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BOEREMIA EXIGUA LEAF SPOT: A NEW EMERGING THREAT TO *GOSSYPIMUM HIRSUTUM* L. IN PAKISTAN

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ABSTRACT

Cotton is grown all over the world including Asian countries. Severe foliar disease symptoms were observed on cotton in fields from different districts of cotton zone of the province of Punjab, Pakistan. Infected plant leaves were sampled from various areas and processed for the isolation of the causal organism. Fungal isolates were identified morphologically. The conidia of the fungus were observed oblong to ellipsoid, hyaline, and aseptate, but occasionally 1-septate with dimensions ranging from 2.7 to 7.8 × 1.6 to 3.5 μm. A total of two isolates were characterized further for genetic diversity. Both the isolates showed moderate reactions to the NaOH test for E metabolite. The pathogen was morphologically identified as *Boeremia exigua*. Further, the internal transcribed spacer region (ITS) and TUB gene of the fungal isolates were amplified and analyzed. The sequence of partial ITS and TUB showed 99% homology with the isolates of *B. exigua*. The pathogenicity test was performed on three months old cotton plants by using two isolates of *B. exigua*. After 15 days of inoculation, necrotic spots started developing on the leaves that were very similar to those observed in the field. The fungal pathogen was re-isolated from the leaves of all the inoculated plants, identified morphologically following Koch's postulates. This disease might be a potential threat to cotton production in Pakistan in the future. However, further studies are required to know the virulence, behavior, alternate hosts, and spreading nature of the pathogen.

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INTRODUCTION

Cotton (*Gossypium hirsutum* L.) belongs to the genus *Gossypium* of *Malvaceae* family and is one of the most important seed oil crops in the world (Ali et al., 2009; Farooq et al., 2014). It is a prime source of fiber and plays an important role in the socio-economic progress of many countries as more than 20 million farmers cultivate it as their main cash crop globally (Khan et al., 2022). It is also the major cash crop of Pakistan with

second most important in terms of area after wheat and rice, and being a cash crop, plays significant role in country's exports and foreign exchange (Anonymous, 2021). Annually, about 3 million hectares have been under cultivation throughout Pakistan and decreasing as its production is affected by many biotic and abiotic factors (Razzaq et al., 2021). It is important in agriculture as well as the textile sector of the economy. It contributes about 0.6 percent to the GDP and 3.1 percent

of the value added in agriculture. The crop was planted during 2020-21 on an area of 2,079 thousand hectares (Anonymous, 2021). Pakistan ranked fourth in cotton production after China, USA and India. Cotton is the chief source of fiber, feed and edible oil (Abdullah and Khan, 2022; Brown and Khan., 2022). It is hot area crop which requires water after succession of time for all stages of growth (Revathi and Hemalatha, 2014). Cotton is attacked by many insects and abiotic factors as well as viral, fungal and bacterial diseases which ultimately affect the yield (Kumar et al., 2021). The most affected part of cotton is the leaf which includes about 80% of disease of the foliar and the most destructive disease on foliar is leaf spots caused by fungi (Revathi and Hemalatha, 2014).

Boeremia exigua (Desm.) Aveskamp, Gruyter & Verkley (basionym: *Phoma exigua* Desm.) (Aveskamp et al., 2010) is spread worldwide in agriculture, horticulture and forestry. Taxonomically, *B. exigua* is a group of more than ten species (Aveskamp et al., 2010; Berner and Bruckart, 2015). Thus, morphological determination is often supported by molecular methods. Many methods for identifying the types of *Boeremia/Phoma* are based on genes or combinations of genetic sequences such as the ITS and β -tubulin genes (Aveskamp et al., 2010; Berner and Bruckart, 2015; Kowalski et al., 2019).

Cotton production is greatly influenced by biotic factors that causes significant yield losses (up to 10-30%) (Tarazi et al., 2020). In the recent study, irregular wide ranged leafspots on cotton leaves were observed in different cotton growing areas of Punjab. Morphological and molecular characterization of the causal organism was conducted to know the potential causal organism and its epidemiology. It is initial study regarding this new disease and required further deep study to know the nature and epidemic potential of this disease. Modern molecular biology technologies have great potential to enhance the cotton yield and fiber quality. It is very important study, as this disease is not reported from Pakistan before but it might be a threat in future due to virulence of this pathogen.

MATERIALS AND METHODS

Field Survey and Sample collection

During June, 2021 field surveys were conducted in the fields of cotton zone. A total of six districts of Punjab were scouted for leaf spot. It was observed that a leaf spot of different pattern (Figure 1) was present in

Layyah, Muzaffargarh and Multan districts in the Punjab province of Pakistan. However, disease incidence was zero in the areas of Vehari, Sahiwal and Bahawalpur. Disease incidence percentage was calculated by using formula described by Noordzij et al. (2010).

