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Article

Aspergillus tubingensis Causes Leaf Spot of Cotton (*Gossypium hirsutum* L.) in Pakistan

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Abstract: Cotton (*Gossypium hirsutum* L.) is a key fiber crop of great commercial importance. Numerous phytopathogens decimate crop production by causing various diseases. During July-August 2018, leaf spot symptoms were recurrently observed on cotton leaves in Rahim Yar Khan, Pakistan and adjacent areas. Infected leaf samples were collected and plated on potato dextrose agar (PDA) media. Causal agent of cotton leaf spot was isolated, characterized and identified as *Aspergillus tubingensis* based on morphological and microscopic observations. Conclusive identification of pathogen was done on the comparative molecular analysis of CaM and β-tubulin gene sequences. BLAST analysis of both sequenced genes showed 99% similarity with *A. tubingensis.* Koch's postulates were followed to confirm the pathogenicity of the isolated fungus. Healthy plants were inoculated with fungus and similar disease symptoms were observed. Fungus was re-isolated and identified to be identical to the inoculated fungus. To our knowledge, this is the first report describing the involvement of *A. tubingensis* in causing leaf spot disease of cotton in Pakistan and around the world.

Keywords: *Aspergillus tubingensis*; CaM gene; Koch's postulates; cotton

1 Introduction

Cotton (*Gossypium hirsutum* L.) is the major fiber and cash crop and it is generally known as "silver fiber". Cotton is also considered to be the core strength of the economy of Pakistan [1], China, USA, India and Pakistan are the world's prominent cotton growing countries. Pakistan is third largest exporter and fourth major producer of cotton [2]. Apart from being a good source of fiber, cotton seed is also used for the extraction of oil, in different parts of the world. Moreover, different parts of cotton plant are being used in traditional medication. For many years, young shoots have been used to treat asthma. Crushed seeds are being used to cure bone ache and blossoms have been used to treat earache [3]. A phenolic aldehyde, Gossypol possesses distinct antiviral, anti-cancerous, spermicidal, antimalarial and anti-parasitic activities [4,5].

Aspergillus tubingensis belongs to class "Ascomycota" and it is known as black *Aspergillus* specie. In 1934, Raoul Mosseray described *A. tubingensis* as a black *Aspergillus* species, for the first time [6]. This species is found all over the world, grows on fresh parts as well as predominantly on dead plant material [7]. *A. tubngensis* is morphologically very similar to *A. niger*, which makes it hard to identify them based solely on structural data. However, *A. tubingensis* could microscopically be distinguished by its production of seclorotia, which shows a characteristic white to pinkish color. The seclorotia production by species of *A. tubingensis* is not always observed in the species of *A. niger* [8]. *A. tubingensis* have the ability to produce ochratoxin (OTA), a very dangerous toxin to human and animal health. It has been

reported to cause skin infection [9] and lung diseases [10]. In *A. tubingensis*, when the development of conidia starts, white colored mycelium starts turning black. Its hyphae and conidiophores are uniserate. It also possesses light brownish to brownish red conidial mass [11]. *A. tubingensis* have been reported to be involved in damaging agronomically important food crops. It causes disease on leave and fruits of different plants. It also has the ability to synthesize numerous mycotoxins which are harmful to poultry, livestock, fish and human kind [12,13].

Yield of upland cotton is affected most notably by cotton leaf curl virus (CLCV) in Pakistan. Similarly, some other biotic factors including viruses, insects, bacteria and fungi cause various diseases on cotton and thus affect the yield of cotton crop. While some environmental factors such as heat stress and unavailability of water also contribute towards low cotton yield [14]. Mostly, leaves of cotton are more prone to diseases. Disease may also occur on aerial parts and roots [14].

