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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF THE PATHOGEN ASSOCIATED WITH INFECTED LEAF AND FRUITS OF TOMATO

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ABSTRACT

This investigation describes the morpho-molecular characterization of the pathogen associated with infected leaf and fruits of tomato (*Solanum lycopersicum* L.). The fungal pathogen was identified based on their cultural morphology, microscopic features and analysis of Internal Transcribed Spacer (ITS) region sequences of nuclear ribosomal DNA (nrDNA). Cultural morphology including colour of the young and mature colony, growth pattern, concentric rings etc. and microscopic features including shape and size of microconidia, macroconidia, phialide, shape of apical and basal cells of macroconidia, septation in conidia, inter-septal distance and formation of chlamydospores corroborated the identity with the pathogenic fungi *Fusarium oxysporum*. The phylogenetic analysis based ITS regions sequences of nrDNA of the fungal isolate showed 100% identity with *Fusarium oxysporum*. Thus the ITS of nrDNA sequences validated the morphological data. Pathogenicity test also reconfirmed the identity of the test isolate of *Fusarium oxysporum.*

Key Words: *Fusarium oxysporum*, DAPI, ITS sequencing, *Solanum lycopersicum*.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a widely cultivated vegetable crop of solanaceae family and most common vegetable crop in Bangladesh. It contains nutritive elements such as vitamin A, B, C, calcium, iron etc. almost double as compared to apple and shows superiority with regard to food values (Mohiuddin *et al*., 2007). It is consumed as a raw salad, processed food item such as sauce, ketchup, jam, jelly, pickles, soup etc. Though tomato is native to tropical America, in Bangladesh it is now considered as second cash crop. The area of cultivation of tomato is about 30756 ha with the production of about 414000 tons and average yield 54.7 tons/ha (Biswas *et al*., 2017). At present 6.10% area of

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Bangladesh is under tomato cultivation both in winter and summer (Karim *et al*. 2009).

Tomato plants are susceptible to a number of pests and diseases, including bacterial wilt, early blight, mosaic virus, *Fusarium* wilt etc. Different *Fusarium* species causes different diseases of tomato including *Fusarium* foot rot, *Fusarium* crown and root rot, *Fusarium* crown and stem rot, *Fusarium* wilt of tomato are caused by *Fusarium solani* f. sp. *eumartii*, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. striatum*, and *F. oxysporum* f. sp. *lycopersici*, respectively. Among these diseases wilt is a serious and most important disease of tomato which is caused by *F. oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyder and Hansen (Agrios 1988, Smith *et al.* 1988, Ignjatov *et al*. 2012, McGovern 2015, Iannotti 2018). In an outdoor environment due to high temperature and humidity, *F. oxysporum* f. sp. *lycopersici* can cause 10-90% yield loss of tomato (Kumar and Sharma 2015, Worku and Sahe 2018). *Fusarium oxysporum* f. sp. *lycopersici* is a soil borne pathogen that belongs to the class Hyphomycetes. Since several *Fusarium* species cause diseases in tomato and their morphological and cultural characteristics are quite similar, it is difficult to accurate identification morphologically and there is a possibility of mystifying of paraphylatic taxonomy. Recent bio-molecular techniques, especially Internal Transcribed Spacer (ITS) sequence analysis of nuclear ribosomal DNA (nrDNA) alleviated the difficulty. Morpho-molecular characterization has utmost importance for accurate identification and determination of pathogenic variation of *F. oxysporum* isolates, which has not been previously studied in Bangladesh. To minimize the yield loss and control of the pathogen, proper identification of the relevant pathogen is important. Considering these, the present study was under taken with a view to identify the causal organism of *Fusarium* wilt of tomato by applying morphological and molecular techniques.

