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Morphological and Molecular Characterization of *Fusarium oxysporum* f.sp. Vanilla Inciting Root and Stem Rot Disaease in Vanilla

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A B S T R A C T

Vanilla planifolia is popularly known as Prince of Spices a fleshy perennial liana grown in and around Western Ghats for its natural compounds used in several ice creams, chocolates and beverages. *Fusarium oxysporum* f.sp. *vanillae* is one of the most destructive pathogens causing severe loss to yield and during the survey conducted in 2016, maximum of 25 % incidence was noticed in coorg district. The pathogen was isolated and morphologically identified as *F. oxysporum* based on the conidial, chlamydosporial and cultural characters. The size of microconidia ranged between 5.97 to 8.60 µm in to 2.02 to 4.07 µm in width, most of the isolates did not produce macro conidia. Further to confirm the identity of pathogen, 18S rDNA or ITS region DNA was amplified and sequenced. A phylogenetic tree was constructed using Maximum likelihood showed clearly two distinct cluster which clearly out grouped the *Colletotrichum gloeosporioides* and all other *Fusarium* sp. in one clade. The seven isolates used in the study grouped under *F. oxysporum* clearly separating from other species of *Fusarium.* Futher Tajima's test also showed only six nucleotide differences indicating that the pathogen is *F. oxysporum* f.sp. *vanilla*.

Introduction

India is known for its varied climatic conditions and almost all the crop plants are produced in the sub continent. Among them, spices produced in India have a special privilege intended for both domestic and international market. *Vanilla planifolia* also referred to as Prince of Spices a mysterious spice once enjoyed the second place of market next to Saffron has no more a part of Indian crop production scenario. Vanilla is the only edible belonging to Orchidaceae family, extracts of Vanilla are widely used for flavouring ice creams, certain soft drinks, chocolates and fragrance ingredient in many perfumes (Jadhav *et al.*, 2009). Vanilla production drastically reduced from year 2004 (100 MT) (Anandan, 2004) to 5-10 MT during 2015 (Spices Board, 2017).

Several biotic and abiotic factors accounts for

declining trend of Vanilla production. High incidence of dreadful diseases like Root and Stem Rot (*Fusarium oxysporum* f.sp. *vanilla*), Bud rot (*Phytophthora meadii*), Stem rot (*Sclerotium rolfsi*), Yellowing and immature bean shedding (*Colletorichum* spp.) and some viral diseases (Necrosis and Mosaic) (Pearson *et al.,* 1991, Grisoni *et al.,* 2010) further reduced the production.

Some root rot disease resistant species of vanilla are known, including *V. pompona*, *V. phaeantha* Rchb. f. and *V. barbellata* Rchb. f. but poor quality and short lengths of beans that do not meet commercial criteria and plants often flower sparsely and tend to drop their fruits before maturity. Several species of *Fusarium* such as *F. decemcellulare*, *F. fujikuroi*, *F. graminearum*, *F. mangiferae*, *F. napiforme*, *F. oxysporum*, *F. polyphialidicum*, *F. proliferatum*, *F. pseudocircinatum*, *F. semitectum*, *F. solani* and *F. subglutinans. F. oxysporum* were reported by Pinara *et al.*, (2010) upon isolation from infected plants, but only *F. oxysporum* was found to be pathogenic. Hence, the present study aims to record the incidence of RSR diseases in two major vanilla growing states and to elucidate the pathogen associated with the disease in India by molecular phylogeny.

Materials and Methods

Survey, isolation and morphological characterization of pathogen

A Survey was conducted during August to October (2016) in two different states namely Karnataka and Tamil Nadu to assess the incidence of wilt and leaf spot disease in Vanilla. Leaves showing the symptoms of leaf spot were assessed as per the severity grade of 0 - 4 and the per cent disease index was calculated (Faisal *et al.,* 2014). The incidence of wilt was recorded as number of plants infected to number of plants observed, later on converted to percentage.

