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S. DUTTA, A. BASU, P. K. MAJI AND T. K. BAG

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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New leaf spot disease caused by *Alternaria burnsii* on Mokara orchid in India

S. DUTTA[1], A. BASU[1*],P. K. MAJI[1] AND T. K. BAG[2]
[1]Department of Plant Pathology,Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia -741 252, West Bengal
[2]Division of Plant Pathology, Indian Agricultural Research Institute, Pusa, New Delhi-110012

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Mokara is a high value tropical orchid, gaining huge popularity due to its great aesthetic value. In January 2018, small necrotic spots having variable size from circular to oval with depressed brownish centers surrounded by a thin yellowish halo was observed on *Mokara* sp. of orchid in a nursery at Hooghly District, West Bengal, India (22.90°N and 87.76°E). Two nurseries were selected for the fixed plot survey. The Pallishree nursery of Hooghly district during the survey in August and January month, *Mokara* sp. was found moderately infected with Alternaria leaf spot having disease incidence and disease severity of 12.5% & 17.33% respectively. The fungus was isolated from the diseased leaves and tentatively identified as *Alternaria burnsii* based on morphological characteristics such as conidia, conidiophore and sporulation pattern. The partial nucleotide sequences of M3H isolate infecting Mokara Orchids were deposited in the GenBank and assigned with accession number of MT158683 (575bp). The 18S gene in SSU region sequence was used to carry out BLAST alignment search tool of NCBI GenBank database. Blast analysis of the sequences of ITS region depicted 99.81% nucleotide sequence identity (NSI) with *Alternaria burnsii* strain CBS 107.38 (Accession number: NR136119) from India.This is the first report of *Alternaria burnsii* as the causal organism of leaf spot disease in *Mokara* orchid in India.

Keywords: *Alternaria burnsii,* Leaf Spot, Mokara, new report,Orchid

INTRODUCTION

Orchids are one of the most beautiful, delicate and exotic ornamental flowers. They are members of the Orchidaceae family, which is the world's largest and most sophisticated flowering plant family with about 25,000-35,000 species classified into 600-800 genera and accounting for 6.8% of all flowering plants in India (De and Medhi, 2015).High humidity and low temperatures along with plentiful rainfall make the entire North-eastern India (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and Tripura) an orchid hotspot(Panda and Mandal, 2013).Orchids are regarded for their great variety in size, shape, form, and colour, as well as their attractiveness and long shelf life up to ten weeks (De and Medhi, 2015). They are widely planted for commercial cut flower and potted plant production.

*Correspondence:mtvbasu@rediffmail.com

Orchids are prized for their exquisite blossoms and extended vase life, and they command a high market price both nationally and internationally (Pant *et al.* 2013). Orchids are found in different climatic conditions and are capable of growing in soil, tree trunks, rock surfaces and dead organic matter. On the basis of growth behavior Mokara orchids are classified as monopodial (stems having a vertical growth, non-branching, with aerial roots). The tropical orchids grown commercially are *Vanda* spp., *Cattleya* spp., *Dendrobium* spp., *Mokara* spp.,*Oncidium* spp. and *Phalaenopsis* spp.(Hew and Young, 2004).

The most common diseases of orchid plants are caused by fungal species. These might be foliar blights, leaf spots, fungal rots, and flower blights. The detection of plant disease is found critical to combat orchid diseases (Srivastava *et al.*2018). In the past, no attention was paid to studying orchid diseases in India, but as orchids have become more popular in the world floral market

in recent years, more attention is being paid to assess the orchid diseases and adoption of sanitary and phytosanitary measures in accordance with WTO requirements for import and export(Pant *et al.* 2013). Among fungal diseases, black rot (*Phytophthora palmivora*, *P. parasitica*, *Pythium ultimum* and *P. splendens*), anthracnose (*Colletotrichum gloeosporioides*), orchid wilt (*Sclerotium rolfsii*), petal blight (*Botrytis cinerea*), rust (*Uredo* sp.), leaf blight (*Fusarium oxysporium*), Sclerotinia white rot (*Sclerotinia sclerotium*) and leaf spots caused by species of *Fusarium*, *Cercospora*, *Alternaria*, *Pestalotia* and *Haplosporella* are the most common.

Based on the above background concept, a research work considering characterization of economically important fungal plant pathogens infecting tropical orchids under West Bengal conditions of India was proposed.

