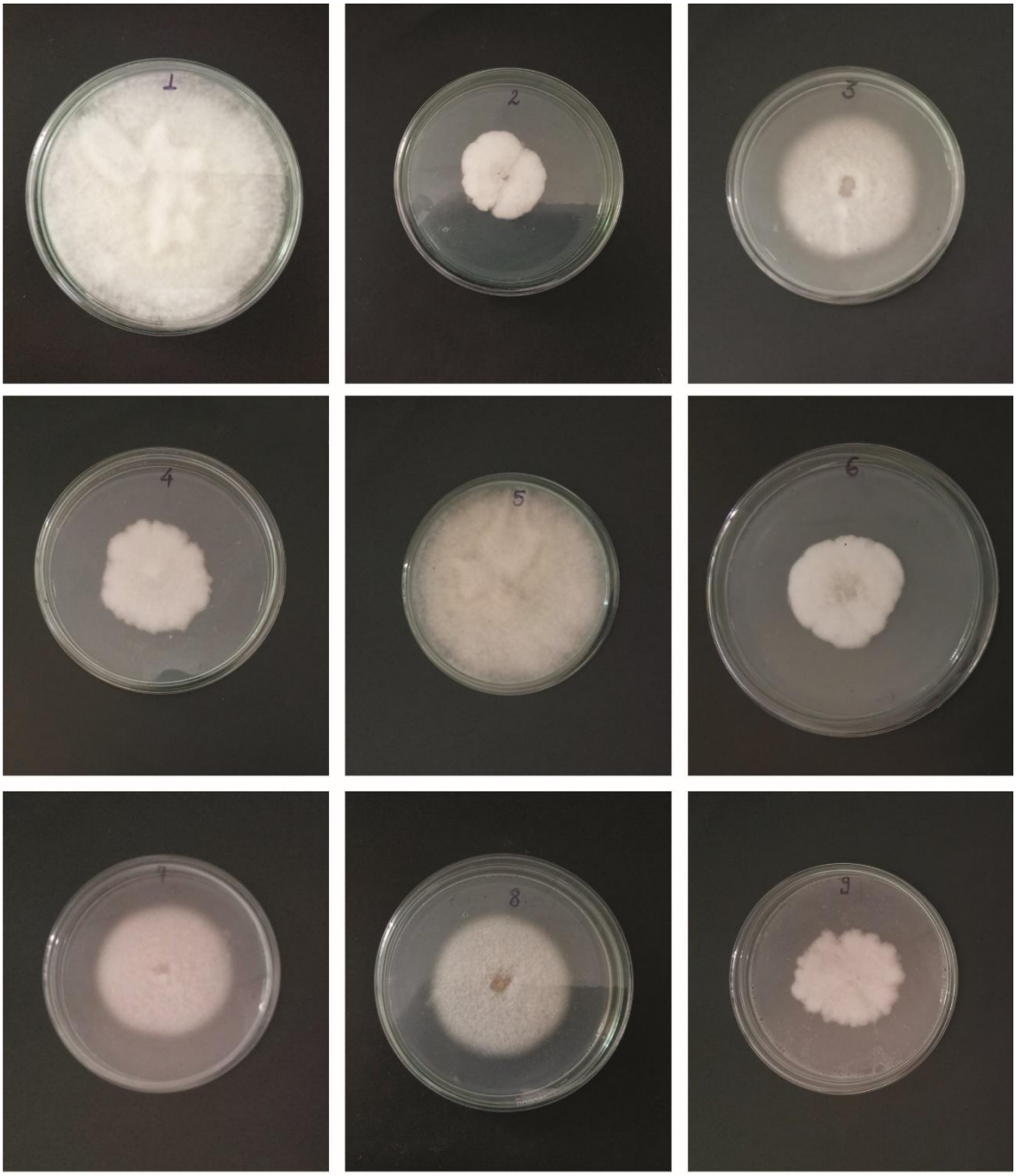


Fig.4 Colony characteristics of *Fusarium* species



Genetic diversity analysis by using RAPD marker

Genetic diversity of *Fusarium* species isolates of pigeonpea was analyzed using RAPD marker in this study. Several RAPD primer were screened with the genomic DNA of *Fusarium* species. From these RAPD primers, almost 50 % primers yielded polymorphic pattern and fingerprinting profile. These primers were used to study genetic diversity analysis. Average 6.37 number of total amlicons were produced in RAPD DNA fingerprint profile. Out of

which OPB-07 and OPD-13 produced each of eight numbers of polymorphic amplicons. The study shows that each primer has differentiating the isolates at genetic level (Table 3). A total of 20 RAPD primers were screened, of which 8 primers were polymorphic. Whereas remaining primers failed to amplify any DNA fragments therefore, were not used to perform PCR amplification. Some primers yielded a single band (monomorphic), hence were not useful to do the genetic comparison studies.

The dendrogram generated by using NTSYS-pc analysis based on scoring of RAPD marker data which separated eighteen isolates of *Fusarium species* in to four major cluster (Fig. 3). The phylogenetic analysis was assessed using similarity matrix depicted on RAPD fingerprint data. Dendrogram analysis revealed four distinct clusters, the Cluster-I comprised isolates namely FU-1, FU-2, FU-3, FU-4, FU-5, FU-16, FU-17 and FU-18 together as a major cluster with 65% genetic similarity to each other. Whereas, Cluster-II comprise FU-6, FU-9, FU-10, FU-11, FU-12 and FU-13 together with 59% genetic similarity with members assembled in Cluster-II. Cluster-III consisted isolates FU-7, FU-8 and FU-15 which showed 59.40% similarity with each other. Cluster-IV comprised by distinct isolates FU-14 with has 45% similarity was arranged in between 45% to 80%. Among *Fusarium* species isolates under study whereas genetic diversity was ranged in between 20% to 55%among isolates of *Fusarium* species. The most genetically similar isolates were found to be FU-2 and FU-18, while most distinct isolates was found as FU-14. The OPB7 and OPD13 primers produced most polymorphic amplification profiles.

In the present investigation eighteen isolates of *Fusarium* species from Maharashtra and Karnataka were analyzed. Based on morphological and pathogenicity analysis the identity of all isolates was confirmed. The molecular analyses showed isolates member FU-1 and FU-2 are most distant from origin. The isolates FU-2 and FU-18 are closely related and FU-18 is close to the origin.

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