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ORIGINAL ARTICLE

Cotton leaf blight disease caused by *Alternaria alternata* in Sudan

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Abstract

Genetically modified Bt cotton (*Gossypium hirsutum*) leaves with typical symptoms of Alternaria early blight disease resembling that of tomato and potato were observed in the main cotton growing schemes in Sudan. Symptoms on leaves appeared as either brown 2leaf spot with gray centers or leaf blight with concentric rings. Pathogenicity tests using isolates with both symptoms showed that the isolated fungi were highly pathogenic to both *G. hirsutum* and *G. barbadense* cotton varieties. *Alternaria alternata* isolated from infected tomato and potato leaves with early blight symptoms was included for comparison. Microscopic examination showed that the mean length of conidia from cotton, tomato and potato isolates ranged from 26.25 to 45.45 µm, while the width ranged from 9.56 to 13.64 µm. The mean number of transverse septa among all isolates was 3.4 to 5.7 and the peak length ranged from 3.75 to 7.8 µm. Based on morphological characteristics the two isolates from cotton were identified as *A. alternata.* Genomic DNA was extracted directly from fungal cultures grown on potato dextrose agar (PDA) plates using a Zymo Research Quick DNA kit. A species-specific primer using the internal transcribed spacer ribosomal DNA (ITS rDNA) PCR scoring indicated the presence of *A. alternata* using primer pair ITS4/ITS5. Amplifications of the internal transcribed spacer region of 600 bp revealed 100% identity of the isolated fungus from cotton with *A. alternata* from tomato and potato. These data oblige us to reconsider the presence of *A. alternata* in the four main cotton growing schemes in Sudan while these symptoms have always been described for tomato and potato early blight disease.

Keywords: *Alternaria alternata*, conidia, *Gossypium* spp., internal transcribed spacer (ITS), morphological variability, pathogenicity

Introduction

The genus *Alternaria* is one of the most important fungi responsible for leaf blight symptoms in many cultivated crops. *Alternaria alternata* (Fr.) Keissler is a pathogen that infects numerous hosts. It causes early blight of tomatoes (*Solanum lycopersicum* L.) and potatoes (*Solanum tuberosum* L.) and other members of the family Solanaceae (Pryor and Michailides 2002; Khalid *et al.* 2004*;* Maiti *et al*. 2007). *Alternaria macrospora* is considered the main causal agent for leaf spot disease in *Gossypium barbadense* cv. Pima but not in *Gossypium hirsutum* cv. Acala. On the other hand, *A. alternata* is capable of producing leaf blight symptoms in both cotton species but is found mainly on *G. hirsutum* plants (Bashan *et al.* 1991). When both cotton species were naturally infected by both pathogens together, the number of *A. alternata* spores on the leaf surface was greater than that of *A. macrospora* (Bashan *et al*. 1991). The symptoms caused by this pathogenic species on tomato and potato leaves are brown spots with characteristic concentric rings that may coalesce to form large lesions and eventually cause leaf death. Under favorable conditions, the

disease can cause severe defoliation, yield reduction and lower the quality of the marketable crop (Kemmitt 2002).

Recently, characteristic brown leaf spots and leaf blight symptoms with concentric rings similar to those of tomato and potato early blight disease were observed in Bt cotton (*Gossypium hirsutum*) in many geographic areas in Sudan. However, these symptoms had not been observed earlier in any of the local *G. barbadense* and *G. hirsutum* cotton cultivars. The symptoms developed on Bt cotton leaves, bracts and bolls. They first appeared as small, circular brown, gray-brown to tan spots which varied in size from 3–10 mm. Mature spots had dark concentric rings or dry, dead, gray centers which often cracked and fell out. Plants were most susceptible late in the season.

Alternaria leaf blight is an important disease of cotton in many parts of the world. In China, the disease has resulted in a significant reduction of 20–50% in yield and poor fiber properties in recent years (Zhao *et al*. 2012). Yield losses of the diseases can also be as high as 20% in the USA (Zhao *et al*. 2012). In Sudan the disease has not received much attention but since it is emerging as an important disease in Bt cotton, more research will be done in the near future to evaluate its economic importance.

The internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit has become the primary genetic marker for molecular identification in many groups of fungi especially *Alternaria* (Sharma *et al*. 2013). The identification of fungi to the species level has been based mainly on the use of variable ribosomal-DNA (rDNA) ITS regions. The non-coding ITS region consisting of ITS1, the 5.8S rDNA and ITS2, ITS3 and ITS4 should produce highly sensitive assay as the target sequence for amplification, because of its high copy number in the fungal genome as part of randomly repeated nuclear rDNA (Guo *et al*. 2004). The objective of this study was to classify *Alternaria* isolated from Bt cotton to the species level and to investigate any genetic diversity between *Alternaria* isolates from cotton compared with *Alternaria alternata* from tomato and potato.

Materials and Methods

Isolation of the fungus

Infected Bt cotton leaves variety Seeni1 with characteristic symptoms of *Aternaria* leaf spot (Fig. 1A) and leaf blight (Fig. 1B, C, D) were collected from four cotton schemes (El-Suki, Gezira, El-Rahad and New Halfa) in Sudan. Infected leaf lesions were cut into small discs (3–5 mm), surface sterilized with 1% solution of sodium hypochlorite for 1 minute followed by three rinses of sterilized distilled water. The discs were plated on PDA and incubated at 25 ± 2°C for 7 days. Cultures were similarly prepared from infected tomato and potato leaves with early blight symptoms. All cultures were purified using single spore cultures and then transferred to potato dextrose agar (PDA) medium for storage and subsequent studies. Microscopic identification of all isolates to the genus level was made under a light microscope. Further identification to the species level was performed according to *Alternaria* conidial characteristics using the Ellis *Alternaria* key (Ellis 1971).

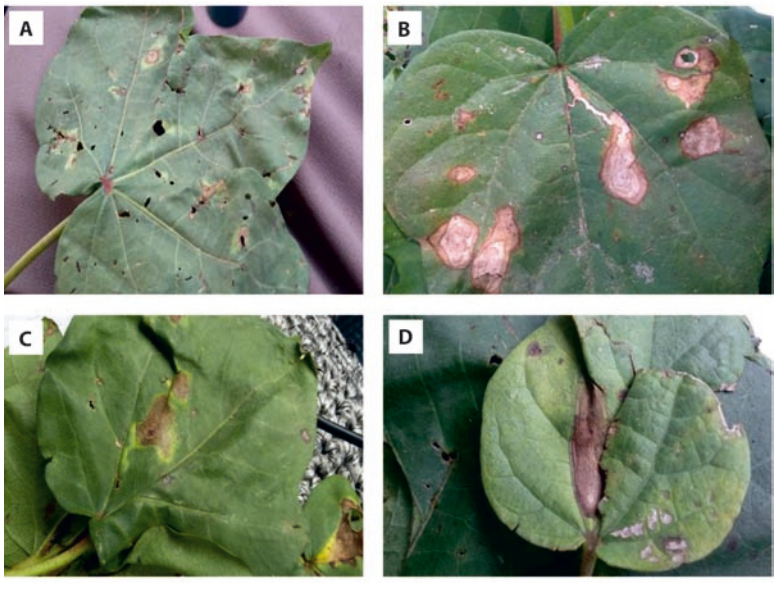

Fig. 1. Symptoms of *Alternaria alternata* on cotton. A – leaf spot, B, C, D – leaf blight

Morphological and cultural variability

Alternaria isolates from infected cotton leaves collected from different areas in Sudan were grouped according to their morphological character similarities in two isolates to represent leaf spot and leaf blight symptoms. Isolates from cotton, tomato and potato were subjected to detailed morphological and cultural characteristics viz., colony appearance on PDA, conidia shape, size (length and width) and beak length. The numbers of transverse septa were also recorded and means of all measurements in micrometers (µm) were calculated. The morphological characters were measured in 50 conidia of each isolate under a microscope with a high power objective using ocular and stage micrometers.

