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FIRST REPORT OF *CORYNESPORA CASSIICOLA* (BERK. & CURT.) LEAF SPOT OF BITTER GOURD IN PUNJAB, PAKISTAN

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ABSTRACT

Bitter gourd (*Momordica charantia* L.) is a vital vegetal crop. It is very eminent amongst the crops in South East Asia. Diseases of Bitter gourd instigated by fungi are globally very common. Presently, an investigation was steered to find the infectious diseases of some vegetables in vicinity of the University of the Punjab, Lahore and bitter gourd plants were found to be septic with fungal leaf spots. The diseased sections were assembled for the isolations, sanitization and documentation of isolated strain. The recognition and documentation was supported microscopically for morphological characterization and genetically by the nucleotide sequencing of amplified ITS1/ITS4 and BT_2a/BT_2b region of rDNA. *Corynespora cassiicola* (Berk. & Curt.) was identified as the leaf spot pathogen of Bitter gourd. After that the pathogenicity potential of causal agent was confirmed by re- isolating the fungal pathogen with the artificially immunized leaves of test plant using disconnected and intact leaf method. The study indicated the first report of *C. cassiicola* as a leaf spot pathogen of bitter gourd in Pakistan and manifests the need for the management of pathogen.

Key words: Characterization, *Corynespora cassiicola*, Identification, *Momordica charantia*, Pathogenicity.

INTRODUCTION

Momordica charantia L. is a fast growing vine that is tropical and sub-tropical and belongs to cucurbitaceae family consisting of 130 genera and 800 species **(**Radford *et al*., 1968). Throughout the Pakistan it is grown as a summertime vegetable on an area of about 6107 ha with the yearly production of 56949 tons (MNFSR, 2015). Its nutritional value is same as other cucurbits. Bitter gourd has great medicinal value as it is used in curing infectious diseases and diabetes (Khan and Anderson, 2003; Dey *et al*., 2006; Lako *et al*., 2007). A large number of pathogens are known to induce leaf blight diseases on catholic limit of host plants. Diseases of Bitter gourd triggered by pathogenic fungi are very communal worldwide (Ahrazem *et al*., 2000; Murakami *et al*., 2005; Anonymous, 2009; Shakoor *et al*., 2011; Abid *et al*., 2017; Rehman *et al*., 2022). The crop production can be improved by the management of disease inducing microorganisms. Therefore, the current research is concentrated on the leaf spot diseases of Bitter gourd investigated by fungal pathogens that are ultimately distressing economy of Pakistan because for better arrest of leaf spot fungi of Bitter gourd it is essential to precisely classify the fungi concomitant with leaf spot disease.

MATERIALS AND METHODS

Investigation and assortment of diseased samples

A survey was steered to study the diseases of vegetables during 2018. Leaves of bitter gourd were found to be infested with spots. Data regarding the shape, size, color, appearance of spots and lesions on leaves was recorded and all the plants were directed for the study of disease causing organism. Photography of all septic leaves along with their plants was done as a record for reference.

Isolation and Purification of Pathogen

Malt Extract Agar (MEA) fungal growth medium (2%) was made to segregate and identify pathogen. To isolate fungal pathogen, the infected leaves were cut into approximately 3 mm^2 sized pieces at the site of lesion along with some healthy leaf tissue and immersed in sodium hypochlorite solution (1%) for 5-10 min for surface disinfection. About 4-5 sanitized leaf sections were placed onto MEA plates and reared at 25 $\pm$ 2 $^{\circ}$C. Mycelia of the fungi emerging from inoculated leaf sections were transmitted to the fresh plates of MEA and left to propagate at 25 $\pm$ 2 $^{\circ}$C for distillation of fungal cultures. Purified cultures were stockpiled at 4 $^{\circ}$C.

Identification and Characterization of Pathogens

Screened fungal strains were grown on MEA at 25 ± 2 °C for a week and primarily recognized on morphological bases and that were further identified or confirmed on the basis of nucleotide sequence exploration of Internal Transcribed Spacer (ITS) sequence of rDNA and partial beta tubulin gene (Shafique *et al*., 2022) (Table 1).

Table 1. Details of the primers utilized for amplification of ITS or β-tubulin gene.

