Mycopath (2018) 16(2): 101-110 **Research Article**

Morpho-molecular characterization and pathogenicity of *Fusarium* species causing fruit rot of bell pepper

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Abstract

Fusarium fruit rot of bell pepper was found prevalent 100% in all the visited greenhouses and open fields during the month of February (greenhouses) and May (open fields) 2016-2017 in Attock, 10.5% to 18.3% with the average 13.63%. However, the disease incidence in open fields varied from 7.3% to 15% with the average of 12.04%. Standard protocol was followed for the isolation of pathogen from symptomatic fruit samples collected from the study area. Single-spore isolation technique was employed for obtaining pure culture of each *Fusarium* isolates. Based on morphological characteristics *viz.* colony color and appearance, phialides, and shapes and dimensions of conidia (micro-conidia, meso-conidia, macro-conidia and chlamydospores), twenty-nine isolates (72.5%) were confirmed as *Fusarium incarnatum*, whereas the remaining 11 isolates (27.5%) were confirmed as *Fusarium proliferatum*. Pathogenic nature of all the isolates was evaluated on the artificially inoculated bell pepper fruit following 0–5disease rating scale and percent disease severity index (DSI). All the isolates were able to cause disease symptoms, however10 isolates with 93-100% DSI were ranked as highly virulent. Translation elongation factor (EF-1α) gene of these isolates belonging to both species was sequenced, submitted to GenBank (for accession numbers) and grouped into separate clades (*F. incarnatum* and *F. proliferatum*) employing Mega7 software.

Keywords: Bell pepper, EF-1α, fruit rot, *Fusarium incarnatum*, *Fusarium proliferatum*.

Introduction

Bell pepper (*Capsicum annuum* L.) is a high value cash crop worldwide and generally cultivated in greenhouses and open fields. Many biotic and abiotic factors cause significant reduction in yield. Among biotic factors; *Fusarium* fruit rot is one of the most prevalent and serious constraint. Since 2000, the internal fruit rot of bell pepper has emerged as an important disease worldwide (Frans *et al*., 2017). The disease affects bell pepper grown in both greenhouses and open fields (Kline and Wyenandt, 2014). Infection initiates when spore of the pathogen enters the flower stigma via air or insect vector such as bumblebees and pollinating bees (Kharbanda *et al*., 2006).

Typically, internal fruit rot symptoms included the appearance of whitish-gray hyphal growth on the seeds, placenta and inner fruit wall. The external symptoms on outer surface of fruit occur as greenish to dark brown lesions only in severe infection. The infected fruits generally show rare or few external disease symptoms such as sunken lesions and fruits may not be discarded before transport to market, they might be purchased and consumed (Yang *et al*., 2010). Annual yield losses reported from diverse global positions *viz.* northern hemisphere regions (Canada, Belgium and United Kingdom)between 5% to 50% (Utkhede and Mathur, 2003; Yang *et al*., 2010; Frans *et al*., 2017) Mid-Atlantic regions of USA between 1% and 50% (Kline and Wyenandt, 2014), tropical marine region (Trinidad) between 20% to 40% (Ramdial *et al*., 2016b) and the losses insub-tropic region of Chakwal district, Pakistan was estimated as 11% (Tariq *et al*., 2018).

Bell pepper is grown round the year in green houses and open fields in Pothohar plateau (Rawalpindi division and Islamabad) depending upon the availability of water. The crop is suffering from many diseases including *Fusarium* fruit rot and causes considerable losses in the yield. No detailed study was reported from Pothohar region, Punjab, Pakistan, prior to this work. Consequently, there was a dire need to conduct a comprehensive study for documenting prevalence and incidence of *Fusarium* fruit rot, which is prerequisite for the management of the disease. Keeping in view the fact, the objectives of the present study were to identify *Fusarium* species causing internal fruit rot on bell pepper in Rawalpindi division and Islamabad, Pakistan. Symptomatology and morphological approaches were used to identify the pathogen. Moreover, the pathogenic behavior of all isolates was recorded by artificial inoculation on bell pepper fruits. Symptomology and conventional identification tools are not reliable criteria for the confirmation of the exact species of the pathogen, which is imperative for the disease diagnosis and management. Therefore, molecular tools were also employed in this study for the confirmation of pathogenic species.

