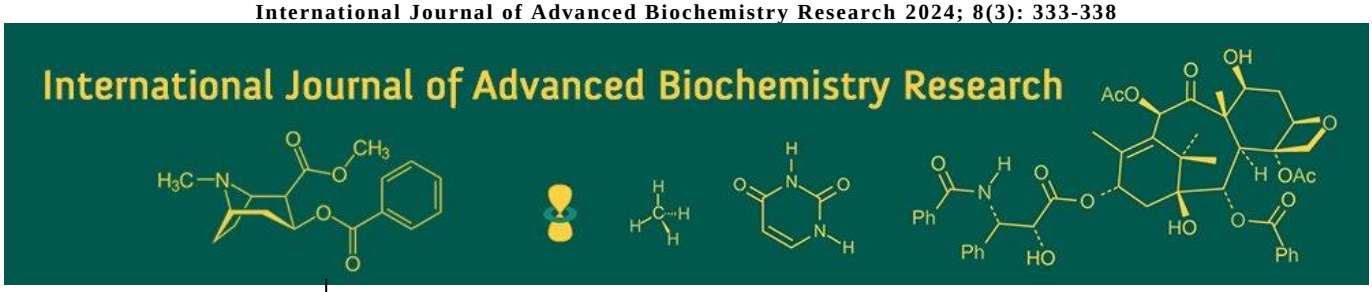

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; 8(3): 333-338
www.biochemjournal.com
Received: 15-01-2024
Accepted: 20-02-2024

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Molecular and morphological characterization of *Fusarium oxysporum* f. sp. *lycopersici* causing wilt disease in tomato (*Solanum lycopersicum*) in Karnataka

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DOI: https://doi.org/10.33545/26174693.2024.v8.i3d.738

Abstract
Tomato (*Solanum lycopersicum* L.) is the one of the most important vegetable crop globally, but its production and quality are known to be largely affected by many pests and diseases. One of the major hindrances to tomato production is the fungal wilt disease caused by *Fusarium* spp. In the current study we isolated, visually examined and characterized the fungal pathogen both morphologically and at the molecular level by employing standard techniques for tissue isolation, microscopic observations and PCR methods. Under the microscope, morphological features like white to whitish pink mycelium with prominent septations, sickle shaped conidia with prominent septations and smooth walled spherical micro conidia were observed. To investigate deeper into their genetic makeup, the molecular characterization of pathogens was done by amplification, sequencing of Internal Transcribed Spacer and phylogenetic analysis. This allowed us to gain insights into the genetic identity of this pathogen. Furthermore, phylogenetic analysis shed light on the evolutionary relationships within the group of fungi we studied. This comprehensive approach provides a deeper understanding of *Fusarium* spp. in the context of tomato cultivation and offers valuable knowledge for disease management and sustainable tomato production.

Keywords: Internal transcribed spacer, phylogeny, wilt disease and tomato

1. Introduction
Tomato (*Lycopersicon esculentum* Mill.) is one of the world's most widely cultivated vegetable crop. Its nutritional powerhouse and economic pillar silently weaving its way into cultures and cuisines around the world. India is one of the leading countries in tomato production growing in an area of 8.42 lakh ha with production of 2069.43 lakh tonnes and productivity of 24.60 t ha^{-1}. The major tomato growing states in India are Andhra Pradesh, Karnataka, Maharashtra and Uttar Pradesh, contributing significantly to the country's total tomato production (Singh, 2023) [20]. Tomato crop is very often affected by several diseases incited by pathogens such as fungi (wilt, blights, anthracnose and spots), bacteria (wilt and canker), viruses (leaf curl and tomato spotted wilt) and nematodes. Among all the fungal diseases that infect tomato, wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.), Snyder and Hans, is one of the most serious and destructive diseases across the world (Sheu and Wang, 2006) [19]. It affects greenhouse and field grown tomato in warm vegetable production areas. The characteristic wilt symptoms appear similar to severe water stress, as the pathogen invades the root epidermis and extends into the vascular tissue and colonizes the xylem vessels by producing mycelium and conidia. Which results in yellowing of leaves and wilting of plants with minimal or complete loss of crop yield. There may be a 30 to 40 % yield loss due to this disease and may reach up to 80 % under severe infection (Kirankumar *et al*., 2010; Kapoor, 1988; Pandey and Gupta, 2013; Asha *et al*., 2013) [12, 11, 18, 2].
Fusarium spp. is ubiquitous soil-borne pathogen, which infects wide range of horticultural and food crops. Which cause destructive vascular wilts, rots, and damping-off diseases (Bodah, 2017) [3].