Sr. no	Gene	Primer name	Primer sequence
1.	ITS	ITS 1	5′-TCCGTAGGTGAACCTGCGG-3′
2.		ITS 4	5′-TCCTCCGCTTATTGATATGC -3′
3	β-tubulin	BT_2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′
4		BTt_2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′

Intensified PCR products were referred for nucleotide sequencing and the subsequent classifications were scrutinized by nucleotide BLAST study. Similarity of structures was noted for the correct documentation of fungal strains. Pure culture of identified fungal pathogen was deposited to the First Fungal Culture Bank of Pakistan at the Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore under specific accession number.

Pathogenicity Confirmation Test

Detached leaf method

To assess the potency of sequestered fungal pathogen, disconnected leaves from the vigorous plant were employed in the petri plates containing filter papers in a manner that the petioles touched the saturated filter papers. Almost 5×10^5 spores mL^{-1} were poured on disconnected leaves and plates were placed at 25 ± 2 °C. Control leaves were treated in the same way but with distilled water. All Petri plates were examined frequently to study development of disease symptoms. After disease occurrence, the pathogen was re-isolated from these leaves for the fulfillment of Koch's pathogenicity hypothesizes.

***In vivo* pot trial method**

Healthy seeds of bitter gourd were sown into earthen pots at the rate of two seeds per pot and placed into growth room at 24-31°C and watered regularly when needed. For the confirmation of pathogenicity, 5×10^5 spores were injected in the stem nodes using a decontaminated syringe. Similar quantity of distilled water was poured in control treatment. To maintain moisture for the germination of spore and development of disease, plants were kept covered for 48 h with the polythene bags. Later on the plants were kept under shadow (at 25 – 26 °C), irrigated regularly and monitored for disease development. Disease incidence and Disease severity was noticed and calculated as the disease symptoms depicted on the plant. To calculate Disease index and Disease severity following formulae were used:

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

$$\text{Disease Index} = \frac{\text{Number of plants in Particular Category}}{\text{Total Number of Plants}} \times 100$$

RESULTS

Study of Diseased Samples

The leaves of *Momordica charantia* L. plants were infected with the spot or lesions. Symptomology appeared on bitter gourd plants was dark brown to blackish lesions or spots ranging from 1-2 mm (Fig. 1). About 40% -60% leaf areas was found to be infected with spots or lesions.

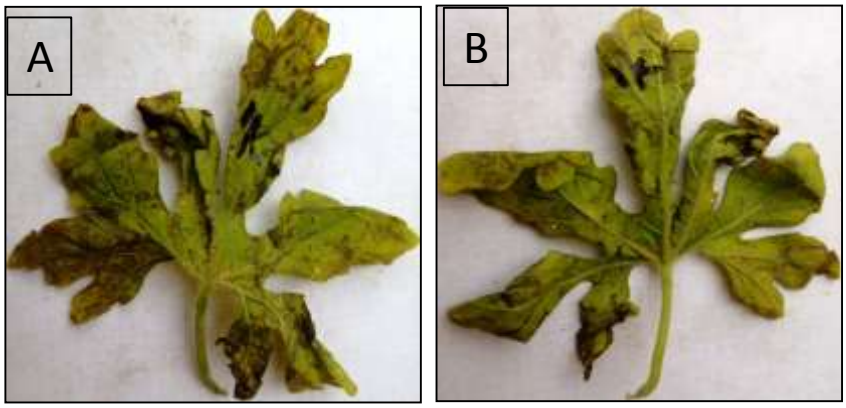

Fig. 1. Bitter gourd leaf infected by *Corynespora cassiicola* from front side (A) from reversed side (B).

Morphological Characterization

Colonies observed on growth medium were 4.0 - 4.5cm, effuse, grey or tanned, reverse black, sparsely hairy; in a dissecting binocular microscope the view of conidiospores was iridescent. Mycelium mostly engrossed; without stroma. Conidiophores pale to light brunet, with almost 9 consecutive tubular proliferations, 110-850µ extended, 4-11µ profuse. Conidia mostly inconstant in form or may be one or in cuffs of 2-6, , obclavate to tubular, straight or bent, sub-hyaline to slightly pale olivaceous brown or tan, flat, with 4-20 pseudosepta, 40-220µ long (up to 520µ in culture), 9-22µ thick, 4-8µ wide at the truncate base (Fig. 2). On the basis of morphology the fungal culture was recognized as *Corynespora cassiicola* ((Berk. & Curt.) Wei, 1950, Mycol). Pure cultures of the isolated pathogen were deposited to the First Fungal Culture Bank of Pakistan, Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore Pakistan, under accession number FCBP1499.

