Plant Archives Vol. 21, Supplement 1, 2021 pp. 2363-2368 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
doi link : https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.387

MORPHOLOGICAL AND MOLECULAR VARIATION OF *FUSARIUM OXYSPORUM* F.SP *LYCOPERSICI* ISOLATES CAUSING *FUSARIUM* WILT IN TOMATO

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of the most important, commercial and widely grown vegetable crop in the world. Tomato plays a critical role in nutritional food requirements, income and employment opportunities for the people. However, its production is threatened by the *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* and productionlossesbetween30%to40%. In the present investigation an attempt has been made to study the morphological and molecular variation of *Fusarium oxysporum* f.sp *lycopersici* isolates. Usual identification of *Fusarium* species based on their micro and macroscopic features and morphological characters alone may lead to incorrect designation. In order to identify the correct species, we amplified the 18S rRNA gene region by PCR, sequenced and analyzed for sequence similarity among the NCBI data through BLAST. Further, PCR amplification of ITS regions was performed using ITS primers. The amplified product of 18S rRNA gene was sequenced and deposited to Gen Bank with the accession numbers.

Keywords: Tomato, *Fusarium* wilt, *Fusarium oxysporum* f.sp. *lycopersici,*18S rRNA, PC, BLAST, ITS, Genbank.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated and popular vegetable crop across the world (Pastoretal, 2012). It belongs to the *Solanaceae* family and it is the most important vegetable after Potato. Tomato is used for consumption due to its high nutritive values, antioxidant and curative properties and it contains Vitamin A, Vitamin C and Vitamin E with 95.3% of Water, 0.07% Calcium and Niacin which have great importance in metabolic activities of humans (Sahu *et al*., 2013). Tomatoes are excellent source of various micronutrients and antioxidants. It has high nutritional values which plays a crucial role in our daily home cooking (Singh *et al*., 2019).

Tomato plants are susceptible to various diseases caused by different agents such as Bacteria, Viruses, Nematode, Fungi and Abiotic factors (Sahuetal, 2013). Among the fungal diseases, *Fusarium* wilt is caused by *Fusarium oxysporum* f.sp. *lycopersici* and it causes economic loss of tomato production in world wide. *F. oxysporum* f.sp. *lycopersici* is a soil borne pathogen, persists in soil for about 8-10 years in the form of chlamydospores as resting structure (Prachi Singh *et al*., 2019). The fungus *F. oxysporum* f.sp. *lycopersici* is exerting pressure on production losses between 30 to 40% and may even raise upto80% if so, climatic conditions favour the growth of the fungus (Nirmaladevi, 2016).

Fusarium oxysporum f.sp. *lycopersici* is a soil borne pathogen with high level of host specificity. There are more than 120 described formae speciales and races within the species. The pathogen produces three types of asexual spores *viz*., microconidia, macroconidia and chlamydospores (Agrios, 1988). Differences in DNA sequences of genes have been used to support morphological identification of *Fusarium* sp. (Harrow *et al*., 2010). A comparison at DNA sequences provides accurate classification of fungal species to elucidate the evolutionary and ecological relationships among diverse species.

PCR based techniques are regularly used for identification, characterization and early diagnosis of microbes and pathogens. Random amplified polymorphic DNA (RAPD) analysis (Williams *et al*., 1990) has been used for identification of fungi. It has been observed to have a high level of variability among many isolates of *Fusarium* by several workers (Pasquali *et al.,* 2005; Balmas *et al*., 2005). The aim of this study was to isolate *F. oxysporum*, to identify it using morphological characteristics and DNA sequencing.

Materials and Methods

Survey to assess the incidence of *Fusarium* wilt of tomato

A rowing survey was undertaken in ten different tomato growing areas of Krishnagiri district in Tamil Nadu. To assess the incidence of tomato wilt randomly 100 plants were selected from each field and the numbers of infected plants were counted and the mean wilt incidence was expressed in percentage. The percent disease incidence was calculated by using the formula (Mayeeand Datar, 1986).

$$\text{Disease Incidence\% (PDI)} = \frac{\text{Number of inf ected Plants}}{\text{Total number of Plants}} \times 100$$

Completely wilted plants were collected to isolate the pathogen along with rhizosphere soil to isolate the antagonistic organisms.

