Mycopath (2018) 16(2): 57-64 **Research Article**

Morphological and molecular characterization of *Fusarium thapsinum,* causing stalk tot of maize in Punjab, Pakistan

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Abstract

Fungi were isolated from diseased maize samples suffering from stalk rot. Six isolates were identified as *Fusarium thapsinum* on morphological and microscopic features. These were further verified by PCR analysis with EF-1α gene amplification. The phylogenetic analysis demonstrated that all the isolates of *F. thapsinum* were more than 98% similar based on the maximum likelihood tree analysis and were grouped into a single major cluster. Pathogenicity test was conducted when maize achieved its physiological maturity stage. Pathogenicity assay demonstrated the ability of all the six isolates to cause infection. Results showed that disease severity index was ranged from 39–47%, reflecting variation in aggressiveness among the isolates. Isolates MN207144, MN200604, MN200605, and MN200606 were found highly aggressive. In the light of previously available documented evidences, it is the first report of *F. thapsinum* associated with stalk rot of maize in Punjab, Pakistan.
Keywords: EF-1α gene, *Fusarium thapsinum,* Maize, Molecular characterization, Stalk rot.

Introduction

Maize (*Zea mays* L.) is the third most grown cereal crop in the world (Gopala *et al.,* 2016). It is cultivated on 140 million hectares with 577 million tons of annual production worldwide (Radma and Dagash, 2013). Globally, stalk rot of maize is a serious and widespread fungal disease with destructive potential (Yu *et al.,* 2017). Estimated yield losses in maize due to this disease are about 10% and by 30–50% in severely affected areas (Li *et al.,* 2010). Several fungal pathogens and colonizers have been implicated with this disease. *Fusarium* species are the most common phytopathogen causing seedling blight, stalk rot and ear rot worldwide (Logrieco *et al.,* 2002). Many species of *Fusarium* such as *F. prolifiratum, F. verticillioides, F. subglutinans* and *F. incarnatum,* were found to occur on maize worldwide and cause substantial yield losses in every growing season (Leslie and Summerell, 2007). These organisms could occur separately or together in the stalk. When these pathogens invade maize stalk prior to physiological maturity, significant yield losses can occur in the form of poor grain fill, premature death and stalk lodging (Shin *et al.,* 2014).

Stalk rot associated fungi infect different parts of the maize plant at all stages of its development from the beginning to end of growing season (Munkvold *et al.,* 1997). These fungi have various natural entry points to get into the host plants such as aboveground nodes, through wounds made by insect or mechanical damage or by direct penetration of the roots and stalk tissues (Sobek and Munkvold, 1999; Munkvold and Gatch, 2002). Stalk rot fungi overwinters in the infected corn residues left on the soil (Naef and Défago, 2006), and function as the main source of primary inoculum for succeeding maize crop (Munkvold, 2003). The disease is characterized by pith decay and discoloration as well as deterioration of inner stalk tissue, directly reducing yield by blocking xylem and phloem vessels and can result in death and lodging of the host plant (Khokhar *et al.,* 2014). Under severe conditions, stalk rot results in premature plant death or lodging of maize (Afolabi *et al.,* 2008).

Physical injuries caused by insects are the common sites for fungal penetration on maize ears and stalks (Miller *et al.,* 2007). One of the major corn borer pests is the European corn borer *Ostrinia nubilalis* that causes significant economic losses throughout the world. This pest not only injures plants and exposes them to fungal infection but also act as a vector for several stalk rot fungi. Infestation starts at the early phenological vegetative stage of the maize plant. First generation larvae feed in the leaf whorls of corn creating irregular shaped holes in the leaves while subsequent stage attacks maize at its reproductive stage (Santiago and Malvar, 2010). *O. nubilalis,* therefore, contribute to the development of stalk rot by tunneling through the stalk tissues, larvae creating entry points for fungal pathogens and cause physiological stress that can predispose maize plant for stalk rot development (Munkvold *et al.,* 1999).