Disease Incidence (%)

$$= \frac{\text{No. of infected Plants}}{\text{Total No. of plants examined}} \times 100$$

The cotton fields in the affected areas were severely affected by foliar disease. Four weeks infected leaves were collected and processed for the isolation and identification of fungal pathogen causing cotton leaf spot. Infected leaf samples were placed into sampling bags and properly labeled. After that, all the collected samples were carried to the laboratory and stored in refrigerator at 4°C till the next procedure.

Isolation, purification and molecular characterization of fungi

Infected leaves were cut into tiny pieces and infected parts were taken separately. Excised leaf samples were washed with 1% sodium hypochlorite (one minute) for surface sterilization. Further, the sodium hypochlorite was removed through washing with sterilized autoclaved water (Topuz et al., 2016). Malt extract agar (MEA) medium was used to inoculate the pieces of necrotic leaves and incubated temperature was 25°C. The media plates were inoculated for a week for the growth of pathogen. Fungal isolates were identified by recording the colony characters and the presence of E metabolites (Aveskamp et al., 2010). For the amplification of internal transcribed spacer (ITS) region and beta tubulin gene, the concentration of genomic DNA was calculated using nanodropper (Thermo Scientific) and diluted at the concentration of 50 ng/ μ l (Kumar et al., 2016). ITS1 and ITS4 primers were used to amplify the internal transcribed spacer region (Lee et al., 1993) using polymerase chain reaction (PCR). For PCR reaction, 25 μ l reaction mixture was prepared using 18.9 μ l nuclease free water, 2.5 μ l PCR buffer, 0.5 μ l of 25mM MgCl₂, 1 μ l 10 mM dNTPs, 0.5 μ l ITS1 primer, 0.5 μ l ITS4 primer, 0.1 μ l DNA Taq polymerase (5U/ μ L, Thermo Fisher Scientific) and 2 μ l DNA template. PCR conditions were used with the initial denaturation for 1 minute at 95°C, 35 cycles of denaturation at 95°C for one minute followed by 1 minute initiation at 50°C and 1 minute elongation time at 72°C temperature. Finally, the reaction was elongated at 72°C for 10 minutes. Similarly, Beta tubulin gene was also amplified. The PCR products

including Internal transcribed spacer region and Beta Tubulin gene were confirmed on 1% agarose gel as compared to 1 kb ladder. Amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, United States). Purified PCR products were sequenced from 1st BASE Malaysia using above-mentioned primer based on procedure (Sanger et al., 1977). Sequences were submitted to the NCBI database for the assignment of Genbank accession numbers.

Pathogenicity Test

The pathogenicity test was performed on three months old cotton plants (Variety FH-142) by using two isolates (FCBP-WA-507 and FCBP-WA-508) of *B. exigua*.

Aqueous conidial suspensions (1.5×10^5 conidia mL⁻¹) were harvested and counted from one week old fungal grown on MEA media and the healthy cotton leaves were treated with conidial suspension. Sterilized distilled water was used to treat the healthy leaves and considered as control. Three replicates for each isolate were used in a duplicated experiment. All the inoculated plants were protected with plastic bags to maintain humidity (60%) for 48 hours and placed in a greenhouse at 25°C. After 15 days of inoculation, symptoms were observed and pathogen was reisolated for confirmation. A disease rating scale (Table 1) was also constructed for comparison.

Table 1: Disease rating scale for comparison.

Disease index	Severity grade	Symptoms	Remarks
0	0	No symptoms	Resistant
1-20	1	Brown spots	Highly tolerant
21-30	2	Necrotic spots on leaves and other plant tissues	Tolerant
31-50	3	tip and marginal drying of leaves surrounded by light brown margin	Susceptible
>50	4	spindle to circular shaped dense brown zonation	Highly susceptible

RESULTS AND DISCUSSION

Disease Incidence

To observe incidence of leaf spot, surveys were carried out in various cotton cultivated areas of the Punjab province of Pakistan viz. Layyah, Muzaffargarh, Multan, Vehari, Sahiwal and Bahawalpur. The disease incidence recorded in these areas is given in the Table 2.

The maximum disease incidence was noticed in Layyah district (34%) followed by Multan (30%). The disease incidence was found in all the districts of Muzaffargarh, Multan, Layyah, Vehari, Sahiwal and Bahawalpur. The least disease incidence was noticed in Vehari which was 10% only. In the present study, the disease incidence was the highest in areas with extreme temperature conditions particularly Layyah and Multan as compared to central mixed areas where the temperature is comparatively low during cotton growing season. It might be due to the nature of the pathogen as it grows at high temperature and humidity.

