Cultural, Morphological, and Pathogenicity Variation in *Fusarium oxysporum* f. sp. *lycopersici* Causing Wilt of Tomato

D. Nirmaladevi[1] and C. Srinivas[2,*]

[1]*Department of Studies in Microbiology and Biotechnology, Jnanabarathi Campus, Bangalore University, Bangalore-560 056, India.*

[2]*Department of Studies in Microbiology and Biotechnology, Jnanabarathi Campus, Bangalore University, Bangalore-560 056, India.*

[*]*e-mail:* srinivasbub@gmail.com

Abstract

Fusarium oxysporum f. sp. *lycopersici* strains were isolated from wilted tomato plants and soil samples collected from different tomato fields in and around Karnataka. A total of 114 isolates named as (MB1-MB114) were subjected to cultural, morphological and pathogenicity studies. Significant variations existed among the isolates with respect to rate and type of growth, colony colour, mycelial growth pattern, sporulation, septation of the conidia, number and pattern of chlamydospore formation. The isolates were studied for their pathogenic variability by inoculating to five different tomato varieties by standard root dip method. The pathogenicity of the isolates could be categorized based on their virulence. The invasive growth of the isolates was assayed by injecting the conidial suspension of the isolates to tomato fruits.

Keywords: *Fusarium* wilt, cultural, morphological, pathogenicity test, virulence.

1. Introduction

Fusarium wilt caused by the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H. N. Hans is known as one of the most devastating diseases of tomato worldwide [1]. It is an important soil inhabiting fungi, and is known to be phylogenetically diverse [2]. Most strains assigned to this species are saprophytic or non-pathogenic. However, plant pathogenic strains *F. oxysporum* cause destructive vascular wilt diseases on a wide variety of crops, often limiting crop production [2, 3].

F. oxysporum isolates are very dynamic and exhibit high variation with respect to their cultural, morphological and pathogenic characters. As in other *Fusaria*, its identification is generally based on morphological criteria such as shape of micro and macroconidia, structure of the microconidiophores and the formation and disposition of chlamydospores [4]. In culture, *F. oxysporum* produces colorless to pale yellow mycelium that turns pink or purple with age. With the exception of grasses and most tree crops, few of the widely cultivated crops are not hosts to pathogenic form of *F. oxysporum* [5].

Individual pathogenic strains have a high degree of host specificity within *F. oxysporum;* it is generally known as a species complex which is assigned to intraspecific groups including formae *specials* (f. sp.) and other forms [6, 7]. The host specificity of isolates of *F. oxysporum* led Snyder and Hansen (1940) to subdivide the species into formae later called formae specials, based on the host or group of hosts, the pathogen is able to infect [8].

Isolates have been divided into more than 120 different formae speciales according to their host range [5]. A particular formae speciales is further divided into physiological races based on the characteristic pattern of virulence on differential host cultivars. A classical gene-for-gene relationship has been proposed to mediate the interaction between *F. oxysporum* races and host cultivars, based on the dominant monogenic resistance traits against known races. This has been confirmed with the cloning of the tomato resistance gene *l-2* that confers resistance to *F. oxysporum* f. sp. *lycopersici* race 2 [9].

As a soil inhabitant *F. oxysporum* can survive extended periods in the absence of the host, mainly in the form of thick walled chlamydospores. Indeed, once an area becomes infected with *F. oxysporum*, it usually remains so indefinitely [10]. The proximity of the host roots induces the dormant propagules of the pathogen to germinate and initiate infection. The ultrastructure of the infection process has been well documented by a series of studies based on the use of light and electron microscopy. After germination, infection hyphae adhere to the host roots and penetrate them directly. The mycelium then advances intercellularly through the root cortex until it reaches the xylem vessels and enters them through the pits. At this point, the fungus switches to a highly peculiar mode of infection, during which it remains exclusively within the xylem vessels, using them as avenues to readily colonize the host [11]. This is mainly accomplished by the production of microconidia, which are detached and carried upward in the sap stream. The microconidia eventually germinate and the mycelium penetrates the upper wall of the vessels producing more microconidia in the next vessel. The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging. Wilting is most likely caused by a combination of pathogen activities such as the accumulation of fungal mycelium and/or toxin production and host defense responses, including production of gels, gums and tyloses and vessel crushing by proliferation of adjacent parenchyma cells [12]. As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. Only when the infected plant is killed by the disease does the fungus invade the parenchymatous tissue and sporulate profusely on the plant surface. *F. oxysporum* thus occupies a highly specific ecological niche, shared by only a few other fungal plant pathogens such as *Verticillum dahlia* and *Ceratocystis ulmi* [10].

