

## Original Research Article

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## Selection and Characterization of the Virulent *Fusarium oxysporum* f. sp. *lycopersici* Isolate Inciting Vascular Wilt of Tomato

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### ABSTRACT

#### Keywords

*Fusarium*, Tomato,  
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*Fusarium oxysporum* f. sp. *lycopersici* [FOL], the pathogen inflicting vascular wilt in tomato, pose a serious threat to the nutritional security. In India, due to lack of proper knowledge on the disease aetiology, the management of the disease becomes difficult. The main objective of this study is to select the most virulent FOL isolate among 7 putative *Fusarium* isolates, including 2 isolates acquired during field survey. The pathogenicity assay revealed the most virulent isolate TOFU-IHBT that exhibited systemic symptom in tomato plants within 5 days of pathogen inoculation. Based on morphological, pathological, and sequence analyses of the ITS region, and by fulfilling Koch's postulates, this isolate was characterized as *Fusarium oxysporum* f.sp. *lycopersici*. This selected isolate can be used in further studies for developing management strategies against FOL.

### Introduction

Vegetables serve as the rich sources of vitamins and nutrients thereby playing an important role in the balanced diet of human beings. Among the vegetables, *Solanum lycopersicum* L., the cultivated tomato is the second most consumed vegetable crop next to potato (Srivastava *et al.*, 2010). Tomato is susceptible to several biotic stresses that greatly limit its production and productivity. Major foliar diseases are gray mold (*Botrytis cinerea*), Cercospora leaf mold (*Cercospora fuligena*), early blight (*Alternaria solani*), bacterial canker (*Clavibacter michiganensis*) and tomato leaf curl disease (*Tomato leaf curl*

*virus*) (Reddy *et al.*, 2005). The main soil borne diseases include *Fusarium* wilt (*Fusarium oxysporum* f.sp. *lycopersici*), bacterial wilt (*Ralstonia solanacearum*), late blight (*Phytophthora infestans*), damping off (*Pythium aphanidermatum*) (Schwarz and Grosch, 2003) and Verticillium wilt (*Verticillium albo-atrum*) (Schaible *et al.*, 1951). Among these, *Fusarium oxysporum* f.sp. *lycopersici* [FOL] (Sacc.) W.C.Synder and H.N. Hans, incident of vascular wilt of tomato, alone causes 30-40% yield loss and in India, under adverse weather conditions, the losses may reach as high as 80% (Nirmaladevi *et al.*, 2016). FOL exists as hemi-biotroph and is closely related to biotrophs than necrotrophs

owing to its biotrophic lifestyle during the initial stages of plant roots invasion (Francia *et al.*, 2007). Although a range of solanaceous crops and weed species are reported to be the symptomless carriers of FOL, tomato is generally regarded as the primary host of FOL (Mc Govern, 2015). In tomato seedlings, FOL causes damping off characterized by yellowing, stunting and wilting. In mature tomato plants, the wilt symptoms include foliar yellowing and wilting which is more evident after flowering and fruit set especially in the hottest day time. Due to the invasion and blockage of distinct sectors of the vascular tissue, the wilt symptoms can show one sided appearance. Vascular discolouration becomes prominent and extends up to the entire stem length sometimes into the petiolar vascular tissue. Symptoms of FOL can be exacerbated by warmer temperatures of approximately 28°C, lower soil pH and the use of ammonium based nitrogen fertilizers (Mc Govern, 2015). Recently, in assessing the aetiology of the disease, besides *F. oxysporum*, the associations of *F. solani* in exhibiting characteristic vascular wilt symptoms in both Himachal Pradesh and Delhi regions were reported (Shanmugam *et al.*, 2017). This warrants a detailed characterization of the pathogen which will help us in formulating suitable management strategies. In this regard, the current work aims at selecting the most virulent *Fusarium* isolate and its characterization, which can later be used for evaluation of various management practices.

