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ISOLATION AND IDENTIFICATION OF A NOVEL LEAF NECROTIC PATHOGEN OF *VIGNA RADIATA* IN PAKISTAN

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Abstract

Mung bean (*Vigna radiata* L.), a herbaceous, annual legume pulse crop, has high nutritive and clinical values. Amongst the factors damaging to the growth and productivity of this pulse, fungi are the largest and the most important cause affecting the plants at all growth stages. In the present study, a field survey was conducted to isolate the leaf spot pathogen from mung beans. Firstly, the disease symptoms were recorded and infected leaf samples were collected. The pathogen, namely *Phoma herbarum,* was isolated and identified on the basis of its morphology as well as genetic characterization by nucleotide sequence analysis of the rDNA internal spacer sequence (ITS) gene. The test plants were subjected to detached leaf assay as well as intact plant trials in order to confirm the pathogenic potential of the isolated pathogen. Afterward, Koch's pathogenicity potential of the identified pathogen was confirmed by the occurrence of the same disease symptoms and re-isolation of the same pathogen from artificially inoculated leaves in both trials. To our knowledge, the study represents a novel isolation of *P. herbarum* as a leaf spot pathogen of mung beans in Pakistan. The pathogen could result in a serious economic impact on mung beans or possibly on other pulses if not managed in time.

Key words: *Phoma herbarum,* Genetic characterization, Morphology, Pathogenicity, *Vigna radiata*.

Introduction

Vigna radiata L. is the most important and valuable pulse crop of Pakistan, belonging to the family Fabaceae (Ali *et al*., 2010) and is universally known as green gram. *V. radiata* is classified into three subcategories: the first is cultivated variety (*Vigna radiata* subsp. *radiata*), while the other two categories are wild (*V. radiata* subsp. *sublobata* and *V. radiata* subsp. *glabra*). The seeds of pulses are valuable nutritional sources and are thought to be alternatives to meat as they contain proteins (20-30% of dry weight) (Imran *et al*., 2016). It is a significant source of bioactive phytosynthetic substances, also important for detoxification purposes and utilizes to stimulate approach, mitigate warm stroke, and diminish swelling in late spring in humen. It is recorded to be valuable in the direction of gastrointestinal disturbance (Espin *et al*., 2007). Additionally, mung beans are generally utilized as a serving of mixed green vegetables or regular sustenance food in Pakistan, India, Bangladesh, South East Asia, and western nations (Fery, 1990). The mung bean is grown in the biggest pulse region in Pakistan, just second to the chickpea. Pakistan imports very high amounts of legumes to cover the gap between demand and supply of pulses (Government of Pakistan, 2010).

Plant diseases reduce the yield and productivity of several crops all over the world including mung beans. Fungi are the most harmful pathogens to mung beans and cause various infections like leaf spots (*Cercospora* leaf spots and *Alternaria* leaf spots, etc.), *Phytophthora* stem blight, powdery mildew, and wilt (Chandrashekar *et al*., 2014; Satyagopal *et al*., 2014a, b) etc. The most important pathogenic groups of fungi which contaminate seeds are Sclerotinia, Colletotrichum, Alternaria, Phoma, Botrytis, Pythium, Fusarium, Rhizoctonia and Ascochyta (Lardner *et al*., 1999; Zhang & Yang, 2000; Elmer *et al*., 2001; Anastasios *et al*., 2005). Fungi of the genera Sclerotinia, Penicillium and Aspergillus may likewise cause financial losses in a few crops including lettuce, mung beans, canola, cabbage, carrots, celery, white beans and soybeans (McLaren *et al.,* 2004). A number of authors have also demonstrated fungal contaminations and their impact on entire crops resulting in the high economic loss (Burgess *et al*., 1997; Phan *et al.,* 2002; Shafique *et al*., 2017). Having observed the above problems of mung beans in Pakistan, the overall objective of the present study is the identification and characterization of the leaf spot pathogen of mung bean plants to reduce the problems of fungal leaf spots on these beans. For accurate identification of the problem, the most trustworthy conservative method is the morphological identification of pathogens at species level. Furthermore, many new molecular techniques are also employed for accurate identification of fungi: viz., analysis of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships within many groups of fungi (Mirhendi *et al*., 2007), or using the mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*., 1996).