Some fungal diseases that have been reported earlier include stem lesions (caused by *Ascochyta gossypii*), anthracnose, seedling blight of cotton (caused by *Alternaria macrospora*), leaf blight (caused by *Cercospora gossypina*), seedling rot of cotton (caused by *Phytophtora* spp., *Fusarium* spp., *Colletotrichum gossypii* and *Aspergillus* spp.), lint degradation (caused by *Aspergillus flavus*), boll rot (caused by *C. gossypii* and *Aspergillus* spp.), Fusarium wilt, *Alternaria* leaf spot, cotton rust (caused by *Puccinia scheedonnardi* and *Phakospora gossypii*), and *Verticillium* wilt (caused by *Verticillium dahlia*) [15]. Different species of *Aspergillus* have also been reported as cotton pathogens. Cotton ball rot and lint rot has been observed to be caused by *Aspergillus flavus* [15]. Among bacterial diseases, bacterial leaf blight (BLB) is the most important bacterial disorder, caused by *Xanthomonas campestris* pv. *Malvacearum* [16]. Crown gall and lint degradation are other major bacterial diseases of cotton [9]. Viruses are the most devastating biotic disease factors of cotton and may result in entire crop loss. Gemini viruses are the most potentially threatening, affecting productivity of cotton crop, worldwide. These are disseminated by white fly (*Bemisia tabaci*). Cotton leaf crumple disease (CLCrD) and cotton leaf curl disease (CLCuD) are known for heavy yield damages [17]. Pests of cotton crop include white flies and leaf insects. Cotton boll weevil infests cotton bolls and causes boll rot and leaves death [18].

The objective of current study was to isolate and characterize pathogen causing leaf spot of cotton. This study also described the pathogenicity of *A. tubingensis* on healthy plants of cotton.

2 Materials and Methods

2.1 Collection of Diseased Samples

In July-August 2018, a severe leaf spot disease of cotton was observed in district Rahim Yar Khan and adjacent areas (28°25’12”N 70°18’0”E). Early foliar symptoms developed along the veins and turned into dark brown lesions. For the identification of disease causing pathogen, cotton leaves with dark brown lesions and necrotic spots were collected in sterile bags and brought to Molecular Plant Pathology Laboratory, Quaid-i-Azam University, Islamabad, Pakistan.

2.2 Isolation of Pathogen

For the isolation and growth of fungus, potato dextrose agar (PDA) media was prepared. For this purpose, 200 gm of potatoes were boiled in one liter of water, until the color of water turned light yellow. Then it was filtered through muslin cloth. In the filtered water, dextrose (20 g) and agar (15 g) were added. This mixture was autoclaved for 15 min at 121ºC and 15 PSI. Pouring of culture media into petri plates (90 mm) was performed and solidified. Selected leaves were surface sterilized with 0.1% $HgCl_2$ for 45 s, rinsed in 70% ethanol for 30 s and washed with autoclaved distilled water, five times. Afterward, 2 mm diseased portion was excised and placed on PDA plates. These plates were incubated at 25 ± 3°C for 6-7 days, under dark conditions.

2.3 Morphological and Microscopic Identification of Pathogen

After 8 days of incubation, prominent mycelial growth could be observed on PDA media. Petri plates were carefully observed from front and back side, to see the morphology of mycelia. For more detailed observation, light microscope and stereoscope were used to see the shape, size, length and width of conidia and acervuli. For light microscopy, one or two drops of lactophenol blue were placed on the slide and pieces of young mycelium, from the margins of fungus culture, were positioned on slide by using mounting needles. Cover slip was placed carefully, avoiding air bubbles. The slide was observed at 100× magnification.

2.4 Molecular Identification of Fungus

For molecular characterization, fungal DNA was extracted, using a standard protocol [19]. Specific calmodulin (CaM) gene was amplified using PCR with specific CMD5/CMD6 primers [20]. Similarly, β-tubulin primers (Bt2a/Bt2b) were also used to amplify β-tubulin gene [21]. PCR reaction mixtures was comprised of genomic DNA (1 µL), *Taq* DNA polymerase (1.5 µL), dNTPs (6 µL), 10× polymerase buffer (5 µL) and each primer (1 µL). The thermocycler was set at 94°C for 4 min, followed by 35 cycles of 94°C for 60 s, 58°C for 1 min, and 72°C for 60 s. After 35 cycles, PCR reaction mixture was kept at 72°C for 10 min for final extension. The resultant PCR product was sequenced and subjected to BLAST analysis in NCBI database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were aligned in MEGA, version 7.0 [22]. Phylogenetic analysis was performed using maximum composite likelihood method with 1,000 bootstrap replications.