## **Materials and Methods**

### **Fungal isolates**

Field surveys were conducted in the tomato fields of IARI Farm during 2016-2017 and *Fusarium* infected plant samples were collected from each of the locations. The fungal pathogen was isolated from the infected tissues by employing standard isolation methods (Riker and Riker, 1936). The infected

tissues were cut into small pieces and surface sterilized with 2% sodium hypochlorite for 30 s. They were then washed in three changes of sterile distilled water and dried between two layers of blotting papers. The bits were further plated on Potato Dextrose Agar (PDA) medium containing 100 ppm streptomycin in sterile Petri dishes and incubated at room temperature (28±2°C). The fungal growth appearing around the bits were sub-cultured on plates containing PDA. These isolates together with other available putative *Fusarium* isolates in the laboratory culture collections (Table 1) obtained from vascular wilt infected tomato were subjected to morphological characterization and pathogenicity assay.

### **Morphological characterization**

Monoconidial culture of *Fusarium* isolates were obtained through single spore isolation technique (Riker and Riker, 1936). To study the morphological characteristics, a portion of actively growing culture discs of the fungus from PDA was picked up with a sterile needle and teased out in a drop of water placed on a microscopic slide. The slide was covered with a clean cover slip taking care to exclude air bubbles. The morphological characters of the fungus were studied using LMI BM light microscope with maximum possible magnification (40x) (Fig. 2).

### **Pathogenicity assay**

The pathogenicity of the fungal isolates was established in a polyhouse under artificial inoculated conditions by root inoculation. The fungal inoculum was obtained from a 7-day old culture on PDA medium. From each Petri plate, the conidial suspension was prepared using 20 ml of sterile distilled water. The bigger mycelial parts were removed by filtering the suspension through a fine nylon mesh. After quantifying the suspension in a hemocytometer, the conidial concentration

was adjusted to  $10^4$  spores/ml using sterile distilled water and used as an inoculum after mixing with 0.05% Tween 80. Apparently healthy seedlings of 25-days old tomato plants (cv. Pusa Rohini) grown under controlled conditions were used for the inoculation. The roots were injured and dipped in the conidial suspension for 3 hours followed by transplanting of inoculated seedlings in sterilized potting mixture. Seedlings inoculated with sterile distilled water served as the control. Four seedlings were used for each isolate as replicates. Disease incidence was recorded after 7 days post pathogen inoculation. The pathogen was re-isolated from the collar region of artificially inoculated plants to confirm Koch's postulates. A highly virulent isolate was selected and molecular characterization was carried out.

### **DNA extraction**

The identity of the virulent pathogen isolate was characterized by PCR amplification of ITS region from the genomic DNA as follows. Five day old submerged culture mycelium, grown in PDB and incubated at  $28 \pm 2$  °C and 180 rpm for 5 days, was used for DNA isolation. In submerged culture, individual colonies of each of the fungal isolates grown on PDA plates were inoculated separately in 100 ml of PDB. The inoculated flasks were incubated on a rotary shaker.

The mycelium was filtered out on a sterile Whatman No. 1 filter paper and washed thrice with sterile distilled water to remove traces of the medium. After air drying, the fungal mass was frozen immediately in liquid nitrogen ( $-196$  °C). The dried mats were ground with a mortar and pestle in liquid nitrogen and genomic DNA was isolated following the standard procedure with some modifications (Saghai-Marooif *et al.*, 1984).