The aim of the present study was to isolate and study the morphological diversity and pathogenicity variability among *F. oxysporum* strains isolated from various agroclimatic regions.

2. Materials and methods:

2.1. Field Survey and Sample collection:

Extensive field survey was carried out in the major tomato growing areas of Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, and Orissa during Kharif 2008-09, 2009-2010. The samples were collected from 72 fields located in 10 districts of Karnataka (Bangalore Rural, Mysore, Mandya, Kolar, Chikkaballapur, Chikmagalur**,** Tumkur, Bellary, Shimoga, Belgaum), 2 Districts of Tamil Nadu (Vellore and Hosur), 2 Districts of Andhra Pradesh (Chittoor and Anantapur), Pune district of Maharashtra, Orissa (Bhubaneswar) and Kerala (Trivananthapuram). Tomato fields were observed, and in each field, five random microplots of 25 x 25m^2 area with at least 50 plants/microplot were examined for disease symptoms. The stem showing vascular discoloration (Fig. 1) along with rhizosphere soil were collected, brought to the laboratory, stored under proper conditions and were used for the isolation of the pathogen.

Fig.1. Brownish discolouration of vascular tissue due to *Fusarium* wilt.

2.2. Isolation:

For isolation of *F. oxysporum* from wilted tomato plant samples, root and stem tissues were washed under running tap water. The plant pieces taken from the lower hypocotyls and upper taproot were surface sterilized in 1% NaOCl solution for 1 to 2 min, rinsed twice in sterile distilled water and dried between sterile filter papers. Pieces of surface disinfected tissues were placed on Potato dextrose agar (PDA). The plates were incubated at 28 ± 2°C for 7-10 days [13]. Isolation from rhizosphere soil samples was done by dilution plate technique. Soil dilutions were plated on PDA and incubated at 28 ± 2°C for 5 days.

2.3. Identification of isolates:

Colonies exhibiting the taxonomic features of *F. oxysporum* were identified according to Nelson *et al*., [3]. Morphological identification was based on characteristics of the macroconidia, phialides, microconidia, chlamydospores and colony growth traits (Fig. 2, 3). The identity of the culture was further confirmed from National Fungal Culture Collection of India (NFCCI, Agharkar Research Institute, Pune). Pure cultures of all the isolates were stored on PDA [14].

Fig. 2. Pure culture of *F. oxysporum* f. sp. *lycopersici* on PDA

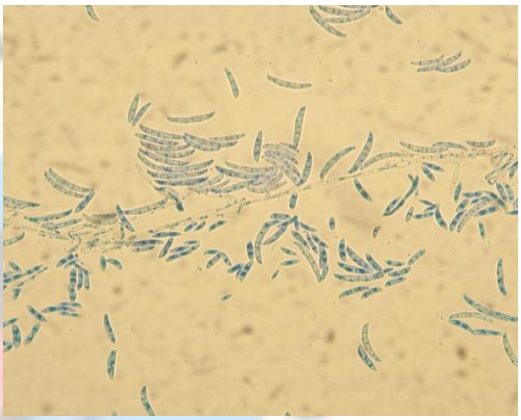

Fig.3. Microscopic view of Macro and Microconidia of *F. oxysporum* f. sp. *lycopersici*

2.4. Morphological and cultural diversity of isolates:

All the isolates were grown on PDA plates at 28 ± 2°C for 9 days. Observation was made on colony colour, mycelial growth pattern, radial growth, sporulation, size of the macroconidia, microconidia and chlamydospores. Based on the above morphological features, the isolates were characterized into different groups [15].

2.5. Pathogenicity testing of isolates:

To determine the formae specials**,** virulence analysis of the isolates was carried out on a set of five tomato cultivars.

