Morphological and Molecular Characterization of *Fusarium* spp. Infecting Tomato in Tamil Nadu

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Abstract

Tomato (Solanum lycopersicum L.), also referred to as the "poor man's apple," is grown all over the world and used in both table cuisine and processed foods. Tomato vascular wilt is primarily caused by Fusarium oxysporum f. sp. lycopersici (FOL), an important contributing pathogen. The earliest signs of the disease progressively appear in the lower leaves, followed by plant withering. According to reports, FOL invades tomato plants, colonises, and turns the vascular tissue dark brown. This discoloration extends to the plant's apex and causes the plants to wilt, collapse, and die. In the present study, twenty one isolates of Fusarium spp. were isolated from different districts of Tamil Nadu. Successful testing of the isolated fungus revelead the pathogenic nature of Fusarium under glass house condition. Characterization of isolates based on the morphological studies revealed they all belong to the genus Fusarium. Molecular analysis based on sequencing of ITS rDNA confirmed that they belonged to Fusarium oxysporum f.sp. lycopersici (Fol). The evolutionary lineages of the pathogen were revealed by phylogenetic analysis using ITS sequences.

Key words: Tomato, Wilt, *Fusarium*, Characterization, ITS

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the world's mostly grown vegetable crops, producing fresh fruits as well as a variety of processed goods [8]. Tomato crops cover an area of approximately 865.0 ('000 hectares) in India, with a total production of 21,056.0 ('000 MT) and an average productivity of 24.3 mt/hectare [3]. The key yield-limiting factors for its cultivation are biotic factors, and one of the most significant and deadly diseases worldwide is *Fusarium* wilt produced by the soil-borne fungus *Fusarium oxysporum* f.sp. *lycopersici* (Fol) [9]. Under ideal circumstances, Fusarial wilt is reported to cause a 60–70 per cent yield reduction in tomatoes [11].

The pathogen persists in soil and contaminated plant debris as mycelim and produces chlamydospores under cooler temperate regions. They enter the tomato plant by penetrating the root tips, wounds from roots or through lateral roots [4].

The pathogen identification methodology employs morphological analyses and serological tests. However, those methods lack sensitivity and specificity and necessitate skilled labour [1]. To address this, molecular techniques like polymerase chain reaction (PCR) must be used to quickly and correctly detect the pathogens. The internal transcriber spacer (ITS) region of the fungal genome must be amplified and sequenced for molecular identification of fungi. Since, populations of the same species have been found to highly variable from one another [5].

The ITS sequences underwent additional phylogenetic analysis to reveal the evolutionary relationships between the strains [8]. In the current investigation, twenty one *Fusarium* spp. isolates from different tomato growing habitats in Tamil Nadu were identified. The isolates were further characterized using morphological and molecular methods under *in-vitro* conditions. Phylogenetic tree has been constructed to investigate the evolutionary relationships between the isolates identified across Tamil Nadu.

2. Materials and methods

2.1. Survey and sampling

Five most important tomato-growing regions of Tamil Nadu were covered by a survey for soil and root sampling in 2021. A total of twenty one isolates of *Fusarium* spp. were obtained from Coimbatore (eight), Erode (six), Dharmapuri (two), Salem (three) and Thirupur (two) districts of Tamil Nadu. All the samples collected were stored in plastic bags and kept at 4°C for further studies.

2.2. Isolation of the fungi

Potato dextrose agar (PDA) medium was used to isolate the fungus. For the isolation of fungus from root sample, the roots were washed under running tap water to remove soil and other debris, chopped into small pieces of about 2-3 cm and rinsed once in 1 per cent sodium hypochlorite solution for 45 seconds followed by three time continuous washing in

sterile distilled water for 30 seconds. The sterilized tissues were then blot dried using sterilized tissue paper and placed in 90 mm petri dishes containing PDA medium and maintained at 27°C until the maximum growth. Soil samples were cleaned to remove stones and debris. Serial dilution of the soil samples were done to isolate the fungus associated with the samples.