Materials and Methods

Survey for collection of diseased fruits

A total of 40 samples of bell pepper with symptoms of *Fusarium* fruit rot were collected during growing seasons from 2016 to 2017 in several localities of four districts of Rawalpindi division (Attock, Chakwal, Jhelum and Rawalpindi district) and Islamabad. The survey and sampling was done in ×+ manner at crop maturity stage in greenhouse during February 2016 and 2017. In open fields, the survey and sampling were carried out in May 2016 and 2017.

Percentage prevalence and disease incidence was calculated following formula;

$$\text{Disease prevalence (\%)} = \frac{\text{Locations showing fruit rot symptoms}}{\text{Total locations observed}} \times 100$$

$$\text{Disease incidence (\%)} = \frac{\text{No. of fruit infected}}{\text{Total no. of fruit assessed}} \times 100$$

Isolation of Pathogen

Five 5×5 mm^2 pieces were taken from the symptomatic fruit rot tissue, surface-disinfected by dipping in sodium hypochlorite solution (1%) for 2–3 min, washed with sterilized distilled water (SDW) three times and dried over folds of sterilized filter paper. The tissues were then aseptically plated on potato dextrose agar (PDA) and incubated at 25±1 °C for five days with 12 hours alternate light and dark cycles. The hyphae developing from the edges of growing colonies were transferred aseptically to PDA plates. Single-spore isolates of each *Fusarium* isolate were obtained.

Morphological characterization

The isolated fungi causing *Fusarium* fruit rot was characterized based on morphological studies and compared with taxonomic keys documented in literature (Leslie and Summerell, 2006). The colony characters *viz.* diameter, color, reverse color, texture and presence of sporodochia was visually noted. Conidial suspension was prepared by flooding the pure cultures in 5 mL SDW. The suspension was mixed in lactophenol cotton blue and observed under microscope (Nikon YS-100). The microscopic characters *viz.* phialides, shapes and dimensions of conidia (micro-conidia, meso-conidia, macro-conidia and chlamydospores) were noted.

Pathogenicity test

The isolates from all locations and morphologies were chosen for the pathogenicity test. The fruit of "Yolo wonder" bell pepper was artificially inoculated. The isolates were cultured on PDA at 25±1 ºC under continuous fluorescent light. Hyphae from 7 days pure cultures were harvested, mixed in 5–7 mL SDW to dislodge the conidia and filtered through muslin cloth. The fruits were surface-disinfested with 1% sodium hypochlorite for 1–2 min, washed with SDW and subsequently placed on sterile filter paper for drying. Three healthy bell pepper fruits per isolate was inoculated with 20µL drop of 1×10^6 spore mL^{-1} adjusted with haemocytometer and incubated at 25±1 ºC for 5 days. Three control fruits per isolate were inoculated with 20µL droplet of SDW. The size of fruit and lesion area was visually calculated (Pakdeevaraporn *et al*., 2005).

The disease severity index for *Fusarium* fruit rot was evaluated according to the development of 0–5 visual disease rating scale (Table 1, Fig. 1). The virulence of the isolates was further evaluated according to DSI (%). Disease severity index was calculated according to the formula:

$$\text{DSI (\%)} = \frac{\text{Sum of all disease ratings}}{\text{Total number of rating} \times \text{Maximum disease grade}} \times 100$$

PCR amplification and sequencing

Based on pathogenicity test, the isolates showing 93–100% DSI value were found highly virulent as shown in Table 2 and further selected for genomic DNA extraction using the phenol chloroform isoamyl alcohol (Raeder and Broda, 1985). Amplification of the translation elongation factor (EF-1α) gene was done using EF1 and EF2 primers (O'Donnell *et al*., 1998b). PCR reactions were conducted in 25 µL volumes consisting of 50 ngμL^{-1} of DNA, 2x Master mix (New England BioLabs, Maine) and 9.5 µL ddH_2O. PCR was performed in a C1000 Touch^{TM} thermal cycler (Bio-Rad Laboratories) as fallows: initial denaturing step at 95 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing for 1 min at 53 °C, extension of 1 min at 72 °C, and a final extension step for 10 min at 72 °C. PCR products were purified with ExoSAP-IT (Affymetrix, California) and sequenced by Genscript Inc. (New Jersey). The consensus sequences of both forward and reverse directions were obtained using the clustal omega (Sievers and Higgins, 2014). The sequences derived from this work were submitted in the GenBank and accession numbers were obtained. Reference sequences of the EF-1α were accessed from GenBank database. The phylogenetic tree construction was performed using the maximum parsimony (MP) based on tree bisection regrafting (TBR) (Nei and Kumar, 2000). The phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis software (MEGA7).