The objective of this study was to characterize isolates of *F. thapsinum* isolated from diseased maize stalks in Punjab, Pakistan using morphological feathers and molecular approaches. Pathogenicity and virulence of all the isolates were also determined. Phylogenetic approach was used to distinguish and analyze the partial sequence of *F.*

thapsinum and other closely related isolates from Asia and Europe.

Materials and Methods

Sample collection

Tissue samples were taken from both diseased and asymptomatic maize plants from Narowal, Lahore, Okara, Sahiwal and Multan. Surveys were typically conducted when crop was at physiological maturity, when stalk rot symptoms are the most likely to appear (Kelly *et al.,* 2017). Diseased stalk sections were packed in paper bags, labeled with necessary information regarding specimen collecting dates, the GPS data and disease position on maize plant (Scauflaire *et al.,* 2011).

Isolation of associated fungi

Collected specimens were observed under stereoscopic microscope and diseased stems with typical stalk rot symptoms were processed further. Three 5 mm diseased sections were cut at 5 cm, 10 cm and 15 cm from the first internode above the brace roots of each stalk sample (Scauflaire *et al.,* 2011). Diseased sections were surface sterilized with 0.5% sodium hypochlorite for 2 min and rinsed thoroughly with double distilled water. Sterilized tissue sections were drained and placed on potato dextrose agar (PDA) media that was amended with streptomycin sulphate (130 g mL^{-1}) to inhibit bacterial growth (Gai *et al.,* 2017). Plates were incubated at 25 °C for two days. The growing fungal hyphae were transferred to PDA media plates and incubated for 7 days at 25 °C. Conidia of *F. thapsinum* were isolated using single spore isolation method and incubated at 25 °C for 7 days. All the fungal isolates were stored at −75 °C in 50% glycerol (Shin *et al.,* 2014).

Microscopic examination and species description

For morphology-based identification, macroscopic and microscopic observations were made using 7-days old fungual cultures grown at 25±2 ºC on PDA. The colonies were identified with the help of key developed by Leslie and Summerell (2007). To differentiate among isolates, the presence or absence of chlamydospores, formation of sporodochia, width and length of 50 random macroconidia and one hundred microconidia were measured (Isaac *et al.,* 2018). In addition, cultural and microphotographs were taken for the use of future reference and comparison in fungal identification.

DNA extraction and Sequencing

Fungal species were also identified based on sequence analysis of the translation elongationfactor 1 alpha region (EF-1α). Fungal DNA was directly extracted from the fungal mycelia of monosporic cultures of all isolates by using PrepMan@ Ultra Genomic DNA purification kit according to the manufacturer's recommendations. The quality and quantity of the extracted DNA were determined at 260 and 280 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Madison, USA). DNA samples were stored at −80 ℃ until further analysis. All monosporic isolates were tested by PCR to amplify Tef region with forward primer EF1 (5′-ATGGGTAAGGARGACAAGAC -3′), and reverse primer EF2 (5′-GGARGTACCAGTSATCATGTT-3′) (O'Donnell *et al.,* 1998). PCR cycling conditions for (EF1/EF2) initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 51 °C for 30 s, elongation at 72 °C for 60 s and a final elongation step at 72 °C for 7 min. PCR products were visualized on a 1% agarose gel, following staining with ethidium bromide. Amplified products were sequenced in both directions with the same primers used for PCR and resulting neocleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov) for identification.

Phylogenetic analysis

Phylogenetic relationships were carried out using the nucleotide sequences alignment of the EF-1α gene among the *F. thapsinum* isolates using MEGA 7.0.14 software (Kumar *et al.,* 2016). Partial sequences of all the *F. thapsinum* isolates were aligned using ClustalW software. A neighbor-joining tree was constructed using 1000 bootsteps replication.