Isolation and identification of *Fusarium oxysporum* f.sp. *Lycopersici*

Typical wilt symptom showing tomato plants were collected from different tomato growing areas of Krishnagiri district and used for isolation of pathogen. The infected root and stem portions were washed in tap water and the tissues showing vascular brown colour discolouration are cut into small pieces. They were then surface sterilized in 1% Sodium hypochlorite ($NaOCl_2$) solution for 30 sec. To remove the traces of Sodium hypochlorite solution the tissues were washed thrice with sterile distilled water and the pieces were transformed to the Petri plates containing sterilized potato dextrose agar (PDA) and incubated at room temperature (28 ± 2°C) for 5-7days. The pure culture of pathogen is obtained by single hyphal tip method (Rangaswami1972).

Morphological and cultural characters of *Fusarium oxysporum* f.sp. *Lycopersici*

Ten isolates of *Fusarium* spp. obtained were compared for variation in respect of morphological and cultural characters on solid medium. Ten days old culture of each isolate was separately inoculated and incubated at 28 ± 2°C for seven days. After the incubation period, fungal radial growth, micro & macro conidia population, colony characters, sporulation and size of micro, macro conidia and chlamydo spores were measured. The characters were compared with those described by Booth (1971).

Molecular characterization of *Fusarium oxysporum*f. sp. *Lycopersici*

(i) DNA Extraction

All the Fungal isolates were grown in100ml of PDA broth for 7days at 28 ± 2°C. The genomic DNA was extracted and purified using the CTAB buffer method modified from Nicholson *et al.* (1996). Fungal mycelium was harvested and 2g of dried mycelium were ground into fine powder in liquid nitrogen using mortar and pestle until it forms dry powder. 200 mg of powdered mycelium was transferred to 2.0 ml Eppendorf tubes and1ml of 20mMEDTA (pH: 8.0) and2% CTAB, mercaptoethanol (0.1) was added, vortexed andincubatedat65°Cfor10minutes. The mixture was transferred to clean tube and chloroform: isoamylalcohol (24:1) was added in equal volume. The mixture was centrifuged at 10,000 rpm for 10 minutes. Equal volume of 5 M NaCl and ice cold isopropanol was transferred to the supernatant taken in clean tube and mixed well. It was incubated at 65°C for DNA precipitation or incubated overnight at -20°C. The content was centrifuged at 13000 rpm for 10 minutes and the pellet was collected by discarding the supernatant. The pellet was washed with70% ethanol twice. 50µl of TE buffer or double sterile water was used for resuspending the pellet (10mM Tris,1mMEDTA,pH 8.0).

(ii) PCR amplification & sequencing of 18sr DNA of *Fusarium* sp.

Genomic DNA was isolated and quantity was measured using Nano Drop Spectrophotometer and the quality was determined using 2% Agarose gel. A single band of high-molecular weight DNA has been observed.18S rRNA gene was amplified by 18SrRNAF and 18SrRNAR primers. A single discrete PCR amplicon band of 700 bp was observed when resolved on Agarose gel.

PCR amplification was carried out to amplify the internal transcribed spacer (ITS) region in the DNA of the *Fusarium* isolates.

Forwardprimer: ITS15'TCCGTAGGTGAACCTGCGG3'

Reverseprimer: ITS43'TCCTCCGCTTATTGATATGC5'

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer in MEDAUXIN SEQUENCING PVT. Ltd. Bangalore. Consensus sequence of 18S rRNA gene was generated from forward and reverse sequence. 18S rRNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed by using MEGA 7. Finally acquiring accession numbers, these sequences were submitted to NCBI (National Centre for Biotechnology Information) gene bank, USA.

Results and Discussion

Survey on the incidence of *Fusarium* wilt of tomato incited by *Fusarium oxysporum* f.sp. *Lycopersici* (fol) in major tomato growing areas of Krishna Giri district

The data presented in table 1 on the survey in different locations of major tomato growing areas of Krishnagiri district revealed endemic nature of the *Fusarium* wilt incidence of tomato. Among the different locations of Krishnagiri district surveyed for tomato *Fusarium* wilt incidence, Uthangarai registered the maximum incidence of the disease (49.47%) followed by Thippampatti with (43.25%), Kollanaikanoor with (38.87%) and the minimum *Fusarium* wilt incidence of (12.56%) was recorded in Arasur.

Similar to the present study Jayanta *et al.* (2018) conducted a survey in four districts of North Eastern Karnataka and the wilt incidence was noticed in all locations surveyed with a range of8.33to 38.66 percent attributed by specific variety.

The maximum wilt incidence of 58.83 per cent was recorded at Sivagiri in Thirunelveli district followed by Pochampalli in Krishnagiri District (55.54%), where as

Vadakadu in Pudhukottai district recorded the minimum of 12.65% PDI (Amutha and Darwin christdhas henry 2017).