Pathogenicity test

The pathogenicity of two purified isolates of *A. alternata* from cotton were tested on Bt cotton variety Seeni1 (*G. hirsutum*) and Barakat (*G. barbadense*) cotton plants and confirmed by Koch's postulates. Cotton plants were raised in pots under green house conditions. Fifteen plants of each variety were sprayed with a spore suspension ($10^6 \cdot ml^{-1}$) of each of the two cotton isolates using a hand sprayer. Inoculated plants were then kept in a humid chamber and observed daily for disease symptoms.

Molecular variability

DNA extraction

Single spore cultures of *Alternaria* sp. were incubated on PDA in the dark at 25°C for 10 to 15 days. About 50–100 mg fresh fungal mycelia, scraped from the surface of the agar plate, was transferred into a tube. Genomic DNA was extracted directly from fungal cultures growing on PDA plates using a Zymo Research Quick DNA – Fungal/ Bacterial Miniprep kit Catalog No D6005. The extracted genomic DNA was quantified for each sample with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA) as well as by gel electrophoresis and dilutions made to a final concentration of 10 $ng \cdot \mu l^{-1}$ and stored at –20°C for further use.

Amplification of ITS region

Sequences for the primers ITS4 and ITS5 region were 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and 5′-TCC TCCGCTTATTGATATGC-3′, respectively. Amplification was performed in a PCR using the following program: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Amplifications were performed in 50 µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 200 mM of each dATP, dCTP, dGTP, dTTP, 25 pmol of each primer, 100 ng of genomic DNA, and 1 unit of Taq polymerase. Amplification products (5 µl of a 50 µl reaction) were electrophoresed in 1.4% agarose gels with 0.5% TBE running buffer (89 mM Tris, 89 mM borate, 2 mM EDTA pH 8.3), stained with GelRed Nucleic Acid Gel Stain and photographed to verify the fragment. Finally, the gels were photographed under UV Transilluminator 2000.

Data analysis

The data was analyzed using MSTSC version and the Duncan Multiple Range Test was used for mean separation.

Results

Morphological and cultural variability

All the isolates from cotton, tomato and potato leaves when cultured on PDA plates had a pale grey to olive appearance during the first 7 days. The color changed with time to dark olive on the upper side of the plate after 2 weeks. The bottom sides of the plates of all isolates were dark black. According to the characteristic *Alternaria* conidia shape, cultures of all isolates were identified as *Alternaria* spp. under a light microscope. Conidiophores were brown, septate, simple or branched and the conidia appeared brown, ovoid or obclavate solitary or in chains. The length of conidia of the cotton leaf spot isolate ranged from 31.5 µm to 72 µm and the width from 9 µm to13.5 µm. The beak length ranged from 4.5 µm to 13.5 µm and the number of transverse septa ranged from 4 to 8. The isolate from cotton leaf blight symptoms displayed conidia 20 µm to 40.5 µm long, 4.5 µm to 13.5 µm wide with beak lengths from 2 µm to 9 µm and 3 to 4 transverse septa. The length of conidia from potato ranged from 27 µm to 58.5 µm while the width range was 9 µm to 18 µm and the beak length was 4.5 µm to 9 µm with 4 to 6 transverse septa. The tomato isolate conidia length was 22.5 µm to 40.5 µm and 6.75 µm to15.75 µm wide while the length of the beak was 4.5 µm and the transverse septa 3 to 5. The means of conidia measurements are presented in Table 1.

The results in Table 1 revealed that the mean lengths of *Alternaria* conidia from cotton leaf spots and potato leaf blight were similar and those of tomato and cotton leaf blight were similar, too. The largest conidia were of the cotton leaf spots with a mean length of 45.45 µm followed by potato conidia (43.76 µm), tomato (29.25 µm) and cotton leaf blight (26.9 µm) (Fig. 2). The widest conidia among the four isolates were that of the potato isolate (13.64 µm), while

Table 1. Mean length, width, beak length and transverse septa of *Alternaria* isolates conidia

