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***Alternaria alternata* causes leaf and fruit blight in makhana**

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Makhana (*Euryale ferox*) is a high-value commodity of nutritional, medicinal and ritualistic significance. North Bihar has occupied a prominent position in terms of both production and productivity of makhana not only

in India, but across the globe. Leaf blight disease on makhana was noticed in April 2018, with a severity of 15–20% in a survey of farmers' ponds in North Bihar. Symptoms of the disease were circular, small, light-brown, necrotic, sunken lesion that later turned into a large, dark, blighted area in the leaves. Blighting of fruits was also noticed during June and July 2018. Blighted fruits were small, distorted and twisted with less seed. *Alternaria alternata* was identified as the pathogen causing the disease based on morphological and cultural characteristics of the culture maintained on potato dextrose agar from symptomatic leaf and fruit samples. The fungus gave rise to greyish to grey-black colonies with obclavate to obpyriform, catenulate conidia in chains. Conidia consisted of 2–5 horizontal and 0–2 vertical septa and measured 15–60 × 5–9 μm in dimension. Molecular confirmation was done by sequence analysis of the internal transcribed spacer (ITS) regions of rDNA using ITS1 and ITS4 primers. Eventually, pathogenicity test inferred that leaf and fruit blight in makhana are due to *A. alternata* infection.

Keywords: *Alternaria alternata*, *Euryale ferox*, leaf and fruit blight, makhana, pathogenicity test.

MAKHANA (*Euryale ferox*), also called gorgon nut, is a floating aquatic plant with large leaves and prickly petioles. It is cultivated only in the wetland ecosystem of tropical and subtropical regions. The wild form of makhana is found in Japan, Korea, Bangladesh, China, North America, Nepal and Russia¹. Bihar, Assam, West Bengal and Odisha are the states in India where it is grown commercially as a high-value commodity². The cultivation of makhana is done in more than 20,000 ha, where Bihar occupies 80% acreage and contributes to more than 90% of the production³. North Bihar occupies a prominent position not only in India, but also in the world in terms of makhana production. Seeds of makhana are popped and eaten after roasting, in addition to being used in the preparation of various kinds of sweets and recipes. Makhana supports the cottage industry and is a livelihood option for thousands of fishing families, besides its high nutritional, medicinal and ritualistic significance.

Makhana plays a vital role in the Indian economy owing to its nutraceutical and cultural value. But like other crops, the production of makhana also faces threats of diseases caused by pathogens in the changing climatic scenario. Root rot, botrytis grey mould, tumour formation and many more diseases affect the production of makhana. The leaf blight disease was observed during April 2018 in North Bihar, during a survey and surveillance programme. It was widespread, involving 35–40% of leaf infection rate and 15–20% disease severity. This was based on the analysis of infected and total number of leaves of available plants (average 8) in every quadrat of 9 m² out of three random quadrates in each of the 120 ponds/fields representing the entire North Bihar. The occurrence of fruit blight was noticed during June–July 2018 at the

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Mandan Bharti Agriculture College, Saharsa (25.88°N lat. and 86.6°E long., altitude 41 m amsl). The major area of makhana cultivation in Bihar, particularly North Bihar, is bearing the brunt of these emerging diseases leading to huge crop yield loss, because of the lack of relevant studies pertaining to identification and diagnosis of leaf and fruit blight disease in makhana. Therefore, the present study was undertaken to identify the causal agent of leaf and fruit blight in makhana.

Tissues of affected leaves and fruits from ten diseased plants of experimental ponds of Mandan Bharti Agriculture College, Agwanpur and four diseased leaves and fruits specimens of farmers' ponds located in North Bihar were collected. Small fragments of symptomatic leaf and fruit tissues were surface-disinfested in 0.5% NaOCl, followed by double-rinsing in sterile distilled water and plating onto potato dextrose agar (PDA) amended with 0.05 g l⁻¹ streptomycin sulphate for isolation of the pathogen. The plate was incubated at 28° ± 1°C with a 12-h photoperiod for six days to obtain pure cultures using the single-spore purification method. Fungal tissues were mounted in water and lactophenol for microscopic examination and the dimensions of 30 conidia per culture were measured from six-day-old cultures. The morpho-cultural growth characteristics of the pathogen were studied for primary identification. The dimensions of the conidia and conidiophores were measured with the help of ocular and stage micrometer mounted on a light microscope. PDA slants were made for the maintenance of isolates.

