3 Biotech (2020) 10:486
https://doi.org/10.1007/s13205-020-02475-z

ORIGINAL ARTICLE

Molecular characterization and race identification of *Fusarium oxysporum* f. sp. *lycopersici* infecting tomato in India

Loganathan Murugan[1,2] · Nagendran Krishnan[1] · V. Venkataravanappa[1,3] · S. Saha[1,4] · A. K. Mishra[1] · B. K. Sharma[1] · A. B. Rai[1]

Received: 3 January 2020 / Accepted: 12 October 2020 / Published online: 24 October 2020

Abstract

Fourteen isolates of *Fusarium* were isolated from wilt affected tomato samples collected from 10 different states of India. Characterization of the fungal cultures based on morphology and sequencing of ITS rDNA revealed that they belonged to *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). Pathogenicity assay on two susceptible tomato cultivars showed all the 14 isolates were pathogenic and categorized in high-, moderate- and low-virulent groups. Differential host assay on Bonny Best (no resistant gene), UC82-L (harboring *I-1*), Fla.MH1 (harboring *I-1* and *I-2*) and I3R-1 (harboring *I-1*, *I-2* and *I-3*) tomato genotypes and PCR amplification with race-specific primers indicated that all the *Fusarium* isolates infecting tomato in India were belonging to race 1. Molecular diversity analysis based on ISSR markers revealed the presence of 3 distinct groups of *Fol* isolates. Abundant diversity was observed among the *Fol* isolates in harboring the virulence-related genes (endo-polygalacturonase gene *pg1* and tomatinases) and toxin production (fumonisin). However, presence of *pg1* does not correlate with virulence and the isolates carrying tomatinase 4 (*tom-4*) in combination with other tomatinase genes were of virulent group. Detection of fumonisin gene in six isolates of *Fusarium* infecting tomato indicated their toxigenic nature.

Keywords Tomato · Fusarium · Wilt · Race · Characterization

Introduction

Tomato (*Solanum lycopersicum* L.), commonly known as 'poor man's apple', is cultivated worldwide and consumed as table food and processed products. India ranks second in tomato production next to China by producing 19.75 million tones with an average productivity of 25.04 t/ha (NHB 2018). Biotic factors are the major yield limiting constraints for its cultivation, of which *Fusarium* wilt caused by the soil borne fungus, *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) is one of the most important devastating diseases all over the world (Prihatna et al. 2018). The pathogen persists in the soil without host for many years and can attack the crop when it is grown. Yield loss in tomato due to fusarial wilt is reported as 60–70% under favorable conditions (Ravindra et al. 2015). Microscopic examination of morphological characteristics such as shape and size of micro- and macro-conidia, phialides and formation of chlamydospores, etc., is the most preferred method for identification of *Fusarium* spp., but it requires expertise to detect the pathogen at species level (Khetarpal 2006). Molecular approaches such as PCR with species-specific primers and sequencing of conserved nucleotide regions preferably of

Loganathan Murugan and Nagendran Krishnan authors contributed equally.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-020-02475-z) contains supplementary material, which is available to authorized users.

✉ Loganathan Murugan
logumuruga@gmail.com

[1] Division of Crop Protection, ICAR-Indian Institute of Vegetable Research, Uttar Pradesh, Varanasi 221305, India

[2] Present Address: Division of Crop Protection, ICAR-National Research Centre for Banana, Tamil Nadu, Tiruchirappalli, India

[3] Present Address: CHES, Chettalli, ICAR-Indian Institute of Horticultural Research, Hessaraghatta Lake PO, Bangalore, India

[4] Present Address: Division of Crop Protection, ICAR-National Research Centre for Grapes, Pune, Maharashtra, India

internal transcribed spacer (ITS) regions are being employed to detect fungal pathogen at species level (Baayen et al. 2000; O'Donnell et al. 2000; Singha et al. 2016). Further phylogenetic analysis of ITS sequences showed evolutionary relationship among the strains (Nirmaladevi et al. 2016). A huge variability exists among different strains of *Fusarium* spp., and to study such variability molecular tools such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990), amplified fragment length polymorphism (AFLP) and inter-simple sequence repeats (ISSRs) (Wilson et al. 2004; Guleria et al. 2007) etc., are commonly attempted. Polygalacturonases are cell wall-degrading enzymes produced by fungal pathogens during pathogenesis and among them, polygalacturonase gene 1 (*pg1*) represents the maximum genetic diversity in *Fusarium* infecting tomato (Hirano and Arie 2009). Fumonisin is a mycotoxin produced by fungal pathogens and 11 species of *Fusarium* are reported to produce the toxin which is a major concern in food crops as it harms human and animal health (Divakara et al. 2014). Tomatinases enzymes produced by *Fol* during pathogenesis process are required mainly to detoxify α tomatin and it could be correlated with virulence of the pathogen (Pareja-Jaime et al. 2008). Hence, in the present study for characterization of pan India collection of *Fol*, morphological and ITS sequencing for identification, ISSR analysis for diversity, PCR assays with specific primers of fumonisin gene to know ability of mycotoxin production and, *pg1* and tomatinases genes for classification of virulence have been attempted.

Though several biocontrol agents and chemicals have been tested for the management of *Fusarium* wilt diseases (Song et al. 2004; Manikandan et al. 2010; Elanchezhiyan et al. 2018), complete protection of crop could not be achieved by adopting these methods (Nirmaladevi et al. 2016; Thangavelu et al. 2019). Rather host plant resistance plays a key role in the development of sustainable management. Three races of *Fol* (races 1, 2 and 3) infecting tomato were reported (Reis et al. 2005). Based on the prevailing pathogenic race(s) in the local environment, selection of cultivar(s)/genotype(s) resistance to the particular race is the foremost step in disease management. To the best of our knowledge, there is no comprehensive study describing races prevailing among *Fol* isolates infecting tomato in India. Therefore, it is further attempted to do PCR with race-specific primers and bioassay with differential hosts to define prevalent *Fol* race(s) in India.

