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First report of *Alternaria phragmospora* causing early blight disease of *Lycopersicon esculentum* in Egypt

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Abstract

Tomato is one of the most common vegetable crops in the world. Many diseases would infect tomatoes during the growing season. In summer (August, 2018), from different fields (5 fields) severe brown spots appeared on the leaves, stems, and fruits of *Lycopersicon esculentum* variety Castle rock in the Abu El-Rish region, at Aswan city, Upper Egypt. The pathogen which isolated from different infected parts of the plant was identified as *Alternaria phragmospora* according to the morphological and molecular characterization using primers ITS1 and ITS4. The pathogenicity was tested by the re-inoculation of the healthy tomato plant from the same variety either by spore suspension ($1.2x10^6$ conidia/ ml) spraying or direct mycelium inoculum on the plant surface. Virulent symptoms were observed in the inoculated plant. In the lab, moisture and high temperature increase the disease severity of infected plant while in the field, high temperature (40℃-47℃), excess watering of the plant and condensation of them in patches were recognized and these conditions increased the disease severity and spreading from plant to other. To our knowledge, this is the first detection of the pathogen, *A. phragmospora* which infected *L. esculentum.*

Keywords: Early blight, *Alternaria phragmospora*, *Lycopersicon esculentum*, Egyptian tomato

1 Introduction

Lycopersicon esculentum is a member of the family Solanaceae which comprises short-lived perennial herbaceous plants. Fruits of tomato have high nutritive values and variable uses (Tijjani et

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al., 2014). *L. esculentum* nutritive value represents a rich source of minerals, vitamins (A & C) and antioxidant lycopene that contribute to a healthy and well-balanced diet (Awan et al., 2018). Tomato (*Lycopersicon esculentum*) is the most important vegetable crop in Egypt. The crop annual production amounts are around seven mil¬lion tons, which are consumed either fresh or processed (Mansour, et al., 2009). Tomato's diseases are early blight and wilt caused by *Alternaria* and *Fusarium* (Singh et al., 1980; Maiero and Barksdale, 1989). These two diseases cause a severe reduction in yield and high economic losses every growing sea¬son. *Alternaria* is a prevalent fungal genus with distribution as saprophytic, endophytic and pathogenic species. This fungus can grow in variable substrates like seeds, plants, agricultural products, animals, soil and the atmosphere. Species of *Alternaria* are known as serious plant pathogens, causing major losses on a wide range of crops. At least 20% of agricultural spoilage is caused by *Alternaria* species; most severe losses may reach up to 80% of yield (Nowicki et al., 2012; Moghadam et al. 2019). Early blight is an economically important disease of worldwide importance affecting solanaceous crops and their wild relatives (Van der Waals et al., 2004; Chaerani and Voorrips, 2006; Kumar et al., 2007).

The main causal agent of early blight is generally considered to be *A. solani*. The disease was recognized in the solanaceous plant-like eggplant, tomato and potato (Neergaard, 1945) as well as non-solanaceous plants (Akhtar et al., 2011).

This work aims to characterize the early blight pathogen in tomato by isolation, microscopic and molecular identification and confirm the pathogenicity by re-inoculation of the pathogen in the healthy tomato plant and estimation of the plant disease severity.

2 Materials and Methods

Samples collection

In the summer, the severe disease appeared on the leaves, stem, and fruits of *Lycopersicon esculentum* variety Castle rock, in the Abu El-Rish region, at Aswan city, Upper Egypt. Tomato plant with symptoms was collected from different fields in the area and kept in sterilized plastic bags. All collected samples were transported to the laboratory and the pathogen was isolated immediately (Abdel-Motaal et al., 2010).

Isolation of pathogen from infected leaves, stems, and fruits

Leaves, stems, and fruits with severe symptoms were surface sterilized in ethanol (75%) for 10 s and NaClO (1%) for 1 min, finally swilled three times with sterile water. Small pieces were aseptically cut and incubated on potato dextrose agar (PDA) for 7 days at 28 °C. Once visible mycelia appear from the plant pieces, single hypha was transferred and cultured on fresh PDA plates and incubated at 28 °C then examined by microscope (Van Emden, 1970).

Morphological and molecular identification of the pathogen

Isolated fungi were identified based on their morphological characteristics according to Van Emden, 1970; Ellis, 1971; 1976; Moubasher, 1993. Molecular characterization of the pathogen was done by the sequencing of rRNA gene with the help of Solgent Company, Daejeon South Korea. Fungal DNA from seven days of incubated fungi was extracted by CTAB method (Gontia-Mishra et al., 2014). The rDNA was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the reaction mixture, ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC

TCC GCT TAT TGA TAT GC -3'). The purified PCR products were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. These bands were eluted and sequenced with the incorporation of dideoxynucleotides (dNTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers. Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. MegAlign (DNA Star) software version 5.05 was used for phylogenetic analysis of sequences (White et al. 1990).