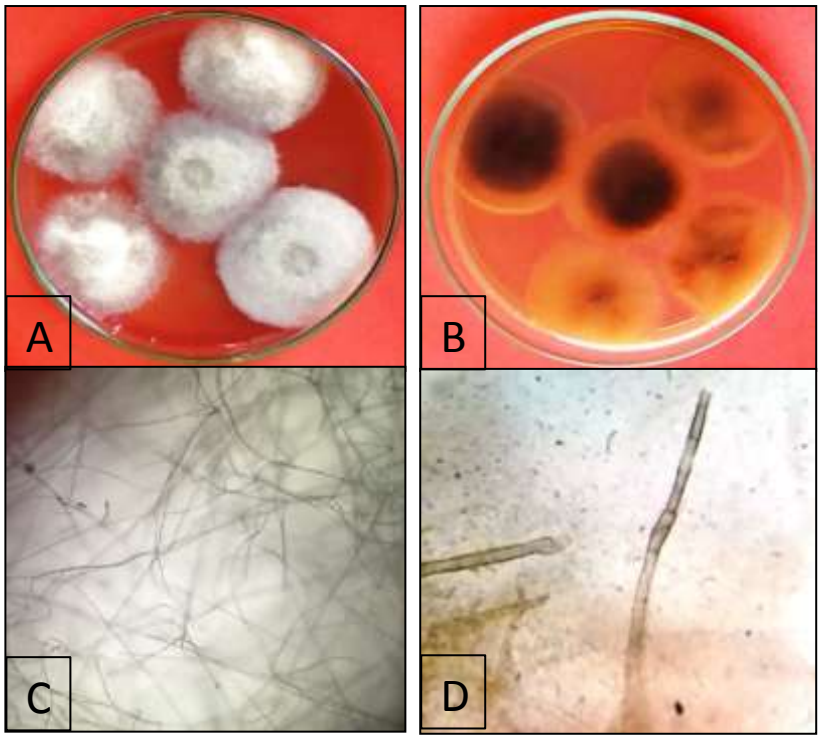

Fig 2. Pure culture of *Corynespora cassiicola*. Colony from front side (A) reversed side (B) and under stereoscope (C). Mycelium under microscope (D)

Genetic Characterization

For the Genetic characterization, total fungal genomic DNA was isolated (Fig 3) and used for the amplification of ITS and Bt_2a regions of rDNA. The resulting PCR products of about 600 bp were led for

nucleotide sequencing. Consequential structure was when explored by BLAST, it showed homology with 98% to *Corynespora cassiicola* (KP735615.1) isolate LM-2 beta-tubulin gene (Fig 4). Pure culture of *Corynespora cassiicola* was placed to FCBP and accession number assigned FCBP1499 and ITS resulting sequence results when analyzed by BLAST, it showed 99%homology to *Corynespora cassiicola* (KU174390.1) isolate ABS48_BE1 (Fig 5).

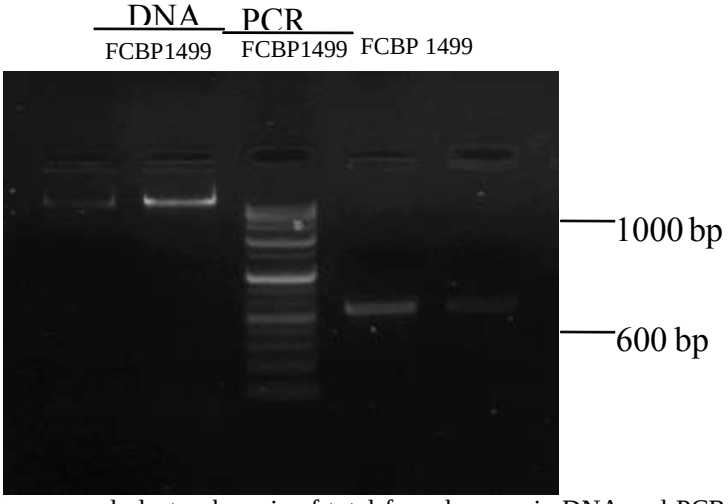

Fig. 3. Agarose gel electrophoresis of total fungal genomic DNA and PCR amplified ITS region and Beta-Tubulin amplified region (*Corynespora cassiicola*).