Results and Discussion

Symptomatology and survey

Symptoms caused by *Fusarium* on outer surface of fruit appeared as discolored, greenish to light brown, water-soaked lesions covered with a white-light gray mold more common upon calyx. Typically, *Fusarium* fruit rot symptoms included the appearance of whitish-gray hyphal growth on seeds,

placenta and inner fruit wall (Fig. 2).

The mean disease prevalence of *Fusarium* fruit rot was found 100% in visited greenhouse (January 2016 and 2017) and open fields (May 2016 and 2017). However, significant variation in mean incidence was observed at various localities. Based upon two years mean data in greenhouse (Fig. 3), the highest mean incidence of *Fusarium* fruit rot was recorded in tehsil Chakwal (18.3%), followed by Gujar Khan (15%) and Rawalpindi (12.3%). Whereas, the lowest incidence was recorded in Islamabad (10.5%). The mean incidence for *Fusarium* fruit rot in open fields was the maximum in Chakwal (15%) followed by Rawalpindi (14.1%) and Jhelum (12.8%). The lowest incidence was in tehsil Taxila (7.3%) (Fig. 4).

Morphological characterization

Colonies of *F. incarnatum* on PDA were white to light beige, reverse beige or pale cream, reaching a maximum of 83mm diameter in 7 days at 25±1 ºC. Aerial mycelium white, medium fluffy to fluffy. Sporodochia were light orange in color and obvious in colonies of six isolates only. Microscopic examinations showed the presence of hyaline and septate hyphae. The isolates produced monophialidic and polyphialidic conidiophores. Microconidia were hyaline, obovate or pyriform and 0–1 septate, and 3.8–4.5×3.1–3.9 µm. Mesoconidia were hyaline, fusoid, spindle-shaped, usually 3 septate and 11.3–14.4×3.6–4.3 µm. Macroconidia were hyaline, cylindrical, slightly curved, tapered at the apex, 3-5 septate and 29.7–34.3×3.8–4.5 µm (Fig. 5).

Colonies of *F. proliferatum* on PDA were initially white and becoming tinged in purple gray, reverse dark purple, reaching a maximum of 79 mm diameter in 7 days at 25±1 ºC. Aerial hyphae were light gray and medium fluffy. Sporodochia were not obvious on culture plate. Hyphae were hyaline and septate. In this study the isolates produced monophialidic and polyphialidic conidiophores. Microconidia formed abundantly, hyaline, single-celled, oval to club-shaped and have a flattened base and 5.7–9.7×2.6–3.4 µm. Macro-conidia were hyaline, slightly sickle shaped to straight and 3-5 septate and 25.9–40.9×1.8–3.8 µm. Chlamydospores were absent in all the isolates (Fig. 6).

Pathogenicity

The isolates representative of the two colony types (*F. incarnatum* and *F. proliferatum*) showed typical symptoms of *Fusarium* rot on detached bell pepper fruit. After 5 days, white mycelial growth surrounded by water-soaked, greenish to light brown necrotic lesions appeared identical to the symptomatic fruits observed in the greenhouse and open fields. However, no symptoms were observed on control fruits. Differences in virulence of isolates was observed among the isolates. The 10 (25%) isolates *viz.* FICW3, FICW5, FICW7, FICW8, FICW10, FICW11, FICW12, FICW15, FICW16 and FICW17 were highly virulent and covered greater than 25% of the fruit area with 93–100% DSI. However, 13 (32.5%) isolates covered only 1–5% fruit area with 20–47% DSI (Table 2). The fungi isolated from artificially inoculated fruits were morphologically similar to the original isolates on PDA and fulfilling the Koch's postulates. No lesions developed in the healthy control fruits inoculated with sterile distilled water.