The infected root stem and leaves Vanilla showing typical rot symptoms were cut into small bits measuring about two mm and surface sterilized in 0.1 per cent mercuric chloride solution for one minute and washed repeatedly thrice in sterile distilled water to remove the traces of mercuric chloride. Then surface sterilized tissues were transferred to sterile Petri plates containing PDA medium under aseptic conditions. The inoculated Petri plates and slants were incubated under at room temperature (25 ± 2ºC) and observations were taken at regular intervals. The pathogen was identified up to species level based on their cultural and morphological characters. A loop full of fungal culture grown on PDA plates were taken on a glass slide and observed with image analyzer under 40 x magnifications for the presence of conidia and Chlamydospore. After confirming the spores, the cultures were purified by single spore isolation technique. The fungus was sub cultured on PDA slants and allowed to grow for seven days at (28 ± 2ºC) and preserved at 4ºC and subcultured under aseptic conditions periodically.

In order to prove Koch's postulates, pathogenicity test was carried out with pathogen multiplied in Sand:Maize media (19:1) so as to get 7X 10^5 cfu/ml, the cultures were inoculated to pot grown vanilla with three replication for each isolate. The fungus was reisolated from the artificially inoculated plants showing typical rot symptoms and the culture obtained was confirmed for its morphology and colony characters.

Morphological characters *viz*., size, colour and shape of the conidia and chlamydospore were observed. Measurements of 50 spores were taken under the image Nikon Eclipse Ci Phase Contrast Microscope at 40x and range were determined. Cultural characteristics of

like pathogen, zonation, colony colour, substrate colour, margin of colony and topography were recorded through naked eye.

Molecular characterization and phylogeny

Genomic DNA was extracted from the suspension culture of *C. musae* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee (1996). To confirm isolates as *F.oxysporum* f.sp. *vanillae* 18S rDNA or ITS region DNA was amplified with primers, ITS1 (5´ TCC GTA GGT GAA CCT GCG G 3´) and ITS4 (5´ TCC TCC GCT TAT TGA TAT GC 3´) to get 450-550 bp amplicon of ITS region. Amplification was conducted in a total reaction volume of 25 µl. The PCR settings used were as follows: a hold of two min. at 95 °C, 40 cycles of one min. at 95 °C, one min. at 55 °C and one min. at 72 °C and a final extension of five min. at 72 °C. The PCR products were resolved on two per cent agarose at 50 V stained with ethidium bromide (0.5 µg/ml) and photographed and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

Amplified 18S rDNA was purified from each reaction mixture by agarose (1.2 %, w/v) gel electrophoresis in TBE buffer containing 0.5 µg of ethidium bromide per ml. A small agarose slice containing the band of interest [observed under long-wavelength (312-nm) UV light] was excised from the gel and purified by using a QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California) according to the supplier's instructions. The DNA sequencing was performed at Sci Genome Pvt. Ltd. Cochin, India.

The rDNA homology searches were performed using the BLAST program (Altschul *et al.*, 1990) through the internet server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, USA). Sequences and accession numbers for compared isolates were retrieved from the GenBank database. Sequence pair distances among related and different fungi of the isolate were scored with the Clustal W program and phylogenetic tree analysis was performed with the MEGA 5 (Tamura *et al.*, 2011). Newly obtained sequences were submitted in the GenBank database, New York, USA. List of other sequences used are described in Table 1.

Tajima's relative rate test, the χ^2 test statistic was 0.33 (P = 0.56370 with 1 degree[s] of freedom). The analysis involved 3 nucleotide sequences. A (*Fusarium oxysporum f. cubense isolate EPPI01 EU022522*) and B (*Fusarium oxysporum f. sp. lycopersici strain FOL1 KR071144*), with sequence C (*Fusarium oxysporum f. sp. Vanilla FOV1*) used as an outgroup in Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 420 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Results and Discussion

Vanilla (*Vanilla planifolia* Jacks. ex. Andrew), a fleshy perennial liana cultivated in several tropical countries for natural vanillin, which is used in food and beverages. Vanilla is susceptible to a number of fungal and viral diseases, which cause considerable damage to the beans or to the whole plant resulting in heavy crop losses. Diseases, notably Root and Stem rot caused by *Fusarium oxysporum* f sp. *vanillae* are a major limiting factor that hinders in the crop production). *Fusarium oxysporum* causing root and stem rot is the most important pathogen responsible for severe damage to the cultivation of vanilla.