2.5.1. Tomato cultivars used: The Seeds of five varieties of tomato, susceptible to Fusarium wilt, were obtained from IIHR (Indian Institute of Horticultural Research), Hesaragatta, Bangalore. 1) Arka Abha 2) Arka Alok 3) Arka Meghali 4) Arka Saurabha 5) Arka Vikas

2.5.2. Raising tomato seedlings: The Seeds were sown in protrays filled with coconut pith compost and watered regularly. 20 days old healthy seedlings were selected and used for further pathogenicity assay.

2.5.3. Standardization of inoculum development: One week old *F. oxysporum* cultures, grown on PDA plates, were flooded with about 10ml of sterile distilled water and the conidia were dislodged with a cell spreader, filtered through cheesecloth and counted with a hemocytometer. The concentration was adjusted to 10^6 conidia per ml. For obtaining large amounts of the spore suspension, the fungus was grown on maize (solid substrate). 50g of maize was added to 250ml conical flasks to which 60% of distilled water was added, autoclaved, inoculated with 1ml spore suspension of *F. oxysporum* and incubated for 7 days. Sterile distilled water was added to these cultures and the spore suspension was adjusted to 10^6 conidia per ml. The spore suspension was used for further pathogenicity assay. Solid substrate served as a better medium for obtaining large amount of spore suspension.

2.5.4. Pathogenicity assay on tomato seedlings: Twenty day old seedlings were inoculated by standard root dip method. Conidia of all the isolates were recovered from one week old cultures. Seedlings were removed from the protrays, shaken to remove the adhering particles and washed carefully under tap water. The roots were trimmed with a sterile scissor and were submerged in the conidial suspension for 30 mins. The inoculated seedlings were transplanted to minipots, 15cm diameter, surface sterilized with 0.1% mercuric chloride [16], containing soil and sand 1:1 ratio and incubated in greenhouse where day and night temperatures varied between 25-30°C with 12h light and 12h dark. The severity of the disease was assessed from 2 weeks of inoculation up to 45 days. Symptoms were recorded according to a scale ranging from 1 to 5 [17].

1- No symptoms.
2- Slight chlorosis, wilting or stunting of the plant.
3- Moderate chlorosis, wilting or stunting of the plant.
4- Severe chlorosis, wilting or stunting of the plant.
5- Death of the plant.

The discolouration of the vascular tissue was confirmed by slitting the stem.

2.5.5. Assay of pathogenicity on tomato fruits: To assay invasive growth of *F. oxysporum* strains, tomato fruits were washed under running tap water and surface sterilized by immersion for five minutes in 70% ethanol. After air drying, the epidermis was punctured with a sterile pipette tip and 10µl of the conidial suspension ($5x10^6ml^{-1}$) was injected into the fruit tissue. Fruits injected with sterile distilled water served as control. Inoculated fruits were incubated at 28°C under conditions of 100% humidity. Colonization of the fruit tissue and formation of a mycelial mat on the fruit surface were determined. All pathogenicity assays were performed at least thrice [18].

3. Results and discussion:

3.1. Field survey and sample collection:

Based on the field survey in major tomato growing areas of Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, Orissa and Kerala, it was evident that the incidence of Fuasrium wilt was highly prevalent. In the rating scale highest disease incidence in tomato was recorded in Mysore, Bangalore Rural, Chikkaballapur, Chikmagalur and Kolar districts of Karnataka. The disease was also prevalent in the fields of Chittoor, Anantapur, Vellore, Hosur and fewer incidences was recorded in Pune, Bhubaneswar and Trivananthapuram. A total of 114 strains of *F. oxysporum* were isolated from the wilted tomato plant and soil samples, identified and stored as pure cultures on PDA at 4°C for further studies.

Tomatoes are of great economic importance. Many seed and soil borne diseases are responsible for causing 20 to 30% loss every year. Fusarium wilt disease causes considerable damage to the tomato crop. Severe losses due to the disease have been observed in Assam, Andra Pradesh, Punjab, Haryana and Delhi [19, 20]. Fusarium wilt has been the most devastating disease resulting in 10 to 50% crop losses around the world [21].

