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A New Leaf Spot Disease of Ash-Gourd Caused by *Curvularia geniculata* From Sub-Himalayan West Bengal

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ABSTRACT

Ash gourd, *Benincasa hispida* (Thunberg) Cogniaux is an important crop for its nutritive and medicinal properties. Several fungal diseases have been reported to cause severe damage to ash gourd cultivation in India. Discolored leaf spots (yellow to dark brown) were observed in wax gourd cultivated throughout West Bengal during January to March 2021. In some cases young plants showed severe necrotic spots resulting to death of the plants. Symptomatic leaves were collected for fungal isolation. One of the isolated fungi showed black-grey mycelial mat on potato dextrose agar plate. Ascendant or erect conidiophores were seen with dark brown appearance, which were branched, with geniculate and sympodial elongations. Numerous light brown conidia were observed on the conidiophore. Under light microscope conidia were oblong to cylindrical, curved to varying degrees, tapering at both ends and with three to four transverse septa. The fungus was identified as *Curvularia* species based on morphological characteristics. The organism was consistently isolated from the artificially inoculated and infected leaf samples. Thus Koch's postulates were confirmed. The ITS region of 18S, 5.8S and 28S ribosomal DNA were amplified with one primer set for molecular identification of the fungus. The amplicon was cloned and the sequence was submitted to GenBank (accession no. OP919566). Sequence similarities confirmed the organism as *C. geniculata.* According to the present study *C. geniculata* is being reported as a pathogen of ash gourd for the first time.

Keywords *Benincasa hispida*, *Curvularia geniculata*, Leaf spot disease.

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INTRODUCTION

Ash gourd, *Benincasa hispida* (Thunberg) Cogniaux of family cucurbitaceae is an annual climbing crop that is grown substantially for its fruits. It is native to Asian countries. In Asia it is honoured for its nutritive and medicinal properties (Purohit *et al.* 2019, Palamthodi *et al.* 2019). *B. hispida* is also known as kundur fruit, chalkumra, winter gourd, ash gourd, winter melon, white gourd, wax gourd, tallow gourd, Chinese preserving melon, ash pumpkin, and (alu) puhul. It is grown for its large sized fruits and eaten as green vegetable (Busuioc *et al.* 2020, Soliman *et al.* 2020, Islam *et al.* 2021). According to scientific reports, *B. hispida* contains many important nutritious substances, including vitamins, amino acids, organic acids, natural sugars and mineral elements (Andrias

et al. 2019, Purohit *et al*. 2019). The fruit can be used as an ayurvedic medicine for peptic ulcer, diabetes mellitus, urinary infection, hemorrhages from internal organs, lung disease, hiccups, asthma, internal bleeding, insanity, epilepsy, and other nervous disorders (Islam *et al.* 2021).

Several fungal diseases of severe nature have been reported from ash gourd cultivated in India. Some of the major diseases are as follows. Didymella black rot and *Colletotrichum anthracnose* were reported on fruits of ash gourd are infected by postharvest pathogens such as *Didymella bryoniae* and *Colletotrichum lindemuthianum, C. orbiculare* respectively (Tripathi *et al.* 2022). The blight and rotting in ash gourd were caused by *Alternaria alternata* and *Curvularia lunata* respectively. *Fusarium oxysporum*, *F. pallidoroseum* were responsible for fruit rot. *Alternaria alternata, Botryodiplodia theobromae*, *Chaetomium* spp., *Curvularia lunata, Drechslera tetramera, Fusarium equiseti*, *F. moniliforme* and *F. solani* can be borne by all gourd seeds (Avinash *et al.* 2021). *Podosphaera xanthii* was firstly reported to cause powdery mildew on *B. hispida* in Korea (Choi *et al.* 2021). *Fusarium commune* causing leaf spot of wax gourd was first reported from China (Zeng *et al.* 2020).

In the present study, several diseased ash gourd fields in Dhupguri, West Bengal, India were surveyed during January to march 2021 and one leaf spot symptom of severe nature was found. The symptoms appeared as yellow spots which later became deep brown to circular to irregular necrotic spots leading to falling of leaves. Black-brown banding pattern on the edges was also observed. These lesions often coalesced to form wide necrotic areas. In severe cases the plants died. Considering the severity of the disease the present study was conducted to identify the causal organism (following morphological and molecular techniques) of the disease and also to confirm the Koch's postulates to establish the organism as a pathogen of the plant.