### **Molecular characterization**

**Fig.1** Cultural characteristics of the putative *Fusarium* isolates used in this study

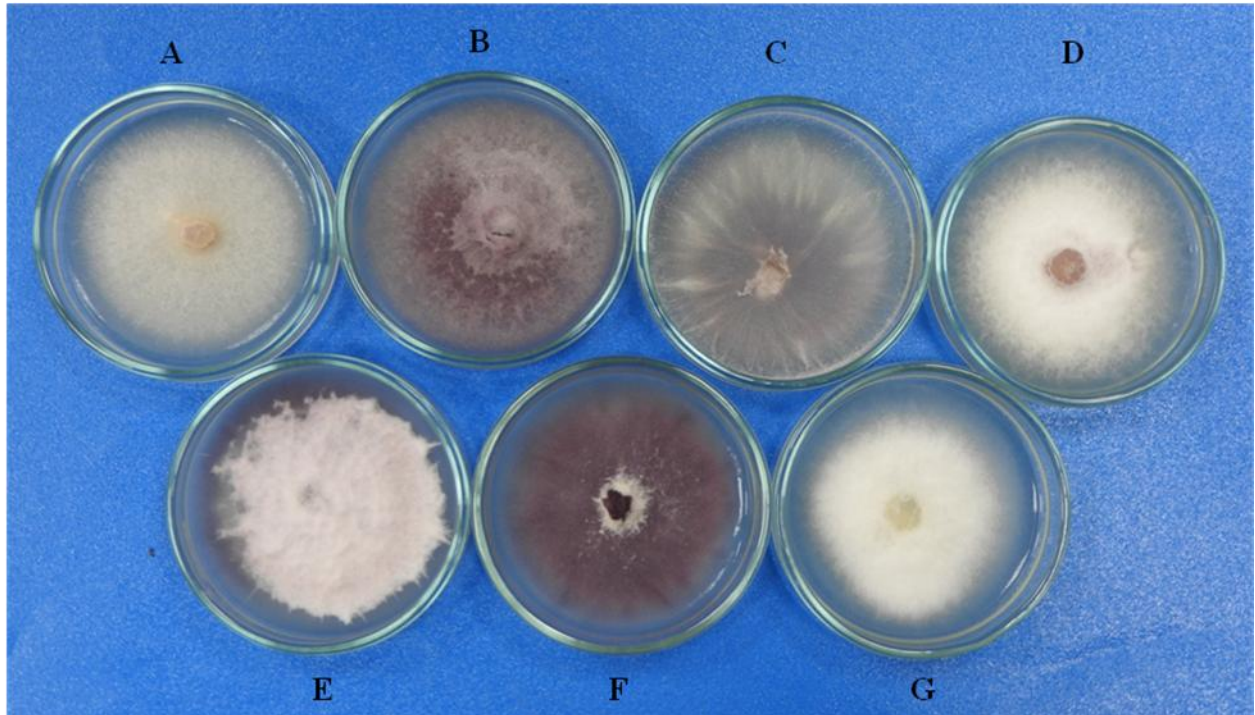
To characterize the pathogen isolates, a region of the nuclear rRNA gene containing the ITS regions 1 and 2 and the 5.8s rRNA gene was amplified from the genomic DNA by PCR using the primer combinations ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') to get an amplicon size of 500-550 bp (White *et al.*, 1990). Amplifications were carried out in 50  $\mu$ l reaction volume consisting of 10X buffer, 5.0  $\mu$ l; 2 mM dNTPs, 2.0  $\mu$ l; 3 U/ $\mu$ l *Taq* DNA polymerase, 1.0  $\mu$ l; 100 ng/ $\mu$ l of each primer, 2  $\mu$ l; 50-100 ng template DNA, 3  $\mu$ l and H<sub>2</sub>O 32.0  $\mu$ l in a Biorad thermalcycler using the PCR conditions 94 °C for 4 min (initial denaturation), 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 2 min (extension).

The total number of cycles was 35, with the final extension of 72°C for 10 min. The amplified products (50  $\mu$ l) were size separated on 1.2% agarose gel. The PCR products were purified, ligated with pGEM-T easy vector (Promega) by following manufacturer's protocol and transformed into competent *Escherichia coli* strain DH5 $\alpha$  (Fig. 4).

The transformed cells were inoculated in Luria Bertani broth and from the overnight grown culture, plasmid isolation was carried out by using Promega plasmid miniprep system.

