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# UNVEILING THE *Fusarium proliferatum* ASSOCIATION WITH FICUS DIEBACK FROM PUNJAB, PAKISTAN

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Several fungal plant pathogens are posing serious threat to the *Ficus* spp. grown as an ornamental plant for landscaping world over. Dieback disease is one of the major limiting factors in *Ficus* spp selection and cultivation as an ornamental plant. Typical Dieback disease symptoms were recorded on Ficus plantation from different commercial ornamental plant growing nurseries and several other landscape areas of Punjab, Pakistan. A prospective etiological study was planned and conducted using certain basic and molecular methods. Frequently associated *Fusarium* spp. were tested for pathogenicity confirmation using Koch's postulates *on Ficus benjamina* L. Among these tested fungal isolates, FMB-45-Fi was proved as principal cause of dieback on *Ficus* species. The pathogenic isolate was subjected to molecular characterization by exploring three genomic regions (ITS, TEF1-α and RPBII). Molecular data validated the pathogenic isolate as *Fusarium proliferatum*. Here, we documented *Fusarium proliferatum* as causative agent of dieback in Ficus spp. in Punjab Pakistan and it is a first record to the best of our knowledge.

**Keywords**: Ficus spp., Decline, *Fusarium proliferatum*, Fungal infection, Etiology.

## INTRODUCTION

*Ficus* spp. as an ornamental component of landscape have great attraction due to their beautiful foliage, cultivated in tropical and subtropical regions, belong to *Moraceae* family, having 40 genera and more than 800 species. (Neal, 1965; Rahman *et al.*, 2013). Mostly these plants are evergreen, while few species are deciduous. Plants of *Ficus* spp are trees, climbers, shrubs, liana and few are herbs. Few of *Fiscus* species are shrubs while most of them are ornamental plants (Henley and Poole, 1989). Ficus species found in Pakistan include Ficus elastica, F. marcophylla (Banyan), F. benghalensis L. (Barghad), F. lyrata, F. nitida, F. religiosa L. (Peepal), *F. rumphii* Blume (Pilkan), *F. racemosa* L. (Gular), (Sheikh, 1993; Hussain *et al*., 2011 Samia *et al.*, 2016). One of the most elegant and beautiful species of *Ficus* is *F. benjamina*. (Neal, 1965).

Several plant pathogens posing threat to the *Ficus* like Crown gall, Myrothecium leaf spot, Xanthomonas leaf spots, Pseudokmonas leaf spot, Corynespora leaf spot, Wilt, Anthracnose, Dieback, Southern blight, Rhizoctonia root rot, Pythium and Phytophthora infections (Norman and Ali, 2018). Among these diseases, dieback is major threat to *Ficus* spp. Benschop *et al*., 1984 reported death of various dieback disease affected plants of *F. benjamina* caused by *Phomopsis cinerescens*. Similar disease on *F. caria* and *F. benjamina* caused by *P. cinerescens* was recorded by ( Ellett, 1979; Hudler, 1979; Anderson and Hartman 1983). In south-eastern Iran, *Nattrassia mangiferae* was proved as pathogen causing trunk canker and dieback on *F. religiosa.* (Mirzaee *et al*., 2002). The branch canker and dieback caused by *Botryodiplodia theobromae* and *Phomopsis* species of trees in tropical and subtropical forests in Egypt have been recorded (Mansour, 1986; Attia and Saber, 1995; Atia *et al*., 2003; Haggag, 2010; Kamhawy, 2011; Ismail *et al*., 2012). Twig dieback in *Ficus benjamina* in Tocantins and Minas Gerais, Brazil, induced by *P. cinerescens* was recoreded first time in 2005 (Lima *et al*., 2005).

So far, there is no official data available on dieback disease (incidence and severity) assessment and etiology on *Ficus* species in Pakistan. Keeping in view the seriousness of dieback issue as an emerging and rapidly spreading disease on *Ficus*, present study was planned and accomplished with the objective, to determine the cause, owing to their probable assistance in discovering the etiology by using certain basic and molecular methods for detection of pathogens associated with die back of *Ficus*.