MATERIALS AND METHODS

Plant sampling and fungal pathogen isolation: For field survey three different ash gourd fields of Dhupguri, (latitude 26.582132º and longitude 89.005142º) West Bengal were surveyed and diseased leaf samples showing leaf blight or deep brown necrotic spots were collected in sterilized zip bag. Some healthy leaves were also collected as control sample. Ash gourd leaves showing disease symptoms were taken and the affected parts with some of the healthy part (5 mm in size) were carefully cut out with a sharp blade. The cut portion of diseased explants were then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 30 seconds and subsequently washed with sterilized distilled water for three consecutive times to remove traces of $HgCl_2$. The surface sterilized explants were then transferred aseptically onto potato dextrose agar (PDA) and incubated at 28±1°C for 3 days. After 3 days the mycelia of the isolated fungi, if found pure, was taken as inoculums and transferred to fresh PDA slants in several replicates. The tubes were then kept in incubator at 28±1°C for 7 days. After seven days some of the slants were kept as stock culture in refrigerator at 4°C. For better sporulation purpose the rest of the slants were kept in room temperature.

Light microscopy: The fungal isolate was studied for morphology of conidia and hyphae taking the help of Leica Application Suite V4.4 microscope (Singapore) equipped with Leica MC 120 HD digital camera (Singapore). For microscopic observations, mycelia of the fungi were taken in microscopic slides from pure sporulated culture and stained with cotton blue in lacto-phenol. The slides were mounted with cover glass, sealed and were observed under microscope. The fungal cultures were morphologically identified and observed thoroughly for different morphological characters.

Inoculum preparation: Inoculum was prepared from the conidial suspensions of the test isolate which were initially grown for sporulation on PDA medium (both in test tubes and petridishes) for 10-12 days at 28±1°C. Some of the tubes/plates were filled with sterile distilled water and conidia were dislodged by gentle brushing with an inoculating needle. The resultant solution was strained through a muslin cloth to separate the conidial suspension from the mycelial fragments. The concentration of the conidial suspension was adjusted to 1×10^6 spores ml^{-1} by adding sterile distilled water using a hemocytometer.

Table 1. Region of collection of leaf samples of ash gourd.

Place of sampling	Disease	Isolate code	Time of sampling	Location Latitude	Longitude
Daukimari, Dhupguri	Leaf spot	Dh/F/1	20-01-2021	26 °5815’	89°0057’
Bamni, Dhupguri	Leaf spot	Dh/F/2	15-02-2021	26°5882’	89°0205’
Debpara, Dhupguri	Leaf spot	Dh/F/3	08-03-2021	26°5821’	89°0051’

Growing of plants for experiments: Ash gourd plants (Cultivar : Namdhari seed) were grown in the Departmental experimental garden under natural condition of light and watering.

The detached leaf inoculation technique: Detached leaf inoculation technique of (Dickens and Cook 1989) was followed for inoculation of healthy leaves. The leaves were collected from plants which were 2 months old. The leaves were kept in sterile plastic trays (16 cm × 10 cm) on a moist blotting paper and covered with a glass plate. Twenty µL conidial suspension drops (5 drops per leaf) of the test fungi were mounted on either side of the midrib. In control sets only sterile distilled water was sprayed. The control and inoculated leaves were kept at room temperature (28 ± 2°C) with relative humidity of 85% and with a 12 hr dark/night cycle.

Pathogenicity, re-isolation and assessment of disease: Pathogenicity was determined on detached leaves after 24, 48 and 72 h post inoculation by the presence or absence of symptoms. The inoculated leaves showing symptoms were counted and the percentage of lesions was calculated by : Total no. of lesions formed out of total no. of mounted drops × 100. In addition, mean diameter of lesions were also recorded after 24, 48 and 72 h of inoculation.

Re-isolation and confirmation of Koch’s postulates: The leaf piece (1 cm^2) containing well developed symptoms were taken from the artificially inoculated experimental leaves and were subjected to surface sterilization. The surface sterilized piece of leaf was placed in a sterile PDA slant and incubated for 10 days. After incubation the reappeared fungal mycelia and conidia were observed under microscope. If the re-isolated fungi and inoculated fungi found to be same then the fungus was considered as pathogen of the host by fulfilling the conditions of Koch’s postulates.

