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ALTERNARIA ARBORESCENS, IDENTIFIED AS A LEAF NECROSIS PATHOGEN OF *VIGNA RADIATA* IN PAKISTAN

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ABSTRACT

Vigna radiata (mung bean) is a most cultivated legume crop having high nutritive and clinical value. A survey was accompanied to isolate leaf spot pathogen from mung bean plants. Infected leaf samples were collected and a novel pathogen, *Alternaria arborescens* was isolated and identified on morphological and molecular basis. Molecular identification was done using nucleotide sequence analysis of rDNA internal spacer sequence (ITS), partial glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and elongation factor (EF) regions. Morphological characters demonstrated grayish-black, woolly, concentric rings on Malt Extract Agar (MEA) plates. The conidiophores were well defined; septate with terminal and sub-terminal branches having tan to brown, short ovoid or ellipsoid, and 7-11 µm sized conidia with transverse septation. In molecular characterization, BLAST analysis of the rDNA-ITS region of the pathogen, *A. arborescens* exhibited maximum (99%) homology with other *A. arborescens* GenBank strains. Similarly, 100% homology was found with partial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and translation elongation factor. Afterward, Koch's pathogenicity aptitude of the identified pathogen was confirmed by the occurrence of the same disease symptomology and re-isolation of identical organisms from artificially inoculated leaves in the *in vitro* and *in vivo* trials. The study signifies the novel documentation of *A. arborescens* as a leaf spot pathogen of mung bean in Pakistan. The manifestation of this pathogen could result in a serious economic impact on mung bean or might be a possible pathogen of other pulse crops if not managed in time.

Keywords: *Alternaria arborescens,* Genetic characterization, Leaf spot disease, Morphology, *Vigna radiata.*

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INTRODUCTION

Mung bean (*Vigna radiata* L.) is an imperative widespread, herbaceous, annual legume crop of Pakistan and is regularly recognized as a green gram. China, Korea, Bangladesh, Nepal, Sri Lanka, India, and Pakistan are the main cultivating areas of mung bean (Fuller and Harvey, 2006; Batzer *et al.*, 2022). Seeds of pulses are profitable nutritional source and are thought to be alternatives to meat as they contain proteins (20 to 30% of dry weight) (Hirtzer *et al.*, 2021). The mung bean seeds contain 1.3% fats, 24.2% protein contents, and 60.4% starches; phosphorus (P) 340 mg and calcium (Ca) is 118 mg for each 100 g of seeds (Imran *et al.*, 2016). Besides, mung bean seeds contain two times greater protein content as compared to maize seeds which contain about 7 - 10% protein content (Chen *et al.*, 2014) is altogether noticed higher in mung beans than customary root crops (Kudre *et al.*, 2013). Mung bean is developed in the biggest pulse region in Pakistan just second to chickpea. Plant diseases reduce the yield and productivity of several crops all over the world including mung bean. Yield loss due to the absence of plant security measures changes from 46 to 96% contingent upon any crop varieties. Abiotic stresses are the most damaging factor concerning the development and profitability of pulse crops (Dinsa *et al.*, 2022). Biotic stresses include insects, weeds, nematodes, causal organisms of diseases, allelopathic chemicals, and so on. Among these, fungi and viruses are the biggest and the most critical pathogens influencing all parts of the plant of the food legumes (Pande, 2009). Fungi are the most harming pathogens to mung bean and cause diverse infections like leaf spots (*Cercospora* leaf spots and *Alternaria* leaf spots etc.), *Phytophthora* stem blight, powdery mildew, and wilting (Chandrashekar *et al.*, 2014; Satyagopal *et al.*, 2014a, b; Shafique *et al.*, 2022; 2023) etc. Crop productivity can be enhanced by suppressing causal agents of disease with improved management strategies (Pandey *et al.*, 2019). Thus, the leading purpose of current research is the identification of foliar fungal pathogen/s related to leaf spots of mung bean plants that are ultimately distressing the economy of Pakistan.

MATERIALS AND METHODS

Field Survey and Sample Collection: A field survey of the Institute of Agricultural Sciences, University of the Punjab Lahore, and its premises was conducted in October 2017 to study diseases of field crops. The leaves of mung bean plants infected with leaf spot symptoms were collected. Data was collected regarding the shape, color, and size of the leaf spots. Photographs of infected plant leaves with disease symptoms were taken as reference. Severely infected plants were selected to study the causal organism of this disease. Three severely infected leaves per plant were selected, and preserved in sterilized sampling bags. These samples were then preserved at 4 °C in the lab for further studies.