Alternaria isolates	Length [µm]		Width [µm]		Beak length [µm]		Transverse septa	
	mean	range	mean	range	mean	range	mean	range
Cotton leaf spot	45.45 a	31.5–72.0	11.03 b	9.0–13.5	7.80 a	4.5–13.5	5.70 a	4.0–8.0
Cotton leaf blight	26.90 b	20.0–40.5	9.56 b	4.5–13.5	3.75 c	2.0–9.0	3.40 d	3.0–4.0
Potato leaf blight	43.76 a	27.0–58.5	13.64 a	9.0–18.0	5.80 b	4.5–9.0	5.20 b	4.0–6.0
Tomato leaf blight	29.25 b	22.5–40.5	10.91 b	6.75–15.75	4.50 c	4.0–5.0	3.90 c	3.0–5.0
CV%	20.30		25.50		28.10		15.56	
LSD	4.66		1.82		0.99		0.45	

Means with different letters within a column are significantly different at ≤ 0.05

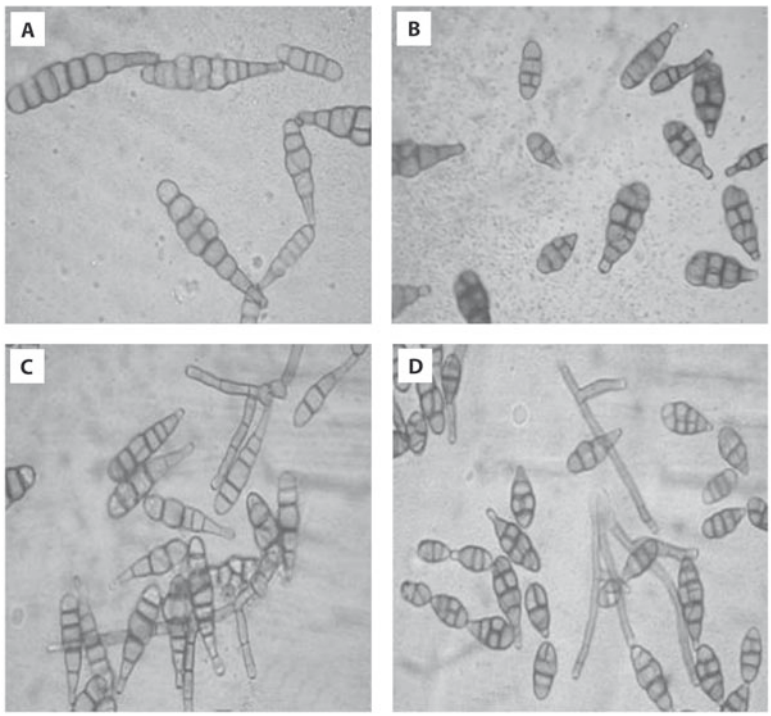

Fig. 2. Shape of *Alternaria* conidia under microscope X40. A – cotton leaf spot, B – cotton leaf blight, C – potato leaf blight, D – tomato leaf blight

the other isolates were similar in conidia width. The longest conidia beak among the isolates was that of cotton leaf spots conidia (7.8 µm) and the shortest was of cotton leaf blight conidia (3.75 µm). There were no significant differences between length, width and beak length of conidia isolated from tomato and cotton leaf blight symptoms. The highest number of mean septa in conidia (6) was in the cotton leaf spot isolate followed by potato (5), tomato (4) and cotton leaf blight (3) septa.

Pathogenicity test

Bt cotton variety Seeni1 (*G. hirsutum*) and Barakat *(G. barbadens),* when artificially inoculated with the two cotton isolates, developed brown leaf spots with concentric rings similar to the original symptoms (Fig. 3). All inoculated plants of both varieties showed identical symptoms and the fungus *A. alternata* was consistently re-isolated from infected leaves, fulfilling Koch's postulate.

Fig. 3. Symptoms on cotton seedlings after inoculation with *Alternaria alternata*

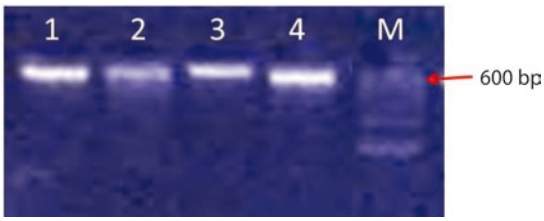

Fig. 4. Agarose gel showing amplification products from polymerase chain reaction using primer pair ITS4/ITS5 amplified a single 600 bp band; Lanes of *Alternaria* isolate: 1 – cotton leaf spot, 2 – cotton leaf blight, 3 – potato leaf blight, 4 – tomato early blight, M – marker at 100 bp

Molecular identification

Amplifications of the ITS region using primer pair ITS4/ITS5 resulted in a single PCR product of approximately 600 bp (Fig. 4) for all the isolates and in all the repetitions conducted with fresh DNA samples.