Jagraj Singh *et al.*(2018) also concluded that sandy soil supported highest wilt incidence in water melon, tomato and marigold and the least incidence was found in silt clay soil. Similar to the present study, Narenderetal. (2015) conducted survey in important tomato growing areas of Himachal Pradesh and recorded the wilt incidence ranging from9.90 t o 27.65 %.

Isolation and cultural characteristics of various isolates of *fusariUm oxysporum* f.sp. *Lycopersici* (FOL) from major tomato growing areas of Krishnagiri district

(i) Colony characters and mycelial growth

The isolates of *F. oxysporum* f.sp. *lycopersici* showed variation with respect to colony characters. The Colour of the isolates varied from white to pale pink and pinkish. Most of the isolates produced fluffy to moderately fluffy cottony aerial mycelium (Table 2). Among the isolates of *F. oxysporum* f.sp. *lycopersici* the maximum mycelia growth (90.00mm) was recorded by the isolates Fol_3 isolated from Uthangari. The minimum mycelial growth of 74.35mm was produced by the isolate Fol_6 isolated from Arasur (Table2).

Rajendran *et al.* (2018) reported that the pathogen produced different colony colors *viz.*, Light pink, Pink, Dark pink, Creamy white, pale white with pink and the mycelial growth pattern showed two different pattern namely adherent smooth and fluffy growths.

Similarly, Amutha *et al.* (2017) reported that such groupings have been done with respect to *F. oxysporum* f.sp. *lycopersici*. In past studies, Nath *et al.* (2017) mentioned that the colony colors were purplish white, Whitish orange, Creamy white, cottony white. Similar such groupings have been done with respect to *F. oxysporum* f.sp. *cubense* by Sanjeev kumar(2008).

(ii) Conidial population

All the *F. oxysporum* f.sp. *lycopersici* isolates varied in their ability to produce micro and macro conidia on PDA. The isolate *F. oxysporum* f.sp. *lycopersici* (Fol_3) produced the maximum conidia population of 2.7 / ml ($\times 10^6$). The minimum conidial population of 0.5 /ml ($\times 10^6$) was produced by the isolate Fol_6 isolated from Arasur (Table 2).

In past studies, the size of micro conidia ranged from 3-4 x1-2 µm to 11-10 x1-2 µm with 0-1 septate andsizeofmacroconidiavariedfrom13-15x3-4µmto24-26x4-5µm3-4 septate (Padvi *et al.* 2018).Narendra Kumar *et al.* (2015) reported that the isolates of *F. oxysporum* f.sp. *lycopersici* variable with respect to their conidia size.

(iii) Molecular analysis

In the present study the results obtained from PCR amplification of ITS region with primer pair of *ITS1* and *ITS4* produced a gene product of 700 bp confirming the isolates to be *Fusarium* sp (Plate 1).

Among the 10 isolates 5 were sequenced. The sequences of isolates *viz.*, Fol_1, Fol_3, Fol_5, Fol_7, Fol_{10} were identified as *Fusarium* sp, *Fusarium* sp, *Fusarium brachygibbosum, Fusarium longipes* and *Fusarium chlamydosporum* through BLAST search in NCBI website (ww.blast.ncbi. nlm.nih.gov/Blast). The sequences were deposited in the GenBank with the accession numbers MT565599, MT565600, MT565601, MT565602 and MT565603.

Similar to the present investigation Nirmala devi *et al.* (2016) reported that using primers ITS 1 and ITS 4, the amplified region specifically ranged from 334 bp to509 bp fragment from all *Fusarium* species isolates. BLAST analysis of ITS sequences of tomato wilt pathogen showed 100% similarity with *F. oxysporum* and *F. solani* causing foot rot of tomato was first observed by Bowen on the Central Queensland coast in 1975 (Vawdrey and Peterson 1988). The isolates associated with tomato wilt, were identified molecularly as *F. oxysporum*, *F. circinatum, F. andiyazi* and *F. fujikuri. F. falciforme* is also causing Foot rot and wilt of tomato in Mexico (Vega-Gutiérrezetal, 2019). Sundaramoorthy *et al.* (2013) identified that the *Fusarium* isolates *viz.*, ATF04, ATF05 and ATF19 as *F. fusarioides* casing wilt complex disease in tomato through BLAST searchofITS1 andITS4in NCBI.

Molecular identification based on Internal Transcribed Spacer (ITS) region and TEF-1α gene sequencing some species of *Fusarium* causing tomato wilt pathogen identified are *F. incarnatum-equiseti*, *F. graminearum*, *F. acuminatum* and *F. solani* (Asma Akbar *et al.* 2018). The isolate of *F. oxysporum* was sequenced and submitted in NCBI database and it's based on the closest match of BLAST analysis, it showed 100% homology with *F. oxysporum* (Amutha Kuppusamy *et al.* 2018).