Sequencing of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) was done for molecular identification of the fungus. ITS region of nuclear rDNA was amplified using ITS-1 and ITS-4 primer pairs^{4,5}. The isolated fungus was grown on potato dextrose broth at 28° ± 1°C. The collected mycelial mats were allowed to dry completely after six days of incubation on pre-autoclaved filter paper. Liquid nitrogen was used to grind dried mycelial mats and cetyltrimethyl ammonium bromide extraction method was used for the extraction of DNA⁶. The DNA was amplified with the help of ITS-1 (5'-TCCGTA-GGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTT-ATTGATATGC-3') primers. QIA quick PCR purification kit (Qiagen Inc., CA, USA) and the specified protocol were used for amplification of DNA by PCR. It was cycle sequenced in both directions using the Genome Lab™ Dye Terminator Cycle Sequencing Quick Start Kit on a Genome Lab™ GeXP Genetic Analysis System (Beckman Coulter Inc, CA, USA) directly. Accession number was acquired after submission of the obtained sequence to the National Center for Biotechnology Information (NCBI) GenBank. The phylogenetic tree was constructed using ITS-5.8S rDNA sequences of five identified, isolated strains of *A. alternata* and 12 other available strains of *A. alternata* on NCBI. Among the isolated strains, three were from the leaves (ALT 1_519 bp, ALT 2_558 bp and ALT 3_558 bp), and two were from the fruits (Alt₄_565 bp and

Alt₅_558 bp). Multiple sequence alignment was run using MUSCLE in MEGA X (ref. 7). The aligned sequence file was saved in mega format to construct the phylogenetic tree by neighbor-joining method with bootstrap test (1000 replicates). Basic Local Alignment Search Tool (BLAST) confirmed the identity of the sequences.

The inoculation of leaf and fruit was done by three leaf blight and two fruit blight isolates respectively, for pathogenicity tests. Conidial suspension (20 ml) containing 10⁶ conidia ml⁻¹ was inoculated on ten healthy leaves of 35-days-old makhana plants by pin-prick method⁸. The conidial suspension was injected using a modified hypodermic needle at the prick point and the leaves were covered by moist sterilized cotton swab along with sterilized aluminium foil. Fruit inoculation tests were performed after spraying four healthy fruits with 20 ml of a 10⁶ conidia ml⁻¹ suspension under natural daylight conditions. The fruits were then covered with thin plastic aerated bags. After five days of inoculation, foil and plastic bag were removed from the leaves and fruits respectively, and they were exposed to natural humidity regime. Similarly, ten leaves and four fruits of makhana plants were inoculated and sterilized distilled water was used as a control. The tested leaves and fruits were monitored daily for the appearance of symptoms. Fungi were reisolated on PDA from the developed lesions on inoculated leaves and fruits. A comparative study of their morphological and cultural characteristic was done with the original isolates.

The leaf blight disease affected an adequate number of makhana plants at the experimental pond in Mandan Bharti Agriculture College, during April 2018, resulting in large losses. There were symptoms like potassium deficiency in the beginning, which initiated from the leaf margin as a circular, small, light-brown necrotic, sunken lesion with a circular ring or target board pattern and a yellow halo that led to complete necrosis and drying of the affected leaves (Figure 1 a). The leaf blight was more detrimental in the early stage of the plant, where it greatly hindered growth and development. At first, the old leaves were affected, but with the passage of time most of the leaves became blighted, excluding a few upper leaves. The development of fruit blight was noticed only in case of infection in the pedicel. The infected fruits became small, distorted and twisted (Figure 1 b). The fruits had less seed, were wrinkled, small in size, grey to black in colour and had low viability.

