ORIGINAL RESEARCH ARTICLE

Morphological and Molecular Characterisation of Fusarium wilt pathogen isolated from Sunhemp

Abstract

Fusarium wilt, caused by *Fusarium oxysporum f. sp. crotalariae* (Kulkarni) Subram. is a significant disease affecting sunhemp (*Crotalaria juncea*) production, leading to substantial yield losses. To understand the pathogen's biology and enhance management strategies, comprehensive investigations were conducted under laboratory and field conditions at the University of Agricultural Sciences, Dharwad, during 2019-20. The pathogen was cultured on various solid media, with Richard's agar identified as the most conducive for fungal growth, while potato dextrose agar, Czapeck's dox agar, and Richard's agar facilitated good sporulation. Notable variation was observed in colony morphology, including color, texture, margin, growth nature, and sporulation across the different media. Molecular characterization confirmed the pathogen as closely related to *F. udum*, with a 99.79% similarity to an isolate with the GenBank accession number MK575503.1. These findings provide valuable insights into the pathogen's growth characteristics and genetic identity.

Key words: *Crotalaria juncea, Fusarium oxysporum f.sp.crotolariae* (Kulkarni) Subram.,

1.Introduction

Sunhemp (*Crotalaria juncea* L)., is a fast growing annual green manure crop which is native of India. It is a vital source of natural fibre and recognized as one of the promising indigenous raw material which is used for manufacturing of high quality tissue paper, twines, ropes, fishing nets. It is used as fodder and fiber and suited for almost all the parts of India. Link diagnosed the generic concept of *Fusarium* in 1809 for the first time with the primary character being the presence of the distinctive banana-shaped conidia well known to all researchers. The genus *Fusarium* is having wide host range including crop and non crop species. The wilt of sunhemp caused by *Fusarium udum* f. sp. *crotalariae* (Kulkarni) Subram (Mitra, 1934). The pathogen mainly survives in the crop stubbles as it is a facultative parasite. The pathogen produce sporodochia which is pink coloured and on which enormous micro and macro conidia are produced. Spores and fungal hyphae block the xylem vessels of the infected part leading to the

death of the plant. The pathogen was dispersed through irrigation, rain water and displacement of host debris from one place to another. The disease infects young seedlings, but the highest mortality has been observed during flowering and pod development stages indicating that the incidence of wilt was more during the reproductive phase of the crop. The disease incidence increased with high temperature and high moisture as reported by Saxena (1989). Rainfall during last fortnight of October was found to be conducive for the spread of this disease. Wilt incidence was found to be more in the low-lying areas. Mosaic and wilt in sunhemp reduce the fibre yield to the tune of 10 to 12 per cent and 40 per cent respectively. But, 60 per cent of yield loss can be caused by wilt alone under favourable conditions. Li *et al.* (2012) reported that Sunhemp grown in wet soil is susceptible to *Fusarium* spp. and causes significant yield losses. Morphological and molecular characterization of the Fusarium wilt pathogen in Sunhemp is essential for accurate identification, understanding pathogen diversity, developing resistant cultivars, and implementing effective management strategies. Keeping this in mind, research was carried out with the objective to conduct morphological and molecular characterization of the Fusarium wilt pathogen in Sunhemp.

2.Materials and Methods

2.1. Isolation of the pathogen from infected sample

The root and lower stem parts of infected mature sunhemp plants showing typical symptoms of the disease were used for the isolation of pathogen. The standard tissue isolation procedure was followed to isolate the pathogen. The infected parts were cut into small bits and surface sterilized with 0.1 per cent sodium hypochlorite solution for 60 seconds and washed serially in sterilized distilled water to remove the traces of the chemical if any and then transferred to sterilized Petri plates containing potato dextrose agar (PDA). The Petri plates were incubated at room temperature (28 ± 1°C). Further, the pure cultures of the fungi were obtained by single spore isolation method.