MATERIALS AND METHODS

Plant materials and experimental site: "Ratan" (BARI Tomato), an adopted tomato variety in the Chittagong region of Bangladesh was selected for the present study. Seedlings were collected from the Regional Agricultural Research Station, Hathazari, Chittagong and grown in the Botanical garden of Chittagong University. For planting seedlings 1×20m seed bed was prepared with mixing proper amount of N, P, K fertilizer and cow-dung with the field soil. The experiment was conducted during November–January 2017. One hundred seedlings were planted in well prepared seed bed at 60 cm distance (between

seedlings to seedling and row to row). Slice of infected leaves and fruits of field plants were selected for isolation of pathogenic fungi.

***Isolation of pathogen*:** Infected leaves and fruits were collected from the field in airtight sterilized polyethylene bags, labeled properly, brought to laboratory and used for isolation of pathogen. Isolation of fungus was done following tissue planting method (Chakraborty and Chatterjee 2007). Dust particles from the surface of the infected explants were cleaned thoroughly under running tap water and then subjected to 0.5% $NaOCl_2$ solution for 5 minutes with occasional agitation. These were then washed three times with sterile distilled water keeping in a laminar-airflow cabinet. A loaf of infected parts of the leaf and fruits were taken out with a sterile forcep and cultured on the surface of the solidified potato dextrose agar (PDA) medium amended with Amoxicillin (500 g/L) to suppress bacterial growth. The cultures were kept in a growth chamber maintaining 25-28°C temperature. Within 3-4 days fungal mycelium came out from the cultured explants. Pure culture was raised by successive sub-culturing the hyphal tips from the fast growing regions of the fungal colony on freshly prepared PDA medium.

***Cultural characteristics of isolated fungi*:** Both surface and reverse colours of the young and mature colony of the fungal isolates were recorded. Colony appearance and colony growth rates were determined according to the techniques of Currah *et al*., (1987). Growth rates were determined by averages based on three replications.

***Microscopic features*:** Microscopic features of the fungi isolated from the infected tissues were studied according to Sharma (2004) and Mandhare *et al*., (2011). Nuclei number in vegetative cells and conidia was investigated by modified method of Shan *et al*., (2002).

***Isolation of DNA and PCR amplification of ITS regions*:** One hundred mg of mycelial mat was taken from the surface of PDA plate and subjected to extraction of genomic DNA following the protocol described by Liu *et al*., (2000). The PCR amplification of ITS regions of 5.8S rRNA gene was achieved using the primer ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and Primer ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′). The PCR reaction was performed in 50 µl reaction mixture containing 1 µl genomic DNA (Con. 25-65 ng/µl), for each primer of 1 µl (con. 10 pmol), 1 mM of dNTPs, 10× PCR buffer with 1.5 mM $MgCl_2$, and 1U Taq polymerase. The thermo-cycling conditions consisted of an initial denaturation at 95ºC for 5 min followed by 32 amplification cycles denaturation

at 94ºC for 30 sec, 1 min at 54ºC for annealing, 50 sec at 72ºC for extension, and a final extension at 72 ºC for 5 min. The sequencing of PCR product was done by ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystem, CA, USA). The amplified PCR products were run on 1.5% agarose gel in Tris-acetate buffer. The gel was stained with ethidium bromide, visualized on an UV-transilluminator and photographed in the gel documentation unit (Alpha Innotech Corp, USA). The sizes of the PCR products were determined by comparison with standard 100 bp or 1 kb molecular marker.

***Phylogenetic analysis*:** The sequences were analyzed using the gapped BLASTN (https://blast.ncbi.nlm.nih.gov) search algorithm and phylogenetic tree was constructed using the MEGA BLAST version 7 software after aligning the sequences with CLUSTAL W version 1.83. The ITS region sequences (~600 bp) of the fungal isolate were deposited in the NCBI GenBank database under the accession numbers MN219649 for *Fusarium oxysporum* f. sp. *lycopersici* (http://www.ncbi.nlm.nih.gov/btast).

***Pathogenicity test*:** Twenty days old 10 healthy seedlings of "Ratan" (BARI Tomato variety) were planted in pots and kept in sunny place of the Botany Department of Chittagong University. Artificial injuries were created on leaf, stem and fruits of the pot plants, inoculated with isolated fungal culture and covered with perforated polythene bags with maintaining 5 uninoculated plants as control. Plants were monitored for the development of disease symptoms. Pathogens were re-isolated and identified from diseased parts of randomly selected five test plants after 25 days to confirm the pathogenicity.