MATERIALS AND METHODS

Monitoring of disease and collection of infected samples

The survey was conducted in the fixed nursery of four districts of West Bengal namely Nadia, Hooghly, Jalpaiguri and Darjeeling for the assessment of the prevalence and the level of incidence of various orchid diseases. The period of survey was from August, 2017 to February, 2018 and from August, 2018 to February, 2019. Disease severity was recorded in the scale of 0-5 proposed by Handayati and Hanudin (2004).The infected portions showing symptoms of diseases were collected separately and brought to the laboratory and were observed under microscope by preparing the slides from the infected areas.

Isolation of Alternaria burnsii

Alternaria burnsii was isolated from the leaves of tropical orchids showing leaf-spot symptoms. Isolation was done within 1-2 days of the collection of the samples. The samples were thoroughly washed in running tap water for 10 min. There after segmented tissues were searched, cut and surface sterilized by using the solution of sodium hypochloriteide (NaOCl, 0.1%) for 5- 10 seconds. Cut tissue portions were rinsed 5-6 times in distilled water and immediately placed on PDA and incubated at 27±1^{0}C for 4-5 days. Mycelial growth of the fungi was isolated by tissue segment method (Woo *et al*. 2010) and hyphal tips were transferred into the fresh PDA slants. Fresh culture was maintained throughout the course of the investigation by sub-culturing the fungi at 30 days interval and storing at 5^{0}C in refrigerator.

Pathogenicity tests of Alternaria burnsii

Pathogenicity test of the fungus was carried out by detached leaf method described by Vannak *et al.* (2015), Joko *et al.* (2011) respectively. Healthy leaves were collected, leaf surface was rinsed with sterilized distilled water, and air dried in the laminar airflow and placed in sterilized Petri plates. Unpricked leaves and needle pricked leaves were placed separately in the different Petri plates. Discs of 0.6 cm diameter were obtained from 15 days old mycelial growth of the fungus and also from uninoculated PDA plates incubated at 27± 1°C. The needle pricked leaves in the Petri plates were artificially inoculated with discs of fungal mat and only agar bit was inoculated in the Unpricked leaves respectively, which were used as control. These were placed in the sterilized tray which were then covered with transparent polythene packets and observed regularly for the development of symptoms.

Characterization of Alternaria burnsii Morphological and Microscopic identification

For morphological study temporary mounts were prepared and were placed in the compound microscope for studying hyphae (width), pycnidial (conidiomata) dimensions, conidial length and breadth. Photograph of the spore were taken with a binocular microscope fitted with Moticam 3.0 MP camera and measurements were done by using the software AxioVision (Rel. 4.8).

Molecular Detection by 18s rRNA Method

The identification of fungal culture was examined by isolation followed by extraction of genomic DNA from the 7 days old fungal culture. PCR amplification of 18S rRNA gene and sequencing

was done as described by Dinsdale *et al.* (2010). Genomic DNA was extracted from each specimen as per manufacturer's instruction (Invitrogen, USA). PCR amplification using 20 ng of fungal DNA was carried out in Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). The amplified PCR products were excised from the gel and purified using the XcelGen DNA Gel/ PCR Purification mini kit (Xcelris genomics, India) following manufacturer's instruction. DNA sequence was obtained through Sanger dideoxy sequencing method from Chromus Biotech Laboratory, India. Multiple alignments of all the nucleotide sequences of targeted specimens were conducted using Clustal W, and analysis was done in Mega-X (Kumar *et al.* 2018). The tree was verified using the maximum likelihood (ML) model and a total 1000 of bootstrap replicates were performed. The tree was processed through iTol(version 6.4.3) for graphical representation. All the nucleotide sequences of fungus isolates were submitted to the NCBI GenBank database.