2.5 Pathogenicity Analysis

Pathogenicity of isolated pathogen was confirmed following Koch's postulates. For this purpose, fungus was grown in Czapek media and shaken for 5 days. Then the mixture was filtered through muslin cloth to separate fungal spores. The fungal filtration procedure was performed in the laminar air flow hood and a specific conidial suspension (10^6 conidia mL^{-1}) was maintained. For the induction of disease, 10 healthy leaves were surface sterilized and inoculated with conidial suspension. Control leaves were treated with sterile distilled water. The inoculated leaves were placed in plant growth chamber at 28°C and 70% relative humidity. Five days post-inoculation, obvious disease symptoms could be observed. Leaves of control plants remained healthy. From these self-inoculated leaves, disease causing pathogen was isolated and grown on PDA media. Physiological and microscopic characteristics of the isolate were observed.

3 Results and Discussion

Diagnosis of leaf spots is always challenging because these are caused by different air-born biotic and abiotic factors. This study was based on the diagnosis of leaf spots in which initial symptoms on diseased cotton leaves were observed as typical small, multiple dark brown spots along the midrib (Fig. 1(A)). Primary symptoms usually developed along leaf veins and asymmetrical spots gradually increased in size and number and turned dark brown, eventually [23]. These spots were different than usual bacterial leaf blight of cotton. After the incubation of sterilized diseased leaf parts on PDA media, a rapid whitish mycelial growth was observed. After 6 days of culturing, net-like fungal colony (6-7 cm in diameter) was observed which was black in the center and its edges were white with powdery texture (Fig. 1(B)). On the back side of petri plate, mycelia were yellowish in color (Fig. 1(C)). This was a typical growth pattern of *A. tubingensis* [21]. Previous studies have also described the same growth pattern of *A. tubingensis* on Czapek yeast extract agar culture (CYA) media [24] and PDA [25].

A. tubingensis usually shows similar growth pattern to *A. niger* on PDA. So, it was imperative to identify this pathogen by microscopy and gene sequencing. Microscopic observation at 100× magnification, revealed uni-sereate conidiophores, which were yellow in color and enclosed by greenish phialides layer. These conidiophores were 21 µm to 35 µm in length and 33 µm to 35 µm in diameter. Each phialide was bearing globose to sub-globose, dark brown conidia. Initially conidial surface appeared

to be even but slowly produced some thorns. The conidia were 2.4 µm to 4.8 µm in diameter (Fig. 1(D)). All these morphological and microscopic observations proved this pathogen to be *A. tubingensis* [26].