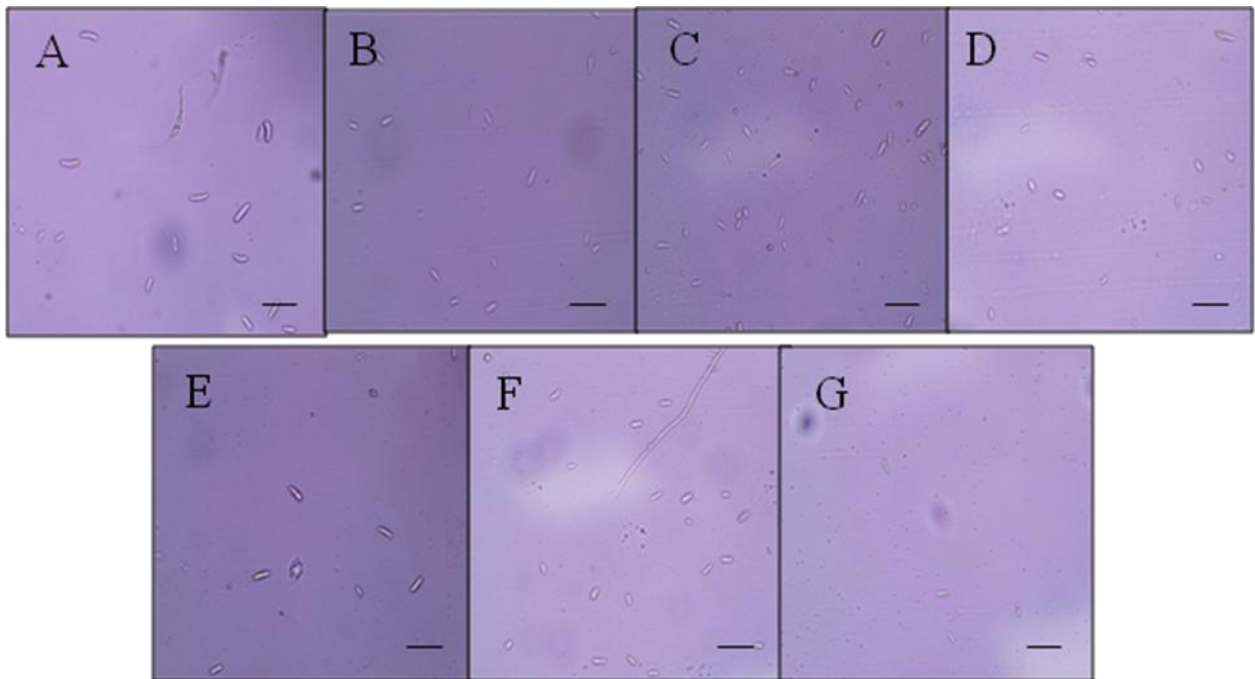
### **Sequencing, homology and phylogenetic analysis**

About 100 ng of plasmid (double stranded) DNA was used as template for sequencing PCR. A sequence similarity search was done using GenBank BLASTN of National Center for Biotechnology Information (NCBI). Sequences of closely related taxa were retrieved and phylogenetic analysis was performed using MEGA5.0 software.