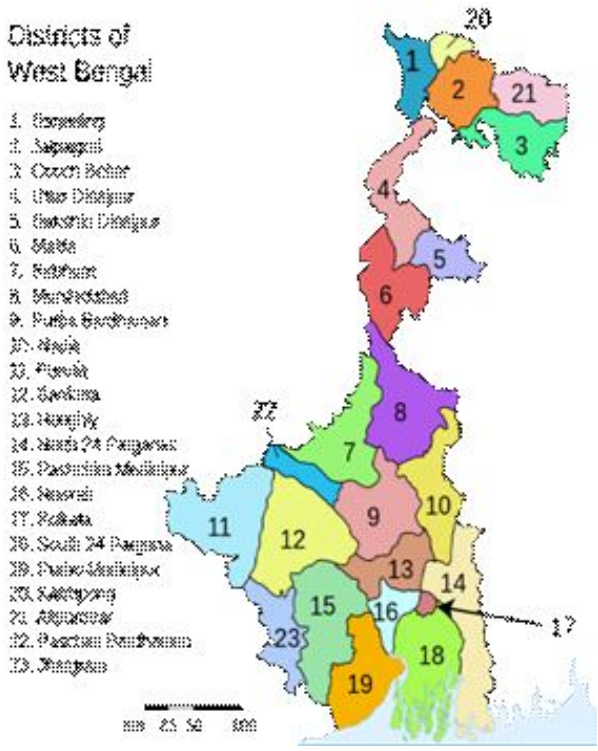

Fig. 1: Locations (1, 2, 10 and 13) in the West Bengal state of India, from where the samples were collected

RESULTS AND DISCUSSION

Leaf spot disease symptom and severity

Fixed plot survey was conducted in the nurseries of four districts of West Bengal namely Nadia, Hooghly, Jalpaiguri and Darjeeling for assessment of the prevalence and the level of incidence of various orchid diseases from August, 2017 to February, 2018 and August, 2018 to February, 2019 (Fig.1). In Hooghly district (22.90°N and 87.76°E) two nurseries were selected for a fixed plot survey. The Pallishree nursery of Hooghly district during the survey in August and January month, *Mokara* sp. was found moderately infected by Alternaria leaf spot having disease incidence & disease severity of 12.5% & 17.33% respectively.

Macroscopic observation

The symptoms manifested were small necrotic spots having variable size from circular to oval with depressed brownish centers surrounded by a thin yellowish halo.Pathogenicity tests performed in the laboratory confirmed that the isolated fungus *Alternaria burnsii* was capable of forming characteristic leaf spot lesions in the

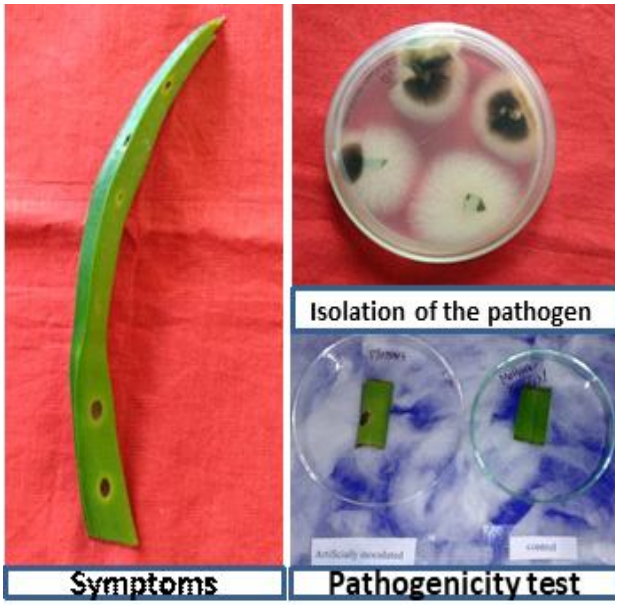

Fig.2: Symptoms and establishment of pathogenicity test of *Alternaria burnsii* isolated from *Mokara* sp**.**

detached leaves of *Mokara sp.*(Fig.2).To establish Koch's postulates, *Alternaria burnsii* isolates were then subsequently reisolated from the periphery of the typical symptoms observed on the detached leaves. However, no symptoms were noticed on leaves kept as control. The symptoms were produced on leaves after 7 days of inoculation.

Characterization of Alternaria burnsii

Morphological and Microscopic characterization for diagnosis of A. burnsii

Ten days old PDA Petri-plates were incubated at 27± 1°C and sporulation was observed (Fig.3). The result reveals that, the pathogen *Alternaria burnsii* produces septate, filamentous hyphae (width: 2.96 ± 0.34 µm), erect, flexuous conidiophores bearing dark brown to olivaceous, smooth walled or somewhat warty, muriform, conidia having longitudinal and transverse septation with short beak. The length of conidia varies from 22.23 µm to 49.53 µm (average 37.47