Molecular characterization

Amplification of the EF-1αyielded amplicons of 700 bp in length. A phylogram trees of *F. incarnatum* and *F. proliferatum* obtained with the EF gene were analyzed separately and concatenated. The reference sequences were obtained from GenBank with *Fusarium concolor* (NRRL 13459) as an out group. All gaps were treated as missing data and eliminated.

The isolates of *F. incarnatum* under study were grouped into three clades. The four isolates FICW7, FICW10, FICW11 and FICW16 in clade 1 clustering with *Fusarium incarnatum* (MLST 1-c clone spt111) with 100% bootstrap support. One isolate FICW5 in clade 3 clustering with *Fusarium incarnatum* (30-a DPGS-2011 strain FRC R10113) with 100% bootstrap support. However, FICW17 isolate in clade 2 showed the bootstrap support of 51% with clade 1 (Fig. 7). The isolates of *F. proliferatum* under study were grouped into two clades. The three isolates FICW8, FICW12 and FICW15 in clade 1 clustering with *Fusarium proliferatum* (B2) with 100% bootstrap support. One isolate FICW3 in clade 2 clustering with *Fusarium proliferatum* (CBS 131574) with 100% bootstrap support (Fig. 8). The phylogenetic tree of the individual data sets of both *Fusarium* spp. were found similar to the tree obtained from the combined alignment. All isolates of this study showed 100% bootstrap support except isolate FICW17 with low bootstrap support (Fig. 9).

Several *Fusarium* species are associated with fruit rot of bell pepper including; *Fusarium lactis* (Pirotta and Riboni), *Fusarium proliferatum* (Matsushima) Nirenberg (Utkhede and Mathur, 2003; Yang *et al*., 2009), *Fusarium verticillioides* (Saccardo) Nirenberg, *Fusarium subglutinans* (Utkhede and Mathur, 2004), *Fusarium oxysporum* Schltdl. (Van Pouckee*t al*., 2012), *Fusarium solani* (Ramdial and Rampersad, 2010) and *Fusarium incarnatum* (Desm.) Sacc. (Ramdial *et al*., 2016b; Tariq *et al*., 2018). However, our study reports the presence of two *Fusarium* species *viz. F. incarnatum* and *F. proliferatum* were found associated with the fruit rot disease of bell pepper in Pothohar region. In a previous study, *F. solani* was found responsible for postharvest deterioration of bell pepper in Karachi (Fatima *et al*., 2009).

A total of 40 *Fusarium* spp. isolates were obtained from the diseased bell pepper with fruit rot

symptoms. Twenty-nine isolates (72.5%) belonged to *F. incarnatum*, whereas the remaining 11 isolates (27.5%) belonged to *F. proliferatum*. In Trinidad and Tobago (West Indies), eighty-two isolates were identified as belonging to *Fusarium incarnatum-equiseti* species complex (FIESC) (Ramdial *et al*., 2017). In Netherlands, *F. proliferatum* was isolated from 11.1% infected fruits (Hubert *et al*., 2003). Whereas, in Alberta (Canada), *F. proliferatum* was recovered from only 5.4% infected fruits (Yang *et al*., 2009).

The survey was basically carried out depending on visual symptoms. It is anticipated that the disease incidence might be much higher than the observed and reported, as the infected fruits remains symptomless in case of mild infection and show external disease symptoms only in case of severe infection. The highest *Fusarium* root rot disease incidence in Chakwal in both greenhouse and low plastic tunnels as compared to other visited tehsils might be due to high pathogen's inoculum level accumulated by repeated cultivation of bell pepper in the same field. Moreover, it was also shared by the farmer during the survey that bell pepper is the main nursery crop grown for distribution to the adjacent areas since many years. Temperature also plays an important role in pathogen growth, susceptibility of the host and *Fusarium* fruit rot incidence (Rossi *et al*., 2009).The temperature (25-30°C) favoring the plant growth at fruiting stage is also conducive for the growth and sporulation of the pathogen and also reported by Frans *et al*. (2017).

