First Report of *Diaporthe phaseolorum* Infecting Indian Trumpet Flower (*Oroxylum indicum*) from India

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Seed health testing, using the blotter method, revealed some fungal growth on the seed surface of one accession of Indian trumpet flower/Broken bones tree (*Oroxylum indicum* (L.) Kurz) collected from Kokrajhar, Assam, India. The fungus was identified as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. based on morphological characters. Later, the identity was re-confirmed by DNA sequencing using ITS gene sequencing (NCBI Sequence Id: MT154253.1) and a large subunit of rRNA (NCBI Sequence Id: OL798081.1). Literature reveals that *D. phaseolorum* is a destructive pathogen causing severe yield losses in various host crops. However, detection of *D. phaseolorum* in Indian trumpet flower seed followed by pathogenicity on its seedlings confirms that *O. indicum* is a new host record. Being a destructive pathogen of several other crops, such as seed decay and stem canker in soybean, it may pose a serious threat to future cultivation of this herbal plant.

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INTRODUCTION

Oroxylum indicum, a medicinal herb, small perennial, deciduous plant species, is commonly known as 'Sonapatha/ Broken bones tree/ midnight horror/ Indian trumpet flower in India and belongs to the family Bignoniaceae. It is distributed throughout Southeast Asia, including India, Nepal, China, Sri Lanka, Japan, Bhutan, Philippines, Malaysia, and Indonesia. In India, it is cultivated up to an altitude of ~1200 m as an avenue tree throughout the Himalaya foothills, Konkan, Malabar, Eastern and Western Ghats, Coro Mondal, and North East India (Jayaram and Prasad 2008).

Its leaves and stems are edible, and all plant parts, including bark, root, and fruit, are used as Ayurvedic and folklore medicine for the treatment of different ailments such as cancer, diarrhea, fever, ulcer, and jaundice (Singh and Chaudhary 2011). *O. indicum* plant parts include baicalein, chrysin, oroxylin A, and oroxylin B chemicals (Dinda *et al.* 2015). Recent studies indicated several medicinal properties such as anti-inflammatory, antiulcer, hepatoprotective, anticancer, antioxidant, photocytotoxic, antiproliferative, antiarthritic, antimicrobial, antimutagenic, and immunostimulant. Realizing the importance of its pharmacological properties, ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India is conserving *O. indicum* germplasm in the National Genebank, India for its future utilization.

Several plant pathogenic seed-borne fungi, *Fusarium solani*, *Alternaria solani*, *A. alternata*, *Acremonium* sp., *Rhizoctonia solani*, *Torula allii*, *Diaporthe* sp., *etc.* on *O. indicum* seeds have been reported, and are responsible for deterioration in seed quality and considerable economic yield loss (Pande and Gupta 2011; Das and Narzary 2017). Therefore, before conserving the seeds for long term storage (LTS) in the National Genebank, all samples were tested for their health status.

EXPERIMENTAL

Materials

Plant material

The experiment was conducted in the Division of Plant Quarantine, ICAR-NBPGR, New Delhi during 2018 to 2020. Three samples of *O. indicum* were received through the Division of Germplasm Conservation, ICAR-NBPGR, New Delhi, India and collected from different locations.

Seed Health Testing

During seed health testing, all the seed samples were first visually examined and then subjected to the blotter test (modified ISTA seed health testing). For this, three layers of sterilized blotter papers were soaked in sterilized distilled water for about 10 min and then kept in sterilized Petri plates (plastic) with diameter of 110 mm and labelled to maintain the identity of individual samples with number and date of observation. The seeds were surface sterilized by immersion in sodium hypochlorite solution (4%) for 30 s and subsequently rinsed thrice in sterilized distilled water. As the seed size was too large, only two seeds per Petri plate were placed on blotter papers near the periphery at equal distance. Three plates of each accession were incubated at 22 ± 1 °C under alternate cycles of 12 h of fluorescent light and darkness for 7 days and examined on the 8th day under a stereo-binocular microscope (Nikon - SMZ 1500; Nikon Instruments, Inc., Melville, New York, USA) at different levels of magnification, *i.e.*, 0.75× to 11.25× for the presence of seed-borne fungi. The associated fungus was identified *via* colony characteristics, fruiting bodies, and spores under stereo-zoom microscope; slides that used mounting media (lactophenol/ cotton blue) were also prepared and examined under compound microscope (Nikon - Eclipse 80i; Nikon Instruments, Inc., Melville, New York, USA). Isolation of the fungus was also made using a modified isolation technique (Akhtar *et al.* 2014) on potato dextrose agar (PDA) medium to confirm its identity.