Materials and methods

Collection and isolation of *Fusarium* isolates

Wilt-infected tomato plant samples were collected from 14 different locations of tomato growing areas covering 10 states of India (Tamil Nadu, Uttar Pradesh, Assam, Jharkhand, Jammu and Kashmir, Punjab, Haryana, Madhya Pradesh, Bihar and Chhattisgarh). For isolation of *Fusarium* sp. associated with wilt disease of tomato, infected root tissues were washed under running tap water to remove soil adhering on the root surface, cut in to 5–10 mm bits and surface sterilized in 1% sodium hypochlorite solution (Merck, India) for 1–2 min, rinsed three times in sterile distilled water and dried on sterile filter paper. Then sterilized tissue bits were transferred on to Potato Dextrose Agar (PDA, Himedia, India) plates amended with 50 mg/L streptomycin sulphate (Sisco Research Laboratories, India). The plates were incubated at room temperature for 5 days for mycelial growth of the fungus. From the growing point, mycelial disc was excised and sub-cultured on PDA slants. The culture slants were incubated for 5 days under room temperature and subsequently stored at 4 °C (Norhito et al. 2004) for further use.

Pathogenicity of *Fusarium* isolates

Pure cultures of *Fusarium* isolates were transferred on to PDA in Petri plates and incubated for 7 days at room temperature. Spores were harvested by scraping mycelial mat of fully-grown culture using 10–15 ml of sterile water per plate. The spore suspension was adjusted to approximately 10^6 spores/ml using sterile water and used for pathogenicity test. Twenty-day-old healthy tomato seedlings cv. Bonny Best and DVRT1 were inoculated by standard root dip method. Seedlings were uprooted carefully preserving the root intactness, shaken to remove the adhering particles and washed gently under tap water. The root apex (about 1 cm) was trimmed with a pair of sterile scissors and submerged for 30 min in the conidial suspension for each isolate. Seedlings dipped in sterile water served as control. The inoculated seedlings and control were transplanted to protrays (7 cm diameter) containing sterilized soil and sand 1:1 ratio. One seedling per hole was transplanted and 6 seedlings for each isolate were inoculated. Plants were maintained in a greenhouse where day and night temperatures varied from 25 to 30 °C. Symptoms started to be visible 15–20 days after artificial inoculation. The discoloration of the vascular tissue was confirmed by splitting the stem (Marlatt et al. 1996; Dubey and Shio 2008). Disease severity was calculated using standard methodology (Nirmaladevi et al. 2016). Individual plants were scored for disease grading based on expressed symptoms as 0—healthy plant; 25—initial leaf chlorosis; 50—severe leaf chlorosis and initial symptoms of wilting; 75—severe wilting symptoms and leaf chlorosis; and 100—plant total wilting, leaves completely necrotic. Average score was calculated as disease severity and based on the disease severity, the virulence of each isolate was categorized as low virulent (< 25%), moderate virulent (25–50%) and high virulent (> 50%).

Morphological characterization of *Fusarium* wilt isolates

Pure cultures of *Fusarium* wilt isolates were grown on PDA in Petri plates as described earlier and after 7 days, the cultures were observed for shape and size of conidia and chlamydospores.

Molecular characterization

DNA Extraction

Fusarium isolates were grown in Potato Dextrose Broth (PDB) at 28 °C under static conditions for 7 days. Mycelia were harvested and lyophilized using liquid nitrogen. Later 100 mg of the finely powdered mycelium was used for DNA extraction using CTAB method with minor modifications (Graham et al. 1994) and the DNA samples were stored at − 20 °C for further use.

Species specific PCR assay

Genomic DNAs of *Fusarium* isolates were PCR amplified with *Fusarium oxysporum* species specific primer pair FOF1/FOR1 (Table 1) as described by Prashant et al (2003). The PCR amplification was carried out in 25 µL reaction volume consisting of 2.5 µL 10X PCR buffer, 2 mM $MgCl_2$, 25 mM dNTPs, 1 unit of Taq polymerase (NEB, England), 10 pM of each primer and 50–100 ng of template DNA in the MyCycler thermal cycler (BioRad, USA). The PCR programme carried out was initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR amplicons were resolved on 0.8% agarose gel stained with ethidium bromide and visualized in a UV transilluminator and 1 kb ladder was used as a marker. Amplification patterns were documented using Gel Documentation system (Alpha Innotech, USA).

Table 1 List of primers used for characterization of *Fol* isolates infecting tomato

S. No	Primer name	Sequence (5′-3′)	Target	Reference
1.	FOF1	ACATACCACTTGTTGCCTCG	*Fusarium oxysporum* species specific	Prashant et al. (2003)
2.	FOR1	CGCCAATCAATTTGAG GAACG		
3.	ITS1	TCCGTAGGTGAACCTGCGG	Internal transcribed spacer region	White et al. (1990)
4.	ITS4	TCCTCCGCTTATTGATATGC		
5.	Endo F	CCAGAGTGCCGATACCGATT	*pg*1 gene	Hirano and Arie (2006)
6.	Endo R	AAGTGTTGGTAGGATAGTTG		
7.	tom1-11X	CTCAAGCAGTCAAAATCCCCT	*tom*-1	Pareja-Jaime et al. (2008)
8.	tom1-12H	CGACGAGTTGTGCTACCATCT		
9.	tom2-1	CCACTTCTGCGACACTGCTA	*tom*-2	Pareja-Jaime et al. (2008)
10.	tom2-2	CTGTGTTTCCTGTTCGTTTCC		
11.	tom3-1	CTATCATTGGTCTTGCCCGTT	*tom*-3	Pareja-Jaime et al. (2008)
12.	tom3-2	TGTTGGAGGATACTGCGTCTA		
13.	tom4-1	ATCCCCGTCTATCCCTCCC	*tom*-4	Pareja-Jaime et al. (2008)
14.	tom4-2	AAGAGACTCCAGAATGCGTTG		
15.	tom5-1	TGTGTTGGTCTCGGGTCTTTT	*tom*-5	Pareja-Jaime et al. (2008)
16.	tom5-2	TAGTCTCCTCCATCGCAATAC		
17.	FUM1 F	AGTCGTGCTGACGTCGAG	Fumonisin	Ramana et al. (2011)
18.	FUM1 R	TGCTGCTGTCGCATCATA		
19.	uni-F	ATCATCTTGTGCCAACTTCAG	Universal to amplify races 1, 2, and 3	Hirano and Arie (2006)
20.	uni-R	GTTTGTGATCTTTGAGTTGCCA		
21.	sp13-F	GTCAGTCCATTGGCTCTCTC	Specific to races 1 and 3	Hirano and Arie (2006)
22.	sp13-R	TCCTTGACACCATCACAGAG		
23.	sp23-F	CCTCTTGTCTTTGTCTCACGA	Specific to races 2 and 3	Hirano and Arie (2006)
24.	sp23-R	GCAACAGGTCGTGGGGAAAA		
25.	ISSR3	CACCACCACGC	ISSR	Nirmaladevi et al. (2016)
26.	ISSR9	GAGAGAGAGAGAGAGAGAC	ISSR	Nirmaladevi et al. (2016)
27.	ISSR10	GAGAGAGAGAGAGAGAGAT	ISSR	Nirmaladevi et al. (2016)

