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 ~~(UAS),~~ 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 *Fusarium 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, contributing to more effective management of Fusarium wilt in sun hemp.

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Key words: *Fusarium oxysporum f.sp.crotolariae* (Kulkarni) Subram., *Crotalaria juncea*, ~~Richard's agar, Potato dextrose agar, Czapeck's dox agar~~, *Fusarium udum*, ~~GenBank~~

Introduction

Sunhemp ~~scientifically known as (~~*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. The pathogen mainly survives in the crop stubbles as it is a facultative parasite. The pathogen produce

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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 (Mitra, 1934). 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. By integrating these approaches, researchers and farmers can better manage Fusarium wilt in Sunhemp, ensuring sustainable crop production and reducing economic losses.

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2.1. Materials and Methods

2.1. Isolation of the pathogen from infected sample

The infected 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.

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2.1.1. Identification of the pathogen

The morphological characters of the fungi such as mycelium, macro and micro conidia, chlamydospores were studied under microscope. The morphological characters of each fungus were compared with the characters of the respective fungi as described earlier by various scientists and the pathogen was identified as *F.udum* f. sp. *crotalariae*.

2.1.2. Preparation of giant culture of *Fusarium udum* f. sp. *crotalariae*

Sorghum seeds were used as substrate for giant culture preparation. The substrate was prepared by mixing 200 g 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* ~~f. sp. *crotalariae*~~ 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 ~~*Fusarium* *F.*~~ *udum* ~~f. sp. *crotalariae*~~ 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/microscopic field (10X) and conidial length and breadth in different culture media were recorded. The data on radial growth was analyzed statistically. ~~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).~~

2.3. Isolation of DNA and PCR Amplification for molecular characterization

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Analysis of chlamydospores: type, location?

Isolation of DNA from the *F. udum* ~~f. sp. *crotalariae* was~~ done by following standard CTAB (Cetyl Trimethyl Ammonium Bromide) method with certain modifications (Murray and Thompson, 1980). ~~For molecular characterization, T~~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. Cultural and morphological characteristics on different solid media

~~Cultural characters of pathogen were studied on eight different solid media *viz*., Czapeck's dox agar, potato dextrose agar, oat meal agar, potato carrot agar, Richard's agar, Sabouraud's dextrose agar, V8 Juice agar and corn meal agar~~ (Fig 2). All the media used differed in their growth and cultural characters of the pathogen (Table 1.)

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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 2. 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.

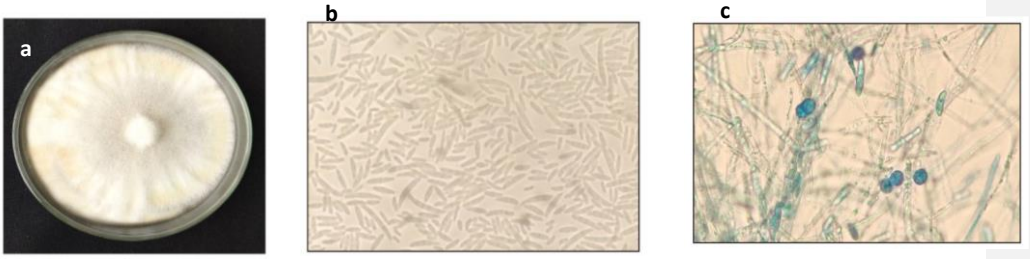

Fig 1. a) Pure culture of *Fusarium udam f.sp.crotolariae* b) Macro and Micro Conidia c) Chlamydospores

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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
8. Oat meal agar

Fig 2. Morphological characteristics of *Fusarium* on different growth media

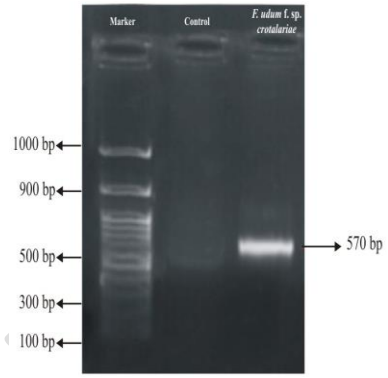

Fig 3. Amplified ITS rDNA region of *Fusarium udam f.sp.crotolariae*

Table 1. Morphological characters of *Fusarium udum* f. sp. *crotalariae* 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)

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3.2. Molecular identification of pathogen

3.2.1. Sequencing and analysis

F. udum f. sp. *crotalariae* 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~~. To overcome this inadequacy molecular method of ITS region sequencing was done. The full length of ITS rDNA region was amplified with ITS 1 (5'-TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') as universal primers for fungi.~~ The DNA amplicon was observed at the region of 570 bp in *F. udum* f. sp. *crotalariae* (Fig.3). The amplified products were checked on 0.8 per cent Agarose gel electrophoresis~~. The PCR product of pathogen causing fusarium wilt in sunhemp was sequenced by using forward and reverse primers at Biokart India Pvt Ltd., Bangalore.~~ 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.

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3.2.2. Sequenced DNA data

GCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACT
CTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGAT
CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC
CTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCC
CCCTGCGGGCACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGT
AGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTG
AATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

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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 *Fusarium udum* of about 99.79 per cent having GenBank accession number of MK575503.1 and NCBI BLAST hit result was *Fusarium udum* isolate (Table 2). The molecular sequencing conformed that the pathogen associated with wilt of sunhemp was *Fusarium udum* f. sp. *crotalariae* with maximum

of more than 98 per cent similarity with other isolates of *Fusarium udum* which were deposited in NCBI.

Table 2. Homology table for molecular identification of Dharwad isolate of *Fusarium udum* f. sp. *crotalariae*

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~~ 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 ~~National Center for Biotechnology Information~~ (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. Cruz *et al.* (2018) and Olalde-Lira *et al.* (2017) also recorded the similar results.

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