Isolation and Purification of Pathogen: The isolation of fungus was carried out using 2% Malt Extract Agar (MEA; pH 6.5; Merck (Germany)). Preserved diseased leaves collected during field survey were washed and cut into 3-4 mm sized pieces of infected leaves along with a bit of healthy part. For sterilization, NaOCl solution (1%) was used for 2 minutes following 3 to 4 washings with distilled water. The leaves were dried and 3 pieces were placed on each MEA plate aseptically and incubated at 25 ± 3 °C for 3-5 days. Petri plates were examined repeatedly for emerging mycelia development from the boundaries of the diseased sections. After 3-5 days of incubation, growth of mycelium emerging from the edge of leaf pieces was observed in Petri plates. The fungal colonies with black color were observed as pathogens of mung bean leaf spot disease. This pathogen was transferred into freshly prepared Petri plates comprising medium and kept at 25 ± 3 °C for 5-7 days in an incubator. Pure fungal cultures were then preserved at 4°C.

Identification and Characterization of Pathogen: The purified pathogen was identified using two methods. First was the microscopic characterization of morphology and the second was the molecular characterization to analyze nucleotide sequence with the help of three sets of primers of Internal Transcribed Spacer (ITS) sequence of rDNA, partial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor (EF) gene.

Morphological Characterization: To study the morphology, a seven-day-old fungal culture was observed under a stereoscope and microscope. Macroscopic study of fungal colony revealed the following characteristics; front and reverse color of colony, colony diameter (cm), and mycelium texture type. Microscopic characters observed were; the color and shape of the conidia, attachment of conidia to conidiophores either in chain form or clusters, attachment of conidiophores to the mycelium, mycelium color and size, septate or aseptate, texture of mycelium wall. Based on morphological characters; the culture was identified and submitted to the First Fungal Culture Bank of Pakistan, IAGS, PU, Lahore, and assigned a specific accession number.

Molecular Characterization: Nucleon reagent method (Shafique *et al*., 2022) was used to isolate fungal genomic DNA. Pure plates of 7-day-old culture were taken and scratched carefully without disturbing the medium. The quality of extracted fungal genomic DNA was observed on 1% agarose gel. DNA bands were observed in UV light.

PCR Amplification and DNA Sequencing: To determine the sequence of extracted DNA, three sets of primers ITS1/ITS4, gpd1/gpd2, and EF1-728F/EF1-986R were used along with *Taq* polymerase and appropriate buffer. Details of the primers used for ITS, GAPDH, and EF1 gene amplification are given in Table 1.

Table 1: List of primers used for molecular characterization of isolated pathogen.

Sr. No	Primer	Primer name	Sequence (5' - 3')
1	ITS (Forward)	ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'
2	ITS (Reverse)	ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'
3	GAPDH(Forward)	gpd1	5'-CAA CGG CTT CGG TCG CAT TG-3'
4	GAPDH (Reverse)	gpd2	5'-GCC AAG CAG TTG GTT GTG C-3'
5	EF (Forward)	EF1-728F	5'-CAT CGA GAA GTT CGA GAA GG-3'
6	EF (Reverse)	EF1-986R	5'-TAC TTG AAG GAA CCC TTA CC-3'

Commercially available 2X Amp Master ™ *Taq* polymerase (GeneAll Biotechnology Co, LTD; USA) was utilized for the amplification using in a total volume of 30 µL consisting of 2X Amp Master ™ Taq 15 µL; 8 µL deionized water, and 5 µL extracted DNA sample, forward primer 1 µL (from 10 pmol/µL stock), reverse primer 1 µL (from 10 pmol/µL stock) and 10-50 ng DNA. Thermo cycler PCR machine was installed with the reaction steps details. The first step of denaturation was done at 90 °C for 30 sec. The second phase was annealing at 60 °C for 30 sec. Elongation was the last phase at 72 °C for 1 minute and later the final extension

for 10 minutes at 72 °C. All these 3 steps were repeated for 35 cycles for significant amplification of fungal DNA. After that gel electrophoresis was carried out on 0.8% Agarose gel (5 µL of PCR product and 1µL of 6x gel loading dye) to visualize the PCR product and to determine band size compared to DNA standard Marker (1 Kb) at 100 V for 50 min. amplified PCR products were observed by a UV transilluminator. Gene products of the correct size were sent for nucleotide sequencing. DNA sequencing data was examined by the Basic Local Alignment Search Tool (BLAST) to identify specific fungal species.

Pathogenicity Confirmation Test

Detached Leaf Method: A fresh culture plate of pathogen was used to make spore suspension. Ten mL of tween 80 solution (0.9% NaCl and 0.1% Tween 80) was poured into the plate and left for 20 minutes. With the help of spatula, the mycelium was scratched properly without disturbing the medium layer. After that suspension was diluted with the addition of more tween 80 solution (app. 2 mL) and the inoculum was adjusted to 5×10^5 spores/mL by using a hemocytometer. To determine Koch's postulates, five sterilized Petri plates were taken, each lined with 2 filter papers and moisturized with 2 mL distilled water. Fresh healthy leaves were detached near the petiole end from mung bean plants, washed thoroughly, and dried. The leaves were placed into a Petri plate on the surface of the filter paper in a pattern that the leaf base touched the moisturized filter paper. Spore suspension (2 mL) was spreaded on the leaf surface of each Petri plate except for 1 set of the plate that was selected as control and incubated at 25 ± 3°C. Monitoring was done to observe the appearance of disease symptoms and photography was done on each stage of spot formation to compare the results with original disease symptoms.