Internal transcribed spacer (ITS)-based characterization of *Fusarium* isolates

Total DNA extracted from fourteen *F. oxysporum* isolates were subjected to PCR amplification using universal primers ITS1 and ITS4 (Table 1; White et al. 1990). The PCR amplification was performed in a total volume of 25 µL as described in species specific PCR assay with modification in annealing temperature (55 °C). The PCR-amplified DNA fragments were purified using QIAquick Gel extraction kit (QIAGEN, Germany) according to the manufacturer's instructions. The eluted DNA fragments were cloned in the pGEMT vector (Promega, USA) and transformed in *E. coli* strain DH5α. Plasmids were isolated from the transformed colonies and sequenced (Eurofins Pvt Ltd., Bengaluru, India). The sequences obtained in the present study were submitted to GenBank.

Genetic diversity among *F. oxysporum* isolates

Genetic diversity of fourteen *F. oxysporum* isolates was studied using three ISSR markers (Eurofins, Bangalore, India). The primers selected for this study were based on the production of polymorphic and reproducible bands for isolates as reported in earlier studies (Nirmaladevi et al. 2016). PCR reactions were performed in MyCycler (BioRad, USA) and the reaction volume was 25 µL consisting of 2.5 µL of 10X PCR reaction buffer, 2 mM dNTPs (each), 25 mM $MgCl_2$, 10 pM of each primer, 1.0 U of Taq DNA polymerase (NEB, England) and 50–100 ng template DNA. For each primer, amplifications were repeated thrice to assure reproducibility of amplification. The PCR cycling conditions were carried out with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 36–45 °C for 1 min and extension at 72 °C for 2 min with a final extension of 72 °C for 10 min. For data analysis, the presence or absence of an allele at a particular locus was scored as 1 and 0, respectively, and the pairwise distance among the strains was calculated from the binary matrix using the Jaccard's coefficient. The resulting distance matrices were used to cluster the strains by the UPGMA (Unweighted Pair Group method using arithmetic means) method using NTSYSpc version 2.0 (Rohlf 1997).

Characterization of *Fusarium* isolates based on endopolygalacturonase, tomatinase and fumonisin genes

Total genomic DNA of different *F. oxysporum* isolates were subjected to PCR using the primers pair Endo F/ Endo R for endopolygalacturonase gene *pg1* (Table 1; Hirano and Arie 2006), tom1-11X and tom1-12H for *tom1*, tom2-1 and tom2-2 for *tom2*, tom3-1 and tom3-2 for *tom3*, tom4-1 and tom4-2 for *tom4* and tom5-1 and tom5-2 for *tom5* for tomatinase genes (Pareja-Jaime et al. 2008) and FUM1 F/FUM1R (Ramana et al. 2011) for fumonisin gene (Table 1). The PCR reaction was carried out in 25 µl as described in species specific PCR assay with annealing temperature of 59 °C for endopolygalacturonase gene, 54–55 °C for tomatinase genes and 58 °C for fumonisin gene.

Race identification of *Fusarium* isolates

Race-specific primers were used as described by Hirano and Arie (2006) in the PCR analysis with annealing temperature of 61 °C and their details are given in Table 1. Further, in bioassay with differentials (Bonny Best, UC82-L, Fla.MH1 and I3R-1 were obtained from AVRDC, Taiwan), twenty-day-old seedlings of tomato line Bonny Best (sensitive to *Fol* races 1, 2 and 3); UC82-L (*Solanum lycopersicum*)—resistant to race 1; Fla.MH1 (*S. lycopersicum*)—resistant to race 1 and race 2; I3R-1(*S. pennellii*)—resistant to races 1, 2, 3 were inoculated with all the 14 isolates *Fol* as described by Sánchez-Peña et al. (2010). The plants were observed for the symptom expression up to 20 days after inoculation.

Sequence analysis

The sequences of ITS gene obtained in the present study were compared to different fungal isolates sequences retrieved from the NCBI database using nucleotide BLAST in GenBank. The phylogenetic tree was constructed by MEGA X software (Kumar et al. 2018) using the neighbor-joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

Results

Characterization of *Fusarium* isolates based on spore morphology

The *Fusarium* isolates were characterized based on the culture and spore morphology. The mycelia were white to pink with purple tinge or slightly orange on the PDA medium. All the 14 *Fusarium* isolates were having macroconidia (sickle shape, 3–5 cells), microconidia (slightly oval, single cell) and chlamydospores (globose, terminal or intercalary) with an average size of 10.57 × 2.92 µm, 2.42 × 6.44 µm and 5–11 µm, respectively.

Pathogenicity assay and their virulence

All the fourteen isolates of *Fusarium* inoculated on the susceptible cv. Bonny Best and DVRT 1 were able to produce

typical symptoms of wilt after 15–20 days post inoculation. Different symptoms such as yellowing of lower leaves followed by drooping of leaves were observed in the *Fusarium* inoculated plants. In severe cases, brown discoloration of vascular bundles was observed upon splitting of stems followed by wilting and death of the seedlings. Koch's postulate was proved as they could be re-isolated from the infected tissues. Based on disease severity the fourteen isolates were categorized as highly virulent (7 isolates), moderately virulent (6 isolates) and low virulent (1 isolate) (Table 2).

Species-specific PCR assay

PCR amplification with species-specific primers in 14 *Fusarium* isolates resulted in amplification of ~ 340 bp fragments specific to the *F. oxysporum* in all 14 isolates.