A. TOFU-MM, B. TOFU-CPCT-2, C. TOFU-IHBT, D. TOFU-CPCT-3, E. TOFU-4-TISSA, F. TOXX-POT-1, G. TOFOL-CBE

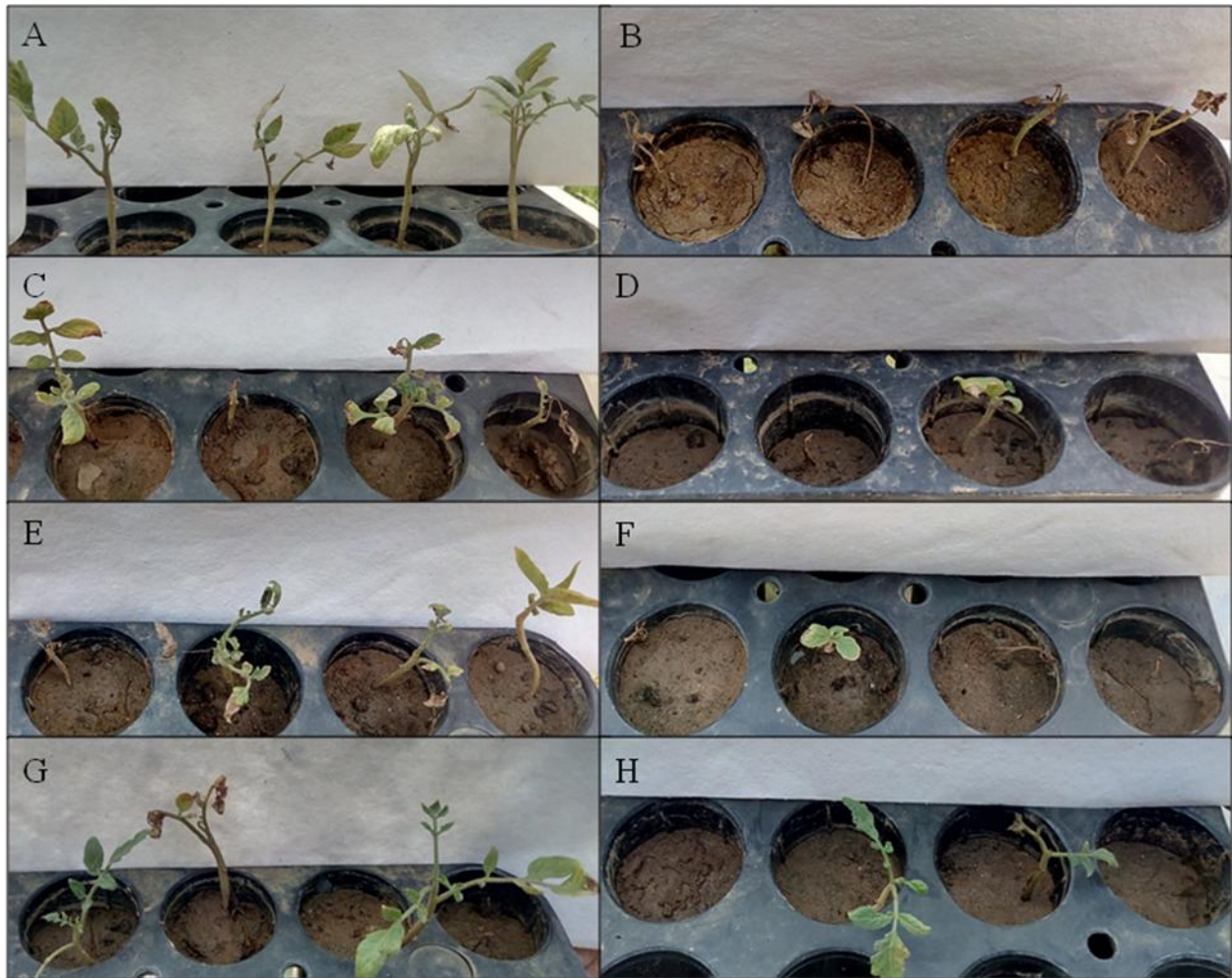
**Fig.2** Conidial characteristics of the putative *Fusarium* isolates



A. TOFOL-CBE, B. TOFU-TISSA-4, C. TOFU-IHBT, D. TOFU-CPCT-3, E. TOFU-MM, F. TOXX-POT-1, G. TOFU-CPCT-2.

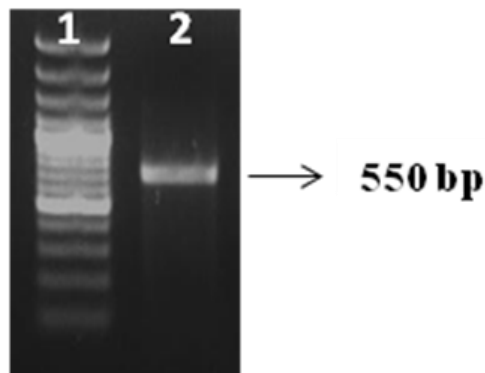
**Fig.3** Pathogenicity of the putative *Fusarium* isolates of tomato on 25 day old tomato cv. Pusa