Pathogenicity and virulence assay

A pot trial was implemented to investigate the pathogenicity and virulence of all the six isolates of *F. thapsinum*. For this purpose, 10% commercial formalin dipped cotton plugs were buried in soil heap at different places and covered with two layers of plastic sheet and left for 3-4 days. After that, soil pile was uncovered and left open for 6 days. Pots were filled with disinfected 700 g soil and 5 surface sterilized seeds of maize per pot were sown at the depth of 2 cm. After germination, pots were thinned leaving one plant per pot. Each treatment was replicated five times. Conidial suspension of each isolate was prepared. Conidia were harvested by scrapping the surface of pure fungal culture with a glass slide and suspended in 30 mL distilled water. Conidial number in suspension was adjusted to 10^4 mL^{-1} conidia by hemocytometer (Marienfeld GmbH). A conidial suspension (2 mL) was injected at 45^0 angle downward into the middle of 3rd internode using sterile needle. For control treatment, 2 mL sterilized water was poured downward into the middle of 3rd internode. The inoculated pots were kept in green house in completely randomized block design. Pots were regularly monitored for disease development. The disease symptoms caused by *F.*

thapsinum isolates were conformed and analyzed on 15th day after inoculation by using 1−9 disease rating scale (ICAR 2012). Stalk discoloration and necrotic lesion were calculated by excising the maize stalk up to the fifth node, measuring the length of discoloration and visible necrotic lesion.

Results and Discussions

Pathogen isolation and identification

A total of six *F. thapsinum* isolates were isolated from infected stalks of maize at six different locations in Punjab, Pakistan. Previously, *F. thapsinum* found to be associated with stalk rot of maize as a pathogen under greenhouse conditions in USA (Jardine and Leslie, 1999). Some isolates of *F. thapsinum* have also been isolated from the maize, associated with ear rot (Madania *et al.* 2013). In the light of the available documented reports, this is the first report of maize stalk infectivity by *F. thapsinum* in Punjab, Pakistan. Stalk rot of maize is a major disease of maize causing severe damage of the crop (Charmley *et al.,* 1995). Based on cultural and morphological characteristics, these isolates were named as *F. thapsinum* (Fig. 2 E1-E6). These isolates produced abundant and septated mycelium, and microconidia were also abundant and produced in long aseptate chains or false heads. Aerial mycelia of *F. thapsinum* were white which became darken as culture grew older. Microconidia were club shaped and some were napiform (Fig. 3F). Conidiogenous cells were monophialitic but occasionally Polyphialdic (Fig. 3 B-E). Chlamydospores were absent in all the isolates. On PDA, all the isolates produced characteristic dark yellow pigmentation, a prominent exception than all other *Fusarium* species (Klittich *et al.,* 1997). Macroconidia were straight, slightly curved, falcate with curved and tapering apical cells and with hyaline poorly developed foot shaped basal cells. Morphological and microscopic characters of all the isolates showed resemblance with morphological character of *F. thapsinum* reported by Klittich *et al.* (1997).

Molecular analysis

After performing PCR amplification of the TEF-1α gene, a band size of approximately 700 bp further validates the specificity of the EF1/EF2 primers pair. This gene is highly suitable for phylogenetic tree analysis because it exhibits a high level of polymorphism when compared to other protein coding genes such as calmodulin, beta-tubulin and histone H3. Thus, gene has become the most powerful and widely accepted molecular marker for single locus identification in *Fusarium* spp. (Geiser *et al.,* 2004; Alastruey-Izquierdo *et al.,* 2008). The nucleotide sequences of the purified amplicon of the 6 isolates of *F. thapsinum* were compared with the sequences of *F. thapsinum* in the gene bank database using the BLASTn utility search. The sequences obtained were submitted to gene bank data base under accession no MN200606, MN200605, MN200604, MN200603, MN207144 and MN228489 for EF1-α gene. Results revealed that four isolates shared sequence homology from 99.71% to 99.86% with *F. thapsinum* and two isolates from Sahiwal and Multan shared 100% homology with *F. thapsinum* (Table 1).