Despite significant economic losses caused by the disease, there has not been an effective method for controlling this disease. In order to assess the disease incidence, survey was conducted in major vanilla growing states of Southern India at 10 different location, the wilt incidence ranged from 0-25% and maximum incidence of wilt was located Madikeri taluk of Coorg district with 25 % disease incidence followed by Sirsi. Certain other diseases, the leaf spot incidence were also recorded and maximum PDI was observed in Sirsi (Table 2 and Fig. 1). *Fusarium oxysporum* was recovered from diseased roots and stems of vanilla cultivated in India, pathogenicity tests using sand maize media on healthy Vanilla plants kept in pots confirmed the pathogenicity of the *F. oxysporum* isolates. Similar results were found in a survey conducted at China (Xia-Hong, 2007), Indonesia (Pinaria *et al.,* 2010), and in India (Vijayan *et al.,* 2012) they all demonstrated that *F. oxysporum* is the principal species causing RSR of vanilla worldwide. Symptoms of the RSR include the browning and death of the underground roots either dry or watery based on moisture content of the soil (Alconero, 1968). The aerial roots normally remain healthy until they propagate rapidly and touch the soil. The destruction of the roots system hinders in the supply of water and food to the aerial parts of the plant leading the plants to shrivelling and silently death. The symptoms also include the drop down of tender tips, yellowing of leaves and stem and the shrivelling of the stem (Fig. 2) due to the lack of nutrients. Nam *et al.*, (2005) surveyed for Fusarium wilt in strawberry in Korea during 2001 to 2003 and recorded almost thirty per cent incidence. The difference in the disease incidence is mainly attributed to the natural environment conditions prevalent in the growing region. A morphological character serves as vital tool in identification and classification of the fungus. In the present study, spore size and chalmydospore characters were used for identifying the fungus (Table 2 and 3; Fig. 3, 4, 5). The isolates showed variation in the colony colour from Whitish to Pinkish colour with predominantly whitish pink colour, in all the colonies the substrate colour remain white except FOV4. With respect tom margin and topography all the isolates has wavy margin and FOV2 alone showed flat topography while all other showed raised. All the isolates except FOV4 showed pinkish pigmentation while the FOV4 showed no pigmentation. All the seven isolates were not growing in similar trend, isolate FOV@ grown maximum to 90 mm on tenth day, while FOV4 showed only 65 mm growth. The size of microconidia ranged between 5.97 to 8.60 µm in to 2.02 ot 4.07 µm in width. The variations in the conidial size were noticed in all the isolates. Cottony profused pinkish color growth of *Fusarium* spp. was observed by Adiver (1996). Variation in the colour of the mycelium and the shape of the conidia were also observed by Kulkarni (2006) and Kishore (2007) in *F. oxysporum* from carnation and gerbera respectively (Table 4).

The polymerase chain reaction (PCR) method has been developed for the *in vitro* amplification of nucleic acid sequence and has been used to detect a number of plant pathogens based on the specific nucleotide sequences. This method is highly sensitive and capable of detecting even a single copy of DNA molecule (Henson and French, 1993). ITS region of *Fusarium* sp was amplified with primers ITS1 and ITS 5 to get 450 bp amplicon of ITS region (Fig. 6). The amplicon was sequenced and the same was submitted to Gene bank. The results confirmed with the findings of Abd-Elsalam *et al.*, (2003) and they reported that the amplification of ITS region of *Fusarium* sp yielded 400-500 bp amplicon.

Table.1 List of species used in the study for constructing phylogeny

S.No.	Species	NCBI Accession
1	*Fusarium oxysporum* f. sp. *vanillae* isolate DL-1-1	AY383320
2	*Fusarium oxysporum* f. sp. *vanilla*	AY380575
3	*Fusarium oxysporum* f. sp. *vanillae* isolate NayR128	KT261749
4	*Fusarium oxysporum* f. sp. *vanillae* isolate 22ma140	JQ975403
5	*Fusarium oxysporum* f. sp. *vanillae* FOV1	MG905419
6	*Fusarium oxysporum* f. sp. *vanillae* FOV2	MG905420
7	*Fusarium oxysporum* f. sp. *vanillae* FOV3	MG905421
8	*Fusarium oxysporum* f. sp. *vanillae* FOV4	MG905422
9	*Fusarium oxysporum* f. sp. *vanillae* FOV5	MG905423
10	*fusarium oxysporum* f. sp. *vanillae* FOV6	MG905424
11	*Fusarium oxysporum* f.sp. *vanillae* FOV7	MG905829
12	*Fusarium oxysporum* f. *cubense* strain ATCC 96285	EF590328
13	*Fusarium oxysporum* f. *cubense* isolate EPPI01	EU022522
14	*Fusarium oxysporum* f. sp. *lycopersici* culture FCBP:1561	MG136705
15	*Fusarium oxysporum* f. sp. *lycopersici* strain FOL1	KR071144
16	*Fusarium verticillioides* strain FS7	KF031434
17	*Fusarium verticillioides*	KJ801959
18	*Fusarium udum* isolate FU-11	KT895918
19	*Fusarium udum* isolate Faizabad	KC859450
20	*Fusarium udum* isolate SN-1	DQ641266
21	*Fusarium solani* strain bxq637	EF117321
22	*Fusarium falciforme* isolate FSS	KJ679357
23	*Microdochium nivale* strain 200120	KT736210
24	*Colletotrichum lindemuthianum* isolate CL05	KJ939273