## MATERIALS AND METHODS

***Isolation of fungal isolates associated with diseased samples*:** For this study, surveys of Punjab were conducted during 2016-17 to document the disease data regarding severity and incidence of dieback/declining of perennial

ornamental plants by the research team of Fungal Molecular Biology (FMB) Laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad under the Higher Education Commission funded project #2762 entitled "Etiology and integrated management of declining perennial ornamental plants". Infected and healthy portion of roots and stem collected from different surveyed areas of Punjab, were cut into 2-4 mm small pieces and washed by using tap water for removal of contaminants. These pieces were rinsed with distilled water and then dipped in 1% bleach for 30 seconds, followed by washing with distilled water, thrice for surface disinfection. Surface sterilized pieces were dried by gentle tapping with paper towel. Potato dextrose agar medium (PDA) was prepared (20 g Glucose+250 ml of freshly prepared potato starch+20 g agar agar+1000 ml distilled water), autoclaved (at 121ºC, 15 psi) and then let it to cool. Antibiotic streptomycin was added to PDA medium to avoid bacterial contamination. Four surface sterilized pieces were placed on culture regime under axenic conditions. Culture plates were incubated at 26±2°C under 12 hours alternate light and dark period.

***Purification*:** Purification was done by single hyphal tip technique (Goh, 1999; Aboul-Nasr and Rahman, 2014) and single spore technique (Choi *et al*., 1999) to get pure fungal cultures.

***Morphological characterization of fungal isolates associated with samples*:** Morphological characterization of fungal isolates was done (Barnett, 1972; Webster, 1980 Leslie and Summerell, 2006). Macroscopic characters of fungal isolates, colony pigmentation and colony diameter were observed on PDA medium. Microscopic characters of fungal isolates were observed and recorded using CXR3 LABOMED, USA microscope.

Frequency of the mycoflora associated with processed samples was recorded (Nelson *et al*., 1983). Purified cultures were preserved on solid (agar) slants at 4°C temperature and dry filter paper technique at -86°C in Fungal Molecular Biology Culture Collection, University of Agriculture, Faisalabad (FMB-CC-UAF), Pakistan for further experimentation (Fong *et al*., 2000).

***Pathogenicity test*:** One representative isolate from each group was randomly selected for completion of Koch's postulates for pathogenicity confirmation. Pathogenicity of each isolate was completed on four selected *Ficus* species including *F. benjamina*, *F. nitida*, *F. lyrata* and *F. elastic.*

***Fungal Inoculum preparation*:** A bit from 7 days old culture was transferred to Potato dextrose broth (PDB) and incubated at 26±2°C on shaker incubator for 7 days. Spore counting was done and spore suspension of $1\times10^6$ spores per millilitre was made, using Haemocytometer.

***Seedlings preparation*:** One-year old healthy *Ficus* species seedlings (*F. benjamina*, *F. nitida*, *F. elastic* and *F. lyrata*) were collected from Institute of Horticultural Sciences (IHS), UAF and established in Fungal Molecular Biology (FMB) greenhouse University of Agriculture, Faisalabad (UAF), Pakistan.

***Inoculation of seedlings*:** Pathogenicity of *Fusarium* isolates were assessed on one-year old healthy selected *Ficus* species viz. *F. lyrata*, *F. benjamina*, *F. elastica* and *F. nitida.* Healthy plant roots were dipped in *Fusarium* species inoculum for 1 hour and transplanted in sterilized earthen pots (30 cm diameter) and sterilized soil. Three pots each containing single host plant were used for pathogenicity test. Control treatment was arranged by transplanting healthy *Ficus* plants in sterilized soils in triplicates in the greenhouse of Department of Plant Pathology, University of Agriculture, Faisalabad. Temperature was maintained at 25 ± 2ºC with 70% relative humidity, and 14 h photoperiod under the growth room of Plant Pathology Department. Randomized Complete Block Design (RCBD) with three replicates was used to carry out the experiment. The experiment was repeated twice. Plants were carefully observed on weekly basis for assessment of disease development.