For proving pathogenicity, *O. indicum* seedlings raised from health seeds of the same accession as well as the remaining two accessions were used. Conidial suspension of *D. phaseolorum* was prepared in sterilized distilled water with gelatin (2.0%). Conidial concentration of the suspension was adjusted to $2x10^4$ conidia mL^{-1} using a haemocytometer. Four weeks old seedlings (five of each accession) were spot inoculated by placing 10 µL conidial suspension using micropipette on the adaxial surface of the leaf. Gelatin was used to avoid the overspreading of the conidial droplets. Inoculated seedlings were incubated in a growth chamber at 25±1 °C with >90% relative humidity, 16-hour photoperiod, and 8-hour darkness. Incubated seedlings were observed daily for 2 weeks under stereobinocular microscope to record infection and symptom development.

Re-isolation was made on potato dextrose agar for further identification of the pathogen to compare with original culture used for inoculation.

Molecular Identification

To re-confirm the identity of the fungus, Internal Transcribed Spacer (ITS) region and Large Subunit (LSU) of rRNA sequencing was completed following the undermentioned process. For genomic DNA extraction, mycelial mat was harvested by filtering through sterilized Whatman filter papers. The freshly harvested mat was dried in between filter paper and ground into fine powder using pestle and mortar with liquid nitrogen. Approximately, 300 mg of fine powdered mycelia was used for DNA extraction using the CTAB method (Murray and Thompson 1980). The quality and concentration of the extracted DNA was then assessed by a spectrophotometer (NanoDrop^{TM} 2000; Thermo Fisher Scientific Inc., Waltham, MA, USA) and the DNA concentration was adjusted to 50 ng μL^{-1} for PCR cycle reaction. For the polymerase chain reaction (PCR) cycle, 15 µL PCR master mix consisted of 1x PCR buffer, 50 ng DNA, 2.5 mM dNTPs, 1 U *Taq* DNA polymerase, and 0.2 mM each primer. The universal primer sets used for amplification were ITS (ITS 5: GGAAGTAAAAGTCGTAACAAGG and ITS 4: TCCTCCGCTTATTGATATGC) (White *et al*. 1990), and LSU (LR6: CGCCAGTTCTGCTTACC and LR0R: ACCCGCTGAACTTAAGC) (Vilgalys and Hester 1990). The amplification of the ITS and LSU region of ribosomal RNA was performed with the following PCR conditions in a thermocycler (Genepro, Bioer Technology Co., Ltd., Binjiang, China): initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s for ITS and 55 °C for 50 s for LSU, and extension at 72 °C for 1 min and final extension cycle at 72 °C for 10 min.

Table 1. Details of the Isolates Used in the Phylogenetic Analysis Using ITS Sequences

Sl. No.	Taxon Name	Isolate/Collection No.	GenBank Accession
1.	*Diaporthe phaseolorum*	DpO1	MT154253.1
2.	*D. phaseolorum*	MIF01	KT964565.1
3.	*D. phaseolorum*	Ck14a7	JX436797.1
4.	*D. phaseolorum*	FM1	JQ514150.1
5.	*D. endophytica*	InaCC-F237	AB899789.1
6.	*D. helianthi*	SDH1	MF033502.1
7.	*D. kyushuensis*	---	AB302250.1
8.	*D. yunnanensis*	SAUCC0481	MT199848.1
9.	*D. thunbergiicola*	MFLUCC:12-0033	KP715097.1
10.	*D. discoidispora*	BPL	MH371247.1
11.	*D. longicolla*	AIL	MH371243.1
12.	*D. viticola*	STE-U 5683	AY485750.1
13.	*D. perjuncta*	AR 3461	AY485785.1
14.	*D. miriciae*	BRIP 54736j	NR_147535.*1*
15.	*D. aspalathi*	508	KX769839.1
16.	*D. novem*	CBS 127270	MH864503.1
17.	*D. ueckerae*	17-DIA-117	MK942678.1
18.	*Valsa mali* var. *pyri*	GSZY113	GU174589.1