Rohini



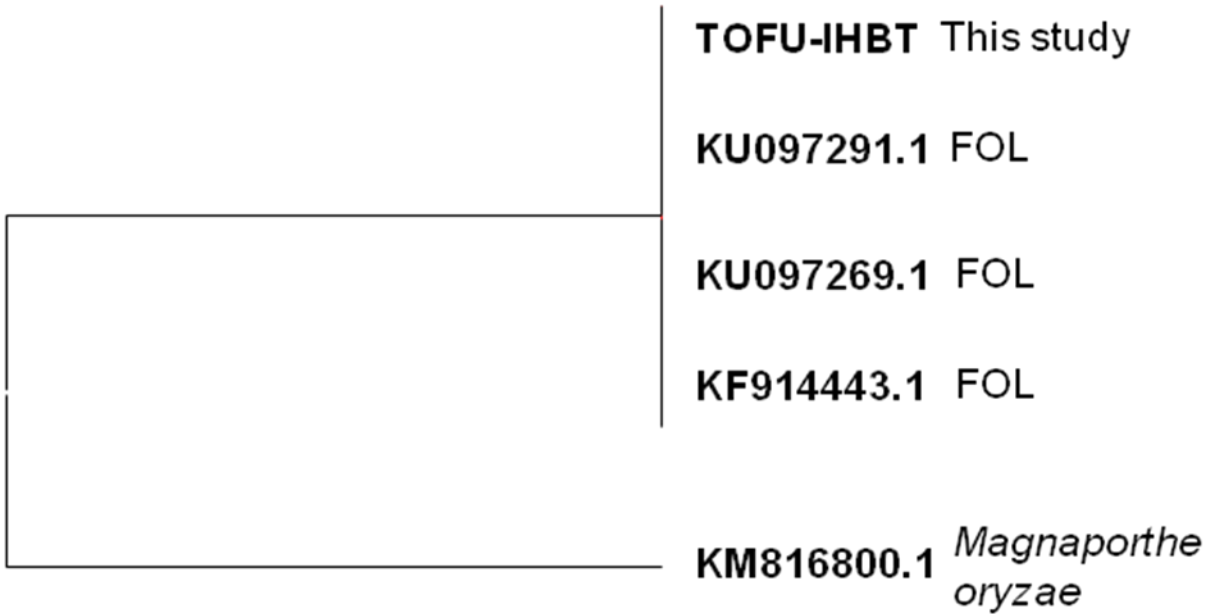
A. Non pathogenic control, B. TOFU-IHBT, C. TOFU-MM, D. TOFU-CPCT-3, E. TOXX-POT-1, F. TOFU-TISSA-4, G. TOFOL-CBE, H. TOFU-CPCT-2

**Fig.4** PCR amplification of ITS region of the highly virulent *Fusarium* isolate TOFU-IHBT. Lanes 1-100 bp ladder, 2-PCR product



**Fig.5** Phylogenetic tree based on ITS sequences, drawn using the neighbor joining method and

showing the relation of the most virulent *Fusarium* isolate, TOFU-IHBT with other *Fusarium* isolates. The isolate name is given after the GenBank accession numbers of each species. Bar, 0.02 substitution per site



**Table.1** List of putative *Fusarium* isolates of tomato used in this study

S. No.	Code	Location	Mode of Acquisition
1	TOFU-IHBT	Barmat, Himachal Pradesh	Laboratory collection
2	TOXX- POT-1	Rajpur, Himachal Pradesh	Laboratory collection
3	TOFU-TISSA -4	Tissa, Himachal Pradesh	Laboratory collection
4	TOFOL-CBE	Coimbatore, Tamil Nadu	Laboratory collection
5	TOFU-MM	MM, Himachal Pradesh	Laboratory collection
6	TOFU-CPCT-2	CPCT, New Delhi	Field survey
7	TOFU-CPCT-3	VRF, New Delhi	Field survey