Table 1: Survey on the incidence of *Fusarium* wilt of tomato incited by *Fusarium oxysporum* f.sp. *lycopersici* (Fol) in major tomato growing areas of Krishnagiri district

Sl. No.	IsolateName	Location	Soil type	Variety	Stage of the crop	Disease Incidence (%)
1.	Fol_1	Hamumantheertham	Clay loam	CO 2	Fruiting	30.54 [e] (33.54)
2.	Fol_2	Irumathur	Sandy Loam	Local	Flowering	34.64 [d] (36.05)
3.	Fol_3	Uthangarai	Sandy loam	PKM 1	Fruiting	49.47 [a] (44.69)
4.	Fol_4	Thippampatti	Clay	CO 1	Flowering	43.25 [b] (41.12)
5.	Fol_5	Puthoor	Sandy loam	CO 2	Fruiting	18.15 [i] (25.21)
6.	Fol_6	Arasur	Clay loam	COTH1	Fruiting	12.56 [j] (20.75)

7.	Fol_7	Kollanaikanoor	Clay	Local	Flowering	38.87^{c} (38.56)
8.	Fol_8	Mittapalli	Red soil	PKM 1	Fruiting	27.26^{g} (31.47)
9.	Fol_9	Kodamandapatti	Clay loam	CO 2	Fruiting	22.45^{h} (28.28)
10.	Fol_{10}	Mathur	Sandy clay loam	PKM 1	Flowering	29.78^{f} (32.88)

* Mean of three publications
* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Table 2: Isolation and cultural characteristics of various isolates of *Fusarium oxysporum* f.sp. *lycopersici* (Fol) from major tomato growing areas of Krishnagiri district

Sl. No.	Isolates	Locality	Cultural characteristics	Mycelial growth (mm)	Conidial population/ml ($\times 10^6$)
1.	Fol1	Hamumantheertham	Moderate Aerial mycelium cottony white to pink colour mycelium	86.76^{bc}	1.9^{e}
2.	Fol2	Irumathur	Aerial with white mycelium	87.56^{ab}	2.1^{d}
3.	Fol3	Uthangarai	Profuse fluffy cottony growth with white to pink mycelium	90.00^{a}	2.7^{a}
4.	Fol4	Thippampatti	Moderate aerial mycelium with white to pink mycelium	89.23^{b}	2.5^{b}
5.	Fol5	Puthoor	Moderate fluffy cottony growth with white mycelium	79.87^{f}	0.8^{i}
6.	Fol6	Arasur	Moderate aerial mycelium with slightly pink mycelium	74.35^{g}	0.5^{j}
7.	Fol7	Kollanaikanoor	Moderate fluffy cottony growth, white to pale pink colour mycelium	87.20^{c}	2.2^{c}
8.	Fol8	Mittapalli	Thin flat with slight pink colour mycelium	85.26^{cd}	1.6^{g}
9.	Fol9	Kodamandapatti	Fluffy white to pink colour mycelium	83.12^{e}	1.3^{h}
10.	Fol10	Mathur	Whitish fluffy growth, slightly pink colour	86.47^{d}	1.8^{f}

* Mean of three publications
* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