Morphological characterization of *Boeremia exigua*

Tiny necrotic spots surrounded by chlorotic halos were visualized on upper side of infected leaves of cotton plants. The necrotic spots were expanded into large areas of 3 to 8

cm in diameter which was distinguished by brown margin and severe infected leaves became chlorotic and abscessed (Figure 1). After 7 days of incubation on malt extract agar, fungal cultures varied in color with olivaceous black in centers with soft and aerial mycelia. *Ascomata* was pseudothecial, sub globose. *Asci* were 8-spored, biseriolate cylindrical or subclavate.

Conidiomata pycnidial were variable in their shape and size, commonly globose or sub globose, superficial and might be immersed into agar plate, solitary or confluent; *ostiole* non-papillate or papillate, lined internally with hyaline cells when mature; *conidiomatal wall* pseudoparenchymatous, multi-layered, outer wall brown pigmented. *Conidiophores* were reduced to conidiogenous cells. *Conidiogenous cells* phialidic, smooth hyaline, ampulliform to doliiform. The conidia were oblong to ellipsoid, hyaline, aseptate, but occasionally 1-septate with dimensions ranging from 2.7 to 7.8 × 1.6 to 3.5 μm (Figure 1 A and B). Isolates showed moderate reactions to the NaOH test for E metabolite. On the bases of these morphological characters, the pathogen was identified as *Boeremia exigua* (Aveskamp et al., 2010; Boerema, 2004).

Table 2: Disease incidence of leaf spot in various districts of Punjab.

Sr. #	Area	Disease Incidence (%)
1	Layyah	34
2	Muzaffargarh	28
3	Multan	30
4	Vehari	10
5	Sahiwal	16
6	Bahawalpur	22

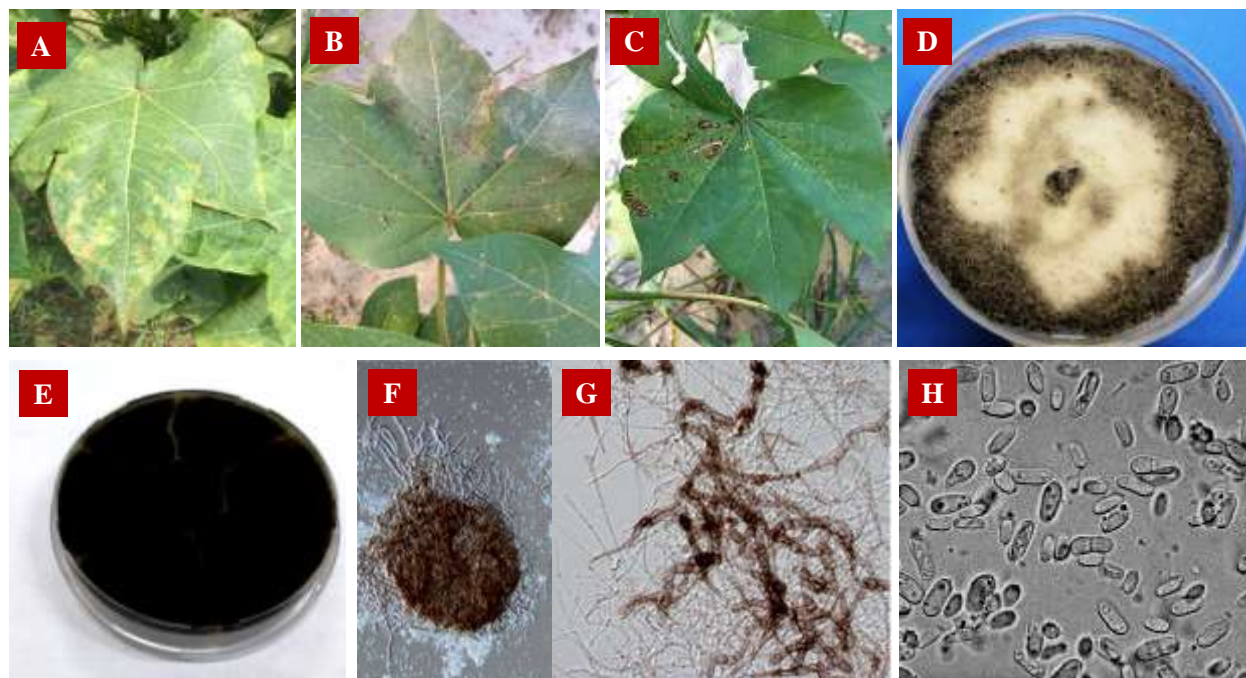


Figure 1. A-C) Cotton plant showing disease symptoms, morphological characterization of *Boeremia exigua*; D) Colony morphology on malt extract agar (MEA); E) Colony reverse on MEA; F) Pycnidia at 10 \times ; G) Hyphae at 10 \times ; H) matured single celled hyaline, oval conidia at 40 \times .