Characterization of *Fusarium* isolates through amplification of ITS region

The ITS regions of fourteen *Fusarium* isolates were amplified using fungal-specific universal primer pair ITS1–ITS4 and obtained ~ 500 bp amplicon in all the isolates. The amplified PCR products were cloned and sequenced. The ITS rDNA sequences of all the fourteen isolates were submitted to NCBI GenBank database and their accession numbers were obtained (Table 2). The BLAST analysis of ITS rDNA sequences showed that the nucleotide identity of the isolates ranged from 98–100% with *Fol* infecting tomato. Phylogenetic analysis using Mega X software by neighbor-joining method with the other selective *Fusarium* species sequences retrieved from the GenBank revealed that the *F. oxysporum* isolates characterized in the present study were formed into two separate groups with *F. oxysporum* f.sp. *lycopersici,* isolates. The five isolates viz., FOL 14, FWT 60, FWT 74, FWT 89 and FWT 20 were closely clustered in one group, while another set of nine isolates (FWT 5, FWT 8, FWT 15, FWT 56, FWT 71, FWT 77, FUS-Co3, FUS VNS 2 and FUS VNS 3) were clustered separately. However, all the fourteen isolates did not group with *F. udum, F. solani, F. graminearum* and *F. culmorum* and the tree was rooted on the outgroup *Alternaria solani* (Fig. 1).

Genetic diversity of *Fusarium* isolates infecting tomato

To study the variability among the *Fusarium* isolates, analysis was carried out using three ISSR markers. All the three ISSR markers (ISSR3:5′CACCACCACGC3′, ISSR9:5′GAGAGAGAGAGAGAGAGAC3′ and ISSR10: 5′GAGAGAGAGAGAGAGAGAT3′) showed high polymorphism among all the isolates (Fig. 2). The amplified fragments of each isolate were scored as 1 (present) or 0 (absent) for three ISSR makers. Co-migrating bands were considered homologous characters. Faint bands and bands showing variable levels of intensity were not considered for scoring. Number of variable bands produced by each marker varied from 12 to 15 ranging between 200 bp and

Table 2 Virulence details of the *Fusarium* isolates used in this study

No	Isolates	Geographic origin	Disease severity			Virulence	GenBank accession no. (ITS based)
			Bonny best	DVRT 1	Mean		
1.	FWT 5	Jammu, J & K	36.67	29.0	32.8	Moderate	KC478624
2.	FWT 8	Jammu, J & K	53.33	73.33	63.3	High	KC478622
3.	FWT 15	Patiala, Punjab	26.0	37.33	31.7	Moderate	KC478640
4.	FWT 20	Karnal, Haryana	37.33	40.0	38.7	Moderate	KC478621
5.	FWT 56	Samastipur, Bihar	93.33	80.0	86.7	High	KC478635
6.	FWT 60	Samastipur, Bihar	80.0	73.33	76.7	High	KC478634
7.	FWT 71	Raipur, Chhattisgarh	40.0	40.0	40.0	Moderate	KC478633
8.	FUS VNS 2	Varanasi, Uttar Pradesh	66.67	80.0	73.3	High	KC478619
9.	FUS VNS 3	Varanasi, Uttar Pradesh	68.0	52.0	60.0	High	KC478629
10.	FWT 74	Ranchi, Jharkhand	40.0	46.67	43.3	Moderate	KC478627
11.	FWT 77	Ranchi, Jharkhand	53.33	66.67	60.0	High	KC478626
12.	FWT 89	Jabalpur, Madhya Pradesh	27.00	40.00	33.5	Moderate	KC478623
13.	FUS CO-3	Coimbatore, Tamil Nadu	66.67	66.67	66.7	High	KC478630
14.	FOL 14	Guwahati, Assam	13.33	16.0	14.7	Low	KC478631
CD					9.12		
CV					10.53		
SE(d)					4.43		

3 kb. In the phylogenetic analysis, *Fusarium* isolates were grouped into three major clusters A, B and C. Cluster A had three isolates (FWT 56, FWT 71 and FWT 74), B had one isolate (FUS-Co3) and remaining 10 isolates were grouped under cluster C (Fig. 3).

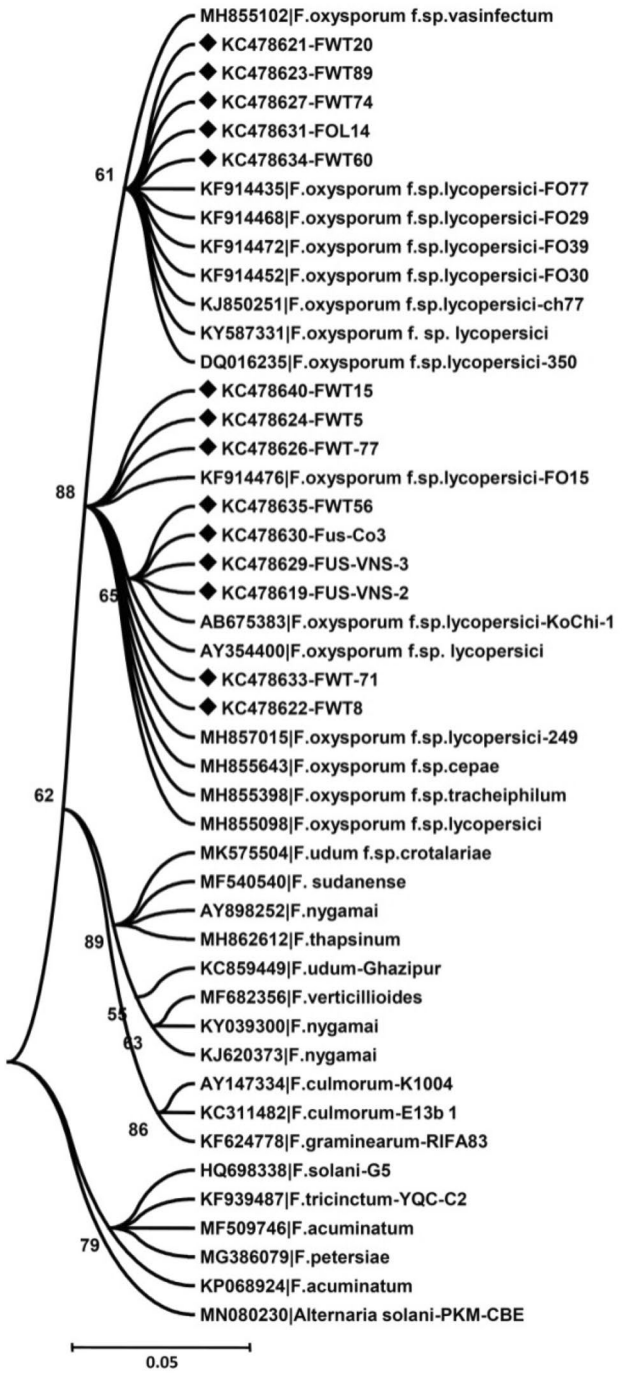

Fig. 1 Phylogenetic analysis of *F. oxysporum* f. sp. *lycopersici* strains based on rDNA ITS sequences by MEGA X software using neighbor-joining method with 1000 bootstrapped replications