Pot trials: *In vivo*, the Pathogenicity test was conducted using pot trials. The field soil was sterilized by fumigation in the field followed by oven sterilization at 40-45 °C overnight. Four (4) pots of 90 mm height and 54 mm width were taken, filled with 120 gm soil into each pot, 3 seeds were sown per pot and watered. The sprouting was started within a couple of days and 1 finger length (3 cm) sized seedling was developed after 4 days of seed sowing. Spore suspension (5×10^5 spores/mL) of fungal strain was made in tween 80 and 5 mL was drenched on each plant's aerial part and near the base tip of stem and covered with paper bags to establish the inoculum on plants. All inoculated plants were covered with polythene bags. The pots were kept in light with 16h photoperiod and 40% humidity at a temperature range from 28-35 °C and watered when needed. Regular monitoring was done to determine the disease pattern of the pathogen. Control plants (i.e., all the 3 plants in each of the 3 replicates) were sprayed with the same amount of distilled water. The pathogen was re-isolated from the leaves exhibiting symptoms and the cultures obtained were matched with the originally isolated pathogen to confirm its identity to accomplish Koch's Postulates within 15 days of pot trials.

Disease incidence and disease severity were observed and calculated by visually observing the first visible symptom for constructing a disease rating scale. Disease severity and disease incidence was calculated using subsequent formulae:

$$\text{Disease Severity} = \frac{\text{Area of tissue affected}}{\text{Total area of tissue}} \times 100$$

$$\text{Disease Incidence (D.I)} = \frac{\text{Number of diseased plants}}{\text{Total Number of sampled Plant}} \times 100$$

RESULTS

Evaluation of Diseased Samples: During the field survey, a total number of approximately 100 plants were observed in the field area, and about 50-60% of plants were found affected by this disease. The infected leaves of mung bean were collected and later studied to evaluate the disease symptoms. Disease symptoms included leaf spots and lesions covering approximately 40-50% leaf area (Fig. 1). On leaves, circular brown spots were appeared, becoming irregular yellowish brown large shiny spots (1-2 mm) and later on converted to dark brown lesions.

Identification of Isolated Pathogens: Pure culture was used to identify the isolated pathogen based on morphological features then molecular characterization was undertaken to confirm the morphological identification.

Morphological Characterization of Fungal Pathogen: Morphological characterization including cultural, macro-, and microscopic features was accomplished using 7 days old pure fungal culture. Noticed characters were used to key out the fungal pathogen by comparing it with authentic taxonomic literature. Fungal colony growth was found to be rapid with grayish-black, woolly, concentric rings on MEA plates. The colony was 4 to 5 cm in diameter. From the front side of culture; the color was initially light gray and turned brown to black at the maturity of culture. The reverse side was dark brown or black (Fig. 2A-B). During microscopic studies, septate-tanned hyphae were observed with varied lengths, with either well-defined and solitary or loosely united sporulating hyphae. The conidiophores were well defined; septate with terminal and sub-terminal branches. Conidia were tan to brown, short ovoid or ellipsoid, and 7-11 µm in size with transverse septation (Fig. 2C-F). A

pure culture of isolated pathogen was identified as *Alternaria arborescens* and deposited to the First Fungal Culture Bank of Pakistan under the accession number FCBP-MB002.