Assessment of disease following whole plant inoculation technique: The test fungal pathogen was inoculated on the leaves of 3 whole plants grown in pots. Three such plants were also sprayed by sterile distilled water. Concentration of the conidia was 1×10^6 spores ml^{-1}. The whole plant inoculation technique of Sinha and Das (1972) was followed. Inoculation was done on four to five leaves of each plant. Conidial suspension was sprayed onto both adaxial and abaxial surface of the leaves. The inoculated and control plants were maintained at 30°/25°C and 90/70% relative humidity in a confined glass chamber with a 12 h day/night cycle. Pathogenicity (disease index) was calculated on 2nd, 4th, 6th, 8th and 10th day post inoculation by the formula of Sinha and Das (1972). The sizes of lesions were categorized into four groups and a value was assigned to each group as follows: Very small-restricted lesions of 1-2 mm diameter = 0.1, Lesions with sharply defined

Table 2. Pathogenicity of the isolated fungi on detached leaves of ash gourd. All values are mean ± standard deviation.

Inoculation surface	Percentage lesion formed Incubation period (hours)			Mean diameter of lesion (mm) Incubation period (hours)		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Abaxial surface	0	65±5	78±7.5	0	8.13±0.70	11.4±0.8
Adaxial surface	0	71.3±6.11	78.6±3.51	0	8.8±0.98	9.6±2.08

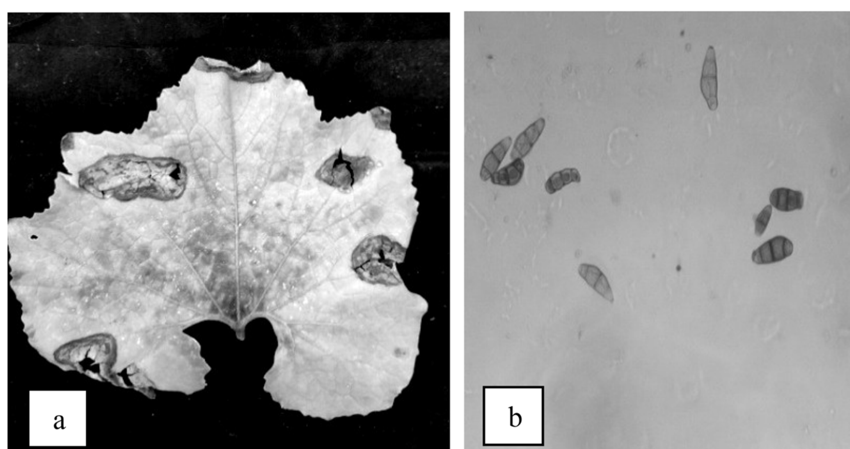

Fig. 1. (a) Naturally infected ash gourd leaf, (b) Spores of *Curvularia geniculata*.

margins of 2-4 mm diameter = 0.25, Slow spreading lesions of 4-6 mm diameter = 0.5; Spreading lesions of variable size (beyond 6 mm in diameter) with diffused margin=1.0. The number of lesions in each group was multiplied by the value assigned to it and the sum total of such values was noted. Disease index was calculated as the mean of observations on three plants per treatment and data was computed as mean disease index per plant.

Molecular identification of fungal pathogen: The total genomic DNA of the fungal isolate/test fungus was extracted from 7-day-old potato dextrose broth cultures following the method of Haible *et al.* (2006). The DNA sample was subjected to PCR using The ITS regions 1 and 4, including 18S, 5.8S and 28S rDNA were amplified by using universal primers ITS 1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS 4 (5`-TCCTCCGCTTATTGATATGC-3`) (White *et al.* 1990). Amplification reaction was performed in a 25 µL reaction mixture containing 2 µLDNA template, 5 µL 5× Taq DNA buffer (containing 100 mM KCl, 10 mM Tris-HCl pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 0.5% Tweenty and 50% glycerol, Promega, USA), 1.5 µL 25 mM $MgCl_2$ (Promega, USA), 1 µL 10 mMdNTPs (Promega, USA), 0.5 µL10 µM each forward and reverse primers (Sigma, USA) and 0.125 µL 5U Taq DNA polymerase (Promega, USA). PCR protocol for the amplification with the ITS 1 and ITS 4 primers was denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. The amplicons were resolved in 1% agarose gels and were visualized on a UV-transilluminator (Genei, Bangalore, India). The purified PCR product of one isolate (Dh/F/2a) was cloned in pGEM-T easy vector (Promega, USA) following the method of Sambrook and Russel (2001) and was sequenced by Biokart India Pvt Ltd (Bangalore, India). The nucleotide sequence of the amplicon was submitted to GenBank after BLASTn analysis (Altschul *et al.* 1997). Phylogenetic tree was generated by neighbour-joining method through Kimura two-parameter in MEGA 6.0 (Tamura *et al.* 2013) following alignment with ClustalW 1.6 (Thompson *et al.* 1994).