3.2. Morphological and cultural variability among the isolates:

All the isolates grown on PDA plates at 28 ± 2°C, were studied for their cultural and morphological characters. Observations on colony colour, mycelial growth pattern, radial growth and sporulation were recorded after 9 days of incubation. The colour and

pigmentation of the isolates on PDA medium varied between white, creamish white to cream, light pink to pink and light purple to violet. On the basis of the mycelial growth pattern, the isolates could be categorized into two groups i.e., fluffy growth and adherent smooth growth. Most of the isolates showed fluffy growth while other isolates revealed adherent growth on the medium. Based on the colony diameter, the isolates were categorized into 3 groups viz., Fast growing (more than 70mm), moderate growing (50-70mm) and slow growing (less than 50mm). Out of the 114 isolates, 85 isolates were rated as fast growing, 19 as moderate growing and 10 isolates were slow growing (Table 1).

Table 1: Grouping of *F. oxysporum* isolates based on radial growth of the colony

Growth of the Isolates MB1- MB114		
Fast growing (Colony diameter more than 70mm)	**Moderate growing (Colony diameter 50-70mm)**	**Slow growing (Colony diameter less than 50mm)**
1,2,3,4,5,6,7,8,9,10,11,12,13,14,15, 18,21,22,23,24,25,26,27,28,29,30,31,33,34,35,37,38,39,40,41,42,43,45,46,47,48,51,52,53,54,55,56,57,58, 59,60,61,62,63,64,65,66,68,69,70,71,72,73,75,77,87,88,90,91,92,93,94,95,96,97,99,100,105,106,110,111, 112,113,114.	16,19,20,32,36,74,76,78,79,82, 84,85,86,89,98,102,102,103, 104.	17,44,49,50,67,80,81, 83,108,109.
85 Isolates	19 Isolates	10 Isolates

The length × breadth of the macroconidia usually varied between 15-37.5µ x 2.5-4µ and that of the microconidia was around 2.5-15µ x 2-3µ among the isolates. The septation of the macroconidia was 3 to 5 and the microconidia were usually aseptate or single septate. The chlamydospores were present at the terminal or intercalary positions, usually single or

in pairs. Sporulation of the macroconidia, microconidia and the chlamydospores varied highly among the iaolates. The isolates thus exhibited a high level of diversity in terms of culture and morphology.

Majority of *F.oxysporum* isolates causing vascular wilts on different crops are morphologically identical and cannot be differentiated from nonpathogenic and saprophytic strains. Hence a huge morphological diversity exists, especially in those isolated from soil. Further identification of the strains has traditionally involved the pathogenicity testing with a set of host differentials appropriate for the formae specials in question. From a diagnostic point of view the separation of the species into formae specials has important diagnostic and quarantine implications [22].

3.3. Pathogenicity testing of *F. oxysporum* f. sp. *lycopersici*:

Variation in symptoms on aerial parts and within the stem tissues of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* was observed. At early stage, 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 brown in colour. In severely infected plants lower leaves dried, ultimately the aerial parts of the tomato plant showed loss of turgidity and drooped down (Fig 4).

Fig.4. The symptomatical variation of *Fusarium* wilt in tomato plants. Disease rated on 1-5 scale.

Pathogenicity of 114 isolates was studied on five susceptible varieties by root cut and dip inoculation method. The isolates were categorized into 4 groups viz., highly pathogenic,

moderately pathogenic, weakly pathogenic and non pathogenic based on the symptomatological variations in the test tomato varieties, whereas noninoculated tomato seedlings showed no symptoms (Table 2).

Table 2: Grouping of *F. oxysporum* isolates based on pathogenicity variation.

Virulence grade	**Isolate number(** MB1- MB114)	**Total number of isolates**
Highly pathogenic	2,12,17,55,66,72,77,82,100,101,102,103,105,107,108,111,113,114.	18
Moderately pathogenic	1,3,4,5,6,7,14,16,19,20,23,24,41,45,51,52,53,54,56,57,58,59,61,62,63,64,65,69,70,71,83,89,92,93,96,97,98,112,104,109.	40
Weakly pathogenic	8,10,11,13,15,18,60,74,75,76,78,79,80,90,91,94,95,99,106,110.	20

The pathogen invades the vascular tissues and grows in the vascular bundles and inhibits water flow causing wilting, ultimately leading to death of the plant [23, 12, 24]. Vessel walls of tomato plant often are coated in an amorphous electron-opaque material [11] and this material includes xylem parenchyma, pit cavities and encrusts intertracheary pit membranes [25]. At later stages, infection is accompanied by the gradual death of xylem parenchyma cells throughout infected plants. Materials produced by pathogen consist of enzymes, growth-regulating compounds, toxins and gummosis [11, 26-31].