Table.2 Survey for occurrence of diseases in Vanilla Gardens

State/ District	Taluk	Locations	No. of fields surveyed	Disease incidence		
				Wilt Range (%)	Leaf Spot Range(%)	Mean (%)
Karnataka						
Uttar Kannada	Sirsi	2	3	10-25	15-25	18.23
	Sagar/Barur	2	2	5-10	5-15	10.00
Kodagu	Virajpet	1	2	12-15	2-10	12.50
	Madikeri	2	3	15-25	2-5	21.00
Tamil Nadu						
Coimbatore	Pollachi (Nursery conditions)	2	2	10-20	--	13.5
	Valparai	1	2	0-5	5-15	9.00

Table.3 Cultural characters of pathogen ten days post inoculation

Isolate	Colony colour	Substrate colour	Margin	Topography	Zonation	Pigmentation	Colony diameter (mm)	Sporulation
FOV1	Pinkish white	White	Wavy	Fluffy	No zonation	Pink	86	+++
FOV2	Violet	White	Wavy	Flat	Single zonation	Pink	90	+++
FOV3	Pink	White	Wavy	Fluffy	Concentric zonation	Pink	80	+++
FOV4	Whitish pink	Yellowish white	Wavy	Raised and fluffy	No zonation	Nil	65	++
FOV5	Pinkish white	White	Wavy	Raised and fluffy	No zonation	Pink	81	++
FOV6	Pinkish white	White	Wavy	Raised fluffy	No zonation	Pink	76	++
FOV7	Pinkish	White	Wavy	Flat	Two zonation	Pink	82	+++

Table.4 Characterization of microconidia, macrocondia and chalmydospore

Isolate	Micrcondia	Macroconidia	Chlamydospore
	Length and breadth (µm)*		Diameter of each rounded of cell (µm)*
FOV1	7.25X3.65	36.77X6.80	6.24-7.75
FOV2	7.02X3.21	-	8.12-10.00
FOV3	5.97X2.27	-	6.48-8.29
FOV4	8.60X4.07	-	5.54-7.65
FOV5	6.81X3.41	-	6.78-8.30
FOV6	7.21X3.21	-	7.7-9.60
FOV7	7.89X2.02	-	7.71-8.14

Table.5 Results from the Tajima's test for 3 Sequences

Configuration	Count
Identical sites in all three sequences	411
Divergent sites in all three sequences	0
Unique differences in Sequence A	2
Unique differences in Sequence B	1
Unique differences in Sequence C	6