***Pathogenicity assessment*:** Disease severity was assessed on weekly basis by visual observation (development of symptoms). Re-isolation of *Fusarium* isolates was done after 8 weeks days (as symptoms appeared) based on the scale (Mukhtar *et al*., 2014).

***Molecular characterization of Fusarium isolate proved as pathogen of dieback disease***

***Molecular Taxonomy*:** Taxonomic identification of fungal isolate was performed at molecular level. Three genomic regions, internal transcribed spacer (ITS) region, partial region of translation elongation factor 1-alpha (TEF1-α) and RNA polymerase II second largest subunit (RPBII) were explored in this study for accurate identification of the fungal isolate. The procedure adopted for molecular taxonomy of studied fungal isolate is given in Table: 1 below.

***Fungal DNA extraction*:** Total genomic DNA of fungal isolate, FMB-45-Fi was isolated by using the protocol described by Plattner *et al*. (2009). Fungal isolate was cultured on potato dextrose broth for fresh mycelial growth. Mycelia were harvested by centrifuging the culture at maximum speed (13200 rpm) for 10 minutes. Harvested mycelia were grind into fine powdery form in liquid nitrogen. Then pre-heated CTAB solution (CTAB, NaCl, 100 mM Tris HCl, 20mM EDTA) were added to it with gentle grinding followed by incubation at 65°C in water bath. The complete protocol adopted for fungal DNA extraction is given in appendix III. The dried pellet was re-suspended into 1XTE buffer (1M Tris HCl, 0.5M EDTA, $d_3H_2O$). The quantity and quality in terms of concentration and integrity of isolated DNA was examined by UV visible NANODROP (8000 Spectrophotometer, Thermo SCIENTIFIC) and gel electrophoresis (using 1% agarose gel) respectively.

***PCR analysis*:** PCR analysis was performed to amplify the partial genomic regions of taxonomically important loci using gene specific primers given in the table 1. PCR analysis was

carried out in Veriti™ 96 wells thermocycler of Applied Biosystems with reaction volume 25µl. The reaction mixture constituted of Phusion High Fidelity PCR master Mix (Thermofisher), primer pair, template DNA and $d_3H_2O$. The thermal profile for amplifying each locus was optimized using gradient PCR.

**Table 1. Primers of ITS, TEF1-α and RPBII loci used in this study for molecular taxonomy**.

| Sr. | Genomic region | Primer pair | References |
|---|---|---|---|
| 1 | ITS | ITS1-F/ITS4 | White *et al*., 1990 |
| 2 | TEF1-α | EF-1/EF-2 | O'Donnell *et al*., 1998 |
| 3 | RPBII | fRPB2-cF/fRPB2-11aR | Liu *et al*., 1999 |

***Elution of DNA fragment*:** The amplicon of required size for each genomic region was eluted using Gel purification kit (FavorPrep, Favorgen Biotech Corp.) by following the protocol provided by manufacturer. The products of PCR were resolved on 2% agarose high-resolution gel (ACTGene) along DNA ladder through gel electrophoresis. The gel was excised at the place of required amplicon from its brink. The gel slice was treated with gel extraction kit to elute DNA fragment for sequencing (Eurofins Genomics DNA sequencing services), USA.

***Phylogenetic analysis*:** Generated sequences were trimmed and aligned using bioinformatics tools, Gblocks server. Homology of each high-quality trimmed sequence was searched using BLASTn. Phylogeny was determined using neighbour joining method by using MEGA 7 software package (Kumar *et al*., 2016). Sequences were lodged into the NCBI (National Center for Biotechnology Information) database to get GenBank accession numbers.

## RESULTS

***Morphological characterization of Fusarium isolates associated with dieback samples*:** *F. oxysporum produced* Macroconidia, short to medium, straight to slightly curved comparatively slender and thin walled white to pale in colour having rare or non-existent sporodochia with usually 3 septation. Microconidia were oval, or kidney shaped without septation having abundant aerial mycelium. *F. proliferatum* produced whitish aerial mycelium on PDA medium. Macroconidia (sporodochia) were slender, thin-walled comparatively straight having pale orange to tan in color and 3-5 septation. Microconidia were rare having flattened base-shaped without septations. *F. solani* produced white to sparse cream mycelium on PDA medium.