Discussion

Early blight disease caused by the fungus *A. alternata* is one of the major foliar diseases that affect tomato and potato crops in Sudan and causes major yield losses in most of the cultivated areas. In this study, the fungus *A. alternata* also infected cotton plants and caused leaf spot/ blight diseases which were detected in almost all the cotton growing schemes in Sudan. The disease has been reported in all cotton growing countries of the world (Venkatesh 2014). The symptoms of *Alternaria* blight on Bt cotton leaves observed under natural conditions appeared late in the season as brown lesions with dark concentric rings surrounded by a yellow halo or leaf spots that were brown with gray centers. These findings were similar to those of Maas *et al.* (2012) who reported *A. alternata* as a late season disease. As lesions expand, they typically exhibit concentric zonation and the necrotic tissue overlaps with other lesions to form larger lesions. These symptoms were similar to those described in cotton by Ephrath *et al.* (1989) and in potato (van der Waals 2001; Olanaya *et al.* 2009). The leaf blight symptoms found on cotton were very similar to those symptoms exhibited by tomato early blight disease in Sudan (Ahmed *et al.* 2006). At least two species of *A. alternata* and *A. macrospora* are capable of producing symptoms commonly known as *Alternaria* blight of cotton and are common in cotton crops around the world (Kaur and Aggarwal 2015). The morphological and cultural variability of the tested isolates in this study agree with the morphological descriptions of *A. alternata* presented by Ellis (1971). The identity of *A. alternata*, the causal pathogen of tomato and potato early blight was confirmed by the morphological description of Ellis (1971). These results concluded that *A. alternata* was the main causal pathogen of leaf blight disease in cotton as well as in tomato and potato. Chelkowski (1992) reported that *A. alternata* can infect Asian cotton (*G. abroreum*) and the upland cotton (*G. hirsutum*). The four tested isolates, cotton leaf blight, cotton leaf spot, tomato and potato leaf blight were very similar in their cultural and morphological characteristics. They showed similar culture color progress, growth rate and conidia measurements despite some variability in their length and the transverse septa number. The mean lengths of cotton leaf spot conidia and potato conidia were the same (45.45 µm and 43.76 µm, respectively). On the other hand, those of cotton leaf blight also resembled tomato early blight isolates (26.90 µm and 29.25 µm, respectively). Similarities between the four isolates were detected in conidia width, beak length and the number of transverse septa.

Bashan (1991) identified *A. macrospora* as the causal pathogen of leaf spot, premature defoliation and yield loss in cultivars of *G. barbadense* in Israel and in the USA. He proposed that *A. macrospora* together with *A. alternata* create a disease composite responsible for *Altemaria* blight symptoms in cotton. In this study we isolated only *A. alternate* from infected cotton leaves. This species had very obvious morphological and cultural characteristics which were different from the conidial morphology of *A. macrospora.* Our results were similar to Laidou *et al.* (2000) who found, based on morphological characteristics of conidia, that *A. alternata* (Nees : Fr.) Keissler caused leaf spots, and boll rot of cotton (*G. hirsutum*) in northern Greece.

The results of molecular variability detected the four isolates DNA bands at approximately 600 bp as *A. alternata* (Hubballi *et al.* 2010). These results support our findings on the morphological variability and description of the fungus. Based on pathogenicity, morphological, cultural and molecular variability, the pathogen that causes leaf spot and leaf blight on cotton is *A. alternata.* The disease is reported to cause serious reduction in yield and quality of cotton (Liu *et al.* 2018). Currently, the disease is very limited and has modest economic impact but as Sudan is a cotton producing country it may pose a threat to cotton production in the future if its control is not carefully planned at this level.

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