3.4. Assay of pathogenicity on tomato fruits:

In the assay of invasive growth on tomato fruits, the isolates tested showed difference in growth when injected into tomato fruits (Fig. 5). Some isolates showed profuse growth, others showed moderate to sparse growth while few isolates failed to grow (Table 3). The difference in invasive growth of the isolates could be correlated to their pathogenicity.

Fig.5. Assay of invasive growth on tomato fruits. A- No growth of *F. oxysporum*, B- Growth of *F. oxysporum*.

Table 3: Grouping of *F. oxysporum* isolates based on assay of invasive growth on tomato fruits**.**

Growth on tomato fruit	**Isolate Number** MB1- MB114	**Total number of isolates**
Good growth	1,2,3,4,6,8,10,11,12,15,17,22,25,35,40,41,52,53,54, 55,58,59,61,72,77,82,83,87,89,92,96,100,103,104, 106,107,108,110,111,112,113.	41
Moderate growth	5,7,14,16,18,20,24,33,39,56,57,62,63,64,66,69,71,80, 94,95.	20
Sparse growth	30,37,45,51,60,65,67,70,74,75,76,78,79,90,91,93,97, 98,99,101,102,114.	22
No growth	9,13,19,21,23,26,27,28,29,31,32,34,36,38,42,43,44,46, 47,48,49,50,68,73,81,84,85,86,88,105,109.	31

It is well known that soil-borne fungal pathogens, including *F.oxysporum*, are able to sense the presence of the plant even before establishing physical contact, most likely through compounds present in the root exudates [32]. These fungi must therefore possess signaling

mechanisms that enable them to sense environmental cues and respond by appropriate changes in gene expression, including those that lead to host recognition, root penetration, breakdown of host defences, proliferation within the host tissue and establishment of disease. Studies from a wide range of pathogenic fungi have converged to define two conserved signal transduction cascades regulating fungal development and virulence: a cAMP-PKA cascade and a mitogen-activated protein kinase (MAPK) cascade [33]. Both pathways are required for pathogenesis and play a crucial role in the formation of specialized infection structures such as appresoria, that are produced by most of the pathogens attacking the aerial parts of the plant. The role of these pathways is less understood in soil borne plant pathogens, since these fungi can usually penetrate roots directly, without the need for fully differentiated infection structures. The MAPK- and cAMP-PKA cascades also operate in *F.oxysporum* where they control a number of key steps during plant infection. The targeted inactivation of *Fmk1*, encoding an *F. oxysporum* f. sp. *lycopersici* MAPK produced mutants that were unable to penetrate the roots of tomato plants and did not produce any disease symptoms [34]. Interestingly, these mutants also failed to grow invasively when injected to tomato fruit tissue, although they grew and sporulated as well as the wild-type strain on artificial media [35].

The present investigation has generated information in terms of cultural, morphological and pathogenicity variability among *F. oxysporum* f. sp. *lycopersici* isolated from various agroclimatic regions. Strains of *F. oxysporum* are often highly host-specific. Virulence has been an extremely useful characteristic for differentiating isolates of *F.oxysporum* into formae speciales. The pathogen is distinct in symptomology, epidemiology and cultivar susceptibility [36]. The cultural, morphological diversity and pathogenicity studies along with molecular methods involving the use of polymerase chain reaction (PCR) will help in better resolution of genetic variation between the strains.

Characterization of the population structure of fungal pathogens is important for understanding the biology of the organism and for development of disease-control strategies [37]. For the elucidation of taxonomic problems, mycologists are still used to relying on the examination of a more or less limited set of characters. On one hand,

morphological and other phenotypical observations are still essential for the valid description of a fungal species, whereas on the other hand, molecular data such as DNA sequences and fingerprints dominate fungal systematics. However, only by combining approaches can the true relationship among different fungal groups be fully elucidated and multidisciplinary studies are the logical answer to meet this challenge [38]. The cultural, morphological and pathogenic variability can be used for further development of local/region specific or even race specific resistant varieties of tomato and in developing disease control strategies. Molecular methods will be used further to resolve DNA polymorphism between these strains.

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e-mail of the authors: nirmaladevi1012@gmail.com, srinivasbub@rediffmail.com