Table 3. Pathogenicity of the isolated fungi on whole plants of ash gourd. All values are mean ± standard deviation.

Fungi	Mean foliar disease index/plant Incubation period (Days)				
	2	4	6	8	10
Curvularia sp.	0.33±0.15	1±0.25	1.16±0.76	2.41±0.99	3.16±0.26

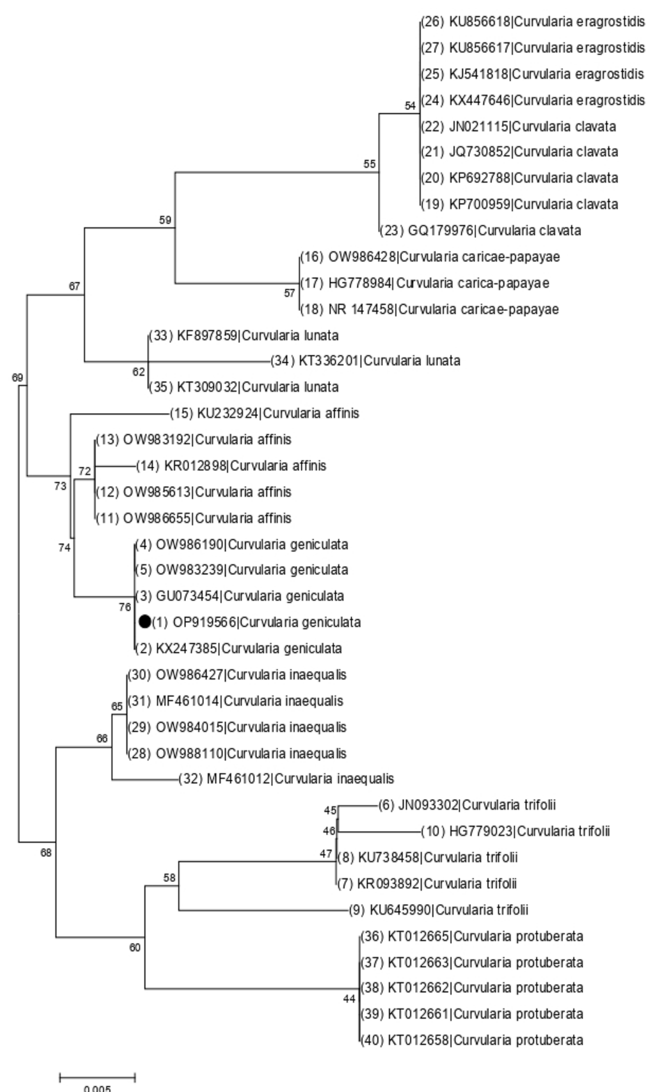

Fig. 2. Phylogenetic tree of the ITS rRNA region of *Curvularia* sp. generated by maximum likelihood method using Kimura-2-parameter model values at the nodes indicate percentage of booster support out of 1000 replicates.

RESULTS

Field survey and isolation of organism: Early disease symptoms in the field were observed as small yellow superficial discoloration on leaves. In severe cases, the discoloration gradually became necrotic and developed into dark brown necrotic spots leading to black patches (Fig.1a). The necrotic spot size ranged from 6-7 mm diameter. Fungal isolates with similar colony morphology were also obtained from infected leaves of the adjoining areas. Three such isolates (coded as: Dh/F/1, Dh/F/2, Dh/F/3) were taken in to consideration for the present study. Details of the isolates are given in Table 1.

Morphological and light microscopic study of the fungal organism: Ten day old fungal culture in potato dextrose agar medium (in petridish) showed dark brown to black, fluffy appearance. Morphological

characteristics of the fungus resembled *Curvularia geniculata* (Katushova *et al.* 2021).

All the three isolates produced erect conidiophores with dark brown appearance, which were branched, with geniculate and sympodial elongations. They formed numerous conidia attached singly or in clusters which were light brown, oblong to cylindrical conidia curved to varying degrees, tapering at both ends, with three to four transverse septa, with one or two central cells larger and darker than the terminal ones (Fig. 1b). Length of the spores varied from 17.5 to 30.0 μm and the width varied from 8.8 to 12.5 μm. The average conidia size was 21.6 × 10.8 μm.