In addition to the losses caused before or during harvest of crop, Some *Fusarium* spp. are also capable of producing mycotoxins in food and agricultural commodities, which makes them inedible (Chandra Nayaka *et al*., 2008; Chandra Nayaka *et al*., 2009; Mudili *et al.*, 2014) [4, 5]. This fungus has numerous morphologically indistinguishable plant pathogenic strains associated with a wide range of vegetable and other crops with high host specificity (Michielse and Rep, 2009) [14]. *Fusarium* spp. is reproducing asexually and its species includes both pathogens and a diverse range of nonpathogens. Pathogenic and non-pathogenic strains of this species can be distinguished from each other with pathogenicity tests, but not with morphological analysis or sexual compatibility studies.

In the recent past, molecular characterization has become popular for identifying species and/or subspecies (Henson and French, 1993) [9] in fungi. Further, for developing effective disease management strategies, the better understanding of biology of fungus is much essential. The present study was carried out to understand the symptomatology, morphology, pathogenicity and molecular characterization of the *Fusarium* spp. isolate causing wilt disease in tomato.

2. Material and Methods

2.1 Sample collection, isolation and identification of fungus.

Tomato plants affected with wilt pathogen exhibiting diagnostic symptoms such as yellowing of lower leaves, drooping, browning and partial yellowing of one side of the plants grown in the farmers field near to Mallenahalli (13.261365, 77.878704), Doddaballapur, Karnataka, India were collected (Fig. 1). The root portion showing the vascular discoloration was cut into small pieces in the laminar wood. Further, infected bits were sterilized with a 1 % sodium hypochlorite solution for a minute and thoroughly rinsed with sterile distilled water to remove traces of sodium hypochlorite solution. Subsequently, the sterilized samples were transferred aseptically into sterilized Petri plates containing potato dextrose agar (PDA) to facilitate fungal growth, and incubated at 27±1 °C. After getting fungal growth, pure culture was obtained by hyphal tip technique. The pure culture was transferred onto PDA slants, incubated at 27 ± 1 °C for a week. The obtained pure cultures were stored at 4 °C for further studies.

2.2 Pathogenicity assay

The identification of forma specialis for the isolated fungi was carried out by the pathogenicity test. One-month-old cultures of the fusarium grown on sorghum grains were inoculated into the sterilized soil and mixed thoroughly to create uniform sick pots under glass house condition. For successful multiplication and establishment of the inoculated pathogen, sick pots were kept undisturbed for 10 days by maintaining optimum moisture. Further, as pathogen took more number of days for establishment, 100 ml of 10-day-old broth culture was added to the same pots on the day of transplantation of seedlings. Later, 20-day-old seedlings of Arka Vikas were transplanted into the sick pots. Seedlings transplanted in pots devoid of inoculum were kept as a control and soil moisture of every pot was maintained at 25 % of its moisture holding capacity by adding water on a weight basis throughout the experiment. Periodic observations were made to study the symptom development. Re-isolation of the pathogen was done after observing the visual symptoms in the infected plant parts and compared with the original cultures.

2.3 Morphological characterization

Morphological characteristics of isolated fungi were studied after 10 days of inoculation by visual observation of colony growth, colony colour, mycelium character (colour and texture) and mycelial growth pattern. Microscopic observation were done for hyphal character and spore morphologies (macroconidia, microconidia, phialides and chlamydospores) produced by the fungus.