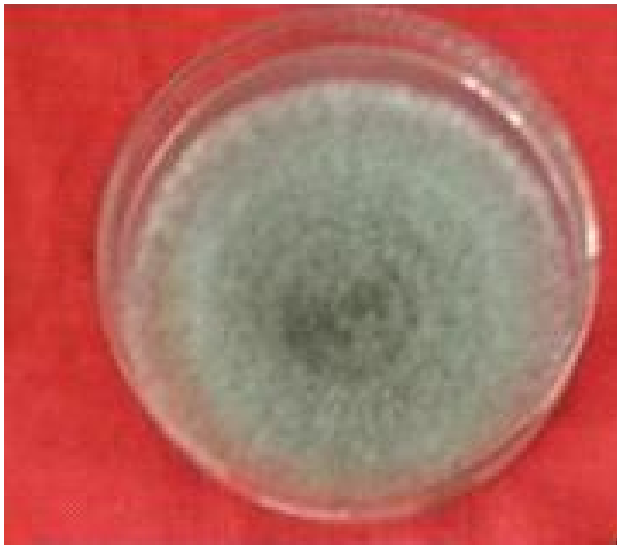

Fig. 3: Cultural Growth of *Alternaria burnsii* on PDA media

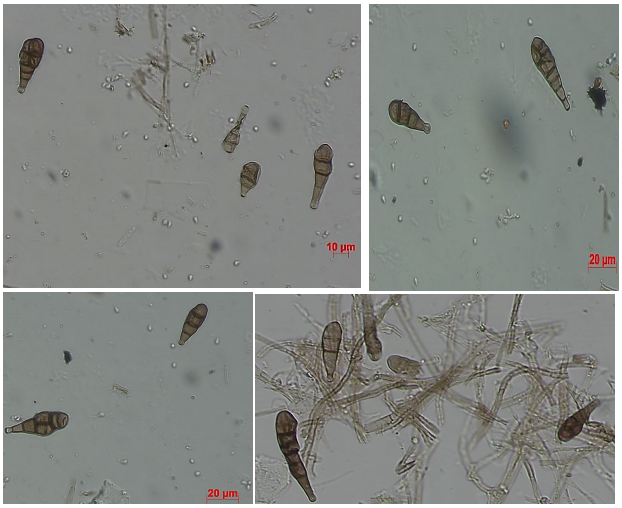

Fig.4: Conidia of *Alternaria burnsii*(10ìm x 20 ìm), produced on Potato Dextrose Agar

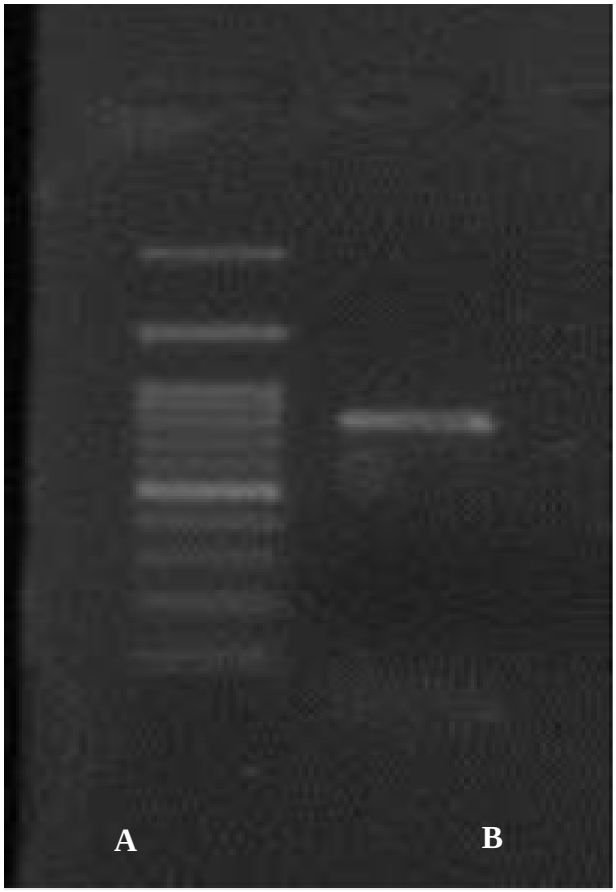

Fig. 5: PCR amplicon of the isolated pathogen *Alternaria burnsii* using universal primers ITS1/ITS4 resolved on Agarose gel showing 790bp amplicon of ITS region of rDNA.
[Lane A: 100 bp DNA Ladder; Lane B: 790bp amplicon (ITS region)]