Characterization of *Fusarium* isolates based on endo polygalacturonase, tomatinase and fumonisin genes

Endo polygalacturonase

Polygalacturonase gene (*pg1*), a virulence determinant gene in *Fusarium*, was amplified through PCR using *pg1* gene specific primer Endo-F/R, to study the pathogenic variation in the isolates of *Fusarium*. Among the 14 isolates of *Fusarium* tested, only 11 isolates (FUS-CO3, FWT 8, FOL 14, FWT 77, FWT 5, FWT 15, FUS VNS 3, FWT 56, FWT 71, FWT 20 and FWT 60) were found to harbor the *pg1* gene (Fig. 4).

Tomatinase

The 14 isolates of *Fusarium* infecting tomato were screened for the presence of different tomatinase genes (*tom-1, tom-2, tom-3, tom-4* and *tom-5*). Amplicon size of ~ 550 bp was amplified in all the isolates for *tom-5* gene, whereas *tom-4* gene with an amplification size of ~ 530 bp was detected in 7 isolates and genes *tom-3* and *tom-1* with ~ 600 bp amplifications were detected in 9 isolates; and *tom-2* gene has been amplified (~ 520 bp) in 12 isolates of *Fol* (Fig. 5).

Fumonisin

Upon screening of fourteen *Fusarium* isolates infecting tomato for fumonisin genes through PCR assay using FUM1 F and FUM1R primers pairs, only six isolates (FWT 56, FWT 60, FWT 71, FUS VNS 2, FWT 89 and FUS CO-3) belonging to the *Fol* infecting tomato collected from Bihar, Chhattisgarh, Uttar Pradesh, Tamil Nadu and Madhya Pradesh resulted in amplicon size of ~ 780 bp.

Race identification

Fourteen isolates of *Fusarium* isolated from tomato were inoculated on the differential lines. All the isolates could infect Bonny Best, which is sensitive to the *Fol* races 1, 2 and 3 but they were unable to infect UC82-L (resistant to race 1), Fla.MH1 (resistant to races 1 and 2) and I3R1 (resistant to races 1, 2, 3). It showed that the all isolates infecting tomato were belonging to race 1. In the PCR analysis for the race identification, universal primer pair uni-F/R was used and obtained PCR amplicon size of ~ 670 bp specific to *Fol* in all the fourteen isolates (Fig. 6a). Further, specific primer pair (sp13-F/R) of races 1 and 3 had also amplified the amplicon of ~ 445 bp in all

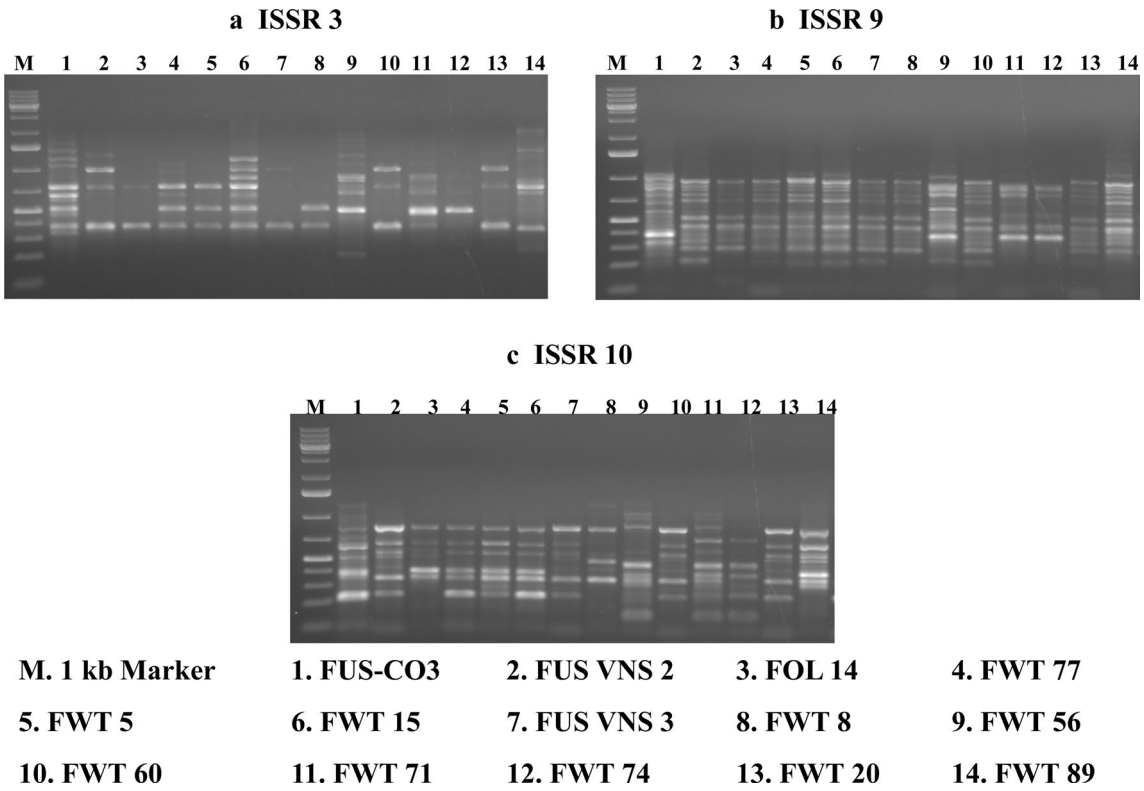

Fig. 2 Polymorphic DNA pattern of *Fusarium* isolates infecting tomato using ISSR markers

14 isolates (Fig. 6b). However, the specific primer pair (sp23-F/R) of races 2 and 3 failed to amplify any of the isolates.