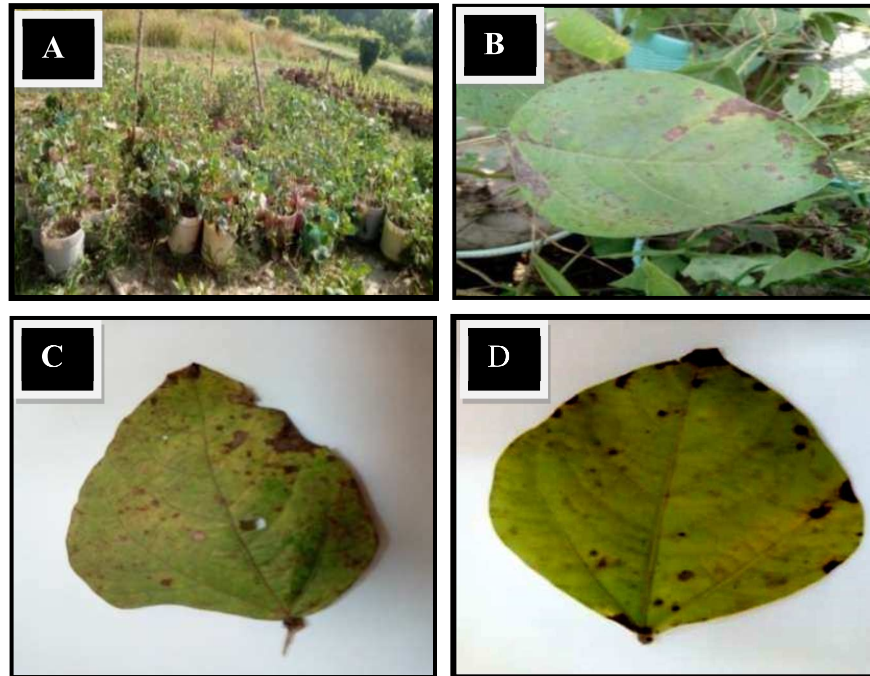

Fig. 1: (A) Field view of mung bean plants (B) Infected leaves (C) leaf spot from the front side (D) from the reverse side.

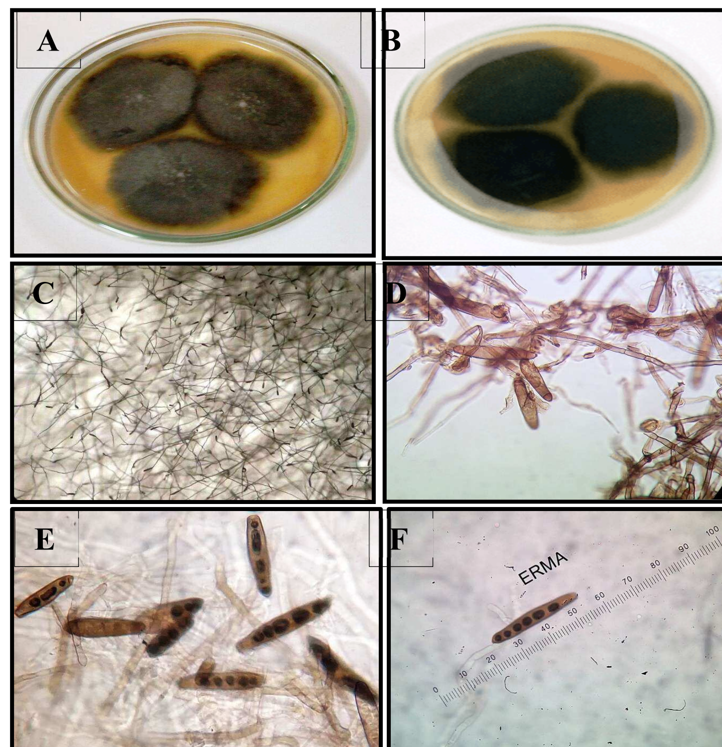

Fig. 2: *Alternaria arborescens* (A) Front side of colony (B) Reverse side of colony (C) Conidial attachment under Stereoscope (D) Mycelia and conidia under microscope (E) Mycelium and conidia under high magnification (100X) of microscope (F) Spore morphology at 40X magnification of microscope.

Molecular Characterization of Fungal Pathogen: Genetic characterization of morphologically identified species was performed by nucleotide sequence BLAST analysis of the rDNA-ITS region, partial GAPDH, and elongation factor gene. All the selected primers successfully amplified the DNA fragment from total genomic DNA (Fig. 3).

BLAST analysis of the rDNA-ITS region of the pathogen, *A. arborescens* exhibited maximum (99%) homology with other *A. arborescens* GenBank strains. Some examples are strain A.arb3 (GenBank ID: MF462297), isolate A10 (GenBank ID: JF802095), PEGT002 (GenBank ID: KC707557), and NAS-3 (GenBank ID: KX768144). The evolutionary relationship of different *Alternaria* species based on the rDNA-ITS sequence was scrutinized by the Maximum Likelihood method (Fig. 4). The associated taxa clustered together shown next to the branches represent the percentage of trees that indicated 99% homology of our strain with different species of *Alternaria* present in Section *Alternaria arborescens*. The analysis intricated 25 nucleotide sequences in MEGA6. This nucleotide sequence was deposited to Genbank under accession number MT367636.