Pathogenicity test

According to Berner et al., (2007) the pathogenicity test was done by the re-inoculation method. The fungus was grown on PDA and incubated at 28 ℃ for 14 days. The harvested conidia were suspended in sterile distilled water at 1.2x106 conidia/ ml, the stem and leaves of L. esculentum variety Castle rock were sprayed by the conidial suspension. Under moist conditions, plants were covered with polyethylene bags and incubated at 30 ℃ for 4 weeks. Under the same conditions, the control plant was sprayed with sterilized distilled water instead of the fungal conidial suspension. In another experiment, healthy tomato leaves and fruits were platted in sterilized Petri dishes with wet sterilized filter paper, mycelia disks of the pathogen were inoculated on the leaf and fruit surface in addition to other plates without pathogen. All plates were incubated at 30 ℃ for 7 days.

Statistical and data analysis

The common diseases symptoms were surveyed and disease severities were estimated randomly on tomato plants. Collected plant samples from fields with clear symptoms in approximately equal sites were brought as method described by Rao et al. 2016.

The Formula in calculating the disease severity is: Percentage of disease severity= *Number of Individual ratings/Number of Plant Assessedx100/Maximum scale*. The maximum rating scale (1-5) has been used for assessment of the disease severity is (Table.1):

Rating Scale	Disease Percentage
1	1-5
2	5-25
3	25-50
4	50-75
5	75-100

The sampling data have been collected from five different fields in Abu El-Rish area. All the collected data were subjected to analysis of variance at an end of the study by using GenStat Release 10.3 (2011) software application.

3 Results

Symptoms

Disease symptoms appeared as obvious circular white patches that developed to dark brown upon sporulation of the fungus on leaves and black patches on fruits. High temperature (40-47°C) in the fields induced plant severing, which caused leaf curling and defoliation, in addition to spreading fungal spore on the stem and infected fruits frequently drop (Fig. 1 A, B, C). Germinated conidia colonized tomato leaves, and then penetrated the leaf tissue through stomata and continued to grow through the leaf cortex, and eventually, the leaf tissue died.