Phylogenetic analysis

Partial sequencing of purified PCR products was performed in the commercial lab. Nucleotide sequence of all the *F. thapsinum* isolates were aligned with different gene sequences form gene bank using the software Clustal X 1.8.3 with default settings. To better understand the relationship of our isolates with rest of the world isolates, phylogenetic tree was constructed with MEGA 7.0.14 software based on maximum likelihood method (Fig. 5). All the six isolates were grouped with sequences reported from Europe and Asia, conforming its identity as *F. thapsinum.* The phylogenetic tree analysis revealed that all the Pakistani isolates were closely related to the isolates from India, Iran, Korea, Brazil, Denmark and Mexico. These results are possibly supported by the fact that biodiversity and occurrence of Fusarium species are influenced by geographic location and climatic conditions (Balmas *et al.,* 2010). It is also an indication of possible molecular diversity in Pakistani isolates that are sharing genomic similarities with rest of the world isolates.

Pathogenicity and virulence assay

All the six isolates had the ability to infect maize plants and showed severe disease development (Fig. 5 E1-E6). Results of our pathogenicity assay conform that isolates of *F. thapsinum* have the ability to cause stalk rot of maize and are in line with the findings of earlier workers on sorghum (Jurjevic *et al.,* 2005; Leslie *et al.,* 2005). Different levels of aggressiveness were observed among the isolates of *F. thapsinum*. The disease severity index (DSI) of all the isolates were ranged from 38.52% to 46.67%. Isolates MN207144, MN200604, MN200606, and MN200606 registered the highest DSI of all the isolates *i.e.* 42.96, 45.19, 45.93 and 46.67%, respectively. These isolates were categorized as highly aggressive isolates. Isolates MN200603 and MN228489 were found to be aggressive with DSI of 38.52 and 39.26%, respectively. Isolates MN200606 (9.59 and 4.79 cm), MN200605 (9.81 and 4.83 cm), MN200604 (10.12 and 5 cm), MN200603 (8.71 and 4.61 cm), MN207144 (9.34 and 4.55 cm), MN228489 (9.12 and 4.72 cm) had produced significantly larger discolored and rotted areas than the control (Table 2). After splitting, inoculated stalk showed internal discoloration, characterized by blackish-brown discoloration of internal pith tissues extending from the point of inoculation up the stalk

towards the panicle. These symptoms were not observed in control treatment. All the isolates of *F. thapsinum* were recovered from the necrotic tissue of the inoculated plants, indicating that this pathogen is also the causal organism of the stalk rot disease on maize plants.

Finally, combined morphological and molecular characterization of the six isolates of *F. thapsinum* isolated from symptomatic stalk led to the following conclusion: 1) All the isolates of *F. thapsinum* have the ability to cause stalk rot of maize, 2) DSI of *F. thapsinum* isolates vary from one region to another possibly due to change in crop growing conditions. The result of the present studies can serve as guidelines and tool for investigations on etiology and management of the stalk rot of maize.