In addition to rDNA-ITS BLAST results, the 100% homology of partial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene from the present study pathogen *A. arborescens* with GenBank strains confirmed the identification of this pathogen. Alignment results revealed absolute homology of our strain with A. *arborescens* strain NB555 (GenBank ID: MH232207), NB157 (GenBank ID: MH232192), NB164 (GenBank ID MH232191), NB150 (GenBank ID: MH232190), NB38 (GenBank ID: MH232179), ICMP 22005 (GenBank ID: KY965829), H9-A (GenBank ID: KY402025), Emory Simmons EGS 39.128 (GenBank ID: GQ180070). This nucleotide sequence was deposited to Genbank under accession number MT628666. The evolutionary antiquity was analyzed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches (Fig. 5). Initial tree(s) for the heuristic search were acquired automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated utilizing Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

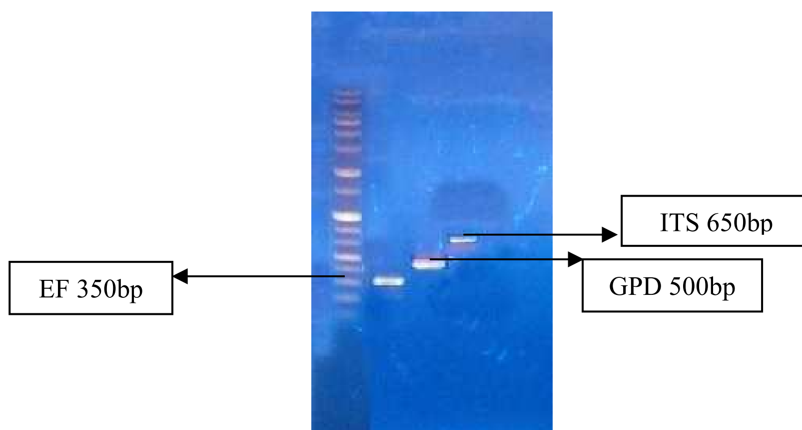

Fig. 3: Agarose gel electrophoresis of *Alternaria arborescens* rDNA-ITS region, Elongation factor, and GAPDH gene.

Finally, the translation elongation factor, which is considered as strong taxonomic marker, from our strains was 100% similar with *A. arborescens* strain PPRI:11433 (GenBank ID: MF381820). This nucleotide sequence was deposited to Genbank under accession number MT628667. The evolutionary history was analyzed using the Maximum Likelihood method based on Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Fig. 6). Initial tree(s) for the heuristic search were attained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. There were a total of 232 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Evaluation of Pathogenicity Assays

Detached Leaf Method: The detached leaf method is a significant tool for studying the pathogenic potential of isolated pathogen. Disease severity was determined by designing a disease rating scale. After 24 hours of

inoculation, disease symptoms were started to develop on leaves in Petri plates. Photography was done on each stage of infection to compare the disease development of the pathogen. Pathogenic infection symptoms observed were leaf spots and lesions (Fig. 7). Infection symptoms on leaves were firstly observed as yellow and then small circular brown spots. These small spots became larger and irregular shiny spots (1-2 mm) and later into dark brown spots lesions following necrosis. After 6 days of inoculation, *A. arborescens* exhibited 20% infection symptoms. However, after 15 days about 90% leaf area was found to be infected by *A. arborescens*.