2.4 Molecular characterization

2.4.1 DNA extraction and PCR amplification

Mycelial disc of isolated pure culture of *Fusarium* spp. was separately inoculated into the conical flask containing PDB (Potato Dextrose Broth) media under aseptic conditions and incubated for seven days at 27±1 ºC. Ten days old mycelial mat was harvested, air-dried and used for the isolation of genomic DNA by following Cetyl (hexadecyl) Trimethyl Ammonium Bromide (CTAB) method (Csaikl *et al.*, 1998) [6]. Extracted DNA was stored at -20°C for subsequent molecular analyses. Polymerase Chain Reaction (PCR) to amplify the Internal Transcribed Spacer (ITS) region of fungi was carried out using general primers, ITS1 Forward Sequence (5'-3') CTTGGTCATTTAGAGGAAGTAA) and ITS4 Reverse Sequence (5'-3') TCCTCCGCTTATTGATATGC. PCR amplification was carried out in 25 µL reaction volume consisting of template DNA (100 ng/ µL), Taq buffer (2.50 µL of 10X), $MgCl_2$ (1 µL of 2 mM), dNTPs mixture (2.50 µL of 1 mM), primer (1.50 µL of 5 pM), Taq DNA polymerase (1.50 µL) and sterile distilled water to make full volume of reaction mixture. PCR was performed using the Proflex PCR system (Carlsbad, California, United States). The amplification was carried out with initial denaturation of 94 °C for 4 minutes followed by 35 cycles of 94 °C for 60 seconds, 55 °C for 45 seconds, 72 °C for 90 seconds and the final extension step at 72 °C for 10 minutes. Following PCR amplification, the resulted products are analyzed by agarose gel (1 % w/w) electrophoresis to confirm their presence and size, often expected to be in the range of 600-800 base pairs for the ITS region. Gels were stained with ethidium bromide (10 µg/mL) and visualized in a UV transilluminator to confirm the amplification of targeted DNA regions. Further, the amplified DNA products were eluted from the gel by using Qiagen gel elution kit (#Cat No.: 28706) and sequenced in both directions at Eurofins Genomics Pvt. Ltd., Bengaluru, India.

2.4.2 Sequencing and phylogenetic analysis

The full-length sequence of ITS region of *Fusarium* spp. isolate was queried in NCBI database using BLASTn tool to find similar sequences available in the database (Altschul, 1990) [1]. The sequences of different *Fusarium* spp. infecting various crops showing more homology with ITS region of the current isolate (Table. 1) were retrieved from the NCBI database and aligned using BioEdit (Hall, 1999) [7] and ClustalW (Thompson *et al*., 1994) [21] programs. To know the evolutionary relationship of the test sequences, a phylogenic analysis was performed by comparison with the sequences retrieved from the NCBI GenBank database (Table 1) using Neighbor-joining method MEGA X software with 1000 bootstrapped replications (Kirankumar *et al.*, 2016) [12].

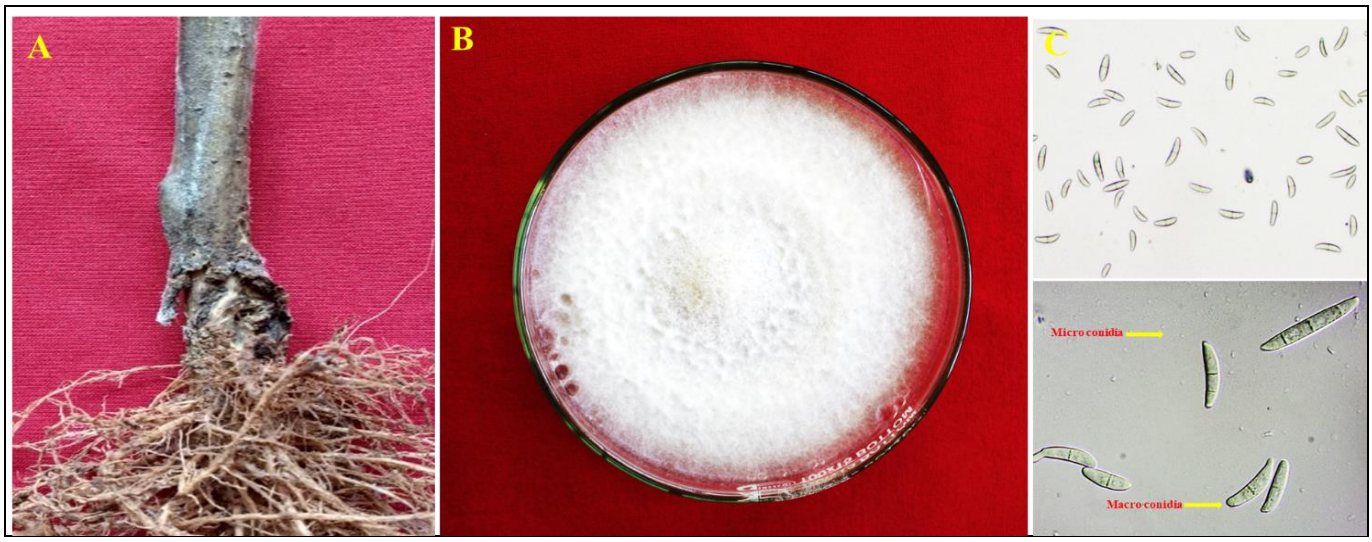

Fig 1: A. Wilt symptoms produced by *Fusarium* spp. in tomato B) Growth of *Fusarium* spp. on PDA C) macro and micro conidia produced by *Fusarium* spp. on PDA

3. Results and Discussion

3.1 Isolation of pathogen

Infected root and stem samples with typical wilt symptoms of *Fusarium* spp. were collected and inoculated onto Petri-plates containing PDA media with the help of sterile forceps and needles under aseptic conditions. The isolation procedure forms the foundational step in research endeavor, allowing for the subsequent morphological and molecular analyses that provide insights into the biology and behavior of *Fusarium* spp. and their role as fungal pathogens in tomato plants (Joshi *et al.*, 2013) [10].