Discussion

Wilt disease of tomato is caused by three races of *Fol*. Races 1 and 2 of *Fol* are distributed worldwide, whereas race 3 has a limited geographic distribution. Several studies were focused on the management of *Fusarium* wilt in tomato through biological and chemical means from India, but success of these methods was very limited due to variation in pathogens. Variation is due to the presence of diversified groups of strains or difference in their virulence or existence of various races. So far, there is no comprehensive study on virulence based on genetic diversity, race differentiation, mycotoxin and toxigenic-related genes of the strains infecting tomato in different parts of the country except one report where few of the characters have been studied with *Fol* isolates collected from five states (Nirmaladevi et al. 2016). Here, we describe morphological, virulence and genetic diversity of *Fusarium* isolates infecting tomato in India collected across the country. The study also showed that isolates varied widely in their morphological characters such as pigmentation, colony morphology, growth and sporulation. Upon inoculation on the susceptible host, all the isolates produced typical wilt symptoms with different severity depicting the pathogenic nature of the isolates. Based on the severity, isolates were categorized into three groups such as low, moderate and highly virulent. Exhibition of different grades of virulence denotes the subsistence of wide range of pathogen population causing wilt disease on tomato in India. Based on disease severity on the host, 42% isolates were classified as moderately virulent and 50% were highly virulent. Similarly, Nirmaladevi et al (2016) reported virulence variation among *Fol* strains infecting tomato-growing areas of a part of India.

Precise identification and genetic characterization of pathogen is necessary for appropriate management of wilt diseases. Studying the variability of *F. oxysporum* from various agro-climatic zones is not only important in the paradigm of breeding for disease resistance but also is necessary for the selection of resistant tomato cultivars. Detection of organism through molecular markers will be robust, less time consuming and cheap with least expertise. Ramana et al (2011) developed species-specific marker to identify the different *formae speciales* of *F. oxysporum.* Further it was validated in several other studies for the identification of different *formae speciales* of *F. oxysporum* (Divakara et al. 2014; Nirmaladevi et al. 2016). To study the genetic relationship of the *Fol* isolates, phylogenetic

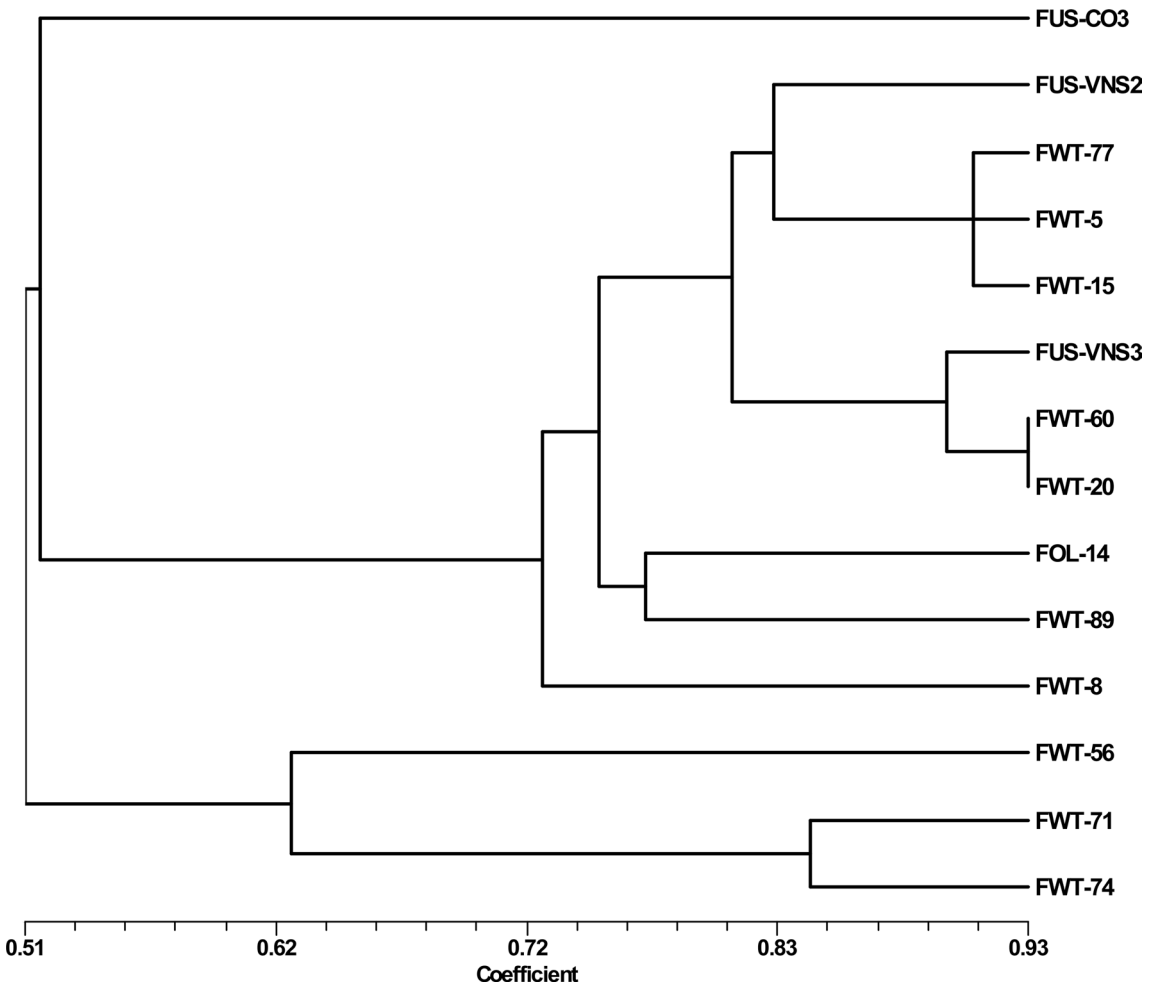

Fig. 3 Dendrogram generated using diversity analysis of *Fusarium* isolates infecting tomato with ISSR marker