2.1.1. Identification of the pathogen

A total of 20 different isolates of fungi were obtained on the media containing PDA. The morphological characters of the fungi such as mycelium, macro and micro conidia,

chlamydospores were studied under microscope and the fungi *F. udum* was identified based on its distinct morphological characters (Leslie and Summerell, 2006; Wang and Dai, 2018)

2.1.2. Preparation of giant culture of *F. udum* to test its pathogenicity

Sorghum seeds were used as substrate for giant culture preparation. The substrate was prepared by mixing 200g crushed sorghum seeds and 50 ml distilled water in 500 ml conical flask and sterilized at 15 psi for one hour for two consecutive days. Flasks were subsequently inoculated with 4-5 discs of seven days old culture of strain of *F. udum* and incubated at 28 ± 1^{o}C for 20 days. During incubation the culture was mixed thoroughly to get uniform growth.

2.2. Preparation of media for cultural and morphological characterization

The morphological characteristics of *F. udum* was studied on Czapeck's dox agar, Oat meal agar, Potato dextrose agar, Potato carrot agar, Richard's agar, Sabouraud's dextrose agar, V8 juice agar and Corn meal agar. All the media were sterilized at 1.1 kg/cm^2 pressure and 121^{o}C for 15 min. The composition and preparation of the above-mentioned solid media were obtained from Ainsworth and Bisby's 'Dictionary of the Fungi' by Ainsworth *et al*. (1983). To carry out the study, 20 ml of each of the medium was poured in 90 mm Petri plates. Such Petri plates were inoculated with 5 mm disc cut from periphery of actively growing four days old culture and incubated at 28 ± 1°C. Each treatment was replicated three times. Observations were taken when the fungus covered Petri plate completely in any one of the media. Observations on radial growth, colour, texture, margin, growth nature of mycelium, number of conidia under microscopic field (10X) and length and breadth of microconidia and macroconidia in different culture media were recorded. The data on radial growth was analyzed statistically.

2.3. Isolation of DNA and PCR Amplification for molecular characterization

Isolation of DNA from the *F. udum was* done by following standard CTAB (Cetyl Trimethyl Ammonium Bromide) method with certain modifications (Murray and Thompson, 1980). The fungal isolate was grown in test tubes containing potato dextrose broth and allowed for

incubation at 28 ºC for 5-7 days. The mycelium was harvested and extraction of genomic DNA was carried out. 10 µl of total DNA solution was loaded on to 0.8 per cent Agarose gel and Electrophoresis to separate DNA. Primers ITS 1 (Internal Transcribed Spacer) (5'-TCCGTAGGTGAACCTGC GG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for amplification conserved ITS region of pathogen. PCR product of the fungi was sent to private company for sequencing. The obtained sequence results were analysed using Basic Local Alignment Search Tool (BLAST) algorithm available at National Centre for Biotechnology Information (NCBI) website.

3. Results and Discussion

3.1. Pathogenicity study

Giant culture of *F. udum* was prepared and applied to pots at the rate of five per cent (w/w) with sterilized soil as described under material and methods. Five sunhemp seeds were sown per pot and were maintained with optimum soil moisture condition. Plants were observed for typical disease development. The results revealed that after 45 days of application of inoculum there was development of typical disease symptoms and noticed as, yellowing of the leaves initially followed by drooping of foliage and finally complete wilting of the entire plant and there was poor pod formation in the wilted plants (Fig.1). Formation of pinkish fungal spore masses was observed on the stem surface near root and vascular discolouration was noticed when the stem was split opened. Symptoms observed in infected plants were consistent with those described in previous studies (Choi *et al*., 2018). Pathogen was re-isolated from such plants and morphological character of re-isolated pathogen was compared with the original culture of the pathogen which was similar to that of original culture. Hence, the causal agent of the disease was confirmed as *F. udum* f. sp. *crotalariae.*

Pathogenicity was in agreement with Wang and Dai (2018) showing external symptoms of the disease from initial yellowing of the lower leaves to wilting of the plant at later stages. Discoloration of vascular bundles first appeared at the crown region and continued upward on one side of the stem. Formation of white to pinkish fungal spore masses on the stem surface at a later stage in sunhemp plant infected with *F. udum* f. sp. *crotalariae.*