Materials and Methods

In the present study, a field survey of the Institute of Agricultural Sciences, University of the Punjab, Lahore, was conducted in October 2017, to study mung bean plants infected with leaf spot symptoms. Photographs of infected plants and leaves with disease symptoms were taken and data was collected regarding shape, color and size of the leaf spots. Three leaves with significant disease symptoms were removed from selected plants, packed into sterilized sampling bags, taken into the laboratory and preserved at 4°C in the lab for further study.

Malt Extract Agar (MEA @ 2% malt extract; 2% agar; pH 6.5) was prepared. The preserved diseased leaves were cut into 3-4 mm sized sections with some healthy portion and their surfaces were sterilized for two minutes in 1% sodium hypochlorite solution followed by several washings with distilled water. Then three sections were inoculated on each MEA plate under aseptic

conditions and incubated at 25 ± 3°C for 3 to 5 days. The Petri plates were monitored regularly for radiating mycelial growth from the edges of the infected sections. The pathogen was then transferred onto freshly prepared MEA plates under aseptic conditions to purify the culture, and incubated at 25 ± 3°C for five to seven days in an incubator. The purified culture was stored at 4°C.

To identify the isolated fungal pathogen two methods were used. The first was a microscopic characterization of the morphology and the second was a genetic level nucleotide sequence analysis using a primer of Internal Transcribed Spacer (ITS) sequence of rDNA. The seven-day-old fungal culture was used to study the morphological characteristics of the pathogen under the stereoscope for macroscopic study while the microscopic characteristics were studied with the help of a microscope. The macroscopic study of the fungus colony revealed the colony color, diameter (cm), and mycelium texture. The observed microscopic characteristics were: the color and shape of the conidia, the attachment of the conidia to the conidiophores either in chain form or clusters, the attachment of the conidiophores to the mycelium, mycelium color and size, and whether septate or aseptate, and the texture of the mycelium wall. The nucleon reagent method was used to isolate the fungal genomic DNA of the fungus isolated from the infected leaves of the mung bean. The Purified plates of 7 day old culture were taken and liquid nitrogen was used to crush the fungal mass. Then, 2 mL of nucleon reagent (120 mM EDTA, 400 mM Tris,(Ph 8), 150 mM NaCl and 1% SDS) and 0.5µL (10mg/mL) of RNAase An enzyme were added to 15 mL falcon tubes and incubated in a water bath for 30 minutes at 37°C. Then 500 µL sodium perchlorate was added and mixed well, followed by the addition of 2mL chloroform. This was again mixed and centrifuged at 4000 rpm for10 minutes. The supernatant containing DNA was isolated and 2 mL of ice cold 96% ethanol was added to it and centrifugation was carried out at 7000 rpm for 5-10 minutes. The supernatant was discarded and the DNA pallet was collected and washed by the addition of 1 mL of 70% ethanol and suspended in 50 µL TE buffer (0.1 mM EDTA, 10mM Tris, Ph 8). A gel electrophoresis was performed to check the quality of the extracted fungal genomic DNA using 1% Agarose gel. To determine the sequence of the extracted DNA, a set of primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') / ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') was used along with *Taq* polymerase and an appropriate buffer. Commercially available 2X Amp Master ™ *Taq* polymerase (GeneAll Biotechnology Co, Ltd) was used for the amplification using a total volume of 30µL consisting of 2X Amp Master ™ Taq 15µL; 8µL deionized water and 5µL extracted DNA sample, forward primer 1µL, reverse primer 1µL and 10-50ng DNA. Then a gel electrophoresis was carried out on 0.8% Agarose gel to determine the size of the PCR product. A single compact band of genomic DNA was visible on the agarose gel. Gene products of the correct size were sent for nucleotide sequencing. The DNA sequencing results were analysed by a Basic Local Alignment Search Tool (BLAST) to find out the exact sequence of the nucleotides of the specific fungal species, leading to the identification of the specific fungal species based on nucleotide homology with corresponding strains in the GenBank database.