2.3. Pathogenicity assay

Among the twenty one isolates, fast growing fungal species (FOL 1) with excessive conidial production was selected for pathogenicity assay. The mass production of fungal inoculum was achieved using sand: maize (ground) medium (9:1 ratio) in an autoclavable polybags. About ten discs from the edge of actively growing four day old fungal culture were inoculated in each bag containing 250 g of sterilized sand: maize (ground) medium. The bags were maintained at 28 ± 2°C for about 15 days for abundant spore production. After incubation, spores were dislodged by pouring 150 ml of water and the spore concentration was adjusted to 6.5×10^5. Twenty five day old seedlings of tomato variety PKM 1 was transferred to the medium sized pots containing red soil: FYM: cow dung manure @ 1:1:1 w/w/w consecutively autoclaved for two days at 121°C @ 15 psi for about 2 hours. The spore suspension was then inoculated in each pot (150 ml/pot) at 15 DAT after disturbing the root portion with sterile scissors for artificially improving the fungus accession to plants. The plants were continuously monitored for symptom expression without much disturbance by maintaining under glass house condition (Rockefellar, Tamil Nadu Agricultural University, Coimbatore). From the infected root portion, the fungus was again reisolated using PDA medium.

2.4. Phenotypic characterization of the fungi

All the isolated samples (twenty one) were identified as *Fusarium* spp. based on their colony morphology, characteristic macro, micro conidia and chlamydospore morphology and conidial measurements. Conidial measurements were carried out using phase contrast microscope (Labomed), at 400 X magnification.

2.5. Fungal Genomic DNA isolation

The total DNA was isolated from fungal mycelial mats using modified CTAB approach [6]. About 100 mg of dried mycelial mats were homogenized with sterilized mortar and pestle. The contents were vortexed for 2 min. and incubated for 30 min. at 65°C. After the incubation, 750 µl of combined chloroform and isoamyl alcohol (24:1 v/v) were added and centrifuged for 10 min. at 10,000 rpm. In the new microfuge tube, the top aqueous phase was transferred and re-extracted with a 24:1 ratio of choloroform and isoamyl alcohol, followed by centrifugation at 10,000 rpm for 10 min. To the upper aqueous (300 µl) phase, 0.5 volumes of 5M NaCl and 2 volume of ice cold isopropanol were added and incubated overnight at -20°C. After centrifugation at 13,000 rpm for 10 minutes at 4°C, the DNA pellet

was air dried and dissolved in 50 µl of Tris-EDTA buffer and kept at -70°C. The concentrations of the genomic DNA of samples were measured using a Nanodrop ND-3300 Fluorospectrometer and gel documentation (NanoDrop products, Thermo Scientific, Wilmington, DE, USA).

2.6. Molecular characterization of the fungi

The Internal transcriber space (ITS) 1 and 4 regions of target fungi were amplified using forward and reverse primers *viz.*, ITS 1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') [12]. A 20 µl mixture containing 50 ng of total DNA, 10 µl of TaKaRa master mix (2X concentration) and 20 pmol of forward and reverse primers was used for the PCR reaction. The reaction took place in a thermocycler (Eppendorf master cycle). The PCR program for amplification of the ITS region included a 5 min. initial denaturation step at 95°C, followed by 35 cycles of 1 min. denaturation at 94°C, 30 seconds annealing at 60°C, and 1 min. extension at 72°C and a 10 min. of final extension at 72°C. Negative control was maintained using sterile distilled water. Amplified products were separated by 1.2 per cent agarose containing ethidium bromide and electrophoresis at 80 V for 1 h and documented in a gel documentation unit (Alpha Imager EC (USA)).

2.7. Sequencing and Phylogenetic analysis

The amplified products were sequenced using Sanger's dideoxy sequencing method and deposited in NCBI GenBank Database. The ITS sequences were edited and aligned using the program Molecular Evolutionary Genetic Analysis software, ver. 7.0 (MEGA7.0). The phylogenetic tree was constructed using the ITS sequences in order to study the genetic relationship between the identified *Fusarium* species. In the present study, evolutionary history was inferred using Neighbor- Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The study isolate F1 was compared with the ITS sequences of other *Fusarium* species available in GenBank database.

2.8. Statistical analysis

The data were statistically analysed using the SPSS version 16.0 (Statistical Package for the Social Sciences) developed by Norman. H. Nie, Dale H. Bent, and C. Hadlai Hull in 1968. The data were subjected to analysis of variance (ANOVA) at two significant levels ($P<0.05$ and <0.01) and means were compared by Duncan's Multiple Range Test (DMRT).