Fig.1. Pathogenicity study of *F.udum* on sunhemp

3.2. Cultural and morphological characteristics of *F.udum* on different solid media

All the media (mentioned in materials and methods) used differed in their growth and cultural characters of the pathogen (Fig. 3, Table 1). Among the different solid media tested highest mycelial growth was observed in Richards agar (90 mm) and V8 juice agar (88 mm) which are statistically on par with each other followed by Sabouraud's dextrose agar (85.00 mm). Minimum radial growth of the mycelium was observed in oat meal agar (45.00 mm) depicted in Fig. 3. Colour of the colony also differed as various shades of white. Whitish colony was seen Czapeck's dox agar, potato dextrose agar and V8 juice agar media whereas, it was milky white on Richard's agar medium. The colony colour was yellowish white on Sabouraud's dextrose agar and on potato carrot agar it was found to be orangish white. Size of micro and macro conidia and chlamydospores varied among of the different solid media used. The micro conidial size was lowest in Czapeck's dox agar which ranged from 7.16 -11.34 × 3.70 - 4.75 µm with average size of 9.72×4.19 µm and the micro conidial maximum size was observed in Sabouraud's dextrose agar which measured 13.44-15.86 × 3.34-3.63 µm with the average size of 14.58×3.48 µm with zero to single septation. Whereas, the macro conidial size was found highest in Richard's agar which measured 31.02-

36.62 × 4.24 - 4.85 µm with average size 33.46 × 4.59 µm and least size was observed in corn meal agar which measured 22.70-31.02 × 4.14-4.66 µm having average size of 26.11×4.38 µm with 2 to 4 septation. The chlamydospore diameter was highest in potato dextrose agar which ranged from 6.58 to 7.41 µm with average of 6.99 µm whereas the lowest was in potato carrot agar which measured 5.65-6.48 µm with a mean of 6.04 µm diameter (Table 2).

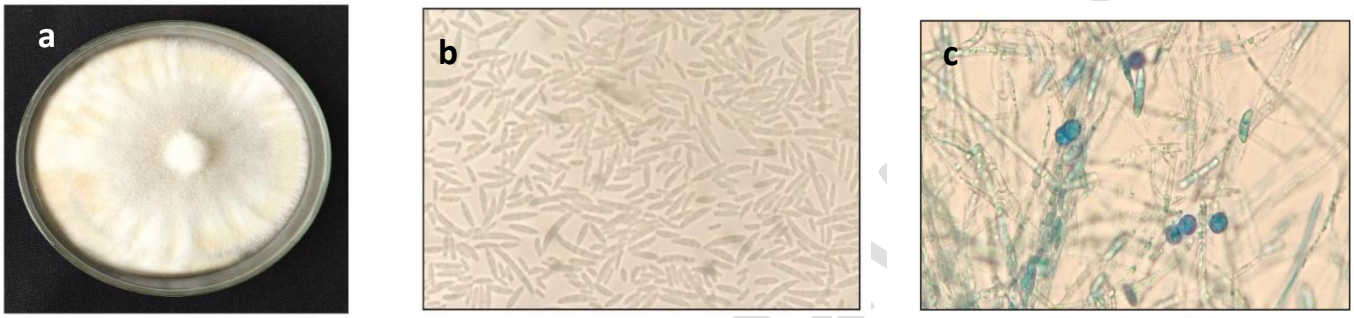

Fig. 2. a) Pure culture of *F.udum* on PDA after 2 days of incubation b) Macroconidia and microconidia (400X) c) Chlamydospores (400x)

Fig. 3. Morphological characteristics of *F.udum* on different growth media 1-V8 Juice agar, 2- Potato dextrose agar, 3-Potato carrot agar, 4-Corn meal agar, 5-Richard's agar, 6-Sabouraud's dextrose agar,7-Czapeck's dox agar and 8-Oat meal agar