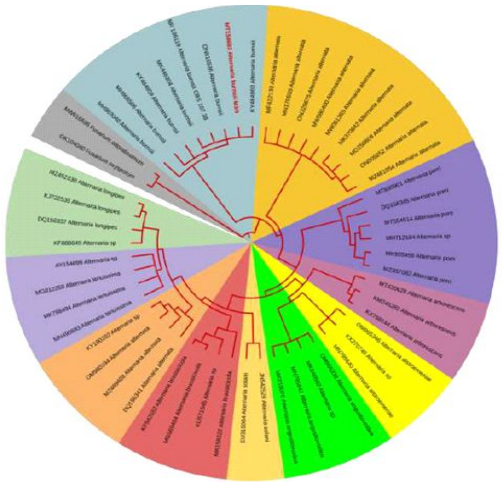

Fig.6: Phylogenetic tree analysis of *Alternaria burnsii*
The maximum likelihood phylogenetic tree obtained from ITS sequence data of the 18S rRNA sequences of *Alternaria species*. The red colour text sequences represent collected sample used in this study, and the rest represent reference sequences obtained from the GenBank database. A total of 53 nucleotide sequences were selected to construct the tree, wherein *Fusarium odoratissimum* MW016595 and *Fusarium oxysporum*(OK104040) were taken as an out-group

±7.14µm) and the breadth varies from 8.18µm to 14.98µm (average 11.60± 1.80µm) where N=100, inter septa length ranges from 3.31-9.63µm (average 5.52±1.51µm) (Fig.4).

Paul *et al.* (2015)also noticed that, conidia of *Alternaria burnsii* were in short or moderately long chains of 2~8 conidia normally, sometimes more; occasionally (uncommonly) branched; normally 16~60 (~90) × 6.5~14 (~16)ìm in size; ellipsoid, long ellipsoid, obclavate or ovoid with 2~11 transverse septa and 2~3 (~4) longitudinal septa; beakless or with a sub-cylindric or cylindric secondary conidiophores (pseudorostrate) with 0~5 septa.

The conidiophores produced by *A. burnsii* were branched, erect, straight, irregularly bend, and geniculate whereas, conidia produced by ten isolates varied widely regarding their size where length vary from 44.92µm to 63.28µm and the breadth vary from 10.84 µm to 24.36 µm. The number of longitudinal or transverse septa, beak length, and sporulation frequency also varied widely (Singh *et al.* 2016).

Molecular identification and phylogenetic tree analysis of the A. burnsii M3H isolate

The molecular identity of the isolated foliar pathogens was confirmed by PCR amplification and sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA by using the universal primer pair ITS1/ITS4 (Fig.5).The PCR product was sent for nucleotide sequencing. The sequence was submitted to GenBank (Accession No.*Alternaria burnsii*- MT158683. BLAST analysis of the fungal sequences showed 98% to 100% nucleotide similarity to the NCBI database.The partial nucleotide sequences of M3H isolate infecting Mokara Orchids were deposited in the GenBank and assigned with accession number of MT158683 (575bp). The 18S gene in SSU region sequence was used to carry out BLAST alignment search tool of NCBI GenBank database. Blast analysis of the sequences of ITS region depicted 99.81% nucleotide sequence identity (NSI) with *Alternaria burnsii*strain CBS 107.38 (Accession number: NR136119) from India. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W which affirmed the same result as mentioned above.

Further, phylogenetic tree was drawn to analyze the phylogenetic relationship of the sequenced genome of the studied fungus with 53 selected 18S rDNA sequences retrieved from GenBank.

Phylogenetic tree was constructed using those 18S gene in Small Subunits (SSU) region sequences which clearly indicated that our isolate M3H (MT158683) was most closely related with NR_136119.1 and clustered together as a single group with other deposited isolates of *Alternaria burnsii* (KY484860 from Oman, ON018536 from Brazil and MK446304 from India)(Fig.6).

CONCLUSION

The most common diseases of orchid plants are caused by fungal species. These might be foliar blights, leaf spots, fungal rots, and flower blights. The disease studied in this investigation was leaf spot caused by *Alternaria burnsii* from Mokara orchids. In West Bengal, most of the orchid growers are non-conventional agriculturists and are mostly unaware of disease problems and their effective management. As no systematic research work has been carried out with special reference to pathogen biology, characterization and effective management strategies for orchid diseases in West Bengal, the orchid growers have to depend on reports from other countries for the information on diseases and their management. Colony morphology of this fungal isolate from the Mokara genus of orchids was studied. The colony of *Alternaria burnsii* on PDA medium was fluffy grey color to whitish and conidia were muriform shaped having longitudinal and transverse septation with a short beak. The molecular identity of the isolated foliar pathogens was further confirmed by PCR amplification and sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA by using the universal primer pair ITS1/ITS4

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