3. Results

3.1. Survey and sampling

A roving survey was conducted to assess the vascular wilt disease incidence in major tomato growing areas of Tamil Nadu. The results revealed the significant disease occurrence in all the districts surveyed. The characteristic symptoms involving yellowing of older leaves

followed by drooping of lower leaves with vascular discoloration in the root region was prominent on all infected plant parts. The wilt incidence was ranged from 15.4 to 48.5 per cent in the different villages. The maximum wilt incidence of about 48.5 per cent was recorded at Thondamuthur village of Coimbatore district, followed by Sennanur village of Coimbatore district with 44.4 per cent incidence during fruiting stage of the crop. The minimum of 15.4 per cent of disease incidence was recorded at Arasur village of Erode district (Table 1).

Table 1: List of *Fusarium* spp. Isolated From Different Places of Tamil Nadu

S. No.	District	Isolate code	Location	Tomato variety	Stage of the crop	% Disease incidence
1.	Coimbatore	F1	Thondamuthur	Sivam	Fruiting stage	48.5^{a} (44.4)
2.	Coimbatore	F2	Boluvampatti	Sahar	Fruiting stage	42.6^{abc} (40.74)
3.	Coimbatore	F3	Sattakalputhur	SS 106	Fruiting stage	38.6^{bcde} (38.41)
4.	Coimbatore	F4	Mathukkarai	Aishwarya	Fruiting stage	35.3^{def} (36.45)
5.	Coimbatore	F5	Valukkuparai	PKM 1	Flowering	29.4^{fgh} (32.83)
6.	Coimbatore	F6	Jalathur	Samporna	Flowering	16.9^{l} (24.27)
7.	Coimbatore	F7	Sennanur	Sivam	Fruiting stage	44.4^{ab} (41.78)
8.	Coimbatore	F8	TNAU	PKM 1	Fruit ripening	16.2^{l} (23.73)
9.	Erode	F9	Thoppampalayam	Sivam	Flowering	40.8^{bcd} (39.70)
10.	Erode	F10	Sathyamangalam	AS 818	Fruiting stage	37.3^{cde} (37.64)
11.	Erode	F11	Gobichettipalayam	AYAAN (TO 7042)	Flowering	17.5^{l} (24.73)
12.	Erode	F12	Arasur	Sivam	Fruit ripening	15.4^{l} (23.11)
13.	Erode	F13	Erangattur	AYAAN (TO 7042)	Fruit ripening	33.9^{efg} (35.61)

14.	Erode	F14	Bhavani	Sivam	Fruit ripening	18.7 ^{kl} (25.62)
15.	Dharmapuri	F15	Kasiampatti	Aishwarya	Flowering	30.2^{fgh} (33.34)
16.	Dharmapuri	F16	Pudur	Sivam	Flowering	25.6^{hij} (30.40)
17.	Salem	F17	Attayampatti	PKM 1	Flowering	20.3^{jkl} (26.78)
18.	Salem	F18	Tharamangalam	Sahar	Flowering	21.6^{ijkl} (27.69)
19.	Salem	F19	Edappadi	Sivam	Fruiting stage	27.2^{ghi} (31.44)
20.	Thirupur	F20	Udumalapet	WS 119	Fruiting stage	24.9^{hijk} (29.93)
21.	Thirupur	F21	Moolanur	AS 818	Fruit ripening	22.1^{ijkl} (28.04)

*Values are mean of three replicates

Figures in parentheses are arcsine transformed values

Means in a column followed by same superscript letters are not significantly different according to DMRT test at $P \leq 0.05$.

3.2. Isolation of the pathogen

The pathogen was isolated from soil and root tissues collected from infected tomato plants using PDA medium by half plate method. After the incubation period, the pathogen produced dense, creamy white to pink fluffy mycelial growth. The pure cultures of the isolates were obtained using single hyphal tip method and refrigerated at 4°C for further characterization.

3.3. Pathogenicity assay

The symptoms of vascular wilt were observed on 15 days after inoculation in tomato variety PKM 1. The significant wilt symptoms *viz.,* yellowing and wilting of lower leaves and young stems, followed by defoliation and browning of vascular tissues and death of entire plant were observed as the disease becomes severed. The reisolated fungus from infected root portion produced same phenotypic characters as that of F1 isolate used for pathogenicity purpose.