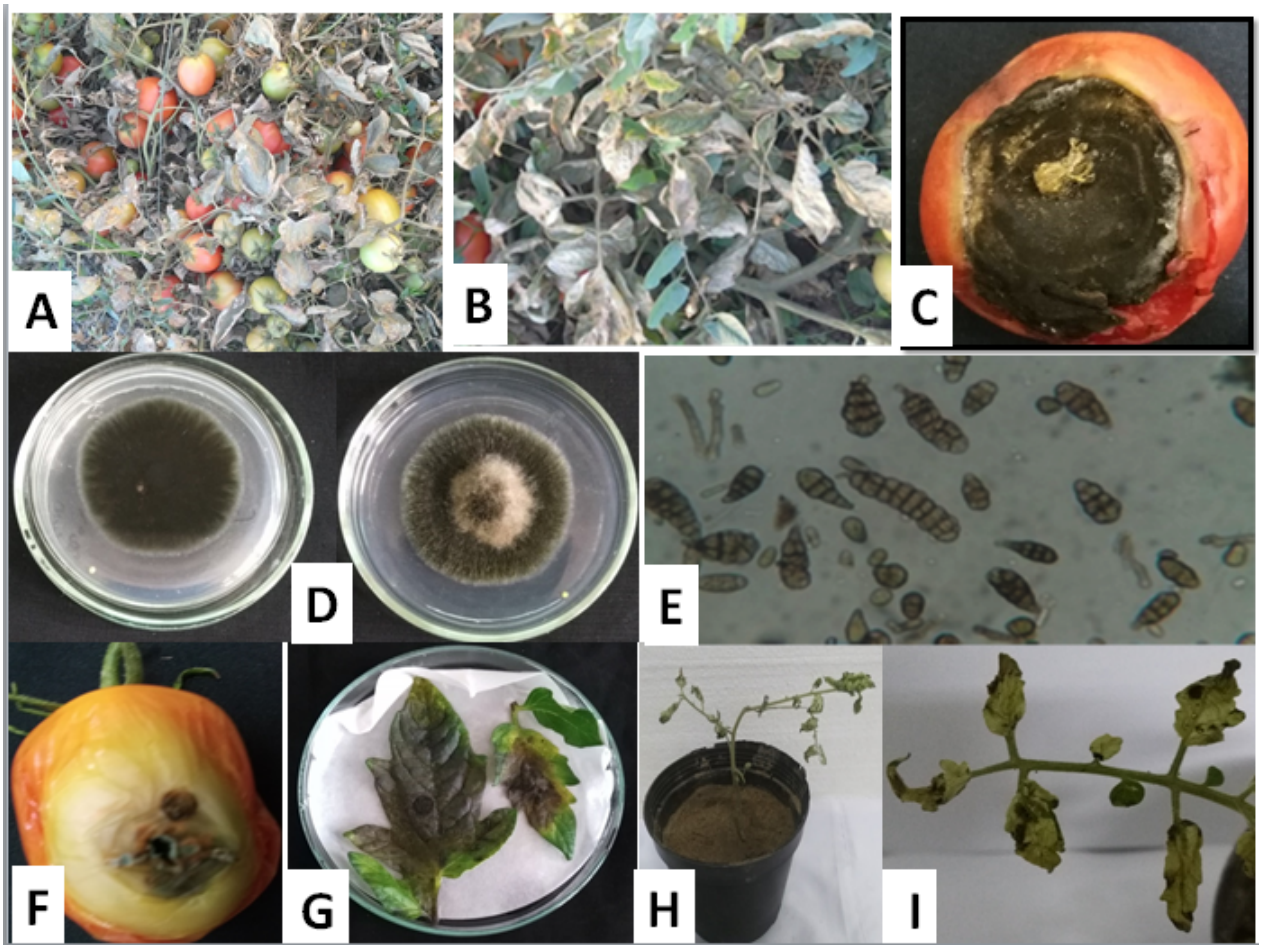

Figure 1. Symptoms of *Alternaria phragmospora* early blight disease in Egyptian Tomato (*L. esculentum*) and morphology of the causative agent *A. phragmospora*. (A, B) natural disease symptoms on the leaves and stem of *L. esculentum* in Abu El-Rish region. (C) The severity of disease by the pathogen in the fruit of *L. esculentum*. (D) Colony, front (right) and back (left) views. (E) Examination of conidia under a light microscope (40X) growing on PDA. (F, G) Pathogenicity test on the fruit and leaves of tomato. (H,I) Brown spots on leaves and stems after spraying the tomato plant by *A. phragmospora* spore suspension.

Morphological characterization of the pathogen

Colonies of isolated fungi on PDA media were dark brownish to black color which were deeply brown to black fundamentally at the colony center, had restricted white margins and black reverse (Fig. 1D). Well-developed septated and loosely branched mycelia (0.5–3.5 µm wide) were recognized. The mycelial wall was brown to deep brown, smooth or slightly rough and unthickened to somewhat thickened. On the septated hyphae, pale brown conidiophores were visible near the agar surface with branched chains of conidia. Slenderly obclavate conidia were abundant, but occasionally elliptical narrow ones would be found. They produced in branched or unbranched chains of varying length, conidia were in short chains cylindrical and with 2-9 transverse septa and longitudinal septum (Fig. 1E). The beak is either short or absent; beaks of more than three cells were not seen. Morphological characteristics matched the *Alternaria phragmospora* (Emden 1970). Yet, there is no report on the disease of *L. esculentum* caused by *A. phragmospora*.

Molecular characterization of Pathogen

For fungal molecular identification, a single spore culture (Leslie and Summerell, 2006) of the

fungus was prepared for genomic DNA extraction and gene amplification (Suarez et al., 2005). The partial 28S rDNA gene and the internal transcribed spacer region (ITS) were amplified using primers ITS1 and ITS4 (White et al., 1990). The ITS sequence showed 93–100% identity with all *Alternaria phragmospora* (NR 135960, MH 859605.1, MK 461012.1) based on the NCBI-BLAST analysis of DNA sequences (Fig. 2). The ITS sequences of *Alternaria phragmospora* were deposited in the Gene Bank database with accession No. LC475453.