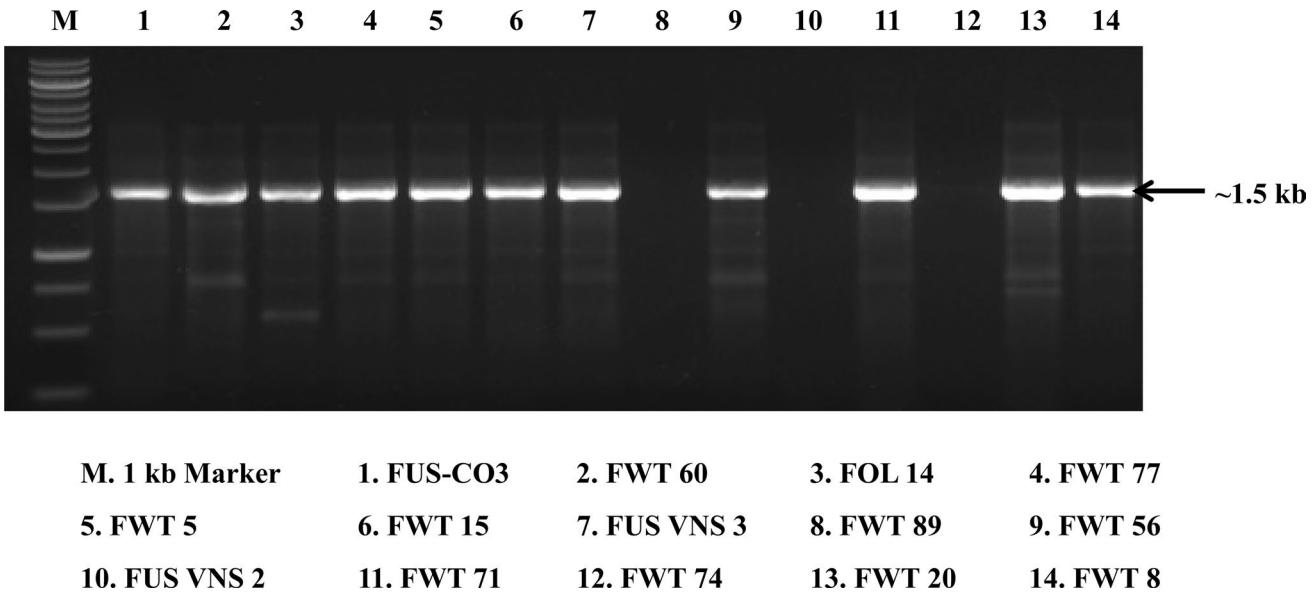

Fig. 4 PCR amplification of *Fusarium* isolates with *pg1* gene primers

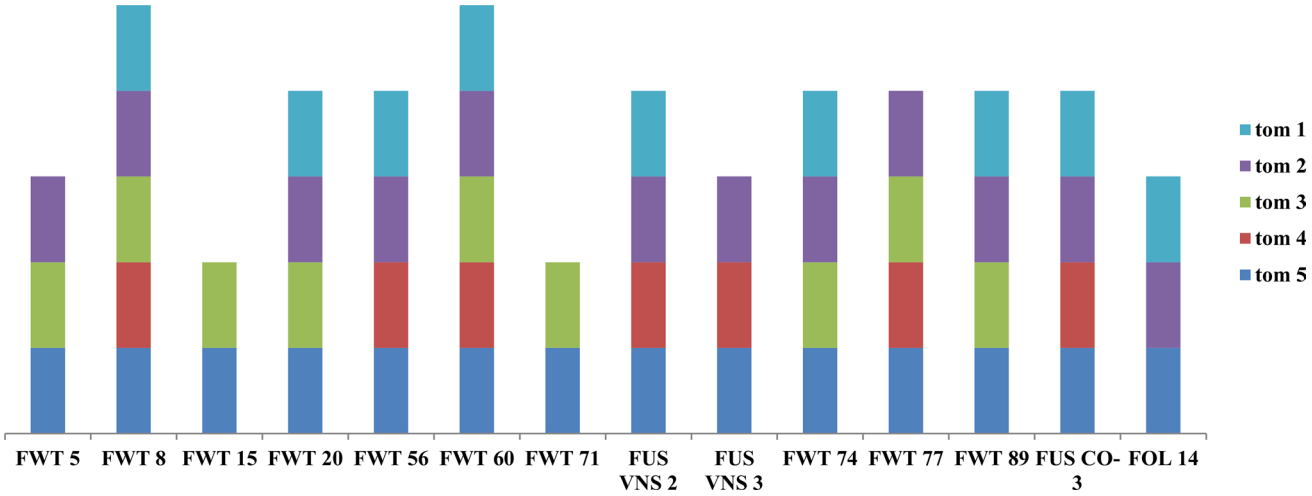

Fig. 5 Distribution of tomatinase genes among the *Fusarium* isolates infecting tomato