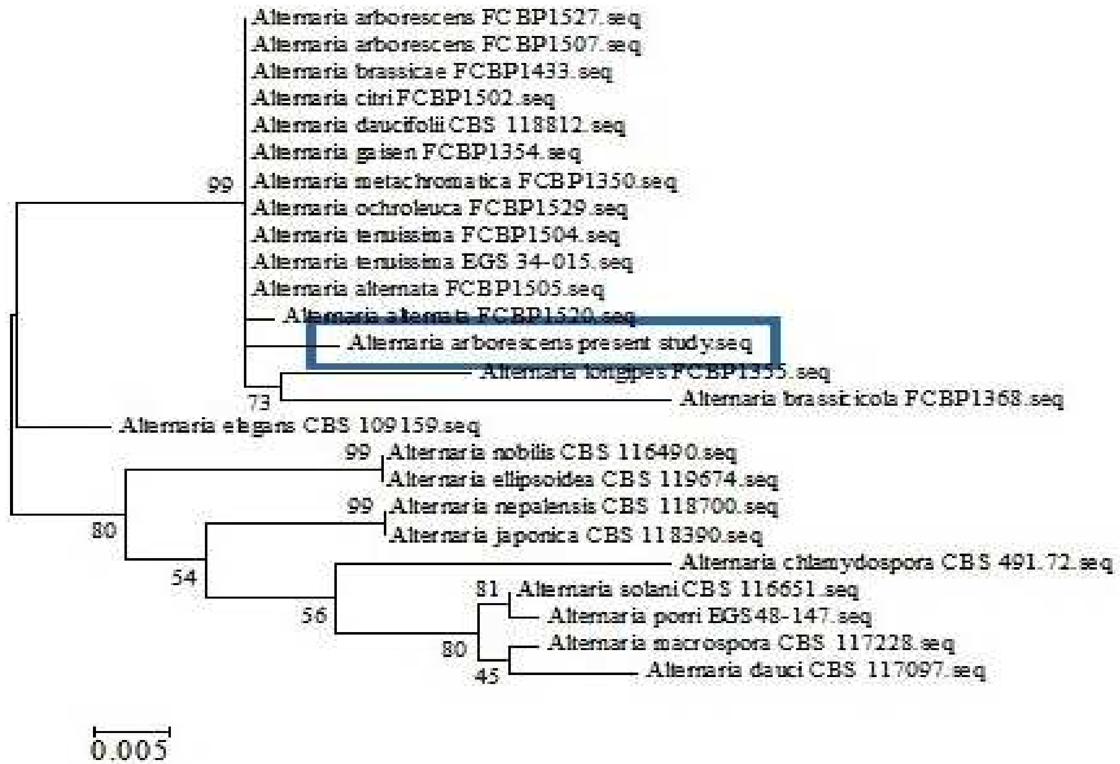

Fig. 4: Molecular phylogenetic analysis of Internal Transcribe Spacer (ITS) region of rDNA of different *Alternaria* species by Maximum Likelihood method.

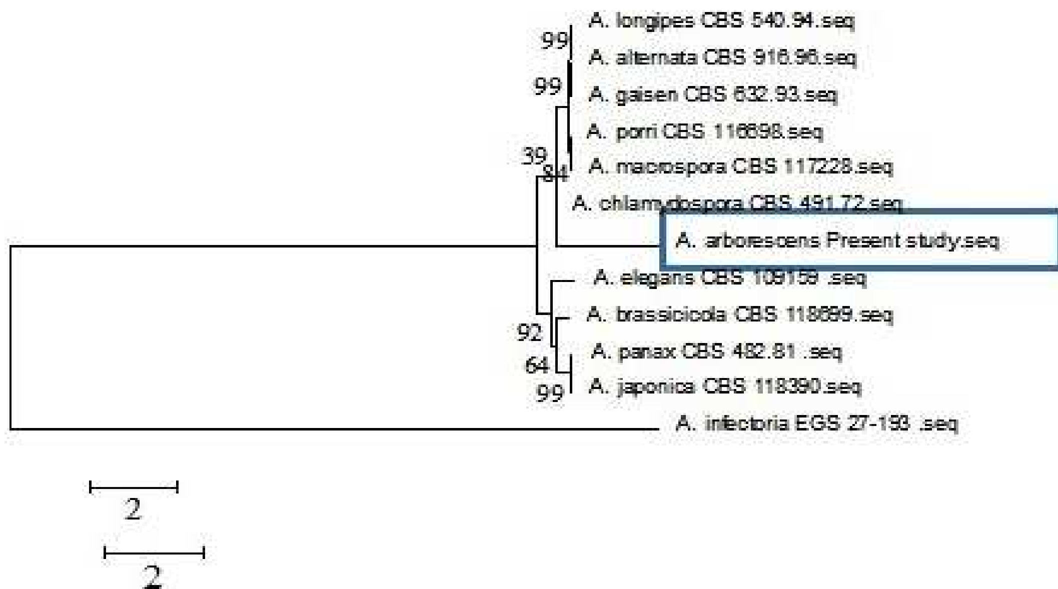

Fig. 5: Molecular phylogenetic analysis of partial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) region of rDNA of different *Alternaria* species by Maximum Likelihood method.

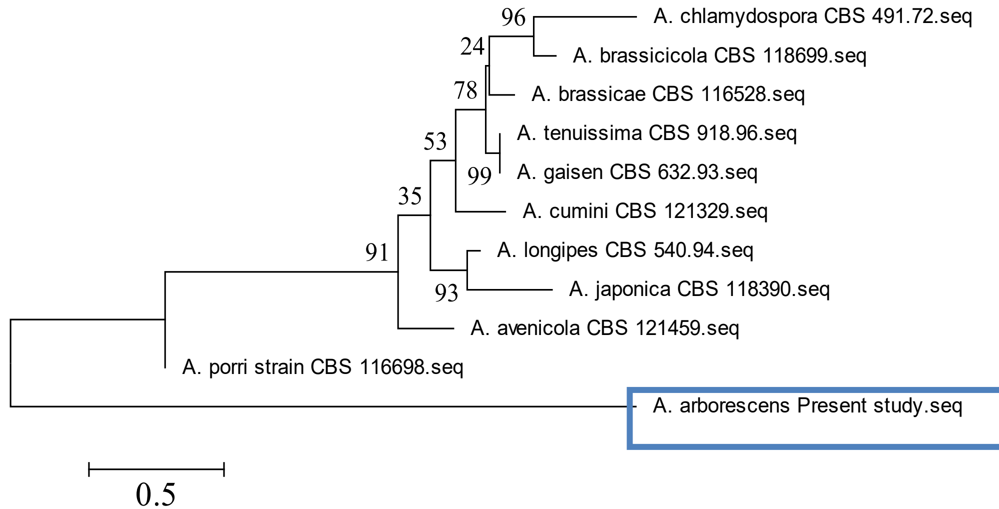

Fig. 6: Molecular phylogenetic analysis of translation elongation factor (EF) region of rDNA of different *Alternaria* species by Maximum Likelihood method.