In an *in vitro* study, the maximum growth and sporulation of FLASC (*Fusarium lactis* species complex) and *F. oxysporum* was observed at 25°C (Scott *et al*., 2010; Webb *et al*., 2015) but *F. proliferatum* showed the maximum sporulation at 30 °C (Marin *et al*., 1999; Samapundo *et al*., 2005). Moreover, *Fusarium* fruit rot may be an internal seed borne disease (Utkhede and Mathur, 2004) and in Pakistan availability of disease-free seeds to the farmers was limited. It seems, if the seeds were treated with an appropriate systemic fungicide, there would have been low or no disease.

Microscopic examinations of *F. incarnatum* showed the presence of microconidia, mesoconidia, macroconidia and chlamydospores in all the isolates. The *F. incarnatum* isolates characterized in our study showed cultural and morphological characteristics similar to those described in previous studies (Leslie and Summerell, 2006). *F. proliferatum* isolates produced microconidia and macroconidia. However, chlamydospores were absent in all the isolates. Sporodochia were not obvious on culture plate. According to Leslie and Summerell (2006) sporodochia often are uncommon or are difficult to find since they can be masked by the mycelium. *F. incarnatum* belonged to *F. incarnatum-equiseti species complex* (FIESC) and includes 30 phylogenetically diverse species (O'Donnell *et al.*, 2009, 2012). Members of FIESC causes plant diseases, human and animal health problems (Jacobs *et al*., 2018). *F. proliferatum* is a member of the *Gibberella fujikuroi* species complex (GFSC), which includes over 40 phylogenetically diverse lineages (O'Donnell *et al*., 2000). *F. proliferatum* has an extraordinary broad host range (Proctor *et al*., 2009).

The pathogenicity tests revealed that all isolates infect the bell pepper fruit and produce typical fruit rot symptoms. Apparently, there was no distinction between the symptoms produced by both species (*F. incarnatum* and *F. proliferatum*), when inoculated artificially on the fruits.

Internal transcribed spacer (ITS) region is unable to resolve *Fusarium* spp. lineages (O'Donnell *et al*., 2013). In general, protein coding genes, for example beta-tubulin (TUB), translation elongation factor (EF-1α), RNA polymerase II subunits (RPB1 and RPB2) have been recommended for *Fusarium* species confirmation (Geiser *et al*., 2004). EF-1α gene, however consists of conserved exonic and variable intronic sequences and is suitable for inferring deep phylogenies, capture more recent evolutionary and speciation events (Stielow *et al*., 2015). The EF-1α gene sequence, with similarity index of 99.4% is a suitable genetic marker for categorizing *Fusarium* species (O'Donnell *et al*., 2015). Morphological grouping of *F. incarnatum* and *F. proliferatum* were consistent with phylogenies derived from molecular data of EF-1α gene. The isolates of both species were grouped into separate clusters.

Conclusion

Fusarium fruit rot was found prevalent 100% in Pothohar plateau. The two species of *Fusarium viz. F. incarnatum* and *F. proliferatum* were associated to cause fruit rot of bell pepper in Pothohar plateau, Punjab, Pakistan. The disease causes significant reduction in yield under favorable conditions. The detailed documentation of disease will help the growers and researchers in the correct identification of disease which is prerequisite for the development of effective management strategies against the destructive disease. Further studies are required to explore the association of reported and other species of *Fusarium* associated with the fruit rot in other bell pepper growing areas of Pakistan.

Acknowledgements

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Fig. 1: Disease diagram representing degrees of infection ranging from scores of 0 to 5 (from left to right) in bell pepper fruit.

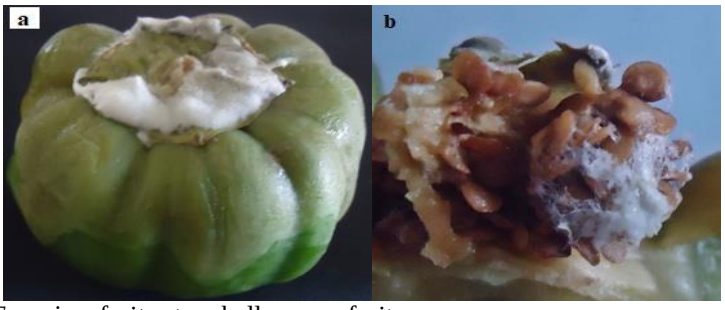

Fig. 2: Symptoms of *Fusarium* fruit rot on bell pepper fruit.