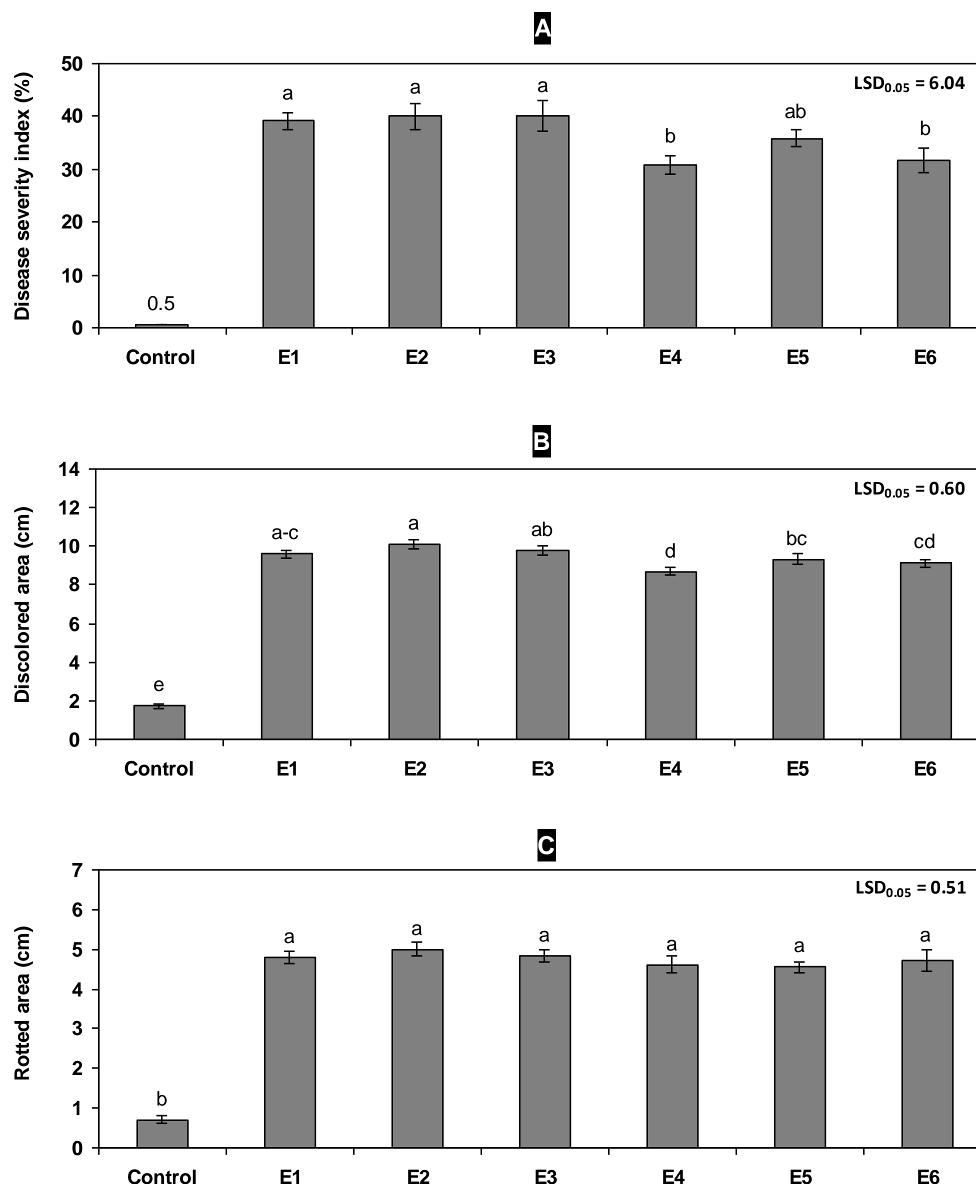

Fig. 1: Disease severity index of stalk rot on maize caused by *F. thapsinum* isolates. **B:** Assessment of discoloration of maize stalks infected with *F. thapsinum* isolates. **C:** Assessment of disintegration of Pith tissues caused by *F. thapsinum* isolates.

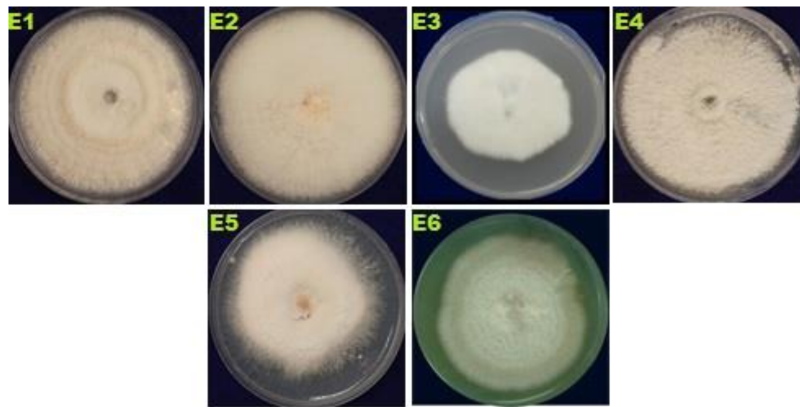

Fig. 2: Cultural characteristics of *F. thapsinum* associated with stalk rot of maize. **E1:** *F. thapsinum* NR; **E2** *F. thapsinum* LH **E3:** *F. thapsinum* OK **E4:** *F. thapsinum* SW **E5:** *F. thapsinum* SW **E6:** *F. thapsinum* MT.