Fig.1 Location of Survey marked with latitude and longitude

Fig.2 Symptom of root and stem rot

a. Drying of leaves and death

b. Complete death of plant

Fig.3 Cultural characters of *F. oxysporum* f.sp. vanilla

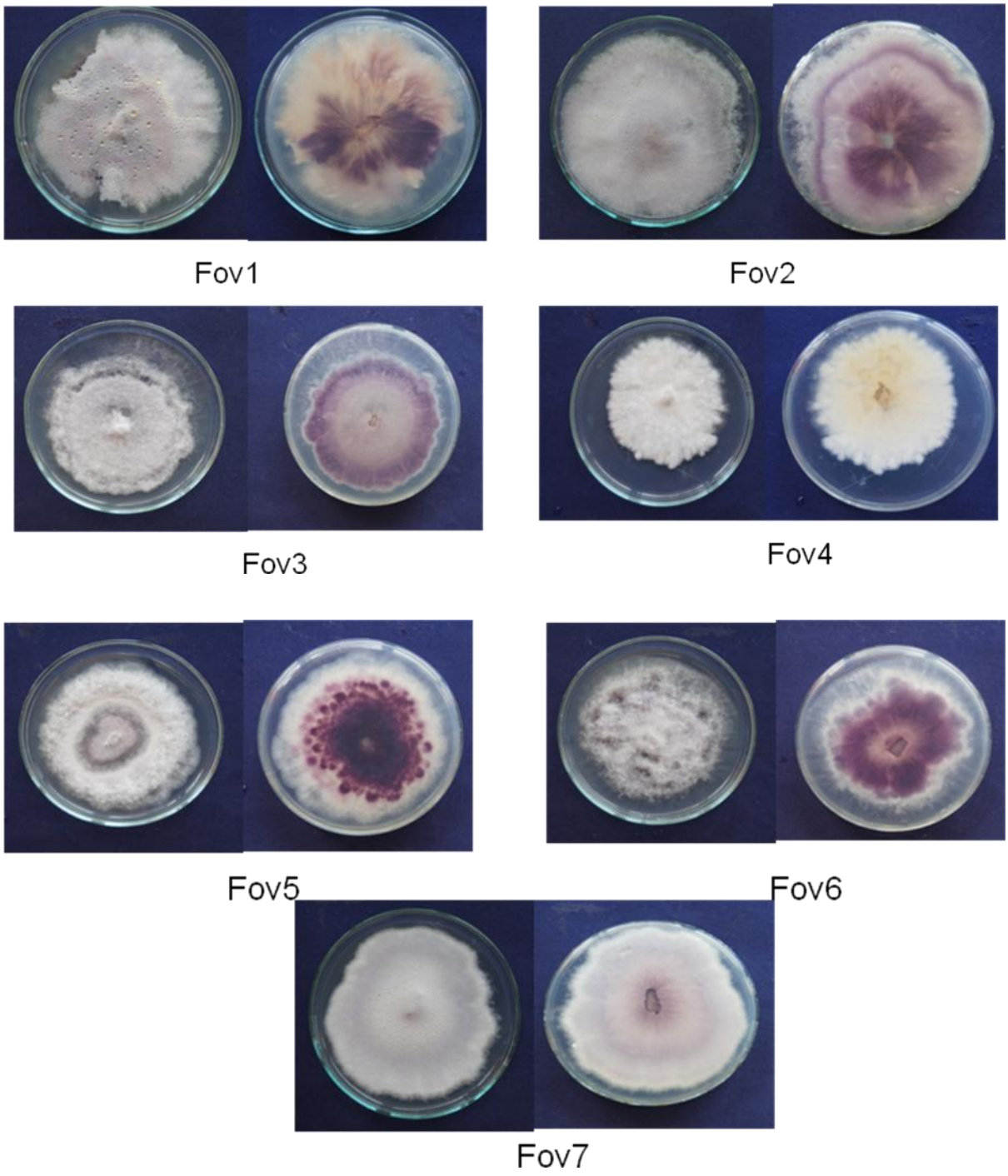
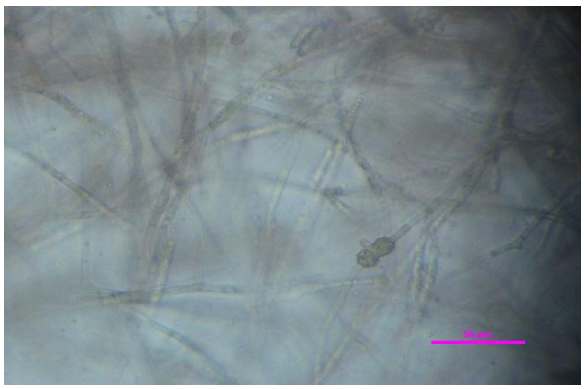
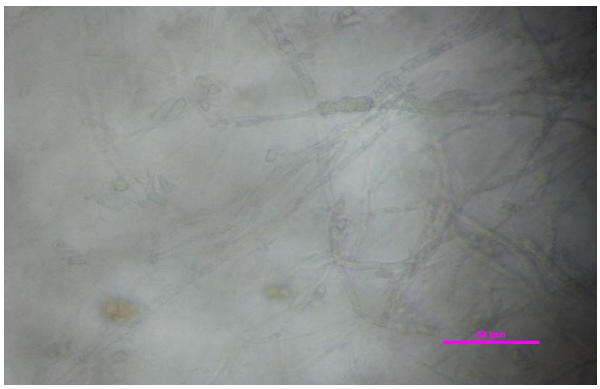