3.4. Phenotypic characterization of *Fusarium* spp.

Microscopic observation revealed that all the twenty one isolates showed slight differences in their colony characters. The colony color varied from creamy white to pinkish

purple. All of them produced small oval shaped, hyaline single or bicelled micro conidia and sickle shaped, hyaline, multicelled (3-5 septa) macro conidia. Macro conidia length ranged from 26.3 – 37.5 µm and breadth was 3.3 – 4.5 µm (Table 2). Both terminal and intercalary chlamydospores were observed.

Table 2. Cultural and Morphological Characters of *Fusarium* spp.

Isolate code	Colony color	Mycelial growth pattern	Growth type	Length of conidia (µm)*	Breadth of conidia (µm)*
F1	Light purple	Adherent growth	Fast	37.51	4.54
F2	Milky white	Adherent growth	Fast	35.90	4.23
F3	Milky white	Fluffy growth	Fast	34.32	4.15
F4	Creamy white	Fluffy growth	Fast	33.42	4.02
F5	Pale yellow	Adherent growth	Medium	30.02	4.11
F6	Creamy white	Fluffy growth	Medium	27.06	3.41
F7	Pinkish purple	Fluffy growth	Fast	36.03	4.42
F8	Creamy white	Fluffy growth	Fast	26.38	3.4
F9	Milky white	Adherent growth	Fast	35.11	4.17
F10	Pinkish white	Fluffy growth	Fast	34.30	4.21
F11	Pale white	Adherent growth	Medium	27.32	3.48
F12	Light yellow	Fluffy growth	Slow	26.34	3.31
F13	Creamy white	Fluffy growth	Fast	32.10	4.11
F14	Milky white	Adherent growth	Medium	27.72	3.51
F15	Milky white	Fluffy growth	Fast	30.17	4.11
F16	Creamy white	Adherent growth	Medium	29.13	3.68
F17	Pale white	Adherent growth	Slow	28.05	3.45
F18	Creamy white	Fluffy growth	Slow	28.52	3.59
F19	Light pink	Adherent growth	Medium	29.43	3.72
F20	Creamy white	Fluffy growth	Slow	28.90	3.65
F21	Milky white	Fluffy growth	Slow	28.71	3.62

*Values are mean of five replicates

3.5. Genetic characterization of *Fusarium* spp.

All the twenty one isolates with positive control taken for PCR amplification yielded an amplicon size of ~ 560 bp except the negative control (sterile distilled water) which showed no amplification under ITS 1/ ITS 4 primer set. Based on the obtained results, all the isolates were tentatively confirmed as *Fusarium* spp. (Figure 1a & 1b).

Figure 1: ITS Amplification of *Fusarium* spp.

Figure1a.

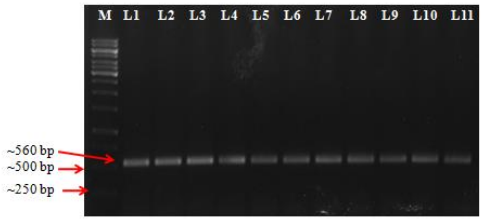


Figure 1b.

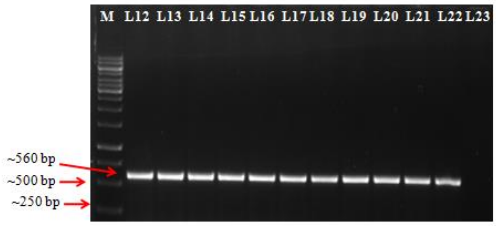
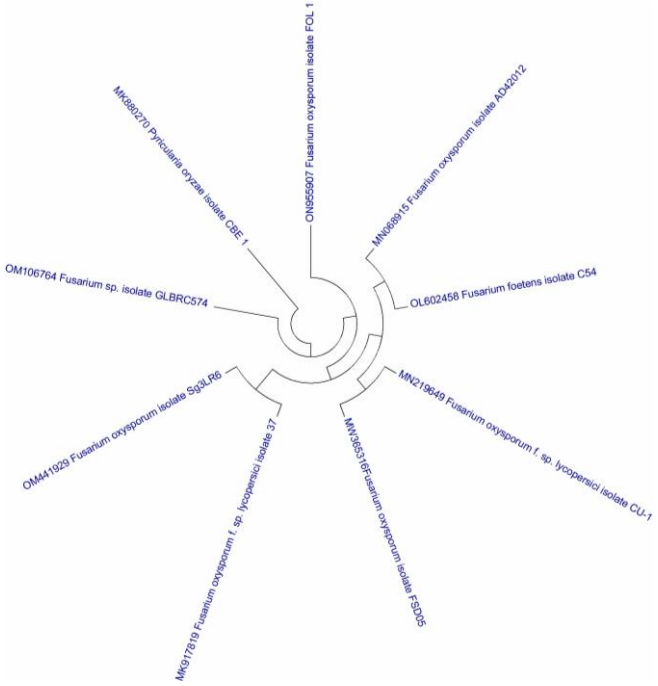