Fungal colonies isolated on PDA from the diseased makhana sample were slow-growing, effused, whitish with pale yellow pigmentation that turned into grey to greyish-black and gave rise to obclavate to obpyriform, dark brown, catenulate conidia in chains, borne on short conidiophores (Figure 2 a). Totally 14 isolates (10 from leaves and four from fruits) were taken from the diseased sample. A week later, it grew into 62.3–81.7 mm diameter. The conidiophores developed were straight or flexuous and pale brown with one or various apical conidiogenous loci. Conidia

($n = 30$) were 15–60 μm long (avg. 32.6; $\text{SD} \pm 4.3 \mu\text{m}$), 5–9 μm wide in the broadest part (avg. 7.2; $\text{SD} \pm 2.3 \mu\text{m}$) with a beak 2.3–6.0 μm long (avg. 3.2; $\text{SD} \pm 2.4 \mu\text{m}$), and 2–5 horizontal and 0–2 vertical or oblique septa (Figure 2 b). These morpho-cultural features and dimensions were similar to that of *A. alternata*^{9,10}. A report of the Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute (IARI), New Delhi, India (identification ID 10.89.18) also confirmed and supported the findings of the present study. The morpho-cultural characteristics of leaf blight isolates designated as ALT 1, ALT 2 and ALT 3, and fruit blight isolates designated as Alt₄ and Alt₅, were recognized according to the variation in cultural characteristics on PDA. These were found to be almost similar based on their morpho-cultural characteristics.

The generated sequences of three strains of the pathogen from leaves (ALT 1_519 bp, ALT 2_558 bp and ALT 3_558 bp) were submitted to GenBank (accession nos MN790781, MN790782 and MN790783 respectively). The sequences of two strains of pathogens from fruits (Alt₄_565 bp and Alt₅_558 bp) were also submitted to GenBank (accession nos MT446226 and MT446227 respectively). Mega BLAST analysis of sequences showed more than 99% homology with *A. alternata* strains MF380866, MH-374280, MH374276, KY609180, MH084279, KY307851 and JX406531. Thus, molecular identification confirmed *A. alternata* as a disease-causing pathogen. *A. alternata* isolate DPS-2 (KU982599.1) was used as the standard strain and *Bipolaris tetramera* (MN547505.1) as an out-group species, while constructing the phylogenetic tree



Figure 1. Symptoms of (a) leaf blight and (b) fruit blight of natural infection in makhana.

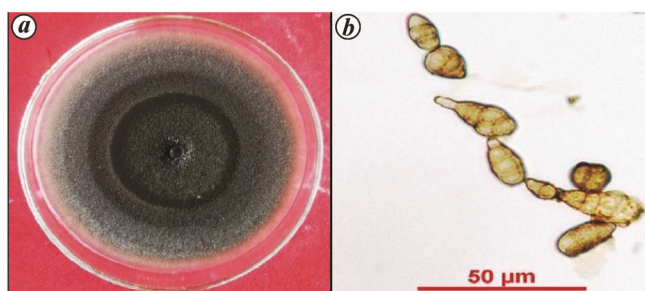


Figure 2. a, Pure culture of *Alternaria alternata*; b, Conidial chain of *A. alternata* (40 \times).

using MEGA X (Figure 3). In Figure 3, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) has been exhibited next to the branches. BLAST for similarities using *Alternaria* sp. showed that the percentage of similarity of all identified strains of *A. alternata* ranged between 97% and 100%. Therefore, the species name allotted was based on the closest BLAST search. In BLAST analysis, ALT 1 isolate of *A. alternata* exhibited 99% similarity with *Alternaria* sp. strain A8 (MK037451.1) and 99.9% similarity with *A. alternata* isolate AaAJB1 (MN960325.1). ALT 2 isolate of *A. alternata* exhibited 99.0% similarity with *A. alternata* isolate DPS-2 (KU982599.1) and *A. alternata* strain ITCC-4312 (MH084279.1). ALT 3 isolate of *A. alternata* exhibited 99.0% similarity with *A. alternata* strain TY198-02 (MT093259.1). Alt₄ isolate of *A. alternata* exhibited 99.0% similarity with *A. alternata* isolate MH1857-1-A and *A. alternata* isolate DT1892-A. Alt 5 isolate of *A. alternata* exhibited 99.0% similarity with *A. alternata* strain ITCC-8201 and *A. carthami* isolate OACr2. The phylogenetic tree revealed that out of five identified isolates of *A. alternata*; four (ALT 1, ALT 2, ALT 3 and Alt₄) fell in one clade and Alt₅ in another.