3.2 Morphological characterization

The culture obtained from samples were having typical colony characters of *Fusarium* spp. described earlier with sparse white cottony mycelium growth with series of morphological transformations, later turning into cream white to whitish pink in colour with appearance of smooth and flat mycelium. Finally, the mycelium turned into whitish mat with pink colored bottom (Fig. 1).

Furthermore, a closer examination of culture revealed its ability to produce the branched conidiophores, which are emerging directly from the mycelium. Conidia borne on these conidiophores were single-celled, ellipsoidal in shape with distinct pigmentation. Further, small unicellular, non-septate, oval or round, straight to curved or slender microconidia arising from simple phialides measuring 2.5µm × 3µm and larger falcate or sickle-shaped multiple macroconidia measured 15-37.5µm × 2.5–4µm with three-septations was observed under the microscopic studies which were the typical morphological feature of the genus, *Fusarium (*Fig. 1). This comprehensive morphological analysis of the culture provides valuable insights into the life cycle and characteristics of the pathogen responsible for wilt disease. In our investigation, the morphology and conidial dimensions of the pathogen isolated from tomato plants showing typical features in conformity with the standard descriptions by Nelson (1983); Leslie and Summerell (2008) [16, 13] for *Fusarium* spp. The conidial dimensions reported in our study, in line with these earlier references, contributing to the validation of the pathogen's identity and provide essential supporting evidence for its classification as *Fusarium* spp. Such congruence with established standards and the corroboration by previous research reinforce the robustness of our observations and the reliability of our morphological characterizations. But many previous reports suggest that the *Fusarium* spp. isolated from the soil may have pathogenic and nonpathogenic isolates. Hence to prove the virulence capacity of the current isolated fungi, it was further evaluated by pathogenicity test.

3.3 Pathogenicity assay

Typical symptoms of wilt disease were first observed 15–20 days after inoculation. In the virulence test, variation in symptoms on aerial parts and within the stem tissues of tomato plants infected with *Fusarium* was observed. Initially, symptoms appeared as yellowing of the lower leaves and in later stages, drooping of the leaves was observed. In severe infection, the pith of the stem was turned to brown colour. In severely infected plants drying of lower leaves with loss of turgidity and drooped symptom was observed leading to the death of the tomato plants. There was no development above-mentioned symptoms observed in the untreated check plants maintained without inoculation of fungal spore suspension (Fig. 2). The symptoms observed in the glass house condition were in conformity with the initial symptoms observed during the sample collection under field condition. Which confirms that isolated pathogen was same and have virulence to cause the wilt disease in tomato plants. The culture reisolated and purified from these artificially inoculated plants showed identical characteristics to the original culture. Which confirmed the pathogenicity of the isolated fungus. The similar kind of infection process and symptoms were observed in the studies conducted by Nirmaladevi *et al.*, 2016 [17].

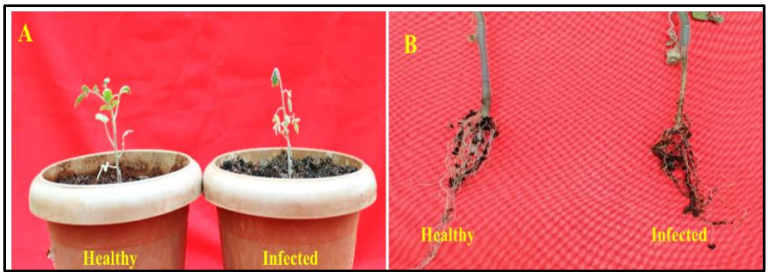

Fig 2: a) Pathogenicity test showing symptomatic expression of *Fusarium oxysporum* f. sp. *lycopersici* in infected tomato b) Wilted symptoms on the tomato plants.