Table 2: Disease rating scale.

Days after inoculum	Percentage of infected area	Status
1-3	1-20%	Highly resistant
4-6	21-40%	Resistant
7-9	41-60%	Moderately susceptible
10-12	61-80%	Susceptible
13-15	81-100%	Highly susceptible

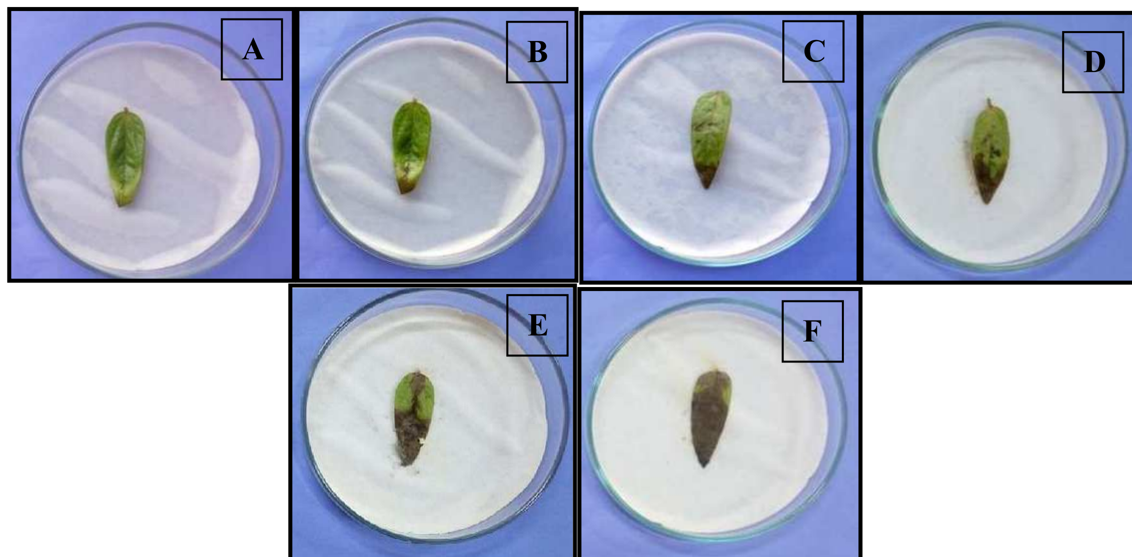

Fig 7: Symptom development caused by *A. arborescens.*

The disease progression curve was designed to evaluate the level of infection of the pathogen (Fig. 8). This curve explicated that *A. arborescens* is a virulent disease-causing organism of mung bean as it exhibited severe disease symptoms in the host plant and hence used for subsequent lab experimentation.

Pot trials: Pathogenicity test was performed by spraying a spore suspension (5 x 10^5 spores/mL) of pathogen from 10 days old culture on 1-week-old mung bean plants in pots. The appearance of infection indications started after 3-4 days. Foliar symptoms were visualized as the yellowing of leaves followed by small brown spots and

angular chlorotic spotting. Later on, leaves became fully necrotic. Results of pot assays showed that the pathogen validated maximum disease severity in the host plant. Initially, the moderate disease severity was induced by *A. arborescens* but after 15 days, the disease severity was found to be 100% with maximum disease incidence (Fig. 9).

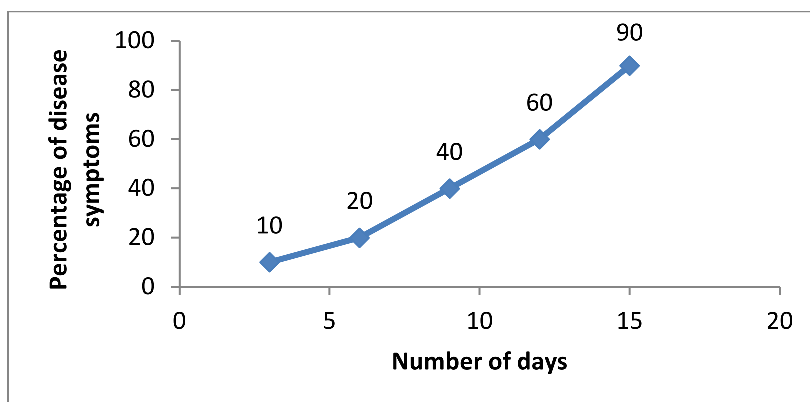

Fig 8: Disease progression curve of *A. arborescens* on mung bean by detached leaf assay.