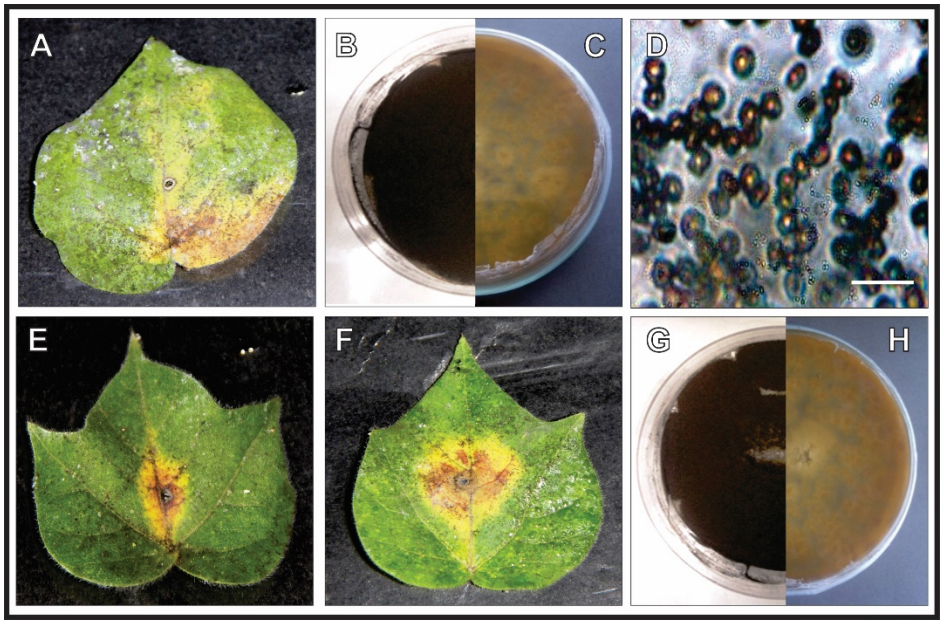

Figure 1: Leaf spot were observed on cotton leaves (A). Diseased causing fungus was isolated in petri plates containing PDA. Petri plates were observed from front side (B) and back side (C). Microscopic observation of fungus at 100× magnification (D). Disease symptoms were observed after 3 days (E) and 6 days post inoculation (F). Pathogen was re-isolated and petri plates were observed from front (G) and back side (H). Scale bar: (D) = 10 µm

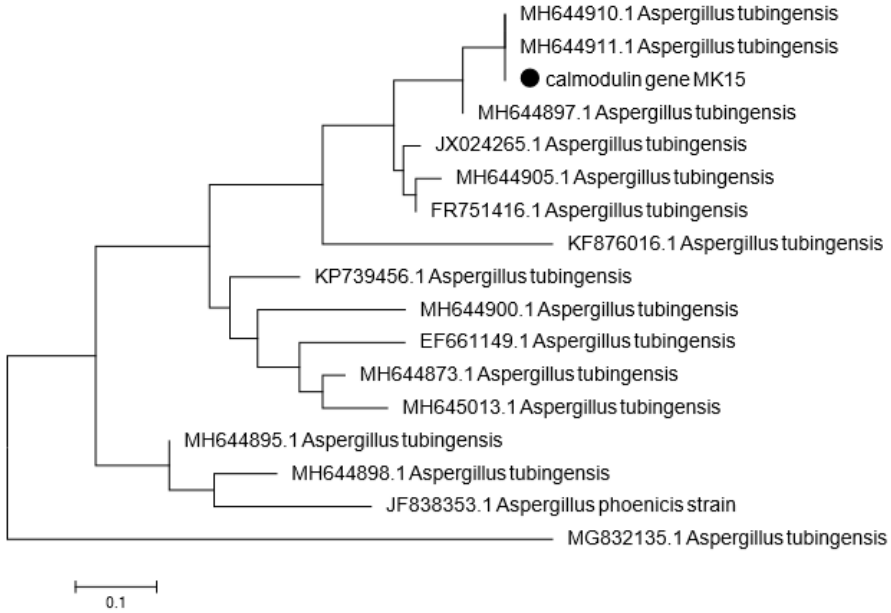

Figure 2: Phylogenetic analysis of *A. tubingensis* (strain MK15) isolated from cotton, with 16 related Calmodulin gene sequences from GenBank

In this study, amplification of CaM and β-tubulin genes also helped us to characterize isolated pathogen. BLAST analysis specified that the CaM gene sequence was 99% similar to *A. tubingensis*