***Pathogenicity of isolated Fusarium species over Ficus species*:** Vein clearing and marginal necrosis was observed on younger leaves and yellowing on older leaves. Latterly, drooping and wilting was observed. *Ficus benjamina* plants showed severe symptoms compared to other species. To fulfill Koch's postulates, re-isolation was done from artificially inoculated plants after 8 weeks. The fungus *F. proliferatum* was isolated frequently as a result. Among all *Fusarium* species were isolated, only *F. proliferatum* started secondary cycles forming macro conidia. Results indicated that other isolates like *F. oxysporum* and *F. solani* might have colonized the host roots as saprophytic fungi. They might have performed a synergistic role for the symptom development. But they were not found in direct association with *Ficus* dieback.

***Molecular characterization of Fusarium isolate proved as pathogen of dieback disease*:** Considering the cryptic nature of *Fusarium sp*ecies, molecular characterization was also performed by direct sequencing the putative DNA barcodes for *Fusarium* to unravel its phylogenetic place in the complex frame of evolution.

In this study, three genomic regions (ITS, TEF1-α and RPBII) were explored for the identification of investigated fungal isolates, FMB-45-Fi. The PCR analysis was performed and DNA fragments of ~684 bp ITS, ~710 bp TEF1 and ~970 bp RPBII were successfully amplified (Fig. 1).

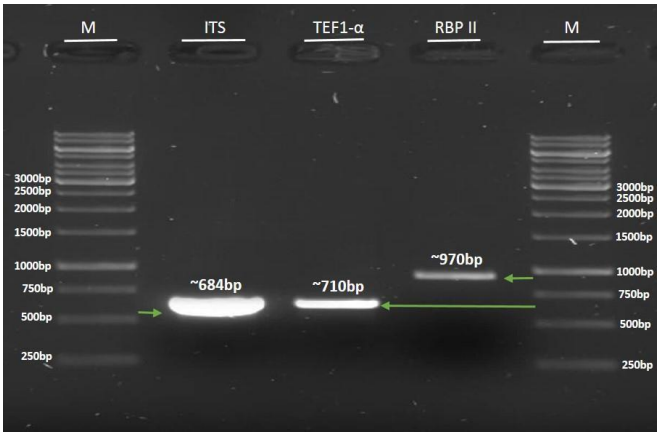


**Figure 1. PCR analysis using ITS, TEF1-α and RPBII based primer pairs yielding amplicons of required sizes. M=1Kb DNA ladder.**

The amplicons of required sizes respective to each primer pair were eluted from gel and were then direct sequenced Eurofins Genomics DNA sequencing services. The generated sequences were trimmed to get high quality sequence using BioEdit v.7.2.6.1 and were queried against NCBI, *Fusarium* MLST and FUSARIUM-ID databases, which showed their homology to *F. proliferatum*. For further validation regarding the status of investigated fungal isolate, the phylograms were constructed to unravel its place in phylogenetic hierarchy.

The generated sequences were supplemented with the sequences available on databases and literature to make individual gene dataset. The dataset of each locus was aligned by ClustalW, a multiple sequence alignment tool and

phylogenetic tree was constructed by Neighbor joining (NJ) method using MEGA 7 software. The phylogram of each locus revealed the position of FMB-45-Fi close to CBS strains of *F. proliferatum*. In each phylogram (Fig 2, Fig 3, Fig 4) this fungal isolate was nested close to *F. proliferatum*. FMB-45-Fi isolate was revealed to be closed to CBS strain 138981 of *F. proliferatum* with 100% bootstrap support (Figure 3) in TEF1-α data set based phylogenetic tree. However, the both were closed to each other with 98% bootstrap support in RPBII data set based phylogram (Figure 4). As documented in literature, there is intragenomic variation in the ITS region of *Fusarium* species and identification of Fusarium species on the bases of ITS region is not considered to be authenticated among fungal molecular taxonomists.