**Table.2** Colony diameter and wilt incidence exhibited by different *Fusarium* isolates

Isolate	Colony diameter (mm)*	Percent disease incidence <sup>#</sup>
TOFU-IHBT	4.00 <sup>a</sup>	100.00 <sup>a</sup>
TOXX-POT1	3.80 <sup>a</sup>	81.25 <sup>d</sup>
TOFU-TISSA-4	3.17 <sup>b</sup>	93.75 <sup>b</sup>
TOFOL-CBE	3.63 <sup>a</sup>	81.25 <sup>d</sup>
TOFU-MM	3.10 <sup>b</sup>	81.25 <sup>d</sup>
TOFU-CPCT-2	3.96 <sup>a</sup>	87.50 <sup>c</sup>
TOFU-CPCT-3	3.67 <sup>a</sup>	93.75 <sup>b</sup>

\*Values are mean of three replications; colony diameter was measured after 5 days of pathogen inoculation when one of the isolates covered the full 40mm diameter glass plate; <sup>#</sup>Values are mean of four replications; disease incidence was measured after 7 days post pathogen inoculation; Means followed by a common letter are not significantly different at 5% level by DMRT.

### Statistical analysis

The data were statistically analyzed using Web Agri Stat Package 2.0 and Duncan's Multiple Range Test (DMRT) was used to compare the treatment means.

### Results and Discussion

Vascular wilt of tomato was widely prevalent to the extent of 10-15% in IARI farms. On isolation of the associated pathogen from vascular tissues of infected stems on potato dextrose agar, both samples from two fields revealed a single type of fungus. On PDA plates, all the putative *Fusarium* isolates, including the two isolates obtained during field survey, demonstrated various colony characteristics ranging from fluffy mycelium to immersed mycelium (Fig. 1). Considering growth pattern, TOFU-IHBT covered the full 40 mm diameter petriplate within 5 days of inoculation while TOFU-MM demonstrated the lowest growth of only 31 mm (Table 2). In some isolates like TOFU-CPCT-2, TOFU-4-TISSA and TOXX-POT-1, pink pigmentation was more evident than in others. In pathogenicity assays, one isolate designated as TOFU-IHBT, representing Himachal Pradesh, that exhibited systemic

infection within 5 days and caused mortality of all the four seedlings, was identified as a highly virulent isolate and used for further characterization (Fig. 3, Table 2). PCR amplification of Internal Transcribed Spacer (ITS) region of the strain TOFU-IHBT yielded 550bp amplicons. In establishing the identity of the strain TOFU-IHBT, by sequencing of its Internal Transcribed Spacer (ITS) region, its sequence displayed 100% identity to the sequences of *F. oxysporum* f.sp. *lycopersici* isolates with Accession No KU097291.1, KU097269.1 and KF914443.1, available in the NCBI database. In phylogenetic analysis, the ITS sequences of TOFU-IHBT was grouped along with other isolates of *F. oxysporum* as a single cluster (Fig. 5). Thus, based on colony morphology, sequence of the ITS region, and Koch's postulates, the highly pathogenic strain, TOFU-IHBT was identified as *F. oxysporum* f.sp. *lycopersici*. Future researches can be directed to determine the role of environmental parameters in disease causation by various *Fusarium* isolates. Also, molecular mechanisms underlying the differences in virulence need to be explored in detail. The identified isolate, TOFU-IHBT can now be used for developing and evaluating various management strategies

including screening of tomato genotypes for *Fusarium* wilt resistance and evaluating the efficacy of fungicides and biocontrol control agents in managing *Fusarium* wilt of tomato.

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