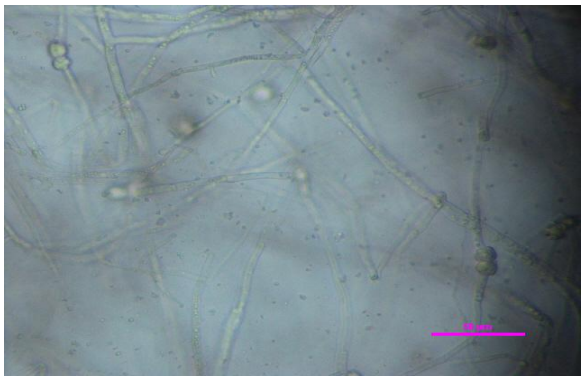
Fig.4 Chlamydospore produced by individual Isolate



FOV1

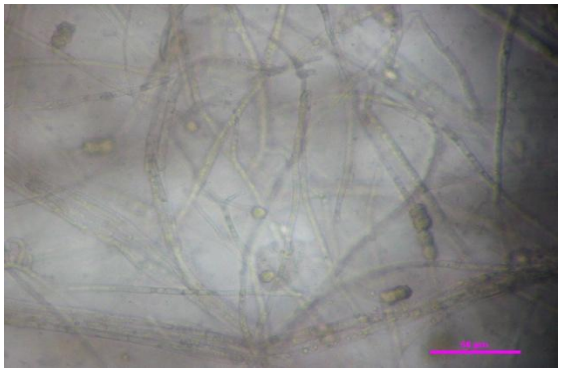


FOV2

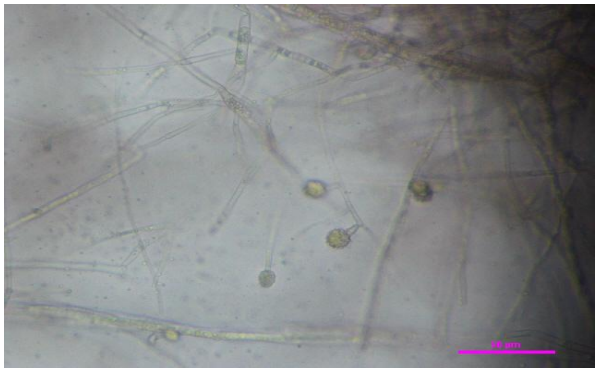


FOV3

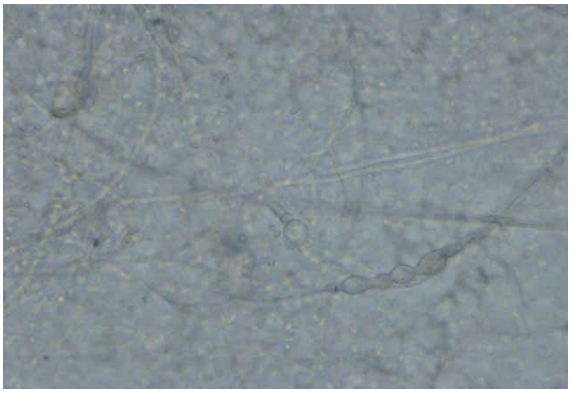