Detached leaf method is a significant tool to study the pathogenic potential of the isolated pathogen. To determine the Koch's Pathogenicity postulates of the isolated strain, sterilized Petri plates were lined with two filter papers in each Petri plate and moistened with 2 mL distilled water. Fresh healthy leaves were detached near the petiole end from the mung bean plants, washed thoroughly, dried and placed into Petri plates on a filter paper surface in such a way that each leaf base touched the moisturized filter paper. Spore suspension (2 mL) @ 5 x 10^5 spores/mL was spread on the leaf surface in each Petri plate while one set of plates was selected as the control and sprayed with water, and incubated at 25 ± 3°C. The plates were monitored on a daily basis to observe the appearance of possible disease symptoms and photographs were taken at each stage of spot formation to compare these results with the original disease symptoms. A disease rating scale was designed, to determine the disease severity. *In vivo*, a pathogenicity test was conducted using pot trials. The field soil was sterilized by fumigation followed by sterilization in a hot air oven at 45°C for 24 hrs. Four (4) pots were taken and filled with 120gm soil in each pot. Three (3) seeds were sown in each pot and watered. Sprouting started within a couple of days and a 1-finger-length (3cm)-sized seedling was visible four days after sowing. Then 5mL of fungal spore suspension (5 x 10^5 spores/mL) was sprayed on each replicate plant's aerial parts and near the base tip of the stem, and covered with bags to establish the inoculum on the plants. The pots were kept in the light with 16 h photoperiod and 40% humidity at a temperature range of 28-35°C and watered when needed. Regular monitoring was done to determine the disease pattern of the pathogens. The control plants were sprayed at the same times with the same amount of distilled water.

Results

During the field survey, approximately 100 plants were observed and about 50-60% of these were found to be affected with leaf spot disease covering approximately 40-50% of the leaf area (Fig. 1). On these leaves, circular brown spots appeared, becoming large irregular yellowish-brown shiny spots (1-2 mm) which later on converted to dark brown lesions.

The morphological analysis revealed that the fungal colony with pinkish white color was a purified pathogen of mung bean leaf spot disease. Within seven days, the colony was found to be growing rapidly with 5.2 cm average diameter. The colony texture was powdery to velvety, and the mycelia were submerged into the medium. From the front, the color was initially white, which then turned to grey with an occasional tint of pink. From the reverse side, the colony color was dark brown to black. Brown diffusible pigments were also readily visible on the reverse side (Fig. II A-B). Under the microscope hyaline to brown septate hyphae were seen. Spores were thick walled, multicellular, dark brown in color, 20-70µm in diameter and inter-mycelial. Chains of brown globose to elongated chlamydospores were present. The conidia were hyaline, unicellular and very small in size (1µm), and ovoid in shape with a bead-like chain appearance. Also in the culture, large numbers of pycnidia were produced that were dark brown in color and of varied sizes. The pycnidia were

regularly globose or sub globose in shape and without any ostiole (Fig. 2, C-F). These observed characteristics were used to distinguish the fungal pathogen by comparing it with authenticated taxonomic literature. Based on the studied features, *Phoma herbarum* was identified as the causal organism of leaf spot in mung beans. A purified culture of this pathogen was deposited into the First Fungal Culture Bank of Pakistan under the accession number FCBP-MF-1539.

The genetic characterization of the identified species was carried out by sequence analysis of the ITS region by nucleotide BLAST analysis. The consensus primers ITS1 forward and ITS4 reverse successfully amplified the DNA fragment of approximately 650 bp and the total genomic DNA as a template. A BLAST analysis of the ITS region nucleotides from the present study revealed 99% homology of *P. herbarum* with many strains of *P. herbarum* in the GenBank. These include *P. herbarum* strain VB8 (Sequence ID: JQ754707) shown in (Fig. 3), XGC-31 (Sequence ID: KY849800), GX8-2 (Sequence ID: KU324835) and JL-14 (Sequence ID: JX867221). This nucleotide sequence was deposited into the Genbank under accession number MT367635. The evolutionary history based on the ITS region of the rDNA was inferred by the Maximum Likelihood method (Fig. 4) based on the Tamura-Nei model (Tamura & Nei, 1993). The percentage of plants in which the associated taxa clustered together is shown next to the branches. The analysis involved 11 nucleotide sequences from different species of the genus *Phoma*. All positions containing gaps and missing data were eliminated. There were a total of 439 positions in the final dataset. Evolutionary analyses conducted in MEGA6 phylogenetic trees placed our strain close to other *P. herbarum* strains in the GenBank hence confirming its identity.