RESULTS AND DISCUSSION

***Disease assessment and recovery of isolates*:** Within three weeks of plantation some of the older leaves started yellowing with brown spots (Fig. 1A, B) and drooping of the lower leaves. Successive leaves also infected and die, often before the plant reaches maturity. As the disease progresses, growth is typically stunted, and little or no fruit develops. After 10-11 weeks fruit setting occurred and these also started to be infected. Similar symptoms of *Fusarium* wilt of tomato were reported by Joshi *et al*., (2013). Almost 40% unripe fruits were found infected with brown spots and got rotten in the infected parts (Fig. 1C). The fungal mycelium came out from the cultured explants within 3-4 days of *in vitro* culture.

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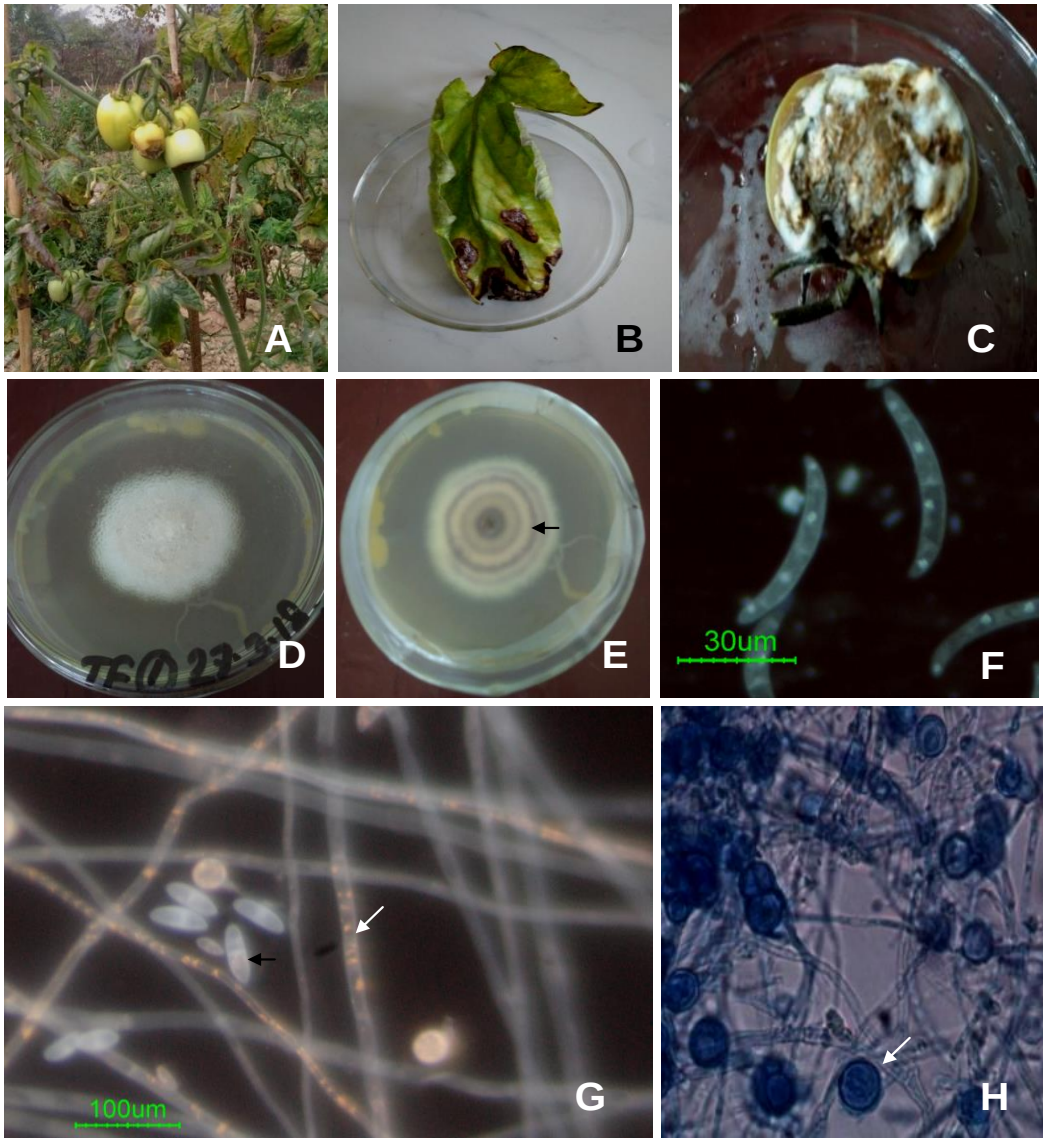