Fig 1a & 1b: M – 1 Kb DNA Marker, L1- F 1, L2 – F 2,L3 – F 3, L4 – F 4, L5 – F 5, L6 – F 6, L7 – F 7, L8 – F 8, L9 – F 9, L 10 – F 10, L11- F 11, L12 – F 12, L13 – F 13, L14 – F 14, L15- F 15, L16 – F 16, L17 – F 17, L18 – F 18, L19 - F 19, L20 – F 20, L 21 – F 21, L 22 – Positive control, L23 – Negative control (Sterile distilled water)

3.6. Sequencing and Phylogenetic analysis

The sequencing results of F1 isolate revealed 99.80 per cent identity with *Fusarium oxysporum* isolate FSD05 (Accession no. MW365316) followed by 99.60 per cent identity with *Fusarium oxysporum* f. sp. *lycopersici* (Accession no. MN219649) isolate retrieved from NCBI database. The F1 isolate was grouped along with various other isolates of *Fusarium* well separated from other isolates retrived from NCBI database (Figure 2). The resulted sequences of all twenty one isolates were submitted in GenBank database and accession numbers were obtained.

Figure 2: Phylogenetic Tree of Pathogenic *Fusarium* spp.



Tree was constructed using Neighbor joining Tree method in mega 7.0 with 1000 boot strap replications.

4. Discussion

The *Fusarium* genus, which causes the vascular wilt, is one of the most prevalent issues in tomato production. In the present study, we have identified and characterized different species of *Fusarium* spp. that place a major role in reducing the tomato yield by causing vascular wilt disease. In the main tomato-growing regions of Tamil Nadu, a field survey clearly shows that *Fusarium* wilt is quite prevalent and omnipresent. The *Fusarium* wilt pathogen *Fusarium oxysporum* was frequently found in soil samples collected from the infected fields as well as in infected plants. The prevalence of *Fusarium* wilt suggests that the disease is a persistent issue, and all widely grown cultivars appeared to be susceptible to it. *FOL* 1 isolate displayed typical wilt signs after being introduced into the susceptible host, proving their pathogenic character. Our results were similar to the work done in which the prevalence of several isolates of *Fusarium* infecting Tomato across India were reported and that pathogenic strains of *FOL* were successful in infecting the susceptible host with varying degrees of infection based on their virulence[7].

Fusarium isolates from various ecosystems of Tamil Nadu differ greatly in terms of culture and morphology, as shown by a comparison of those isolated from infected plants or rhizosphere soils. The fungal growth, colony traits, pigmentation, and sporulation patterns varies hugely. Similarly, morphological differences between the *FOL* strains isolated from vascular wilt infected tomato plants were described [8].

The absolute management of wilt diseases can be achieved, after precise identification and genetic characterization of associated pathogen. With the least amount of skill, molecular markers will enable reliable, quick, and efficient detection of particular organism. Our study

clearly indicated the difference between tested isolates upon genetic level with the application of PCR based detection. Similarly, successful differentiation among the *FOL* isolates based on PCR detection assay was shown [2]. They even succeeded in identifying races of *FOL* with specifically designed primers.

In *Fusarium* systematics, the phylogenetic species concept has just recently been used, but it has the potential to help with taxonomic issues. In our present study, phylogenetic tree has been constructed for F1 isolate using Mega 7.0 software. Similarly, constructed phylogenetic tree using Mega 5.0 software to assess the relationships between the genetic traits of the identified *Fusarium* species were constructed [10].

In conclusion, the present study results stated that, *Fusarium oxysporum* f.sp. *lycopersici*. might be a significant agent contributing to tomato wilt incidence in Tamil Nadu. The morphological and molecular characterization of *Fusarium* spp. revealed major in sights in understanding the nature and occurrence of the pathogen which is of utmost importance before going for management strategies.

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Conflict of interest

Authors have no conflict of interest to disclose

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