Fig. 1. (A) Field view of infected mung bean leaves (B) Leaf spot from the front side (C) From the reverse side.

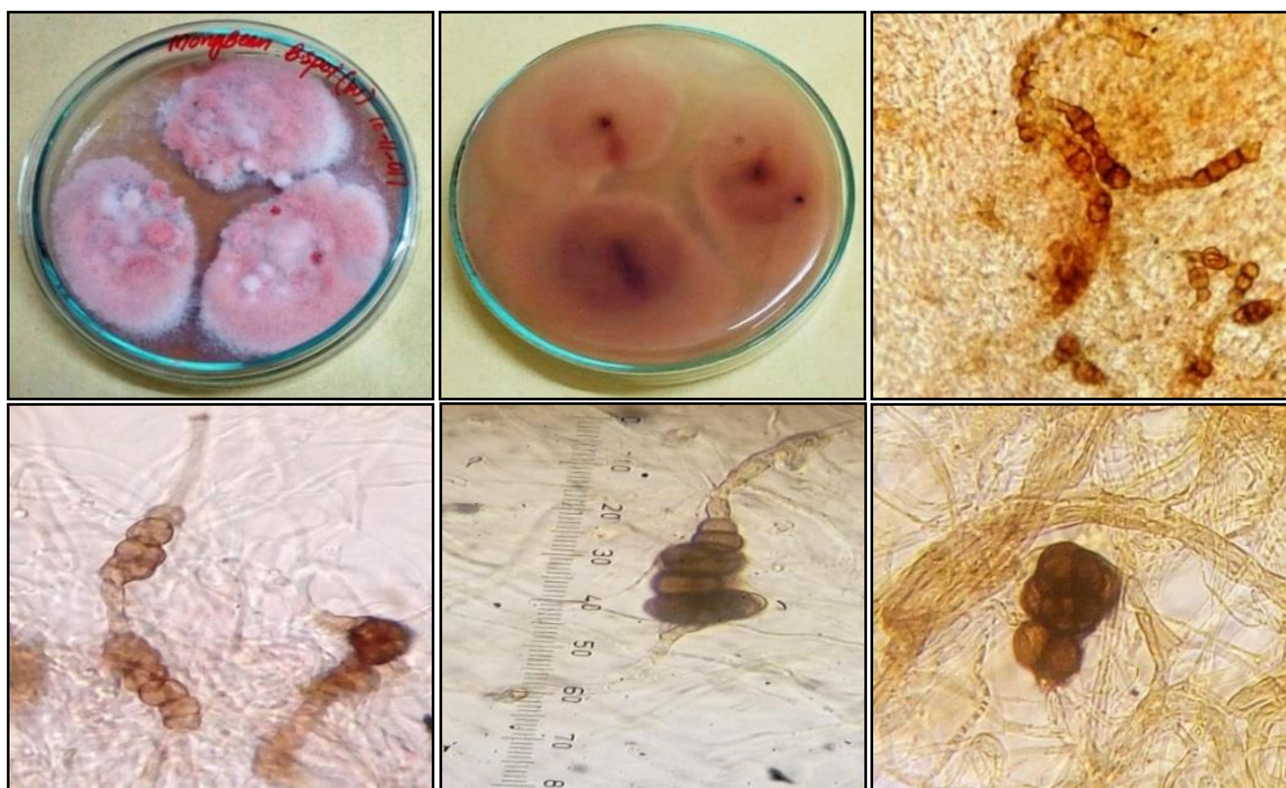

Fig. 2. *Phoma herbarum* (A) Front side of colony (B) Reverse side of colony (C) Arranged chains of Chlamydospores (D) Chlamydospores under higher microscope magnification (E) Spore at 100X microscope magnification (F) Pycnidia.