FIG.1: MORPHOLOGICAL CHARACTERIZATION OF *FUSRIUM OXYSPORUM* F. SP. *LYCOPERSICI*: A) A DISEASED PLANT, B) INFECTED LEAF, C) INFECTED FRUIT, D) A YOUNG COLONY (SURFACE VIEW), E) A YOUNG FUGAL COLONY (REVERSE VIEW), F) FUSOID MACROCONIDIA, G) MICROCONIDIA AND MULTI-NUCLEATE VEGETATIVE CELLS (ARROW), AND H) CHLAMYDOSPORES (ARROW).

***Morphological characterization of fungi*:** Colony characters of the isolates showed fluffy growth pattern with white to brownish white mycelial colour (Fig. 1D). Concentric rings were observed after one week (Fig.1E). The mycelium was hyaline, 8-10µm in diameter, profusely branched and septate (Fig. 1G). The fungus produced three types of asexual spores i.e. macroconidia (Fig. 1F), microconidia (Fig. 1G), and chlamydospores (Fig. 1H). Macroconidium was thin walled, 3–5 septate, boat shaped, multi-cellular, fusoid-subulate, pointed at both ends and measured 35–60 × 3–5 µm. Microconidium was oval-ellipsoid shape, 7–15 × 2.5–3.5 µm in size, and single septate or nonseptate. Chlamydospores were both smooth and rough walled and formed terminally or on an intercalary basis. The vegetative cells were multinucleate while both macro- and microconida contained uninucleate cells. These characteristics were similar to those reported by Nelson *et al*. (1983), Leslie and Summerell (2006), Asante *et al*., (2008) and Rafique *et al*., (2015) in different *Fusarium oxysporum* strains.

***Molecular characterization of isolated fungi*:** Two fungal strains were isolated from diseased tomato plants and subjected to molecular characterization through sequencing and analyzing of ITS regions of the nrDNA (Fig. 2). The BLASTn search of ITS region sequences of the fungus showed 99 to 100% similarity with *F. oxysporum*. The phylogenetic tree based on ITS region sequences of the fungal endophytes and their closely related isolates nested within a clade forming a monophyletic group (Fig. 3). Phylogenetic analysis of the ITS provided a strong support for the recognition of morphologically identified *Fusarium*. The use of phylogenetic analysis in addition to morphological characterization greatly helped in the confirmation of pathogens at species level (Mandhare *et al*., 2011; Rafique *et al*., 2015; Ashwathi *et al*., 2017). This was supported by the concept given by Aoki *et al*., (2003) who suggested that phylogenetic techniques facilitate to identify new species, which is usually difficult and often impossible by using conventional morphological characters. The conventional approaches for identifying pathogenic fungi have limitations as many of the fungi produce similar reproductive structures. Consequently, the broad vegetative criteria for identification have resulted in paraphyletic taxonomy, with various unrelated fungi being grouped together necessitating the application of molecular techniques for accurate identification (Joshi *et al*., 2013; Taylor *et al*., 2016). ITS region sequencing is the common and powerful molecular technique for accurate identification of fungi (Edel *et al*., 1997; Taylor and McCormick 2008; Hirano and Arie 2006; Khosrow 2016). The ITS region has several features that make it a strong candidate for a universal ‘barcode’ for fungal identification. It is easy to

amplify, relatively few primer sets are needed, highly conserved regions, and varies relatively little within species but dramatically between species, and far better represented in GenBank than other loci in fungi (Taylor and McCormick 2008; Saikia and Kadoo 2010).