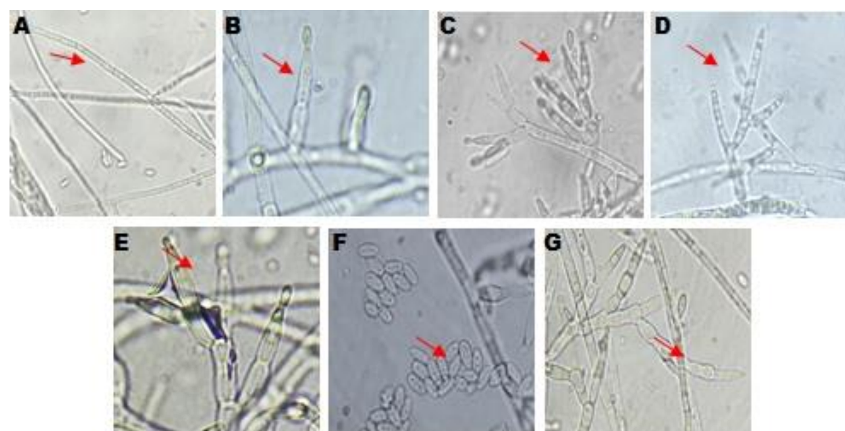

Fig. 3: Microscopic characteristics of *F. thapsinum.* Septated mycelium (**A**), Monophialides (**B-E**), Microconidia (**F**), Microconidia in chains (**G**).

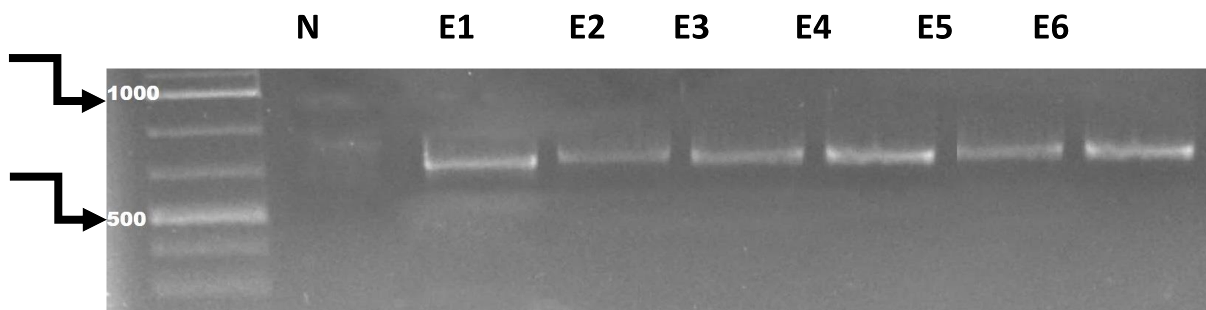

Fig. 4: Agarose gel of amplified PCR products using the EF1 and EF2 primers to detect *F. thapsinum*. **N** = Negative Control; **E1-E6** = *F. thapsium* isolates DNA obtained from diseased stalks of maize collected from different localities of Punjab, Pakistan.

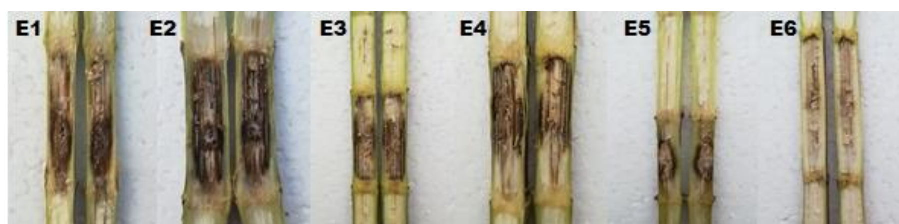

Fig. 5: Symptoms of stalk rot on maize stem due to inoculation of different isolates of *F. thapsinum*.**E1:** *F. thapsinum* NR; **E2** *F. thapsinum* LH **E3:** *F. thapsinum* OK **E4:** *F. thapsinum* SW **E5:** *F. thapsinum* SW **E6:** *F. thapsinum* MT.

