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First report of Twig blight of Lalshak (*Amaranthus gangeticus* L.) caused by *Choanephora infundibulifera* (Curr.) Sacc. in India

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Amaranthus gangeticus is one of the most economically important vegetable and widely cultivated in all the states of India. Due to its enriched nutritional qualities, this vegetable has the potential to play major role in combating malnutrition problem especially for the poor people of Third World countries. In August – September 2015, Twig blight associated with soft rot, was observed for the first time in "C" block farm of Bidhan Chandra Krishi Viswavidyalaya and Kalyani region (22°45'36.00º N, 88°22'12.00º E) of West Bengal, India. Twig blight symptom was characterized by blighting of leaves, blossom and shoot apical meristem (SAM). A symptom appeared with browning of the chlorophyllous tissue which gradually covered with cushiony mat of caterpillar's hair like emerging sporangiola. About 30-35% disease incidence level recorded among the entire surveyed region. Cultural and morphological characteristic clearly indicate the pathogen was *Choanephora infundibulifera*. Molecular detection of isolate A.G – 20 accurately established the causal pathogen was *Choanephora infundibulifera*. ITS – r DNA region of submitted 559 bp sequence shows 100% (JQ724498) homology with publicly available *Choanephora* database. Rainy season (June – October) with the optimum temperature range between 28-30°C and high moisture (80-90 %) level enhances the disease progress. To the based of our knowledge this is the first report of Twig blight of *Amaranthus gangeticus* caused by *Choanephora infundibulifera* in India.

Key words: *Amaranthus gangeticus*, Twig blight, *Choanephora infundibulifera*, ITS, India.

INTRODUCTION

Amaranthus gangeticus L. (Amaranthaceae) the most popular cultivated leafy vegetable used as a major resource of nutrients to the poor people's as well as all the strata of the society in India. All parts of the plant are edible. *Amaranthus gangeticus* has been valued as a promising food for its high nutritional value of both seeds and leaves. Whole plant parts are used as folk medicine to tribal communities. According to Yang and Keding (2009), each 100 g fresh weight edible portion content 3.9 g protein, 1.8 mg vitamin A, 62 mg vitamin C, 358 mg calcium, 2.4 mg ferrous, 0.8 mg zinc. Because of its low production cost, *Amaranthus* is one of the cheapest redish green leafy vegetables in tropical markets (Figure 1 A) and is often described as poor man's vegetable (Varalakshmi, 2004). *Amaranthus gangeticus* exhibit C4 photosynthesis and grow rapidly under heat and drought stress, and they tolerate a variety of unfavourable abiotic conditions, including high salinity, acidity, or alkalinity, making them uniquely suited for subsistance agriculture. One of the major diseases is damping off caused by *Pythium aphanidermatum* and *Rhizoctonia* and very much serious threats in seed beds. During a field survey in August –September, 2015, a new disease Twig blight of *Amaranthus gangeticus* caused by *Choanephora infundibulifera*, was first time observed and recorded at 'C' block farm Bidhan Chandra Krishi Viswavidyalaya, and Kalyani region of West Bengal (22°45'36.00º N, 88°22'12.00º E), India. Monsoon season with 28-30°C temperature and 80-90% relative humidity prefers in disease progress.

MATERIALS AND METHODS

Isolation and morphometric characterization of the causal pathogen

Pathogen was isolated on Potato dextrose agar (PDA) (Saroj *et al.* 2012), under the optimum

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temperature range 28 ± 2ºC (Fig.1 F). Pathogen isolation was done by three ways – i] infected part ii] picking single sporangiola iii] single spore dilution method. Mycelia were hyaline and non septate. Cremish white coloured culture shows yellow pigmentation at maturity due to β-carotene synthesis. Morphometric characterization was performed by micrometric measurement of different parts. A total of 30 isolates of the fungus, were collected from diseased parts of *Amaranthus gangeticus* among which isolate A.G - 20 was most virulent, however, no mycological difference were found among the isolates.