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FCBP1499  8    GATCGGCAATGCCCGTCGACGCGGAACGAC-ACTGACTCCATGCGTCTACAGGCAGCAGA  66
               ||||||||   ||||||||||||| |||||  ||||||||||||||||||||||||||||
LM-2Bt    9    GATCGGCATGCCCCGTCGACGCGG-ACGACAACTGACTCCATGCGTCTACAGGCAGCAGA  67

FCBP1499  67   TTTCTGGCGAGCACGGTCTCGACGGATCCGGCGTGTAAGTTGGCTCTCGTCCGACAGCCC  126
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LM-2Bt    68   TTTCTGGCGAGCACGGTCTCGACGGATCCGGCGTGTAAGTTGGCTCTCGTCCGACAGCCC  127

FCBP1499  127  ACGAGGCCTTTTCGCGACACGAACTAACTGGGACTAGCTACAATGGCACCTCCGACCTCC  186
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LM-2Bt    128  ACGAGGCCTTTTCGCGACACGAACTAACTGGGACTAGCTACAATGGCACCTCCGACCTCC  187

FCBP1499  187  AGTTGGAGCGCATGAACGTCTACTTCAACGAGGTTCGCGGCCACGCTTCTGCTACGCGCG  246
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LM-2Bt    188  AGTTGGAGCGCATGAACGTCTACTTCAACGAGGTTCGCGGCCACGCTTCTGCTACGCGCG  247

FCBP1499  247  ATGCTGACATGCTAACAACAAGCAGGCTTCCGGTAACAAGTTCGTTCCCCGTGCCGTCCT  306
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LM-2Bt    248  ATGCTGACATGCTAACAACAAGCAGGCTTCCGGTAACAAGTTCGTTCCCCGTGCCGTCCT  307

FCBP1499  307  CGTCGACTTGGAGCCCGGTACCATGGACGCCGTCCGTGCCGGCCCCTTTGGTCAGCTTTT  366
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LM-2Bt    308  CGTCGACTTGGAGCCCGGTACCATGGACGCCGTCCGTGCCGGCCCCTTTGGTCAGCTTTT  367

FCBP1499  367  CCGTCCCGACAACTTCTTCTTCGGGCAGTCTGGTGCCGGTAACAACTGGGCCAAGGGTC  425
               ||||||||||||||| ||||||| ||||||||||||||||||||||||||||||||||
LM-2Bt    368  CCGTCCCGACAACTTCGTCTTCGGCCAGTCTGGTGCCGGTAACAACTGGGCCAAGGGTC  426
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Fig 4. BT_2a sequence alignment of FCBP 1499 with the *Corynespora cassiicola* (KP735615.1) isolate LM-2 beta-tubulin gene.

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FCBP1499  10   GGGGCTCGCCCCTTCGAGATAGCACCCTTTGTTTATGAGCACCTCTCGTTTCCTCGGCAG  69
               ||| || ||| ||||||||| ||||||||||||||||||||||||||||||||||||||||
ABS48_BE1  8   GGGACTAGCCTCTTCGAGA-AGCACCCTTTGTTTATGAGCACCTCTCGTTTCCTCGGCAG  66

FCBP1499  70   GCTCGCCTGCCAACGGGGACCCACCACAAACCCATTGTAGTACAAGAAGTACACGTCTGA  129
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48 BE1  67  GCTCGCCTGCCAACGGGGACCCACCACAAACCCATTGTAGTACAAGAAGTACACGTCTGA  126

FCBP1499   130 ACAAAACAAAACAAACTATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  189
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1  127  ACAAAACAAAACAAACTATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  186

FCBP1499  190  GAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAAT  249
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1  187  GAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAAT  246

FCBP1499  250  CTTTGAACGCACATTGCGCCCTTTGGTATTCCTTAGGGCATGCCTGTTCGAGCGTCATTT  309
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1  247  CTTTGAACGCACATTGCGCCCTTTGGTATTCCTTAGGGCATGCCTGTTCGAGCGTCATTT  306

FCBP1499  310  CAACCCTCAAGCCTAGCTTGGTGTTGGGCGTCTGTCCCGCCTCCGCGCGCCTGGACTCGC  369
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1 307  CAACCCTCAAGCCTAGCTTGGTGTTGGGCGTCTGTCCCGCCTCCGCGCGCCTGGACTCGC  366

FCBP1499  370  CTCAAAAGCATTGGCGGCCGGTTCCCAGCAGGCCACGAGCGCAGCAGAGCAAGCGCTGAA  429
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1 367  CTCAAAAGCATTGGCGGCCGGTTCCCAGCAGGCCACGAGCGCAGCAGAGCAAGCGCTGAA  426