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Ph_Mb  1    GGGGCTTC-TGAGTGTACGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTA  59
            |||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||
VB8    1    GGGGCTTCCTGAGTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTA  60

Ph_Mb  60   CGTTTCCTCGGTGGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VB8    61   CGTTTCCTCGGTGGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATC  120

Ph_Mb  120  AGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATC  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VB8    121  AGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATC  180

Ph_Mb  180  GATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCG  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VB8    181  GATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCG  240

Ph_Mb  240  AATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VB8    241  AATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCA  300

Ph_Mb  300  TTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTCAAAA  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
VB8    301  TTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAA  360

Ph_Mb  360  CAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VB8    361  CAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAA  420

Ph_Mb  420  CGACGACATCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGC  479
            |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
VB8    421  CGACGACATCCAAAAGTACATTGTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGC  480

Ph_Mb  480  TGAACTTAAGCATATCAAT-AGCCGGAGGAAA  510
            ||||||||||||||||||| ||||||||||||
VB8    481  TGAACTTAAGCATATCAATAAGCCGGAGGAAA  512
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Fig. 3. Nucleotide sequence alignment of ITS region of rDNA of *Phoma herbarum* isolated from mung beans (Ph Mb) and strain VB8 (Sequence ID: JQ754707). The two sequences are 99% similar.

Phoma digitalis CBS 109179.seq
48
Phoma omnivirens CBS 654.77.seq
Phoma aliena CBS 379.93.seq
47
Phoma polemonii CBS 109181.seq
Phoma multirostrata PD 83-48.seq
100
Phoma glaucii CBS 114.96.seq
80
61
Phoma destructiva var. destructiva CBS 133.93.seq
Phoma saxea CBS 298.89.seq
Phoma herbarum CBS 615.75.seq
Phoma herbarum VB8.seq
99
Phoma herbarum present study.seq
0.05

Fig. 4. Molecular Phylogenetic analysis of different species of *Phoma* based on sequences of the Internal Transcribed Spacer region of rDNA (ITS). The initial tree(s) for the heuristic search were obtained automatically by applying the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The detached leaf method was performed to confirm Koch's Pathogenicity postulates of the isolated strain. Disease symptoms started to appear twenty-four hours after inoculation on the leaves in the Petri plates and these symptoms were found to be affected. The pathogenic infection symptoms observed were leaf spots and lesions (Fig. 5). The infection symptoms observed on the leaves were, firstly, yellow and then small circular brown spots. These small spots became larger and irregular shiny spots (1-2 mm) and later dark brown lesions followed by necrosis. After six days of inoculation, the *P. herbarum* showed approximately 15% infection symptoms. However, after 15 days about 95% of the leaf area was found to be infected. The disease progression curve was designed to assess the level of infection (Fig. 6). The curve indicated that the pathogen *P. herbarum* was a virulent disease-causing organism in mung beans, as it provoked severe disease symptoms in the host plants hence this pathogen was used for subsequent lab experimentation.

In the *In vivo* trials three to four days after the inoculation to the plants, the infection symptoms started to appear. The pathogen showed distinct symptoms on the mung bean plants. Foliar symptoms were observed as the yellowing of leaves followed by small brown spots and angular chlorotic spotting. In the later stages, complete necrosis of leaves was observed. Data analysis of the pot trials showed that the pathogen demonstrated drastic disease severity in the host plants, which became 100% after 15 days (Fig. 7). The pathogen was re-isolated from the leaves exhibiting symptoms and the cultures obtained were compared with the original pathogen to confirm the identity of the pathogen to complete Koch's Postulates.