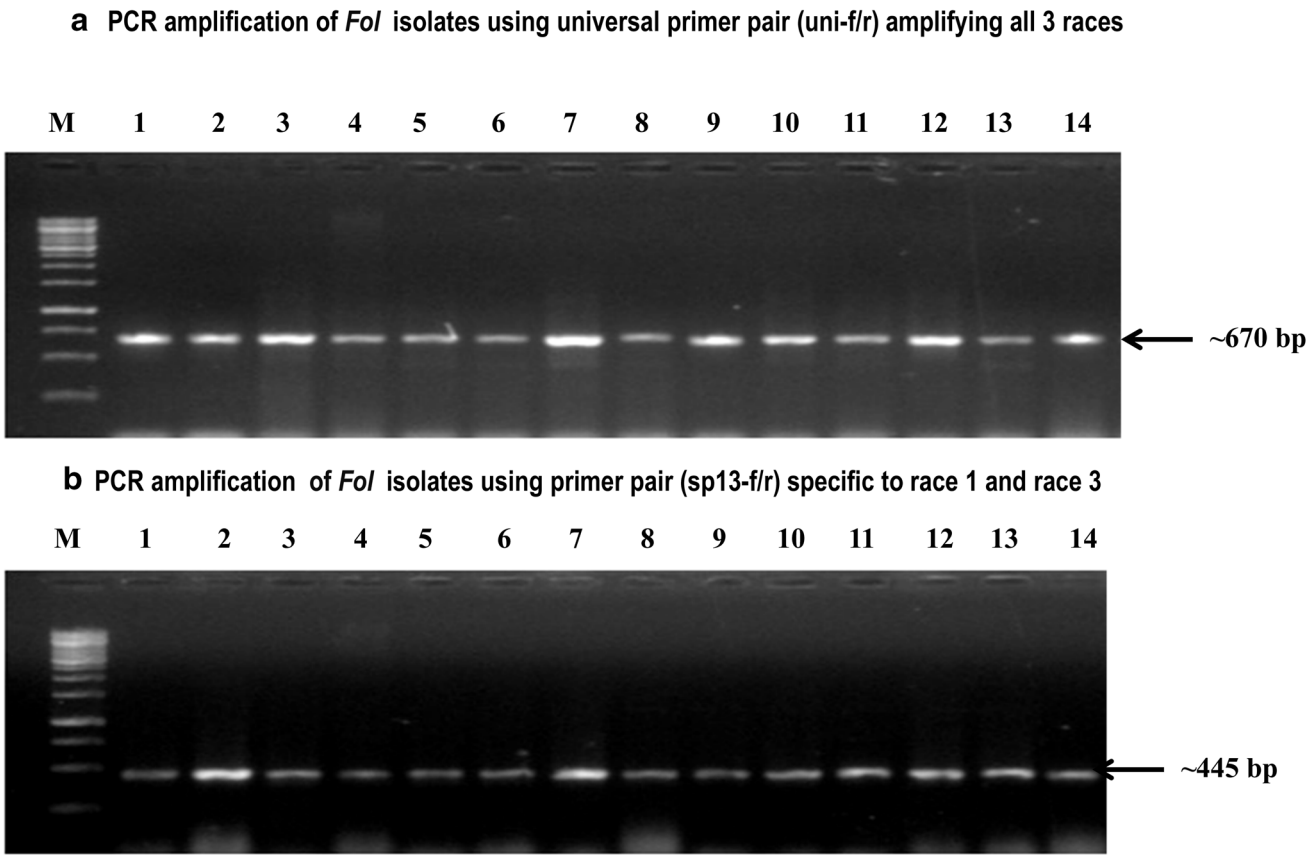

Fig. 6 **a** PCR amplification of *Fol* isolates using universal primer pair (uni-F/R) amplifying all 3 races. **b** PCR amplification of *Fol* isolates using primer pair (sp13-F/R) specific to race 1 and race 3

tree was constructed based on the ITS region. Evolutionary relationship of these *Fol* isolates with the other *formae speciales* was clear in the phylogenetic analysis. Several studies described the *Fol* and other *formae speciales* are not monophyletic in nature (O'Donnell et al.1998; Cai et al. 2003; Nirmaladevi et al. 2016; Srinivas et al. 2019). Similarly, the present study also confirms that *Fol* is polyphyletic in nature as some isolates of *Fol* were grouped together with other *Fol* isolates reported earlier infecting tomato, whereas remaining isolates were forming in another cluster with *Fol* and other *F. oxysporum.*

Fol produces group of pectinolytic enzymes contributing towards the penetration and colonization inside the host plant. The production of polygalacturonase by pathogenic fungi is critical for their success and survival during host infection. Literature showed that loss of *pg*1 activity will be resulting in the decrease of the pathogenicity of the pathogen (Liu et al. 2017). In case of *Botrytis cinerea,* the disruption of endo-polygalacturonase genes *Bcpg1* or *Bcpg2* results in its reduced virulence on different hosts (Have et al. 1998). Similarly, it was showed that *pg1* is required for infection by *Phytophthora capsici* and *Alternaria citri* (Isshiki et al. 2001; Wen et al. 2009). However, some studies also showed that disruption of few polygalacturonase genes (*PG1*, *PG5*, *PGX4*) in fungi did not directly affect virulence in *Fusarium oxysporum* and led to no virulence difference in tomato (Pietro et al. 2003; García-Maceiraet al. 2000). Similarly, in our study also no correlation could be made between *pg1* and virulence among *Fol* isolates as in one case *pg1* was absent in a high-virulent isolate (FUS VNS2), while a less-virulent strain (FOL14) was having it (Supplementary Table 1) which indicated besides *pg1* activity other mechanisms might also be involved in virulence.

Role of tomatinase enzymes in the virulence of *Fol* has been demonstrated by several studies. Over-expressed tomatinase gene (*tom*1) constitutively resulted in increased symptom development. Upon disruption of *tom*1, tomatinase activity reduced only by 25% revealing the existence of additional putative tomatinase genes (Pareja-Jaime et al. 2008). In the present study, we detected five tomatinase genes in 14 different *Fol* isolates through PCR assay using specific primer pairs. Among these, *tom5* was found to be present in all the isolates irrespective of their virulence status (Supplementary Table 1). Tomatinases *tom1* and *tom4* are having more influence on the pathogen virulence. Here, the isolates harboring *tom4* gene were found to be highly virulent. Even though FWT 20, FWT 74 and FWT 89 were harboring all four tomatinase genes except *tom4*, they were categorized under moderately virulent strains. This shows that *tom4* might be contributing more towards the virulence. Moreover, additive effect of different genes can also be observed from this study, i.e., isolates having fewer genes were found to be least virulent than the isolates with more number of genes. This needs further investigation at the expression level of genes.

As of now, there is no study depicting the effect of fumonisin produced by the *F. oxysporum* f.sp. *lycopersici* in tomato plant, fruit and its effect on human upon consumption, although several authors have demonstrated the production of fumonisin by the *F. oxysporum* (Seo et al. 1996; Proctor et al. 2008; Das et al. 2012; Nirmaladevi et al. 2016). Similarly, six fumonisin toxin-producing strains of *F. oxysporum* f.sp. *cubense* infecting banana were identified through PCR assay, which are targeting toxin biosynthetic pathway genes in banana (Das et al. 2012). Nirmaladevi et al (2016) identified 11 toxigenic strains among 31 pathogenic strains of *Fusarium oxysporum* infecting tomato which have potential to produce fumonisin mycotoxin through PCR-based assay. In the present study, fumonisin gene was detected in six isolates of *Fol* indicating that they have the potential to produce fumonisin.

Race identification through both race-specific PCR and bioassay indicated that all the isolates collected from different parts of India were belonging to race 1. This study will help the breeders to concentrate more on developing tomato lines or genotypes resistance against race 1 of *Fol* in India.

Acknowledgment The work was carried out with the financial support of Indian Council of Agricultural Research (ICAR) network project on " ICAR-Out reach programme on Phytophthora, Fusarium and Ralstonia wilt (otherwise called as PHYTOFURA)" operated from ICAR-Indian Institute of Spices Research, Calicut, India.

Author contributions LM, NK and VV have conceptualized study, performed experiments and wrote manuscript; AKM and BKS maintained culture and inoculums required for experiments; SS and ABR critically revised manuscript and facilitated for conducting experiments; all the authors gone through the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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