FCBP1499 430  GTGGCTGCGGGTCGGCGCACCATGAGccccccACACCAGAATTTTGACCTCGGATCAGG  489
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABS48_BE1 427  GTGGCTGCGGGTCGGCGCACCATGAGCCCCCCCACACCAGAATTTTGACCTCGGATCAGG  486

FCBP1499 490  TAGGGATACCCGCTGAACTTAAGCATATCAATAAGTCGGAGGAAAAG  536
              |||||||||||||||||||||||||||||||| |||||||| ||
ABS48_BE1 487 TAGGGATACCCGCTGAACTTAAGCATATCAATAAGCCGGAGGAATAG  533
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Fig 5. ITS sequence alignment of FCBP 1499 with the *Corynespora cassiicola* (KU174390.1) isolate ABS48_BE1.

Analysis of Pathogenicity Assays

Detached leaf method

In detached leaf method the characteristic symptoms of pathogen were observed to appear within 3-4 days on bitter gourd leaf and photographed (Fig. 6). Initially the plants displayed yellowing on the leaves that was turned into chlorosis and slowly it was converted to necrosis and eventually the death of the whole plant. Within 3-10 days, about 30% symptoms appeared due to *C. cassiicola*. The disease progression curve was

plotted for the pathogen which was made very sharp (Fig 7). This disease progress curve ascertained that *C. cassiicola* exhibited severe disease symptoms and proved the virulent pathogen of bitter gourd.

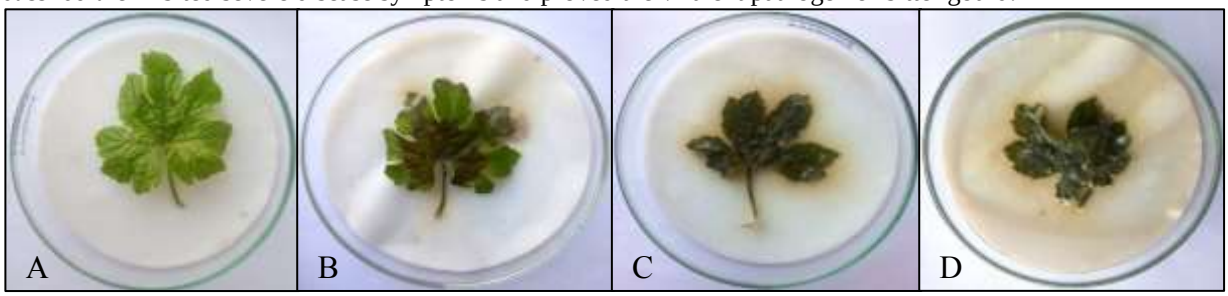

Fig 6. Symptom development caused by *Corynespora cassiicola*.

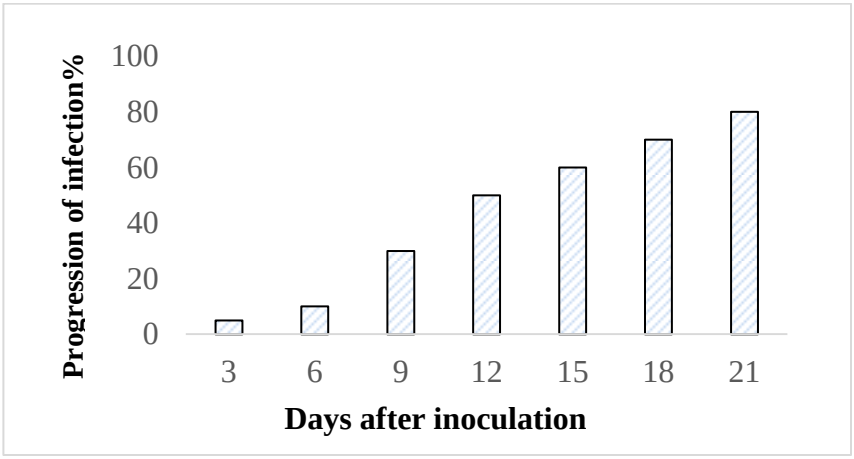

Fig 7. Disease progression curve of *Corynespora cassiicola* on Bitter gourd by detached leaf assay.