(MH644910.1 and MH644911.1). Similarly, amplified β-tubulin sequence was also 99% similar to *A. tubingensis* (KY608858.1). Sequences of both genes were deposited in NCBI database. Previous studies have also identified *Aspergillus* species, on the basis of ITS, β-tubulin (βtub), actin and calmodulin genes [27,28]. This is indeed a more reliable way to distinguish closely related species. In this study, we used Calmodulin (CaM) gene because it is considered to be very reliable for the identification of *Aspergillus* species because its Ca^{2+} binding protein is highly conserved in eukaryotes [29,30]. The calmodulin gene also helps to differentiate various species of *Nigri* [31]. In molecular biology, comparison of nucleotide or protein sequences from the identical or unrelated organisms is very important. Basic Local Alignment Search Tool (BLAST) is the most recurrently used tool for evaluating similarity among sequences. Scientists can now conclude the function of newly sequenced genes, explore evolutionary relationship and predict new gene members of gene families by finding similarities between sequences [32]. Phylogenetic analysis also confirmed the presence of both CaM gene (Fig. 2) and β-tubulin gene (Fig. 3) in the same clade with *A. tubingensis*.

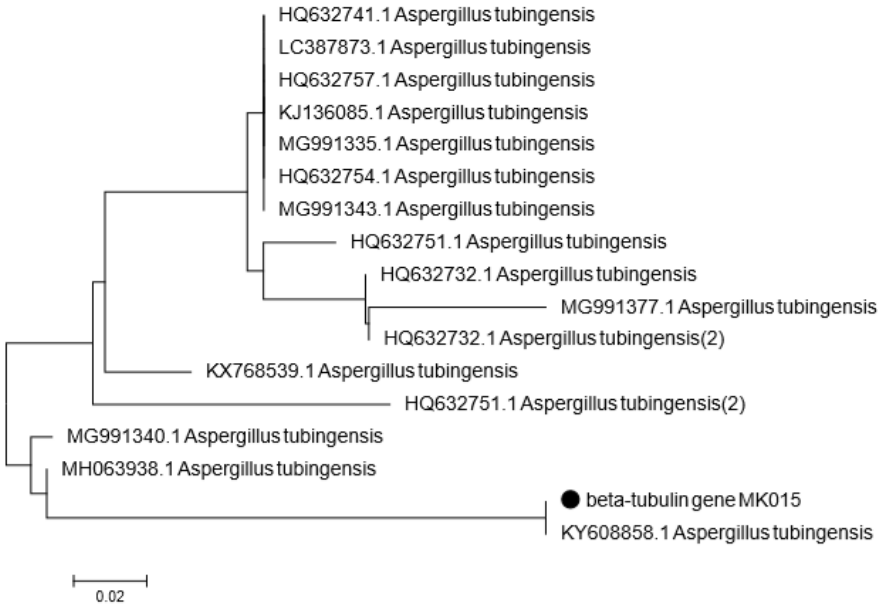

Figure 3: Phylogenetic analysis of *A. tubingensis* (strain MK15) isolated from cotton, with 16 related β-tubulin gene sequences from GenBank

Before claiming *A. tubingensis* to be the disease causing pathogen of leaf spot of cotton, its pathogenicity test was performed, following Koch's postulates. In pathogenicity test, symptoms of leaf spot were less evident in first three days. These symptoms appeared gradually in 1st week and irregular size spots progressed rapidly on leaf surfaces, thereafter (Fig. 1(E)). After 10 days post inoculation, typical symptoms could be observed, which were similar to the symptoms observed in the field (Fig. 1(F)). Fungal cultures were re-isolated from the self-infected leaves on the same PDA media and found to be similar to *A. tubingensis* (Figs. 1(G), 1(H)). In accordance with Koch's postulates, these results confirmed the involvement of *A. tubingensis* in leaf spot of cotton.

A. tubingensis has been formerly reported to cause similar disease on *Jatropha curcas* [23] and *Hellebore* species [21]. In a recent study, this fungus has been described to induce bunch rot of grape [33]. In California, *A. tubingensis* has been reported to cause strawberry fruit rot [34]. Our findings have described a fungal pathogen causing leaf spot diseases. Usually cotton leaf diseases are associated with bacterial and viral pathogens. These results will help farmers and scientist to timely diagnose this disease and use effective control measures.

4 Conclusion

This study helped us to identify *A. tubingensis*, as the causal organism of leaf spot disease of cotton. To our knowledge, this is the first report of *A. tubingensis* causing leaf spot disease on cotton in Pakistan and around the world.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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