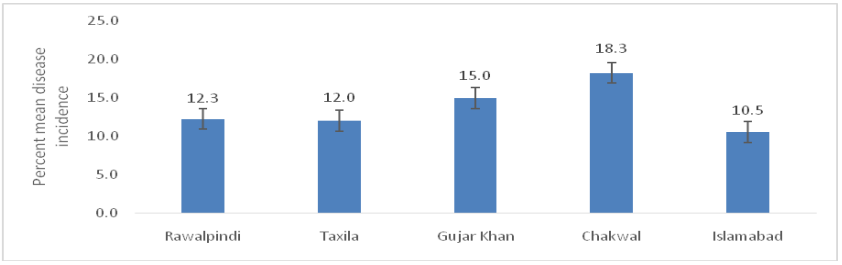

Fig. 3: Mean DI (%) of *Fusarium* fruit rot in greenhouse during January 2016 and 2017.

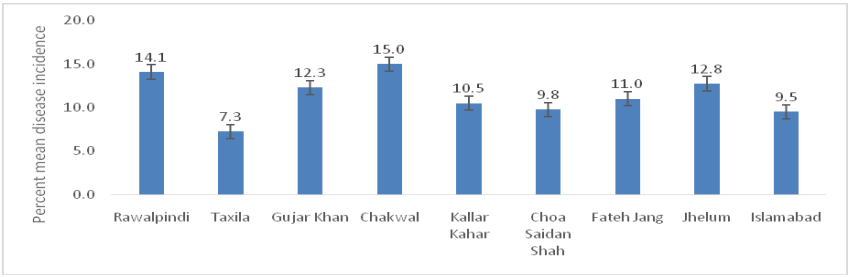

Fig. 4: Mean DI (%) of *Fusarium* fruit rot in open fields during May 2016 and 2017

Fig. 5: 1-week-old culture of *F. incarnatum* on PDA (a, b), conidia (c), phialides (d).

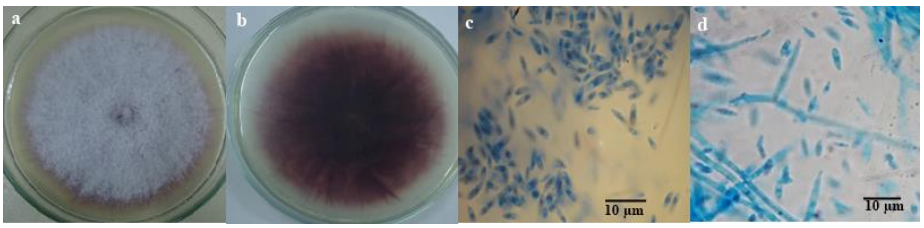

Fig. 6: 1-week-old culture of *F. proliferatum* on PDA (a, b), conidia (c), phialides (d).