In this study, the isolate FMB-0158 (FMB-45-Fi) make strong cluster by 100% bootstrap support with different CBS strains of *F. proliferatum*, *F. globosum*, *F.acutatum*, and *F. fujikuroi* (Figure 2) That's why TEF1-α and RPBII regions of this isolate was explored and characterized. The sequences of investigated genetic region of FMB-45-Fi fungal isolate were deposited to GenBank, which assigned the accession numbers (Table 2).

## DISCUSSION

In the present study, *Fusarium* isolates associated with Ficus dieback were identified on the basis of morphological and molecular characterization. It is a common phenomenon to identify the *Fusarium* under light and electron microscopy by detailed study of the mycelial and spore anatomy. Study of anatomical characters lead to the precise identification of new races. Moreover, spore size, shape, spore type (microspore, chlamydospore, macro spore etc) and colony characters are the major keys of morphological characterization of the *Fusarium* species. (Leslie and Summerel, 2006; Al-Ani and Albaayit, 2018). Molecular characterization has become necessary as well as most valid tool for the confirmation of the species and races of the evolving pathogens.

In the present study we have conducted multigenic analysis to study the phylogeny of *Fusarium* isolates from *Ficus* species. Study of practical applications in Phylogenetic analysis,

**Table 2. List of *Fusarium species* used in this study for constructing phylogenetic trees. FMB: Fungal Molecular Biology Laboratory Culture Collection, University of Agriculture Faisalabad, Pakistan; CBS: Culture Collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands.**

| Species/Isolate | Culture Accession # | GenBank Accession Numbers | | |
|---|---|---|---|---|
| | | ITS | TEF1-α | RPBII |
| FMB-45-Fi | FMB 0158 | MK372915 | MK388902 | MK455902 |
| *Fusarium proliferatum* | CBS138981 | KT716199.1 | KT716210 | KT716196.1 |
| *Fusarium proliferatum* | CBS18433 | MH855401.1 | KU604399.1 | KU604244.1 |
| *Fusarium proliferatum* | CBS18232 | MH855271.1 | KU604395.1 | KU604246.1 |
| *Fusarium zealandicum* | CBS11193 | NR_138298.1 | HQ728148.1 | HM626684.1 |
| *Fusarium solani* | CBS132898 | KF255440 | KF255484 | KF255523 |
| *Fusarium falciforme* | CBS135512 | KM401895.1 | KM401894.1 | KM401892.1 |
| *Fusarium falciforme* | CBS132191 | KF255424.1 | KF255467.1 | KF255510.1 |
| *Fusarium solani* | CBS118931 | JX435204.1 | JX435154.1 | JX435254.1 |
| *Fusarium ambrosium* | CBS57194 | KM231801.1 | KM231929.1 | KM232368.1 |
| *Fusarium neocosmosporiellum* | CBS51771 | KM231804.1 | KM231932.1 | KM232371.1 |
| *Fusarium neocosmosporiellum* | CBS32554 | KM231803.1 | KM231931.1 | KM232370.1 |
| *Fusarium neocosmosporiellum* | CBS56270 | KM231805.1 | KM231933.1 | KM232372.1 |
| *Fusarium striatum* | CBS101573 | KM231798.1 | KM231927.1 | KM232365.1 |
| *Fusarium illudens* | CBS119605 | KM231806.1 | KM231935.1 | KM232374.1 |
| *Fusarium phaseoli* | CBS26550 | KM232375.1 | HE647964.1 | KM232375.1 |
| *Fusarium solani* | CBS102429 | KM231808.1 | KM231936.1 | KM232376.1 |
| *Fusarium petroliphilum* | CBS135955 | KR071702.1 | KU711768.1 | KU604337.1 |
| *Fusarium globosum* | CBS43197 | LT746280.1 | LT746232.1 | LT746345.1 |
| *Fusarium globosum* | CBS42997 | LT746278.1 | LT746230.1 | LT746343.1 |
| *Fusarium fujikuroi* | CBS119855 | KR071669.1 | KU711679.1 | KU604260.1 |
| *Fusarium fujikuroi* | CBS22176 | KR071666.1 | KR071741.1 | KU604255.1 |
| *Fusarium acutatum* | CBS40297 | MH862652.1 | KU604454.1 | KT154005.1 |
| *Fusarium incarnatum* | CBS132895 | KF255437.1 | KF255481.1 | KF255546.1 |
| *Fusarium equiseti* | CBS30794 | MH862468.1 | KR071777.1 | KU604327.1 |

based on molecular techniques has led to the identification and more accurate classification of new species.