Pathogenicity test

The fungus was re-isolated from the cotton leaves of all the inoculated plants, identified morphologically considering Koch's postulates. Necrotic spots developed on the infected leaves were very similar to the symptoms observed during field sampling. Necrotic lesions on inoculated plants were visible after 15 days of inoculation resulting in disease incidence. Disease symptoms were same on inoculated plants in pathogenicity test as in cotton fields of different districts.

Molecular Characterization

Genomic DNA of *B. exigua* was amplified by using PCR with universal primer pairs. These included ITS1/ITS4 and BT2A/BT2B, primers that gave the amplifications of an approximately 475 bp and 215 bp rDNA fragments

respectively (Figure 2). The unique partial sequences of ITS and β -tubulin of *B. exigua* were submitted to NCBI database acquiring the gene bank numbers of ITS sequences (LT838276 and LT838277) and β -tubulin (LT838278 and LT838279). Using the Basic Local Alignment Search Tool (BLAST) the homologous sequences of ITS and β -tubulin sequences were extracted from NCBI database showing more than 98% homology with the isolates of *B. exigua*. Alignment of sequences was done using Mega-X (Kumar et al., 2016). The phylogenetic tree developed on the base of ITS region (Figure 3 and 4) showed that the sequence (LT838276) isolated from *G. hirsutum* have 100% homology with these two isolates MK333925 and MK333929 of *B. exigua* from China which may be due to

similarity in their ecology, the weather conditions and similar host species. Contrarily, the sequence (LT838277) from the same host did not show much homology with any of the clade members. It may be due to difference in its genetic makeup and host. The β -TUB1 and 2 phylogenetic analyses revealed that the sequences LT838278 and LT838279 showed 99% homology with *B. exigua* isolate # KY273917 and KY273918 isolated from Russia and MH732951 and KR653201 from China respectively. The similarity reflects the close

relationship in soil conditions, weather and host species from which they were extracted. Studies done by Chen et al. (2015) revealed that PCR performed with ITS regions (GenBank MT397284) and *tub2* (MT414712) also showed similarity with the sequences present in the GenBank. Similarly, Colmán et al. (2020) also reported that ITS and *tub2* sequences yielded 99.8% and 99.5% homology with sequences of the type species of *B. exigua* var. *exigua* available in GenBank isolated from sweet potato in Brazil.

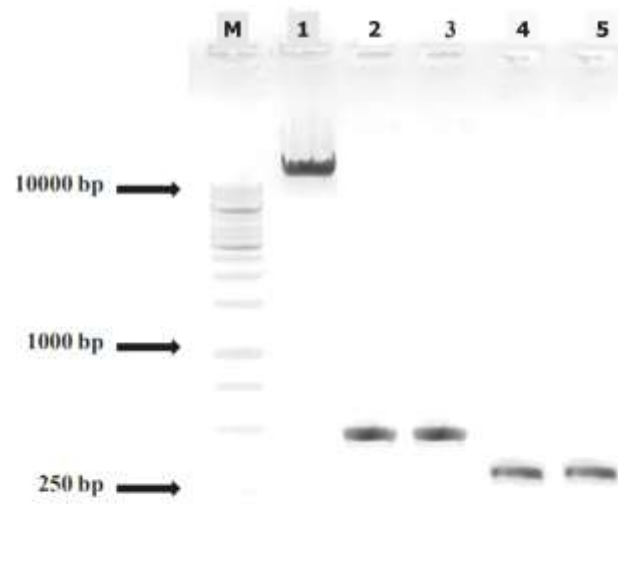


Figure 2. Agarose gel electrophoresis. 1. Total genomic DNA isolated from cotton leaves. 2 and 3. ITS1/ITS4 amplified PCR product of approximately 475 bp. 4 and 5. PCR product of 215 bp using β -tubulin primers. M. DNA size marker.