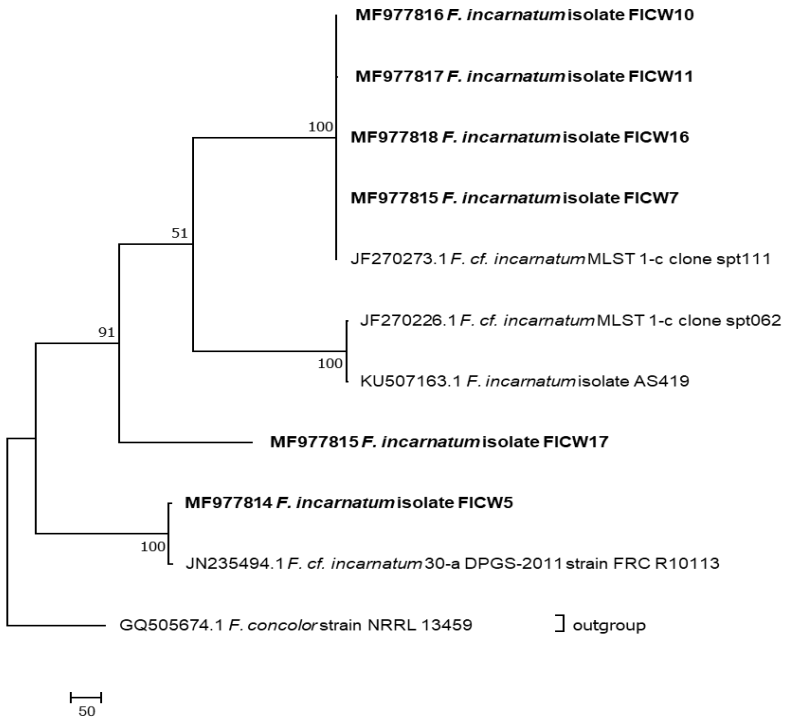

Fig. 7: Molecular phylogeny of *F. incarnatum*, generated in a maximum parsimony analysis tree inferred from the dataset containing the partial DNA sequences of EF1- *α gene*. The most parsimonious tree length was 1378, consistency index was 0.863910, the retention index was 0.877537, and the composite index was 0.762273 (0.758113) for all sites and parsimony informative sites.

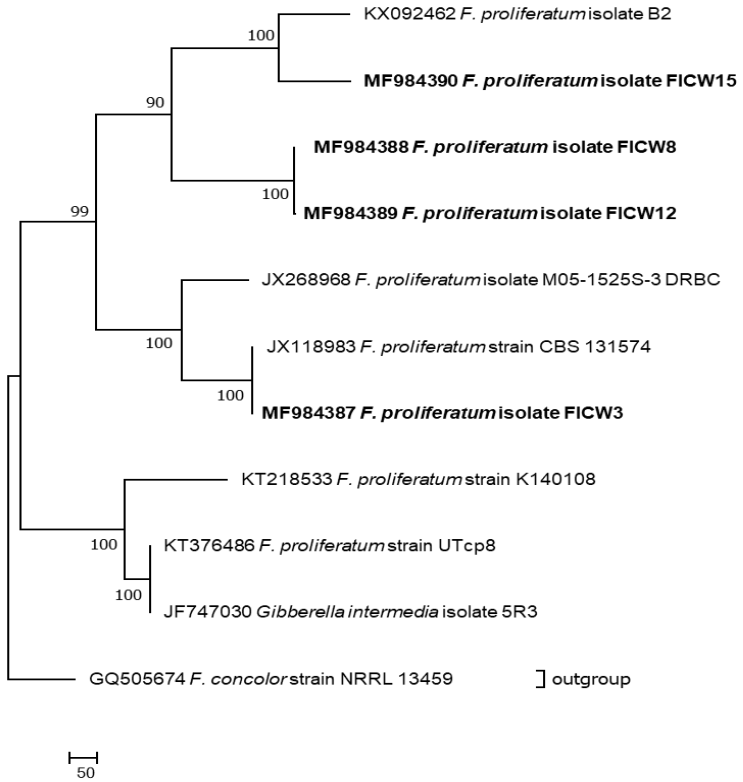

Fig. 8: Molecular phylogeny of *F. proliferatum*, generated in a maximum parsimony analysis tree inferred from the dataset containing the partial DNA sequences of EF1- *α gene*. The most parsimonious tree length was 1920, consistency index was (0.739540), the retention index was (0.761380), and the composite index was 0.563897 (0.563071) for all sites and parsimony informative sites.