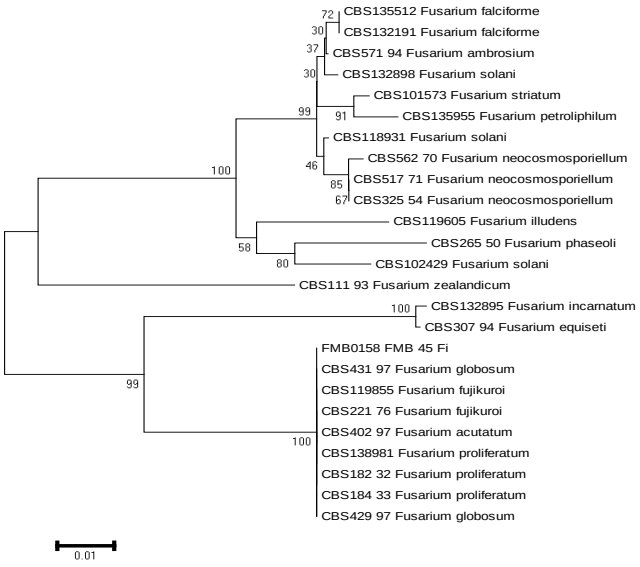


**Figure 2. The phylogenetic tree of individual dataset of internal transcribed spacer (ITS) region, generated with neighbor joining (NJ) method by MEGA 7 software.** This tree resolving the place of FMB-45-Fi in phylogenetic hierarchy by nesting it close to Fusarium proliferatum strains of CBS (Culture Collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands)

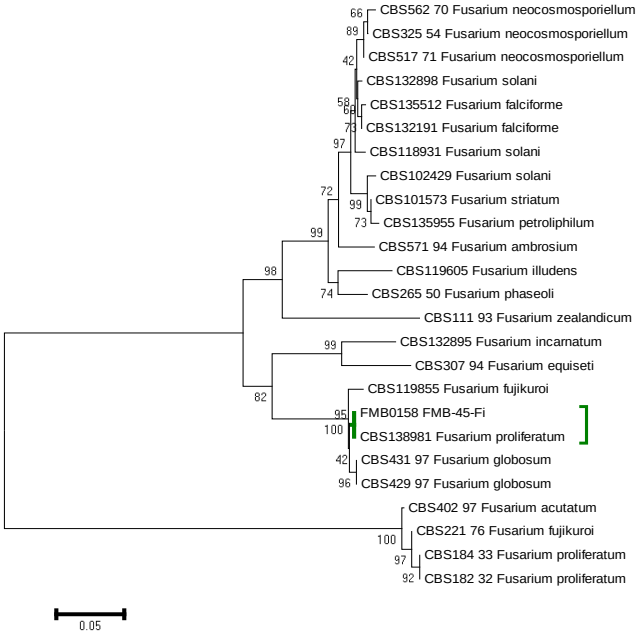


**Figure 3. The phylogenetic tree of individual dataset of translation elongation factor1-α (TEF1-α) region, generated with neighbor joining (NJ) method by MEGA 7 software**. This tree resolving the place of FMB-45-Fi in phylogenetic hierarchy by nesting it close to *Fusarium proliferatum* strains of CBS (Culture Collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands)