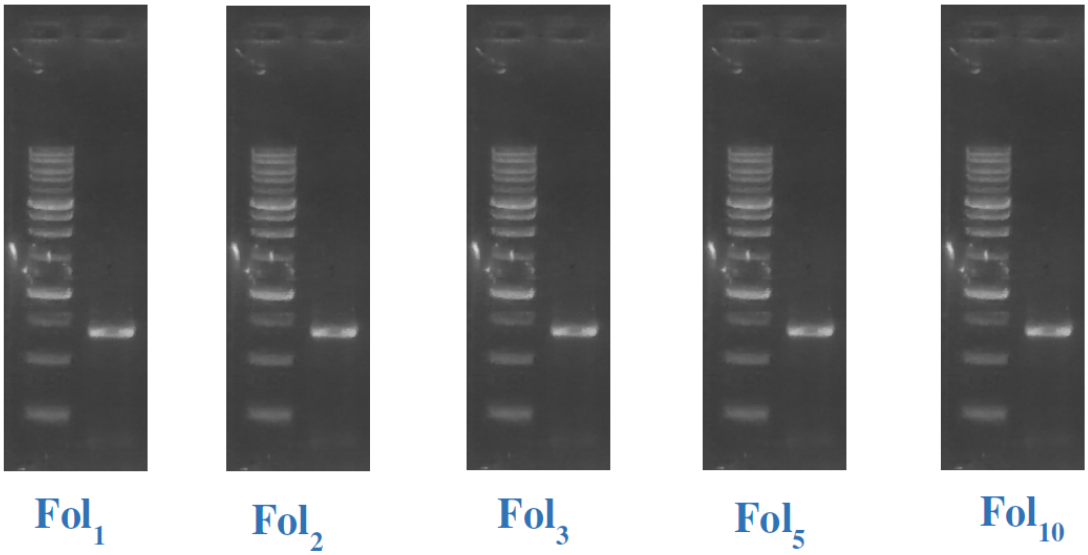

Plate 1 : PCR Amplicons loaded on 2% Agarose Gel

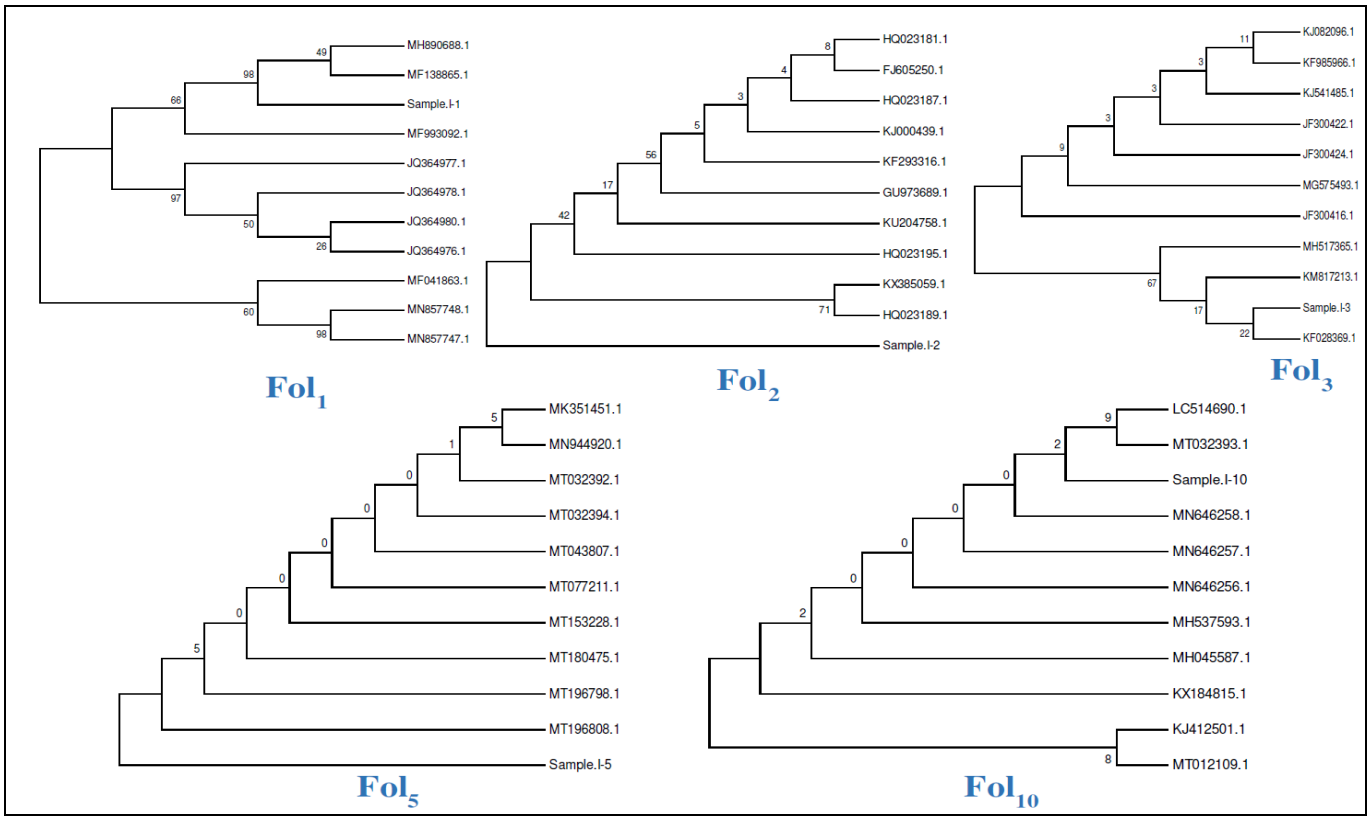

Plate 2 : Phylogenetic tree for different isolates

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