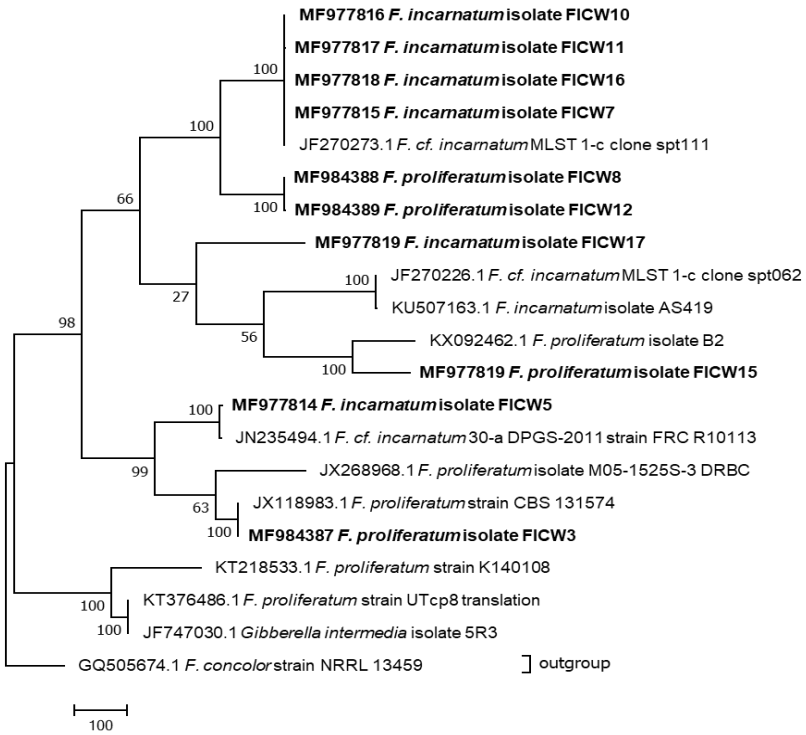

Fig. 9: Molecular phylogeny of *F. incarnatum* and *F. proliferatum*, generated in a maximum parsimony analysis tree inferred from the dataset containing the partial DNA sequences of EF1- *α gene*. The most parsimonious tree length was 2823, consistency index was (0.573858), the retention index was (0.752469), and the composite index was 0.431810 (0.431810) for all sites and parsimony informative sites.

Table 1: *Fusarium* severity scale on bell pepper fruits, symptom description 5 days after inoculation

Disease scale	Description
0	No visible lesions
1	1–2% of the fruit area covered with visible fungal outgrowth
2	3–5% of the fruit area covered with visible fungal outgrowth
3	6–15% of the fruit area covered with discolored necrotic lesions, disease symptoms with visible fungal outgrowth
4	16–25% of the fruit area covered with discolored necrotic lesions, disease symptoms with visible fungal outgrowth
5	>25% of the fruit area covered with discolored necrotic lesions, disease symptoms with visible fungal outgrowth, rotting

Table 2: Percent disease severity index (DSI)

Sr. No	Isolate	Pathogen	DSI (%)
1	FICW1	*F. incarnatum*	80.00
2	FICW2	*F. incarnatum*	60.00
3	FICW3	*F. proliferatum*	93.33
4	FICW4	*F. incarnatum*	46.66
5	FICW5	*F. incarnatum*	100.00
6	FICW6	*F. incarnatum*	60.00
7	FICW7	*F. incarnatum*	93.33
8	FICW8	*F. proliferatum*	100.00
9	FICW9	*F. incarnatum*	60.00
10	FICW10	*F. incarnatum*	100.00

11	FICW11	*F. incarnatum*	100.00
12	FICW12	*F. proliferatum*	100.00
13	FICW13	*F. proliferatum*	40.00
14	FICW14	*F. incarnatum*	46.66
15	FICW15	*F. proliferatum*	100.00
16	FICW16	*F. incarnatum*	100.00
17	FICW17	*F. incarnatum*	100.00
18	FICW18	*F. incarnatum*	20.00
19	FICW19	*F. incarnatum*	46.66
20	FICW20	*F. proliferatum*	33.33
21	FICW21	*F. incarnatum*	73.33
22	FICW22	*F. proliferatum*	20.00
23	FICW23	*F. proliferatum*	26.66
24	FICW24	*F. incarnatum*	80.00
25	FICW25	*F. incarnatum*	80.00
26	FICW26	*F. proliferatum*	46.66
27	FICW27	*F. proliferatum*	26.66
28	FICW28	*F. proliferatum*	73.33
29	FICW29	*F. incarnatum*	60.00
30	FICW30	*F. incarnatum*	73.33
31	FICW31	*F. incarnatum*	86.66
32	FICW32	*F. incarnatum*	33.33
33	FICW33	*F. incarnatum*	60.00
34	FICW34	*F. incarnatum*	33.33
35	FICW35	*F. incarnatum*	73.33
36	FICW36	*F. incarnatum*	26.66
37	FICW37	*F. incarnatum*	40.00
38	FICW38	*F. incarnatum*	60.00
39	FICW39	*F. incarnatum*	60.00
40	FICW40	*F. incarnatum*	33.33

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