3.4 Molecular characterization and phylogenetic analysis

The DNA isolated from the fungus isolated from infected stem portion of tomato plants collected from Mallenahalli (13.261365, 77.878704), Doddaballapur, Karnataka, India was subjected to PCR amplification of ITS region using universal primers ITS1/ITS4. The process resulted in amplification of ~650 bp fragment (Fig. 3). The ITS is the common conserved region which is used for the differentiation of the fungi at species level. Subsequently, the PCR product was sequenced and the nucleotide sequence of ITS region of *Fusarium* spp. isolate in the current study was compared with the representative nucleotide sequences of ITS region of selected fungal species from the NCBI, GenBank database.

The results revealed that the fungi isolated in the current study shared the maximum nucleotide identity of 97.8 % with *Fusarium oxysporum* f. sp. *lycopersici* (KC478622.1) isolate infecting tomato reported from India, The next closest identity was observed with *Fusarium oxysporum* f. sp. *lycopersici* (KC478622.1) infecting tomato reported from India. The lowest nucleotide identity was observed with *Fusarium decemcellulare* (MW851212.1) (Table 2).

Phylogenetic analysis findings provided further evidence by for close clustering of fungi isolated in the current study with *Fusarium oxysporum* f. sp. *lycopersici* (Fig. 4). For identifying the fungal pathogens infecting different crop plants the amplification and sequencing of ITS region followed by sequence analysis was routinely used by several research workers across the world (Hariharan and Prasannath, 2021) [8].

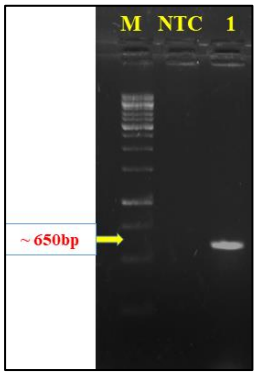

Fig 3: Ethidium bromide-stained agarose gel showing PCR amplicon of internal transcribed spacer (ITS) region M: 1kb ladder; NTC: Non template control; 1 and 2. *Fusarium sp.*

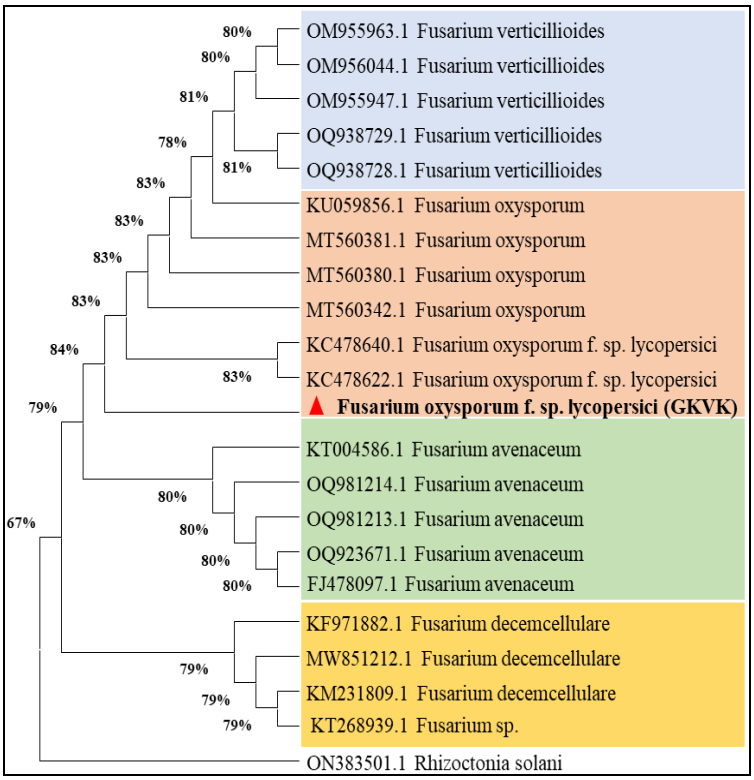

Fig 4: Phylogenetic tree constructed from nucleotide sequences of Internal Transcribed Spacer (ITS) region of *Fusarium oxysporum* f. sp. *lycopersici* isolate infecting tomato with sequences of related species retrieved from NCBI GenBank using Neighbor-joining method available in MEGA X.

Table 2: Per cent nucleotide sequence identities of ITS region of *Fusarium oxysporum* f. sp. *lycopersici* isolated in the current study with other selected *Fusarium* spp. sequences retrieved from NCBI GenBank.