Molecular identification of the causal pathogen

Morphological characterization followed the molecular characterization i.e. the causal pathogen was further processed for molecular detection at its genetic level. Molecular detection of isolate A.G - 20 was confirmed by CTAB DNA extraction method (Doyle and Doyle, 1987). Molecular identification of fungus was performed using sequencing of ITS regions, due to the large ITS data collection for fungi, the extremely variable nature within highly conserved ribosomal coding sequences and the high popularity of studies dealing with fungal identification and phylogenetic relationships (Boysen *et al.* 1996). The ITS region turned out to be an appropriate barcoding marker in Mucorales (Walter *et al.* 2013). Internal transcribed spacers (ITS) of the ribosomal DNA of A.G - 20 isolate were amplified by using the primer pair ITS-1 and ITS-4 (White *et al.* 1990). Amplification was performed with BIO-RAD My Cycler™ thermal cycler (Bio Rad, USA), where the final reaction mixture was prepared with 2.5 µL of 10x Taq buffer containing 15 mM $MgCl_2$, 1.0 µL of ITS1 primer (5 picomolar/µL), 1.0 µL of ITS-4 primer (5 picomolar/µL), 1 µL of 25 mM dNTP mix each, 1.5 U of Taq polymerase (3 U/µL), 16 µL sterile distilled water and 3 µL (20-30 ng/µl) of sample DNA , under the following cycling times: 3 min at 95°C, 35 cycles 30s at 95°C, 30s at 50°C, extension for 1 min at 72°C, with a final extension of 10 min at 72°C. Amplified PCR products were observed in 1.5 per cent agarose gel in 1X TAE buffer and visualized under Gel document unit (BioRad, USA) with ethidium bromide staining technique. The amplified 559 bp product was sequenced by SciGenom pvt limited and submitted in NCBI, Genbank database with BLAST searching tool.

Phylogenetic analysis

Phylogenetic tree was constructed based on internal transcribed spacer sequences using neighbour – joining method (among the closely related taxa of different countries derived from NCBI-Gen Bank *Choanephora infundibulifera* data base), by MEGA version 6 (Tamura *et al.* 2013).

Pathogenicity test

The pathogenicity was established by using two different techniques – IVCI (*In vitro* Cross Inoculation) and spraying of the spore suspension. In the first technique, a 40×25 cm2 plastic tray was used as conserve system (to conserve micro climate moisture level), in which 7-8 cm long seedlings (15 days old, for four sets) were kept on three inversely placed Petri plates (9 cm). A 3% sucrose solution was poured onto the lower plate of each set for minimum nutrient uptake of the seedlings during *in vitro* study. Basement of the whole system (plastic tray) was sandwiched with bilayer moist blotting paper-absorbent cotton layers. Of the four sets, one was used as a control and the other three were inoculated with three different isolates. A pre-grown single mycelial disc of three host specific (A.G – 13, A.G -16, A.G – 20) ware attached 1-2 cm below at a pre-wounded (notched with a finely pointed sterile needle) shoot apical meristem of the seedlings. The inoculation site along with the mycelial disc was coiled with a thin film of moist absorbent cotton. The whole system or tray was covered with transparent perforated polythene sheets (for aeration) and incubated at 28±2ºC and 80-90% relative humidity in a growth chamber. In the second technique, four 15-days old potted seedlings were taken for spraying with spore suspension (1×10^6 spores/ml in sterile distilled water) method. Among the four seedlings (for three replications) one was used as a control and the other three were treated with spore suspension of three different isolates Br-1 (aubergine), A.G-20 (lalshak), M.D-9 (teasle gourd). Treated and control plants were consecutively sprayed with spore suspension and sterile distilled water and finally covered with polythene bags and retained in the growth chamber as mentioned in the first technique.