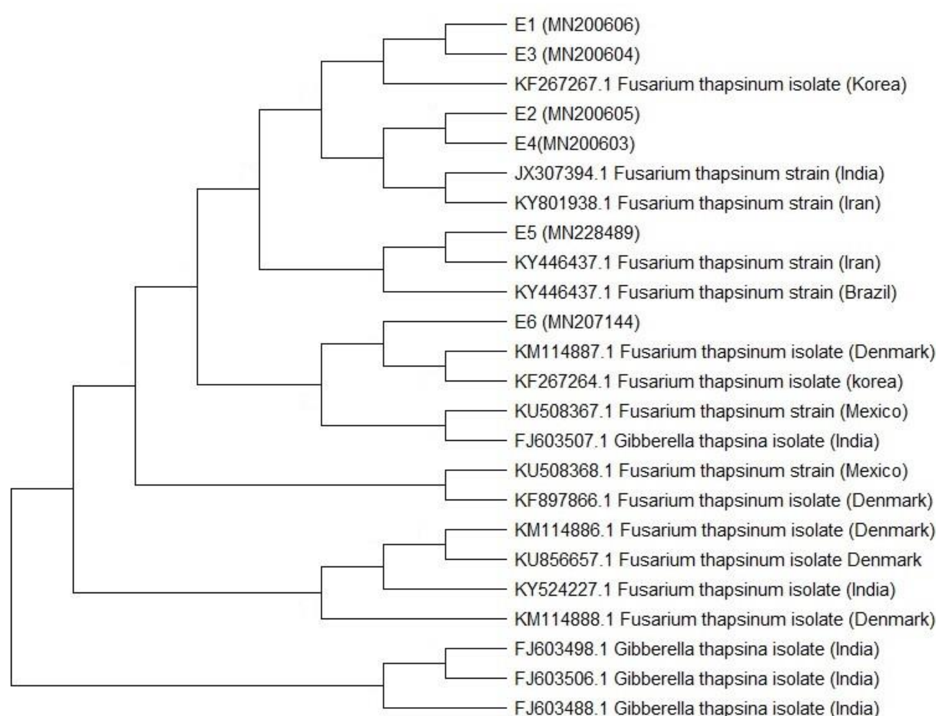

Fig. 6: Phylogenetic dendrogram of 24 *Fusarium thapsinum* isolates including 6 Pakistani isolates and 18 world isolates retrieved from Genbank. The tree was generated by using the Maximum Likelihood method based on the Tamura-Nei model. Missing sequences and gaps were completely deleted and branch swap filtration was strong 1st, 2nd, 3rd and non-coding codons were also included. GenBank accession numbers are indicated with each strain name. Accession number.

Table 1: Summary of molecular identification of six *F. thapsinum* isolates from five different areas in Punjab, Pakistan

Isolate Code	Location	TEF1-α Sequence with best match (GenBank accession)	TEF1-α % identity	Gene bank accession number
E1	Narowal	KF267264	99.86%	MN200606
E2	Lahore	KF267264	99.71%	MN200605
E3	Okara	KF267264	99.86%	MN200604
E4	Sahiwal	KF267264	99.71%	MN200603
E5	Sahiwal	KY801938	100%	MN207144
E6	Multan	KM462997	100%	MN228489

Table 2: Aggressiveness of *Fusarium* isolates against stalk rot of maize under field conditions.

Culture Code	Gene bank accession number	Isolate Name	Disease Severity Index (%)	Discolored area (cm)	Rotted area (cm)	Aggressiveness
Control			11.11	1.73	0.71	Less aggressive
E1	MN200606	*F. thapsinum* NR	45.93	9.59	4.79	Highly aggressive
E2	MN200605	*F. thapsinum* LH	46.67	10.12	5	Highly aggressive
E3	MN200604	*F. thapsinum* OK	45.19	9.81	4.83	Highly aggressive
E4	MN200603	*F. thapsinum* SW	38.52	8.71	4.61	Aggressive
E5	MN207144	*F. thapsinum* SW	42.96	9.34	4.55	Highly aggressive
E6	MN228489	*F. thapsinum* ML	39.26	9.13	4.72	Aggressive

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