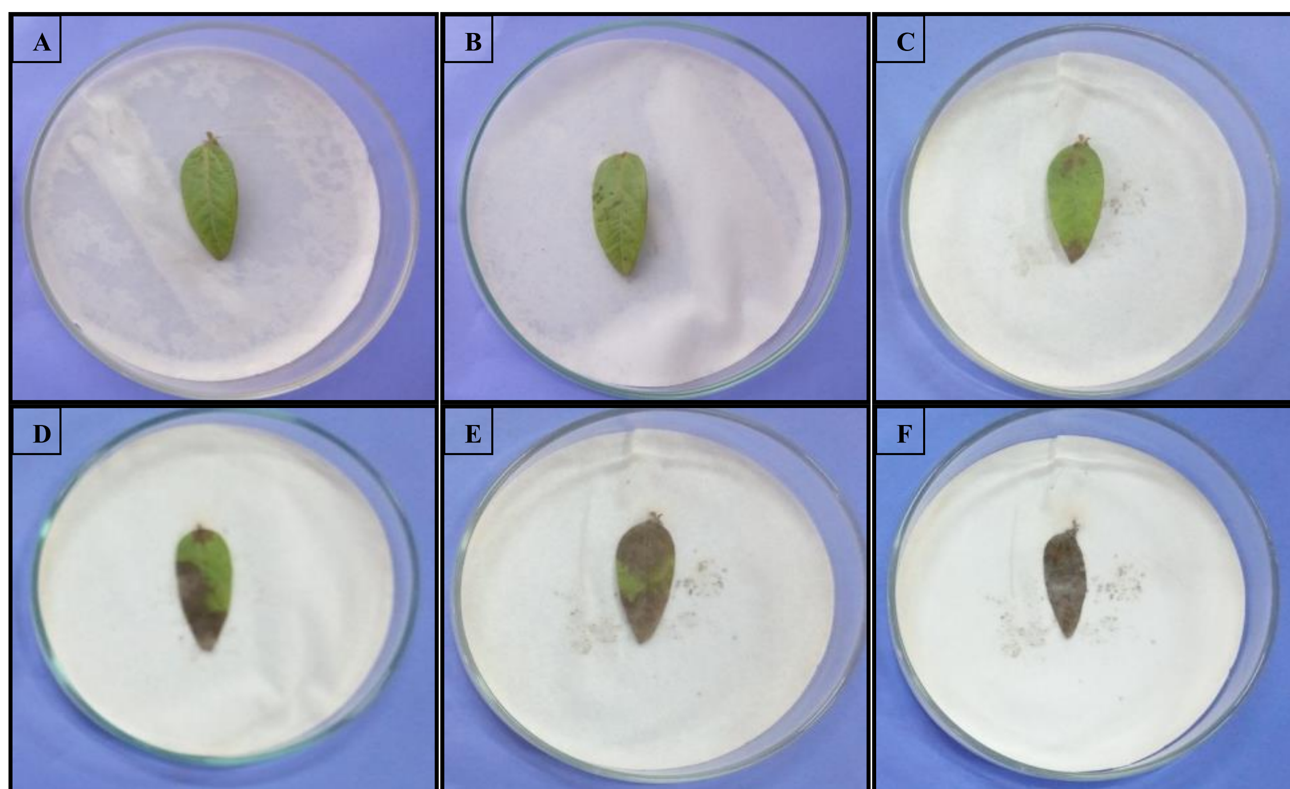

Fig. 5. Symptom development caused by *P. herbarum*.

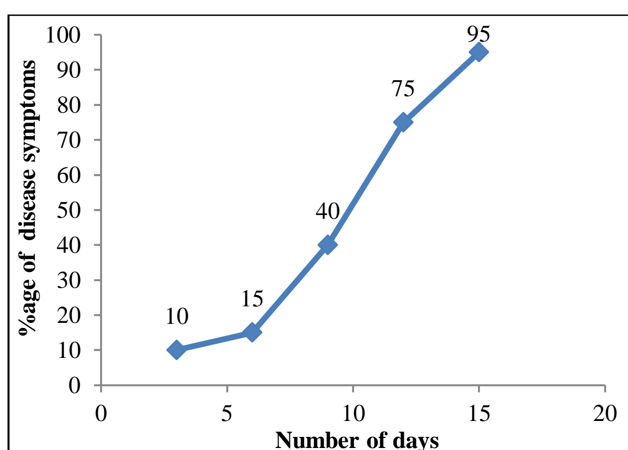

Fig. 6. Disease progression curve of *P. herbarum* on mung beans by detached leaf assay.