Leaf blight symptoms came after 12 days of inoculation and became distinct after 25 days (Figure 4), whereas

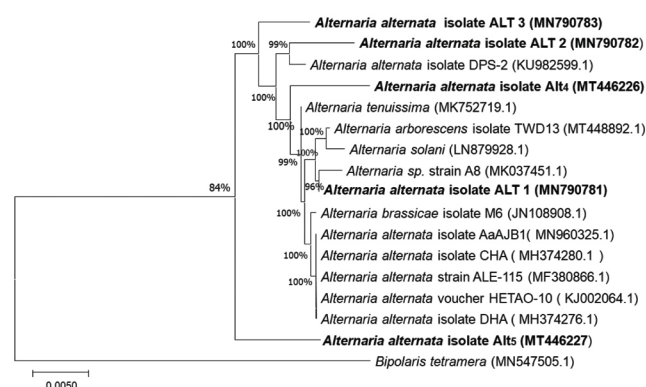


Figure 3. Phylogenetic tree constructed using ITS-5.8S rDNA sequences of three strains of *A. alternata* isolated from leaves (ALT 1, ALT 2, ALT 3) and two strains from fruits (Alt₄, Alt₅) of makhana. *A. alternata* isolate DPS-2 (KU982599.1) was used as the standard strain, whereas *Bipolaris tetramera* (MN547505.1) was used as an out-group species.

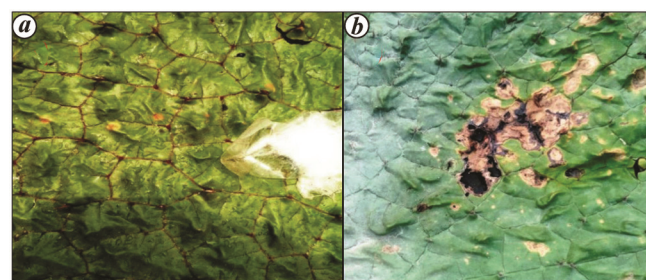


Figure 4. Symptoms of leaf blight after (a) 12 days and (b) 25 days of artificial inoculation in test plant of makhana.

fruit blight was apparent after 14 days. However, the control leaves remained asymptomatic. Culture reisolated from inoculated symptomatic leaves and fruits had similar characters as the initial isolates, but not healthy as control plant tissue. This pathogenicity test confirms that *A. alternata* is the causal agent of leaf and fruit blight disease in makhana. Three leaf blight strains showed fruit infection with various degree of severity. Conversely, fruit blight isolates also showed leaf infection in a cross-infectivity study.

A. alternata species, one of the most common representatives of the genus *Alternaria*, is an opportunistic plant pathogen. As of now, 329 plants have been reported as a host of this pathogen which is responsible for leaf spots, rots and blights on many plant parts^{11,12}. This pathogen is more devastating in makhana. Therefore, an effort was made to diagnose the causative pathogen after a sequential and systematic study. The study of morpho-cultural features is the primary means for the identification of *Alternaria* species¹³. Moreover, most of the *Alternaria* species exhibit considerable morphological plasticity according to cultural conditions. There is also substantial variation in conidial morphology according to size, shape and septation that depend on conidial age. Therefore, molecular methods were included in the study to complement the morphological approaches¹⁴. The isolates were identified as *A. alternata* on the basis of DNA sequence and other relevant data.

Leaf and fruit blight are among the major diseases of makhana, but the causative agent of these diseases was unknown. There was also no effective management strategy to control these diseases. Bihar is the hotspot of makhana production and makhana has become a crop of national importance due to rising demand in the domestic as well as foreign market. In this study we made an effort to identify, characterize and confirm the causal organism of leaf and fruit blight diseases in makhana. The symptoms of leaf blight begin with a circular, small, light-brown, necrotic sunken lesion in the leaves that gradually became a large, dark, blighted area. Blighted fruits were small, distorted and twisted with less seeds. The morphological, cultural and microscopic characteristics revealed that the isolated pathogen was *A. alternata*. This was also evidenced by sequence analysis of the ITS regions of rDNA using ITS1 and ITS4 primers and pathogenicity test. This finding is highly encouraging to devise efficient and specific management strategies against *A. alternata* and to cope with leaf and fruit blight in makhana.

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