Pot trials

Pathogenicity test was performed by spraying one month old bitter gourd plants to runoff with spore suspension of identified pathogen containing approximately 5×10^5 spores mL^{-1} and infection was evident in 3-4 days (Table 2). Foliar symptoms were visualized as the yellowing followed by inward rolling and angular chlorotic spotting of leaves or in advanced phases, thorough necrosis of leaves on one or both sides of the midrib was noticed (Table 2). Data investigation of pot trials publicized that the pathogen demonstrated serious disease severity in host plant which was found to be 100% after 15 days (Fig 8).

Table 2. Pictorial representation of disease rating scale on Bitter gourd plant on the basis of symptoms induced by *Corynespora cassiicola*.

Key scale	**Disease Description**		**Disease severity**
0	No symptoms		0
1	Yellowing started on the leaf		10

2	Wilting with blight spots		30
3	Spots increases on leaf		50
4	Whole plant is infected with spots		70
5	Complete death of plant		100

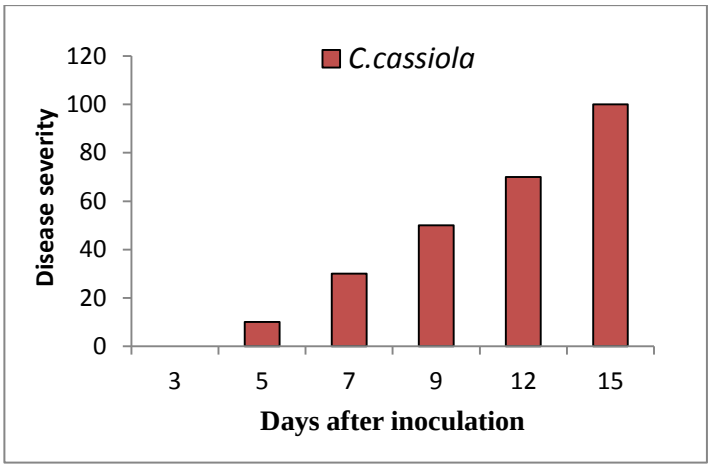

Fig 8. Disease severity of *Corynespora cassiicola* on bitter gourd plant.

DISCUSSION

Bitter gourd (*Momordica charantia* L.) is an important and widespread traditional remedial fruit in many tropical countries. The great threat to significant loss of bitter gourd growers is because of fungal diseases that cause heavy yield losses. Precise identification of the pathogens inducing spot diseases on a particular host plant is imperative. Numerous leaf spot infections ensure analogous biology and consequently identical management alternatives. This study accordingly emphasized the precise identification of the agent causing

leaf spots. Presently, during the field survey of different areas of Punjab the disease prevalence, incidence and disease severity of leaf spot disease of bitter gourd was determined. The similar work was performed by Shazia *et al*. (2003) in which during the survey disease ridden plants were observed in rice and wheat fields of four different regions.

Microscopic characteristics and development of conidia/conidiophores is very crucial for pathogen identification as physical configuration is still deliberated as the utmost persistent system to classify the organism, but errors in identifications are reported (Anderson *et al*. 2006). Consequently, numerous molecular procedures were documented to classify the organisms up to specific level viz., scrutiny of ribosomal DNA (rDNA) composition to find out molecular phylogenetic relationships among different arrays of fungi (Mirhendi *et al*. 2007) or with the help of mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*. 1996). Presently, *Corynespora cassiicola* was identified as a cause of leaf spot of Bitter gourd by analyzing morphological microscopic features followed by genetic characterization from nucleotide sequencing of amplified ITS1-5.8S-ITS4 region of rDNA. Similarly, in a study *Exserohilum rostratum* was reported as a leaf spot pathogen of *Solanum melongena* by Shafique *et al*. (2020).

Finally, Koch's postulates were applied to assess the pathogenicity of *C. cassiicola* on bitter gourd seedlings by detached leaf and pot trial methods. *C. cassiicola* exhibited dark brown to blackish lesions or spots with sharp disease curve by displaying 100% infected leaf area. Working on parallel lines, Conner (2002) used detached leaf method to confirm the pathogenicity of *Cladosporium carygenum* on Pecans. Similarly, pathogenicity of different strains of *F. oxysporum* was confirmed by Koch's postulate on ten different varieties of Chili plant using pot trials. The particular symptoms were apparent after 10 days of inoculation by all the strains. However, strain B of *F. oxysporum* induced the distinctive symptoms within 7 days thus declared as the most pathogenic (Shafique *et al*. 2015). Thus the contemporary research categorically reports an innovative isolation of leaf spot causing pathogen from bitter gourd.

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