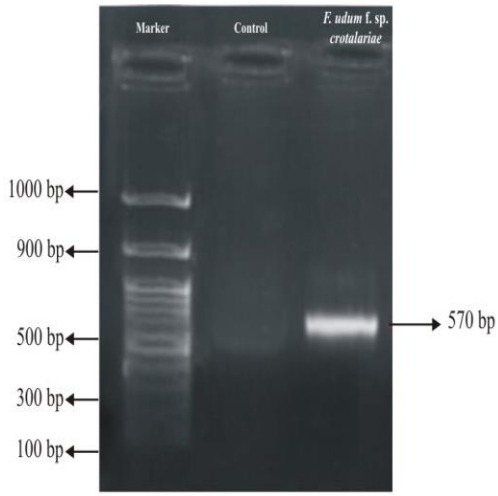

Fig. 4. Amplified ITS rDNA region of *F.udum*

Table 1. Cultural and morphological characters of *F.udum* on different growth media

Sl. No.	Media	Morphological Characters					
		Radial growth of mycelium (mm)	Colour of colony	Texture of colony	Margin of colony	Growth nature	Sporulation
1	Czapeck's dox agar	72.20 (58.18)*	White	Puffy cottony	Irregular	Aerial	+++
2	Oat meal agar	45.00 (42.13)	yellowish White	Cottony	Regular	Aerial	+
3	Potato dextrose agar	76.70 (61.14)	White	Cottony	Regular	Aerial	+++
4	Potato carrot agar	71.40 (57.67)	Orangish white	Cottony	Irregular	Aerial	++
5	Richard's agar	90.00 (71.56)	Milky white	Puffy Cottony	Regular	Aerial	+++
6	Sabouraud's dextrose agar	85.00 (67.21)	Yellowish White	Cottony	Regular	Aerial	+
7	V8 Juice agar	88.00 (69.73)	White	Cottony	Regular	Aerial	++
8	Corn meal agar	64.40 (53.40)	White	Sparse cottony	Regular	Immersed	++
	S.Em. ±	0.65					
	C.D. @ 1%	1.86					

*Angular transformed values

+++ : Good sporulation (> 50 spores per 10X microscopic field)

++ : Moderate sporulation (25 – 50 spores per 10X microscopic field)

+ : Scanty sporulation (< 25 spores per 10X microscopic field)

Table 2. Morphological characters of conidia and chlamydospore of *F.udum* on different growth media

Sl No.	Media	Micro conidia			Macro conidia			Chlamydospore diameter (µm)	Average
		Size[length × breadth(µm)]	Septation	Average	Size[length × breadth(µm)]	Septation	Average		
1	Czapeck's dox agar	7.16 - 11.34 × 3.70- 4.75	0-1	9.72×4.19	24.98-35.53 × 4.12-4.79	2-3	31.09×4.44	6.14-6.75	6.43
2	Oat meal agar	11.52-13.47 × 3.21-3.69	0-1	12.35×3.46	27.24- 35.27 × 3.12-4.89	2-4	31.39×4.01	6.23 -6.98	6.55
3	Potato dextrose agar	9.64-13.55 × 3.05-3.64	0-1	11.13×3.40	26.6-33.93 × 4.05- 5.19	3-4	29.47×4.64	6.58-7.41	6.99
4	Potato carrot agar	7.24-12.83 × 3.42-4.21	0-1	10.21×3.89	27.93-32.08 × 4.29- 4.46	2-3	29.85×4.37	5.65-6.48	6.04
5	Richard's agar	10.48-14.15 × 3.40-4.28	0-1	12.23×3.85	31.02-36.62 × 4.24-4.85	2-3	33.46×4.59	6.35-7.24	6.88
6	Sabouraud's dextrose agar	13.44-15.86 × 3.34-3.63	0-1	14.58×3.48	28.08-34.55 × 3.56-4.30	3-4	31.34×3.90	6.29-7.12	6.56
7	V8 Juice agar	12.17-14.15 × 3.405-3.88	0-1	13.32×3.58	24.52-32.68 × 3.98-5.53	2-3	28.75×4.80	5.82-6.89	6.31
8	Corn meal agar	10.77-13.57 × 2.76 -3.17	0-1	11.97×2.94	22.7 -31.02× 4.14-4.66	2-3	26.11×4.38	6.15-7.06	6.59