Confirmation of Koch's postulates and pathogenicity of the organism: For each fungus isolate detached leaves inoculation as well as whole plant inoculation was done as described in materials and methods. Details of pathogenicity tests are described in Tables 2–3. Irregular yellow spots appeared on the inoculated leaves after three days of inoculation, which turned dark brown to black after 7 days for all three isolates in case of detached leaf inoculation. All the control leaves had no symptoms. In the whole plant inoculations, plants of 'Namdhari seed' variety of ash gourd showed disease symptoms after 6 days of inoculation which were similar to symptoms found in the naturally infected plants. No disease symptoms were evident on the un-inoculated control. After 12 days, fungus was re-isolated from the symptomatic inoculated leaves. The re-isolated fungi were compared with the isolated fungi. All the re-isolated fungi were similar in morphology and spore characteristics. Thus the fungi were considered as pathogen of the ash gourd.

Molecular identification of the pathogen: All the three fungi (established as pathogen following Koch's postulation) associated with leaf spots were detected by PCR using ITS specific primers, ITS 1/ ITS 4 and were further identified by polymerase chain reaction. All the samples showed positive PCR amplification and an expected ~500 nucleotide long sequences containing 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. The sequences of two isolates were identical and one representative isolate, (Dh/F/2) was deposited in GenBank. The sequenced product of the ITS region was found to be 529 nt long (Accession no. OP919566). It showed 100% nt identity (Fig. 2) with *C. geniculata* isolated from human wound from Belgium (Accession no. OW986190), seeds of *Andropogon sorghum* from Indonesia (Accession no.OW983239), an endophytic fungus from *Catunaregam tomentosa* from Thailand (Accession no.GU073454) and leaf of *Sansevieria trifasciata* from Malyasia (Accession no. KX247385).The present isolate formed a cluster with other *C. geniculata* strains from different continents.

DISCUSSION

The isolated and identified pathogen, *Curvularia geniculata* of the present study has not been reported so far as a pathogen of ash gourd. However *C. geniculata* has been reported to be a causal agent for leaf spot in Malaysia rice field (Kusai *et al.* 2016). *C. geniculata* has been reported for the first time to cause leaf spot of maize in India (Manzar *et al.* 2021). In Brazil, *C. geniculata* has been reported to be associated with seeds of Bahia grass, *Paspalum guenoarum* (Gasparetto *et al.* 2017). Several reports of leaf spot diseases by *C. geniculata* are available from literature, such as maize leaf spot in china (Zhang *et al.* 2019). Some of the first reports are on *Microstegium vimineum* (Huang *et al.* 2016) and on bananas in China (Qi *et al.* 2022).

The morphological features of *C. geniculata,* as reported by Kusai *et al.* (2016) are similar to those of our *C. geniculata* isolates. Pathogenicity tests on detached leaf and whole plants confirmed that *C. geniculata* is the causal agent of the leaf spot disease of the present study. Molecular characterization of the fungus through sequencing of the ITS region was done. ITS sequence showed high percentage similarities between 97 and 100 % with GenBank database (http://www.ncbi.nlm.gov). The ITS sequence of two isolates were successfully amplified and one isolate was sequenced. The ITS region with expected sizes, approximately 500–530 bp. Based on BLAST search, the identity of isolates have been shown are shown in one isolate belong to *Curvularia geniculata*. ITS sequence of one isolate was deposited

to Genbank (http://www.ncbi.nlm.gov) assigning accession number.

The phylogenetic tree was constructed and the relationship between fifty Curvularia isolates on the basis of 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence and 28S ribosomal RNA gene, partial sequence has been shown in Fig. 2. This tree has been divided into two main clades and several sub-clades. Isolate Dh/F/2 of the present study has been identified as *Curvularia geniculata* as it clusters together with other *C. geniculata* species. Other *Curvularia* sp. such as *Curvularia affinis*, *Curvularia caricae-papayae, Curvularia clavata, Curvularia eragrostidis, Curvularia inaequalis, Curvularia lunata, Curvularia protuberata* and *Curvularia trifolii* showed cluster among themselves forming cluster specific for each species. Thus, present study reports identification of Dh/F/2 isolate as *C. geniculata* and it is a severe causal pathogen of cultivated ash gourd in Sub-Himalayan West Bengal.

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