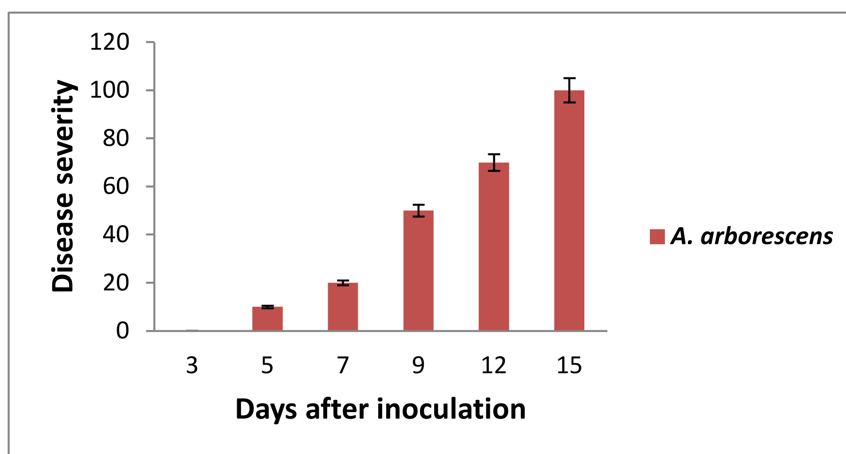

Fig. 9: Disease severity of *A. arborescens* on mung bean plant.
Vertical bars show standard errors of the means of three replicates.

DISCUSSION

Mung bean is a fast-growing hot-season pulse and predominantly contains nutritive protein, calcium, and iron. The yield of agricultural crops is most commonly reduced by plant diseases. In these plant diseases, the harm caused by plant pathogens impacts about 13% of yield losses per annum worldwide (Fletcher *et al*., 2006; Nair *et al*., 2019). Among the number of pathogens, over and above 80% of plant diseases as well as momentous damage to human diet are due to Fungi. Among different constraints; the most distressing disease of mung bean is leaf spot disease which is caused by innumerable mycological pathogens including *Alternaria, Phoma,* and *Drechslera,* etc. (Pandey *et al*., 2019; Shafique *et al*., 2023). Many different modified techniques are used to control these fungi. However, the accurate identification of causal agents is a prerequisite to control fungal diseases (Shafique *et al*., 2021; Shafique *et al*., 2022). The morphological identification of pathogens at the species level is the most reliable conventional method. Moreover, different other molecular techniques are also in practice to identify the fungi i.e., analysis of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships amongst various groups of fungi (Mirhendi *et al*., 2007) or by using the mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*., 1996). In the present study, *Alternaia arborescens* was isolated as a leaf spot pathogen of mung bean and further identified by means of micro and macroscopic analysis for morphology description and genetic exploration from nucleotide sequencing of amplified ITS1, ITS4, gpd1/gpd2, and EF1 region of rDNA. In another study, *Alternaria ochroleuca* was identified as a leaf necrotic pathogen of money plant by evaluating the complete description of macro and

microscopic characters followed by identification using rDNA spacer sequence of amplified ITS1-5.8S-ITS4 region of rDNA (Shafique *et al*., 2017).

After the identification of the pathogen; further, in the current piece of work the pathogenic potential of *A. arborescens* was estimated by the implementation of Koch's postulates using detached leaf laboratory trials and pot trials. The pathogen persuaded the maximum characteristic indications as staining and bruising followed by dark brown spots on the leaves of the mung bean plant and proved very potent as it portrayed a sharp disease progressive curve with 90% of the infected area. In a parallel working; Pettitt *et al*. (2011) developed a detached leaf assay to conclude the pathogenic potential of Pythium isolated from the roots of cut-flower chrysanthemum. Healthy leaves of emergent plants were cut out and injected by the pathogen. Later on, leaves were evaluated for occurrence and magnitude of infection. The pathogenicity was specified by necrosis and was consistently confirmed by appraisals with whole plant inoculations. In another study directed by Shafique and coworkers (2015), the pathogenic potential of 4 strains of *Fusarium oxysporum* was reported on ten dissimilar varieties of chili plants by applying Koch's postulates using pot trials. Specific symptoms were obvious after 10 days of inoculation. Strain B of *F. oxysporum* persuaded characteristic symptoms within 7 days thus as curtained as the most pathogenic. In a similar study, Asghar (2017) appraised the pathogenicity of *Alternaia alternata* and *Cladosporium oxysporum* by employing Koch's postulates both *in vitro* and *in vivo*. Characteristic symptoms of yellowing and dark brown spots on leaves were exhilarated by both pathogens of the cabbage plant and depicted a steep progressive disease curve with 99% and 97% of the infected area, respectively.

Conclusion: Thus, the bioassays in current research conclude that *A. arborescens* was isolated as a novel pathogen from mung bean which produced leaf spot disease in the mung bean plant. It exhibited a sharp progressive disease curve with 90% of infection.

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