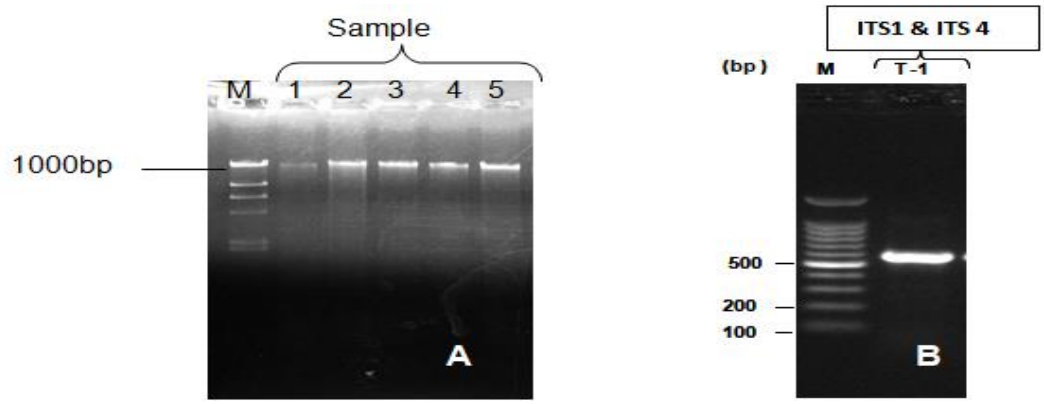

FIG. 2: (A) GENOMIC DNA, AND (B) ITS PROFILES OF *FUSARIUM* ISOLATES (M DENOTES DNA LADDER (MARKER).

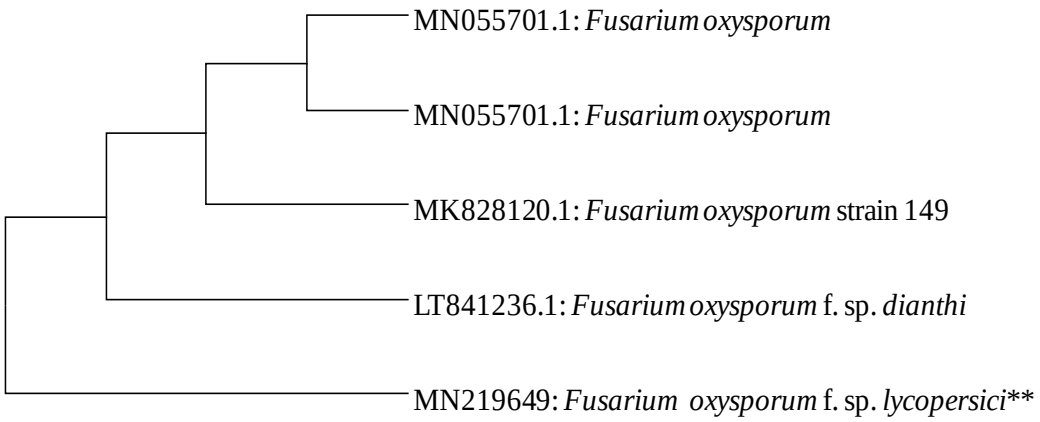

FIG. 3: MOLECULAR PHYLOGENETIC ANALYSIS BY MAXIMUM LIKELIHOOD METHOD. CLADOGRAM SHOWING RELATIONSHIP WITH *FUSRIUM OXYSPORUM* F. SP. *LYCOPERSICI* AND REPRESENTATIVES OF SOME RELATED TAXA BASED ON ITS REGION ANALYSIS [INDICATED THE ISOLATES OF PRESENT STUDY].**

***Conclusion*:** Both morphological and molecular techniques were applied for identification of *F. oxysporum*. The molecular information validated the morphological data. The concurrence between morphological and molecular characterization will facilitate accurate identification of other pathogenic fungi as well.

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