Sl. No.	Accession No.	Isolate name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	KM231809.1	*Fusarium decemcellulare*	100.0																					
2	MW851212.1	*Fusarium decemcellulare*	97.3	100.0																				
3	FJ478097.1	*Fusarium avenaceum*	99.2	97.3	100.0																			
4	KF971882.1	*Fusarium decemcellulare*	98.4	96.6	98.4	100.0																		
5	KC478640.1	*Fusarium oxysporum* f. sp. *lycopersici*	75.2	73.6	74.9	75.2	100.0																	
6	KC478622.1	*Fusarium oxysporum* f. sp. *lycopersici*	75.2	73.6	74.9	75.2	100.0	100.0																
7	MT560381.1	*Fusarium oxysporum*	75.2	73.6	74.9	75.2	99.8	99.8	100.0															
8	MT560380.1	*Fusarium oxysporum*	75.2	73.6	74.9	75.2	99.8	99.8	100.0	100.0														
9	MT560342.1	*Fusarium oxysporum*	75.2	73.6	74.9	75.2	99.8	99.8	100.0	100.0	100.0													
10	KU059856.1	*Fusarium oxysporum*	75.0	73.5	74.7	75.0	99.6	99.6	99.8	99.8	99.8	100.0												
11	OM955963.1	*Fusarium verticilloids*	68.6	67.3	68.4	68.7	90.5	90.5	90.7	90.7	90.7	90.5	100.0											
12	OM955947.1	*Fusarium verticilloids*	68.8	67.4	68.5	68.8	90.7	90.7	90.9	90.9	90.9	90.7	99.8	100.0										
13	OM956044.1	*Fusarium verticilloids*	68.8	67.4	68.5	68.8	90.7	90.7	90.9	90.9	90.9	90.7	99.8	99.6	100.0									
14	OQ938729.1	*Fusarium verticilloids*	68.6	67.3	68.4	68.7	90.7	90.7	90.9	90.9	90.9	90.7	99.8	99.6	99.6	100.0								
15	OQ938728.1	*Fusarium verticilloids*	68.6	67.3	68.4	68.7	90.7	90.7	90.9	90.9	90.9	90.7	99.8	99.6	99.6	100.0	100.0							
16	KT004586.1	*Fusarium avenaceum*	73.7	72.2	73.3	74.0	81.9	81.9	82.1	82.1	82.1	81.9	84.5	84.6	84.6	84.5	84.5	100.0						
17	OQ981214.1	*Fusarium avenaceum*	73.7	72.2	73.3	74.0	81.9	81.9	82.1	82.1	82.1	81.9	84.5	84.6	84.6	84.5	84.5	100.0	100.0					
18	OQ981213.1	*Fusarium avenaceum*	73.7	72.2	73.3	74.0	81.9	81.9	82.1	82.1	82.1	81.9	84.5	84.6	84.6	84.5	84.5	100.0	100.0	100.0				
19	OQ923671.1	*Fusarium avenaceum*	73.7	72.2	73.3	74.0	81.9	81.9	82.1	82.1	82.1	81.9	84.5	84.6	84.6	84.5	84.5	100.0	100.0	100.0	100.0			
20	GKVK	*Fusarium oxysporum* f. sp. *lycopersici*	76.6	75.0	76.3	76.6	97.2	97.8	97.6	97.6	97.6	97.4	88.7	88.9	88.9	88.9	88.9	81.2	81.2	81.2	81.2	100.0		
21	KT268939.1	*Fusarium* spp.	73.7	72.2	73.3	74.0	81.9	81.9	82.1	82.1	82.1	81.9	84.5	84.6	84.6	84.5	84.5	100.0	100.0	100.0	100.0	81.2	100.0	
22	ON383501.1	*Rhizoctonia solani*	36.2	35.4	36.1	36.1	40.2	40.2	40.0	40.0	40.0	39.9	42.5	42.5	42.5	42.3	42.3	42.2	42.2	42.2	42.2	40.4	42.2	100.0

4. Conclusion

In conclusion, our research underscores the importance of integrating both morphological and molecular approaches in the study of plant diseases. The results of the current study clearly indicated the existence of pathogenic strain of the *Fusarium oxysporum* f. sp. *lycopersici* causes wilt diseases of tomato in major tomato growing area of Karnataka, India. The ability to accurately identify and characterize the pathogen responsible for wilt is crucial for the development of effective disease management strategies. These insights are invaluable for agricultural practices, contributing to the sustainability and productivity of tomato crops. The information generated further adds to the information of tomato infecting *Fusarium oxysporum* f. sp. *lycopersici* distribution in different geographical regions of India.

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