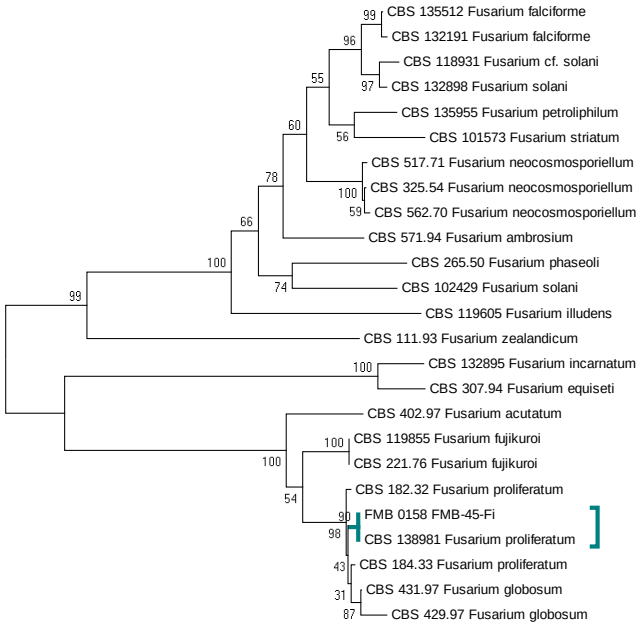


**Figure 4. The phylogenetic tree of individual dataset of RNA polymerase II second largest subunit (RPBII) region, generated with neighbour joining (NJ) method by MEGA 7 software.** This tree resolving the place of FMB-45-Fi in phylogenetic hierarchy by nesting it close to *Fusarium proliferatum* strains of CBS (Culture Collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands)

Phylogenetic and population genetics analysis allows the researchers to compare the alterations that occur at nucleic acid levels. These alterations led to the identification of new races particularly including fungal pathogens. Similarly, *Fusarium* isolates have also been characterized and classified by following the above mentioned techniques (O'Donnell, 1996; Leslie *et al*., 2001; Leslie *et al*., 2005).

In the past, several molecular tools including AFLP, RFLP, PFGE (pulse field gel electrophoresis), isoenzyme (isozymes), SSCPs (singles strand conformation polymorphisms), DNA-DNA hybridization, protein electrophoresis, probes DNA, Microsatellite markers, 28S, ITS region, sequenced portions of the genes 18S and 28s, IGS, rDNA, ribosomal DNA of smaller subunit, gene sequencing of calmodulin, β-tubulin and EF-1α were applied to measure the diversity among *Fusarium* and other microbes (Burns and Palmer, 1989; White *et al*., 1990; Grajal-Martin *et al*., 1993; Bruns *et al*., 1992; Bentley *et al*., 1994; Donaldson *et al*., 1995; Hyun and Clark, 1998; Niessen and Vogel, 1998; Abdel-Satar *et al*., 2003; Khalil *et al*., 2003; Patino *et al*.,

2004; Mirete *et al*., 2004; Gonzalez-Jaen, *et al*., 2004; Patino *et al*., 2006; Wang, *et al*., 2007; Trabelsi *et al*., 2017).

Use of multigene phylogenetic analysis has been previously conducted to identify twenty-four *Fusarium* isolates belonging to *F. incarnatum-equiseti, F. fujikuroi*, *F. oxysporum* and *F. tricinctum* (is in accordance to the current line of work).

Most recently, *Fusarium proliferatum* has been isolated from root rot of Indian mulberry in Vietnam. In accordance to our studies, *F. proliferatum* was also characterized on the basis of ITS region and the TEF1-α gene and best grown over PDA solid media (Nguyen *et. al*., 2019).

Former literature about DNA sequencing of the ITS1 and ITS2 regions of *Fusarium proliferatum* and *Fusarium solani* on black pepper (*Piper nigrum L*.) (Shahnazi *et al*., 2012), also support our approach for the identification of the same pathogen over *Ficus* plants.

In the present study, identification of the virulent strain of *F. proliferatum* (among multiple isolates) was accomplished by sequencing TEF-1 α genes. Similar technique was adopted on Pecan tree (*Carya illinoinensis*) in Brazil, where, 11 Fusarium isolates were recovered and subjected to molecular characterization by sequencing their TEF-1α genes (Lazarotto *et al*., 2014).

***Conclusion*:** *Ficus* plant has become important part of landscaping in Pakistan. *Ficus benjamina* is most affected species from dieback. *Fusarium proliferatum* has been found as the principal cause of dieback in *Ficus* species.

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