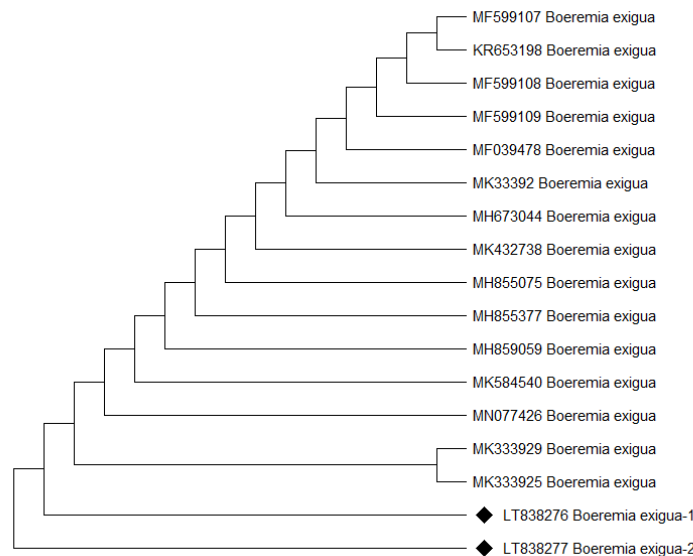


Figure 3. Phylogenetic tree depicting the relationship between different clades of *Boeremia exigua* based on ITS region sequence homology.

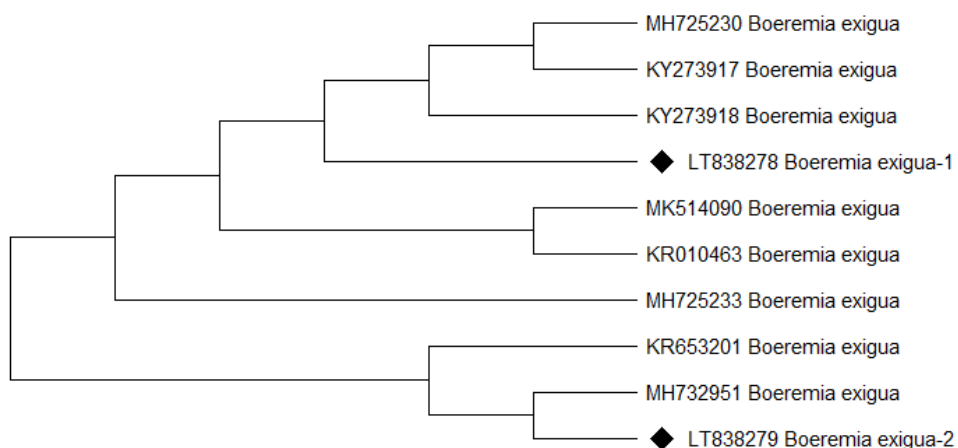


Figure 4. Phylogenetic tree illustrating the relationship between different clades of *Boeremia exigua* on the base of β -tubulin 1 & 2 sequence homology.

Earlier, Farr et al. (1989) reported *B. exigua* on various plants worldwide, but mostly in connection to rots of various organs, and particularly associated with post-harvest diseases. *B. exigua* was also reported to cause leaf spot disease from different parts of world on various plants and trees such as zonate leaf spot of *A. altissima* in Korea (Jung et al., 2022) leaf spot of walnut trees (*Juglans regia*) in China (Wang et al., 2022), white clover in China (Wang et al., 2021), branch blight on walnut in China (Cai et al., 2021), black spot-like symptoms on soybean in Germany (Schaffrath et al., 2021), leaf spots on sweet potato in Brazil (Colmán et al., 2020), leaf spots on *Hydrangea paniculata* in Italy (Garibaldi et al., 2016) and stem and leaf spot on common speedwell in Switzerland (Michel et al., 2018). According to the best of our knowledge and by observing disease incidence and phylogenetic analysis it showed that *Phoma* leaf spot of cotton caused by *B. exigua* is new disease in Pakistan. This disease might be a potential threat to cotton production in Pakistan. *B. exigua* has been demonstrated as the causal agent of black spot of pea and *Boeremia* Blight on *Pyrethrum* in Australia. It has been already reported on different plants in the United States, Italy, Russia and China (Zhao et al., 2017). Further deciphering the virulence of the currently reported isolates, their indigenous host plants and the behavior of *B. exigua* may assist scientists to establish parallels among the inter-playing candidates.

CONCLUSION

It is concluded from the present study that *Boeremia*

exigua is a potential threat to cotton production and can cause severe infestation in future in cotton growing areas as *B. exigua* can survive under harsh environments under field conditions for long period of time.

AUTHORS' CONTRIBUTION

WA and MSH designed the study, WA, SJ and FA executed the experimental work, collected data, AA, HAAK and RK analyzed the data, WA wrote the manuscript, SJ and FA assisted in writing the manuscript, WA and MSH proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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