RESULTS AND DISCUSSION

Twig blight disease syndrome on lalshak (Amaranthus gangeticus)

In August-September 2015, Twig blight of *Amaranthus gangeticus* caused by *Choanephora*

infundibulifera was observed for the first time on 'C' block farm of Bidhan Chandra Krishi Viswavidyalaya and Kalyani (22.9751° N, 88.4345° E) region of West Bengal, India with 30-35% disease incidence level. Later the same disease infestation was recorded in several other districts of West Bengal, India. Symptoms primarily appeared on any susceptible part (leaf, blossom

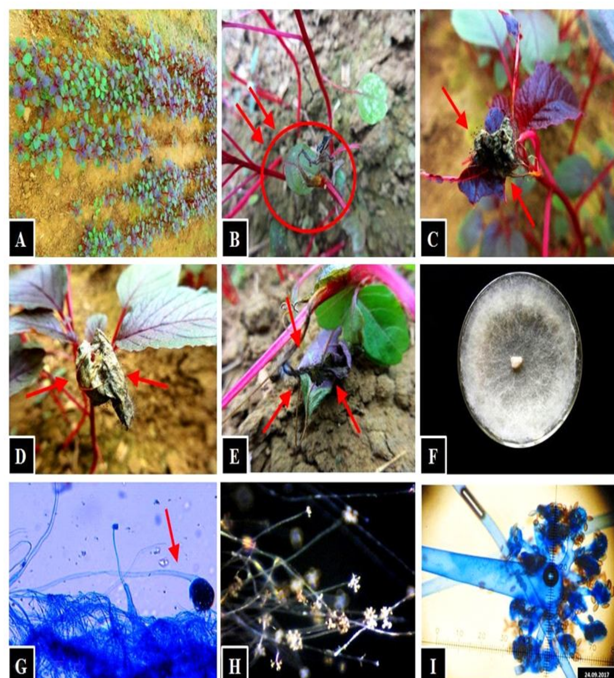

Fig.1:**(A)** *Amaranthus gangeticus* cultivation in experimental plot of Bidhan Chandra Krishi Viswavidyalaya,Kalyani, West Bengal. **(B-C)** Seedling mortality with Twig blight symptom. **(D-E)** Leaf blight. **(F)** Culture of *Choanephora infundibulifera* on PDA. **(G)** Sporangiola formation at cultural condition. **(H)** Cushion of sporangiola under stereoscope. **(I)** A mature sporangiola

or stem) of the shoot system with water soaked lesions. Infected chlorophyllus tissue turned brownish, and leaf, blossom, shoot apical meristem (SAM) blight altogether constituted typical Twig blight symptoms. Black pin head like emerging sporangiola formed a cushiony appearance from the infected part. The infected twig part becomes wilted, blighted or dried up and sometimes blighted part becomes soft rot (Fig.1 B-E).

Cultural and morphometric characterization of the pathogen

Morphological studies reveled that sporangiophores bearing sporangiola were hyaline, unbranched, apically dialated to form a clavate vesicle from which arose dichotomously branched distally placed secondary vesicles. Sporangia were sub-globose in shape and 40.9 – 140.5 µm in size. There are two types of sporangiola i.e monosporus and multisporus sporangiola. Monosporous sporangiola were long, slender, and branched at the apex, with each branch bearing a sporangiophore (Fig.1 G). Multisporous sporangiola were elliptic, fusiform, or ovoid in shape, pediculate, striate, and 7.5 – 28.5 × 6.5 – 15.6 µm in size (Fig.1 H-I). Sporangiospores were elliptic fusiform, or ovoid and ranged between 6.5-12 × 10-25.5 µm in size. Zygospore formation was not observed in this study. Morphological characterization based on shape and striation of sporangiola, were identical with those of *Choanephora infundibulifera* (Curr.) Sacc. The representative isolate A.G - 20 along with its herbarium sample were deposited in NFCCI (National Fungal Culture Collection) and AMH (Ajrekar mycological Herbarium) at ARI (Agharkar Research Institute), Pune, and assigned with the accession number – NFCCI NO – 4018 and AMH – 9822.