FOV4



FOV5



FOV6



FOV7

Fig.5 Microconidal characters of FOV isolates

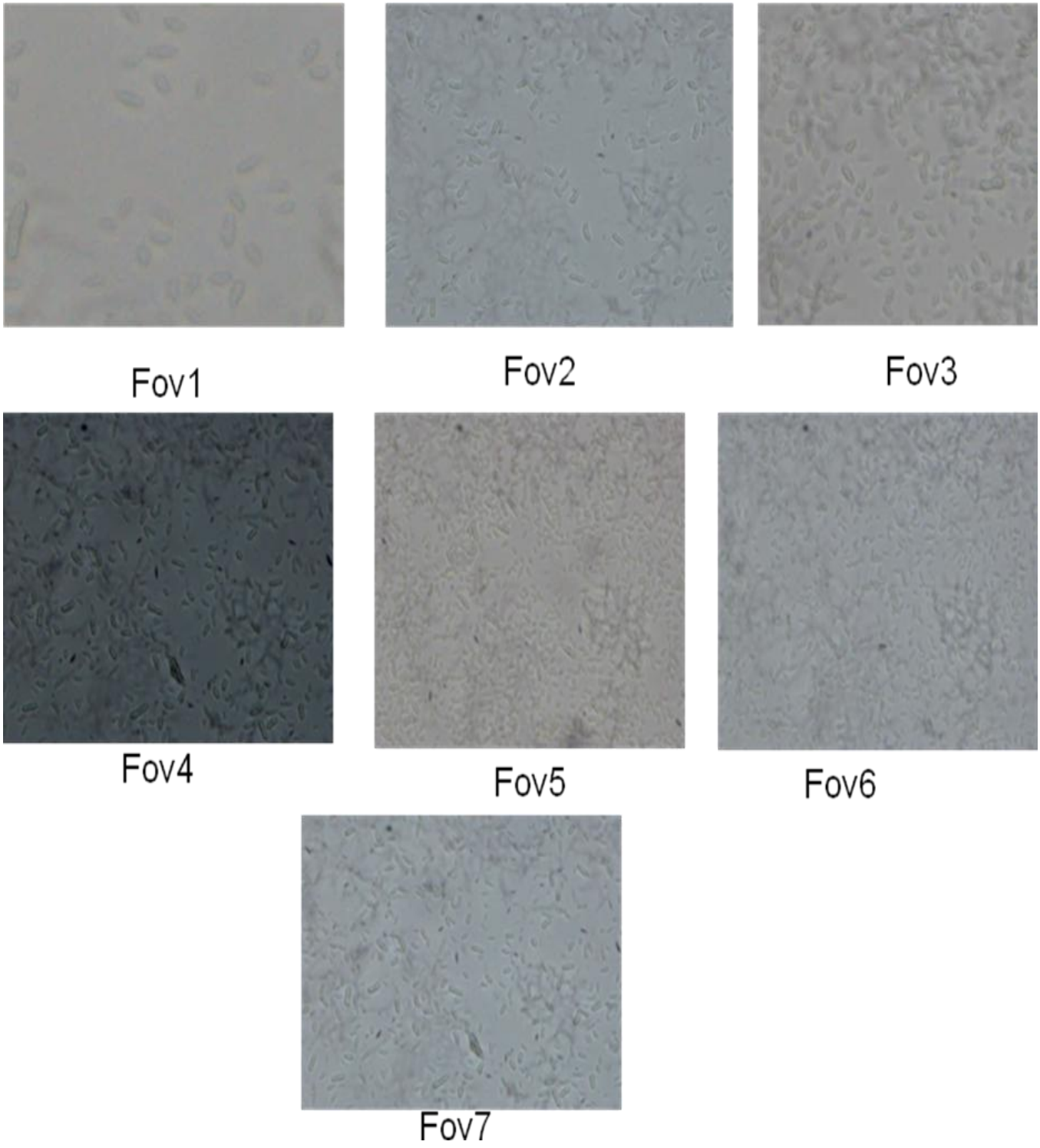
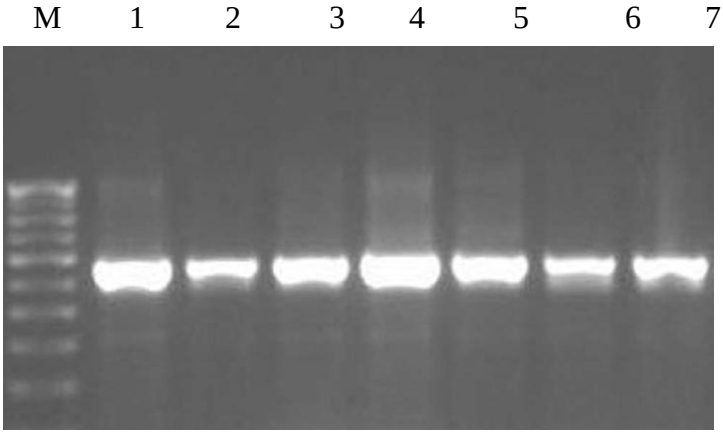
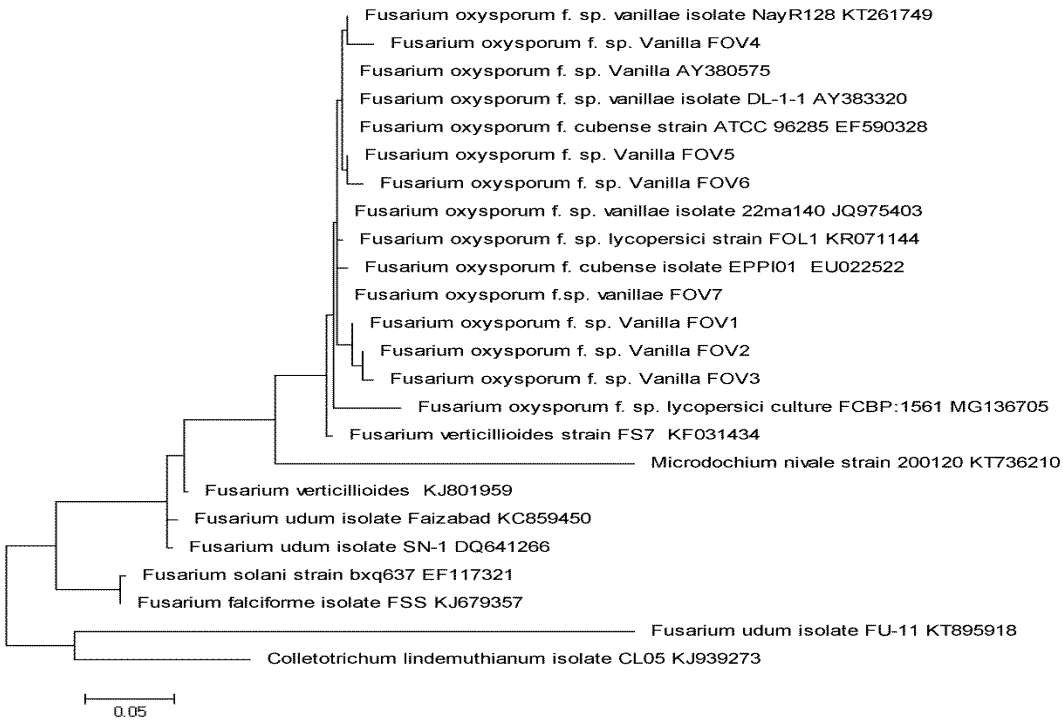