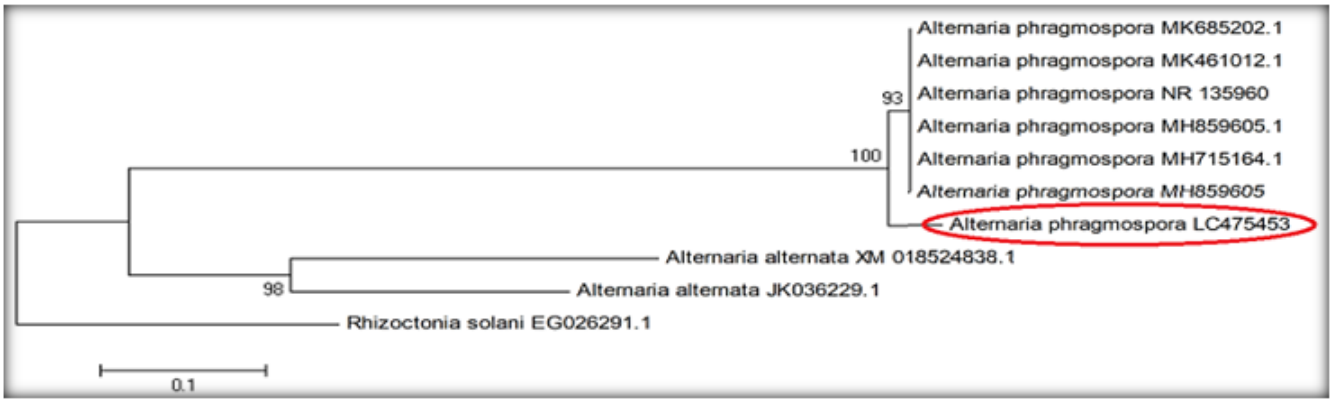

Figure 2. Phylogenetic relationships of isolated *A. phragmospora* and selected fungi derived from NCBI Genbank based on nuclear ribosomal internal spacer sequence (ITS). The phylogram represents the Neighbor-joining method with 1000 bootstrap replication. *Rhizocotonia solani* was the outgroup fungus. Bootstrap values are shown on each branch using MEGA 4 software.

Pathogenicity

In the case of spraying the healthy tomato plant with *A. phragmospora* conidial suspension ($1.2x10^6$ conidia/ ml), small yellow spots appeared after 2 weeks on leaves, which enlarged and turned brown and spreading to stem after 3 weeks (Fig. 1 H,I). Control plants did not develop any symptoms. *Alternaria phragmospora* was re-isolated from the infected plants but not from the control. The second pathogenicity test by inoculation of healthy tomato leaves and fruits by mycelia discs and incubated in sterilized Petri dishes with wet sterilized filter paper. The results indicated maximum lesion length and width on leaves and fruits inoculated with *A. phragmospora* (Fig.1 F, G).

4 Discussion

Early blight, caused by the fungus *Alternaria*, is also known as *Alternaria* leaf spot or target spot. Early blight is common in tomato plantings. Premature loss of lower leaves is the most obvious symptom of the disease and spots frequently merge, forming irregular blotches. Dark, concentric rings often appear in leaf spots. Only a few spots are enough to turn leaves yellow and dry up. The pathogen is spreading to the end of the stem and reaching fruits, causing large, sunken areas with concentric rings and a black, velvety appearance. *Alternaria* is capable to colonize plant parts rapidly (Grzybowska and Kapala, 1976). There are multiple factors for disease severity, including time in the growing season, water availability, and spatial factors (O'hara et al., 2016). Based on field observations and the literature, we formulated three main hypotheses: (1) disease severity was clear during summertime because of high temperature (40-47°C) in this area of Upper Egypt (2) Disease severity in the field was greater in excess water availability by overwatering of

the plant and high soil moisture than moderate water in other fields (3) plant density in patches increase disease severity and fast spreading of the pathogen. Generally, *A. solani* is known as the causal agent of early blight of tomato and later a study indicates that *A. cretica*, *A. elegans*, *A. subtropica*, and *A. tomatophila* (Simmons, 2007) *A. alternata* (Loganathan, et al., 2016) also exists in the virulent form to cause the disease.

In this study, we confirmed that *A. phragmospora* is also capable to cause early blight disease of tomato. *Alternaria phragmospora* was previously reported to be associated with radish seedlings which caused hardly damage to it (Van Emden 1970), and isolated from sugar cane leaves (Gherbawy, 2005). The fungus *Alternaria* species may cause allergies in humans and animals (Breitenbach and Simon-Nobbe, 2002), and the presence of this fungus in plants should be considered a reason for disqualification of the plants as food. To reduce these incapacitates and losses of tomato crops caused by the pathogen *Alternaria*, early control actions should be taken. In our laboratory, we are working in finding biocontrol agents to management tomato early blight disease caused by *Alternaria phragmospora*. The isolated pathogen, *Alternaria phragmospora* has been deposited in Mycology laboratory culture collection, Faculty of Science, Aswan University, Egypt with the accession no., APT-38018.

Conflict of interests

The authors declare that they have no conflicts of interest.

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