The results were found in agreement with the morphological diversity among nine isolates of *F. udum* studied by Rashmi (2016) revealed the highest colony diameter (61.91 to 90.00 mm), pigmentation (white to red) and mycelial growth (flat to raised), colony colour (creamy white to pink), sporulation (excellent to no sporulation) presence or absence of micro conidia and macro conidia, septations in micro conidia (0-1) and macro conidia (3-5) and its size and chlamydospore diameter. These results were also supported by the research findings of Rangaswami and Mahadevan (2001) revealing that the morphology of *F. udum* with macro conidia which are linear, fusoid, pointed at both ends with 3-4 septa and measured 15-50 x 3-5 µm. The micro conidia were small, elliptical and unicellular with one or two septa and measured 5-15 x 2-4 µm. The chlamydospores were spherical to oval, single or in chains in arrangement.

3.3. Molecular identification of pathogen

3.3.1. Sequencing and analysis

F. udum was identified by molecular methods. Only relying upon morphological characters for identification of the pathogen is not an adequate method because of overlapping in morphological characters. The DNA amplicon was observed at the region of 570 bp in *F. udum* (Fig.4). The amplified products were checked on 0.8 per cent Agarose gel electrophoresis. Examination for homology was carried out by using BLAST algorithm programme available in http://www.ncbi.nlm. nih.gov. and the homology table was constructed to identify the fungi.

3.3.2. Sequenced DNA data

The sequenced data is as follows:

GCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACT
CTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGAT
CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC
CTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCC
CCCTGCGGGCACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGT
AGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTG
AATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

DNA sequences of pathogen causing Fusarium wilt in sunhemp was compared using bioinformatics tool NCBI (National Centre for Bioinformatics) BLAST programme. Based on the obtained sequence comparison, ITS region showed the maximum similarity of *F.udum* of about 99.79 per cent having GenBank accession number of MK575503.1 and NCBI BLAST hit result was *F.udum* isolate (Table 3). The molecular sequencing conformed that the pathogen associated with wilt of sunhemp was *F.udum* with maximum of more than 98 per cent similarity with other isolates of *F.udum* which were deposited in NCBI.

Table 3. Homology table for molecular identification of Dharwad isolate of *F.udum*

Primers used	Name of the isolate	Similarity (%)	Host	Place	Accession number
ITS 1 and 4	*Fusarium udum* isolate	99.79	Sunhemp	Uttar Pradesh, India	MK575503.1
	Fusarium udum isolate KA8	99.72	–	Uttar Pradesh, India	KU097297.1
	Fusarium lateritium isolate HYTZ-4	98.11	Peach	China	MT991105.1

The result obtained is in accordance with Singha *et al*. (2016) in which molecular identification of *Fusarium* isolates was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using ITS 1 and ITS 4 primers. The total size of the ITS 1 and ITS 4 regions of the isolates studied varied from 380 to 620 bp. *Fusarium* sequences obtained from amplification of conserved ribosomal ITS region were compared with sequences from (NCBI) database using BLAST 2.0 (http://www.ncbi.nlm.nih.gov). The isolates were identified molecularly as *Fusarium* sp. based on ITS region. Sharma *et al.* (2015) reported the *Fusarium* strain was identified at molecular level by partial sequencing using ITS 4 and ITS 5 and match the existing isolates in GenBank (GenBank accession no. GQ497156.1) by 99 per cent.

4. Conclusion

The cultural and morphological studies of *F. udum* f. sp. *crotalariae* on eight solid media revealed that maximum mean radial growth of the colony was observed in Richards agar (90.00 mm) followed by V8 juice agar (88.00 mm) and Sabouraud's dextrose agar (85.00 mm) which are statistically on par with each other. Minimum radial growth of the mycelium was observed in oat meal agar (45.00 mm). Colour of the colony also differed as various shades of white. Under molecular characterization , the PCR product was sequenced using ITS 1 and ITS 4 primers which were amplified at 570 bp. The maximum identification with 99.79 per cent similarity of pathogen as *Fusarium udum* isolate with GenBank accession number MK575503.1 in NCBI BLAST hit result. This research findings provides valuable insights into the morphological and molecular characteristics of the pathogen.

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