Fig.6 rrDNA amplication of FOV including 18S rDNA, ITS1, 5.8S rDNA and 28S rDNA



M- Marker; 1- FOV1; 2-FOV2; 3-FOV3; 4- FOV4; 5-FOV5; 6-FOV6; 7-FOV7

Fig.7 Maximum likelihood trees for the Fusarium genus and related genera inferred from the rDNA cluster including 18S rDNA, ITS1, 5.8S rDNA and 28S rDNA



Similarly, many workers, Glynn *et al.*, (2006) and McCormick *et al.*, (2006) confirmed the pathogen *Fusarium* sp by amplifying ITS region to get 400-500 bp. Since there is lot of variation in ITS region amplification, hence sequencing of this amplicon was performed. The ITS nucleotide sequences of each isolate were then compared to those in the public domain databases NCBI (National Center for Biotechnology information; www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). In order to generate the phylogenic tree, alignment of ITS DNA sequences was done

using Clustal W program, Phylogenetic tree was created using MEGA 5 Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods (Tamura *et al.,* 2011). The results of the phyogenetic tree constructed by Maximum likelihood showed clearly two distinct cluster which clearly out grouped the *Colletotrichum gloeosporioides* and all other Fusarium sp. in one clade. The seven isolates used in the study grouped under *F. oxysporum* clearly separating from other species of *Fusarium* spp (Fig. 7). Phylogenetic analysis performed based on the ITS sequences helped to reveal the evolutionary relationship of Fusarium spp. (Nirmaladevi *et al.,* 2016). Further in Tajima's test (Table 5) there were 411 identical sites in *F. oxysporum* group with some unique difference of two nutcletide in *Fusarium oxysporum f. cubense isolate EPPI01 EU022522*) and one in *Fusarium oxysporum f. sp. lycopersici strain FOL1 KR071144*), and six difference in *Fusarium oxysporum f. sp. vanilla FOV1*. Thus Tajima's test further confirms in the present study all the isolates belongs to *F. oxysporum* f.sp. *vanillae*

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