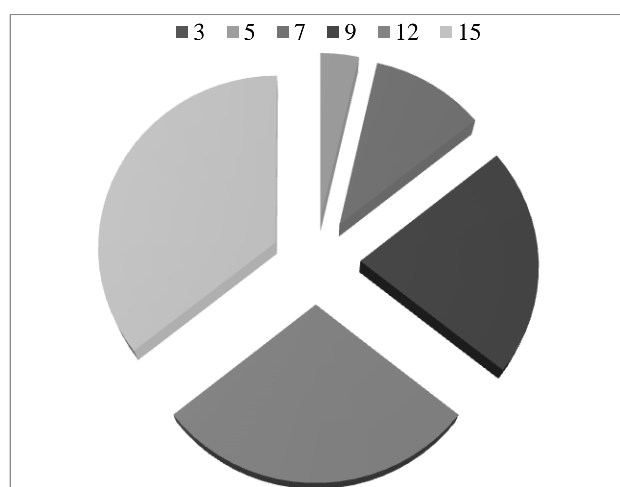

Fig. 7. Disease severity of *P. herbarum* in mung bean plants.

Discussion

The mung bean is a crucial, important and wide-spread, annual legume crop in Pakistan and mostly cultivated by traditional farmers. It has high nutritive and clinical values (Espin *et al*., 2007). Plant diseases reduce the yield and productivity of several crops all over the world including mung bean. Amongst the pressures of a number of diseases on mung beans, leaf spot disease is the most devastating and is induced by numerous fungi including *Alternaria, Phoma* and *Drechslera* etc (Chung, 2012; Chandrashekar *et al*., 2014). A number of modified techniques are practised to manage these fungi. However, for the management of fungal diseases, the precise identification of the causal agent is essential. For accurate identification the most trustworthy conservative method is the morphological identification of pathogens at species level (Shova *et al*., 2020). Furthermore, many new molecular techniques are also employed for accurate identification of fungi: viz., analysis of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships within many groups of fungi (Mirhendi *et al*., 2007) or the mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*., 1996). In the present study, *Phoma herbarum* (FCBP-MF-1539) was isolated as the leaf spot pathogen of the mung bean plants and further identified by means of macroscopic and microscopic study for morphological characterization as well as genetic analysis from the nucleotide sequencing of the amplified ITS1 and ITS4 primers. On the basis of the morphological characteristics followed by identification using an rDNA spacer sequence, *Phoma herbarum* was confirmed as the causal agents of leaf spot in these mung bean plants. In the contemporary lines, *Phoma herbarum* was isolated and identified in Pakistan from the necrotic areas of the leaves of *Cycas revoluta* on the basis of morphological characteristics followed by sequencing of the ITS region of its rDNA (Nayab & Akhtar, 2016). In another study, *Cladosporium cladosporioides* was identified as a leaf-spot-causing pathogen of *Solanum melongena* on the basis of a complete description of the macro- and microscopic characteristics, followed by identification using an rDNA spacer sequence of the amplified ITS1-5.8S-ITS4 region of the rDNA (Shafique *et al*., 2019).

After identification of the pathogenic fungi, later in the present study the pathogenic potential of *P. herbarum* was estimated by applying Koch's postulates using the detached leaf method and pot trials. The pathogen induced the most characteristic symptoms, such as the yellowing of leaves and dark brown spots on the leaves of the mung bean plants. The pathogen *P. herbarum* proved very potent and portrayed a sharp progressive disease curve with 95% of the area infected. These results were in agreement with the work done by Mahmood (2010) who identified a similar disease progression in tomato plants by *A. alternata.* In another similar study, Nayab & Akhtar (2016) appraised the pathogenicity of *P. herbarum* by employing Koch's postulates in pot trials. Characteristic symptoms of yellowing and dark brown spots on leaves were exhibited by the pathogen of the *Cycas revoluta* and depicted a steep progressive disease curve with 99% of the area infected.

To the best of our knowledge, this is the first report of *Vigna radiata* leaf spot caused by *P. herbarum* from Pakistan. This study emphasizes the need for careful management of this pathogen, which could infect other legume crops also.

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