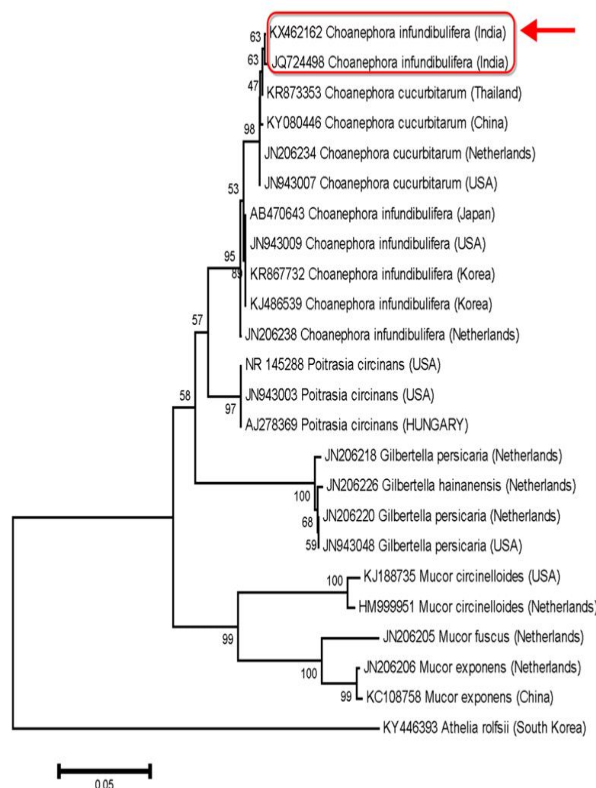

Fig. 2: Molecular phylogenetic analysis (ITS-r DNA) of *Choanephora infundibulifera* with closely related taxa obtained from Genbank by neighbor-joining tree followed maximum composite likelihood method. The nodal significance was evaluated by means of bootstrapping performed using 1000 replicates. Sequence obtained in the present study is marked by (⟵) symbol.

Molecular detection and Phylogenetic analysis of the pathogen

The resulting 559 bp DNA was deposited in GenBank and assigned with the accession number KX462162. A GenBank blast search of the publicly available fungal database showed that the 559 bp sequence of ITS – rDNA region exactly matched with the *C. infundiculifera* (JQ724498) Indian isolate with 100 % similarity. Phylogenetic analysis revealed that isolate KX462162 and isolate JQ724498 were conspecific on the same branch node and very nearer to KR873353 (Thailand), KY080446(China), JN206234(Netherlands), JN943007(USA). Probable evolutionary trends signified that reported isolate may evolved from the source organism *C.cucurbitarum*. Isolate KY446393 act as an out group of this phylogenetic analysis (Fig.2).

Pathogenicity test

Both the host specific isolate A.G -16 and A.G - 20 showed pathogenicity in case of both the techniques (IVCI). But A.G – 20 showed comparatively more virulence than the other isolates. Disease incidence or damage percentage was measured as par Sinclair (1999), 0-9 point scale. A.G – 20 also found to be the most virulent isolate in case of spraying of spore suspension method. Expression of symptoms occurred at 7-10 days of post inoculation in case of IVCI and 10-12 days for spraying of spore suspension. Pathogenicity tests showed similar symptom development as in field conditions. Koch's postulate was established by conducting the pathogenicity test twice, with the re-isolation of the same pathogen in culture media.

Mucorales is also one of the most studied groups in the early diverging fungi. This heterothallic zygomycetous monster increased its host range day by day, and truly a serious threat to the vegetable cultivars under present agroclimatic scenario of West Bengal, India. Our previous research output on this pathogen added two new host index on *Momordica subangulata* subsp. *renigera* (Das *et al.* 2017a) and *Solanum melongena* (Das *et al.* 2017b). The present isolate A.G-20 is not so varied with previously reported isolate M.D-9 (teasle gourd) and Br-1 (aubergine) in morpho-cultural aspects. A pathogenic fungus producing life threatening diseases on different crops with increasing emerging scenario, and to educate farmers on the preventive and control measures especially mixed crop farming. *Choanephora* leaf blight is an issue under high-moisture conditions, so allowing for adequate airflow and a dry plant canopy should aid in disease suppression side by side weeding with proper scientific way is the tough barrier for disease progress. Host susceptibility and preference of food depends on many biochemical factors like total sugar concentration, polyphenol oxidase ratio in the host tissue etc. Previously it was known that plant pathogenic zygomycetes are weak parasites (Agrios,2005), but some zygomycetous fungi evolved its complex adoptability and breaks its traditional boundary for choosing preferable host. To our knowledge, and based on the literature this is the first report and first occurrence of twig blight of *Amaranthus gangeticus* caused by *Choanephora infundibulifera*, in India.

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