The PCR product was run in an agarose gel (1.2%) for 1 h 15 min at a current of 80 Volt with ethidium bromide staining. The amplified product was eluted using QIAquick gel extraction kit (Qiagen) and was sequenced by outsourcing. The fungal sequences of *Diaporthe* spp. obtained from the GenBank database (Tables 1 and 2) were aligned using Clustal W (Thompson *et al.* 1994) and manually curated. Phylogenetic analysis was performed using MEGA 7 software (Kumar *et al.* 2016) and maximum likelihood was constructed by Kimura's two-parameter correction method (Kimura 1980). The fungus *Valsa mali* var. *pyri* for ITS and *Alternaria tropica* for LSU was used as an outgroup.

RESULTS AND DISCUSSION

While monitoring the health status of *O. indicum* seeds using the blotter test at ICAR-NBPGR, New Delhi, seeds of one accession, IC630440, collected from a farmer's kitchen garden from Jhawarbil, Kokrajhar, Assam (located at latitude (N)- 26° 36.960′; longitude (E)- 89° 54.234′; altitude (msl) - 77 m) showed no germination and under stereozoom microscope (Fig. a), there was extrusion of spores in the form of gelatinous cirrus (Fig. 1b and 1c) from ostiolate black pycnidial fruiting bodies from the seed's surface of all the tested seeds. Compound microscopy revealed the presence of two types of hyaline and non-septate conidia, namely alpha (α) and beta ($ß$) conidia in the cirrus. The α conidia were aseptate, ellipsoidal, and bi-guttulate, measuring 5 to 8 × 2 to 3 µm **(**Fig. 1d), whereas, $ß$ conidia were also aseptate and hyaline, but filiform, hooked, and lack guttulae, measuring dimension 18 to 20 × 1 to 2 µm (Fig. 1e). These observations corroborate with the report of Pioli *et al.* (2001), and the fungus was identified as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc.), which is a destructive pathogen causing severe yield losses in various host crops. The identity of the fungus was re-confirmed through ITS and LSU rRNA gene sequencing followed by pathogenicity on *O. indicum* seedlings using spot inoculation with conidial suspension (Fig. 1f). As a result of PCR amplification, amplicons of 600 bp and 1200 bp size were revealed in ITS and LSU of rRNA, respectively, on agarose gel followed by sequencing and BLAST. This resulted in 99.83% sequence identity of ITS gene with NCBI GenBank accession no. KT964565.1. The identity of the fungus was re-confirmed and the sequence was submitted to NCBI (Accession ID: MT154253.1). Phylogenetic analysis of the ITS sequence dataset (Table 1) showed that the strain under study with NCBI GenBank accession no. MT154253.1 was placed within the same clade of *D. phaseolorum* strains (Fig. 2). The other species of *Diaporthe* genus formed separate clades.

Similarly, BLASTN analysis resulted in 99.40% sequence identity of LSU gene with NCBI GenBank accession no. AY346279.1, and the identity of the fungus was re-confirmed. The sequence was submitted to the NCBI (Accession ID: OL798081.1). Phylogenetic analysis of LSU sequence dataset (Table 2) showed that the strain understudy (OL798081.1) was placed within the same clade of *D. phaseolorum* strains (Fig. 3). The culture was finally deposited to the National Agriculturally Important Microbial Culture Collection (NAIMCC), Mau, India (accession number NAIMCC-F-03983). Therefore, association of *D. phaseolorum* with broken bones tree constitutes a new host record for the first time from India and elsewhere.

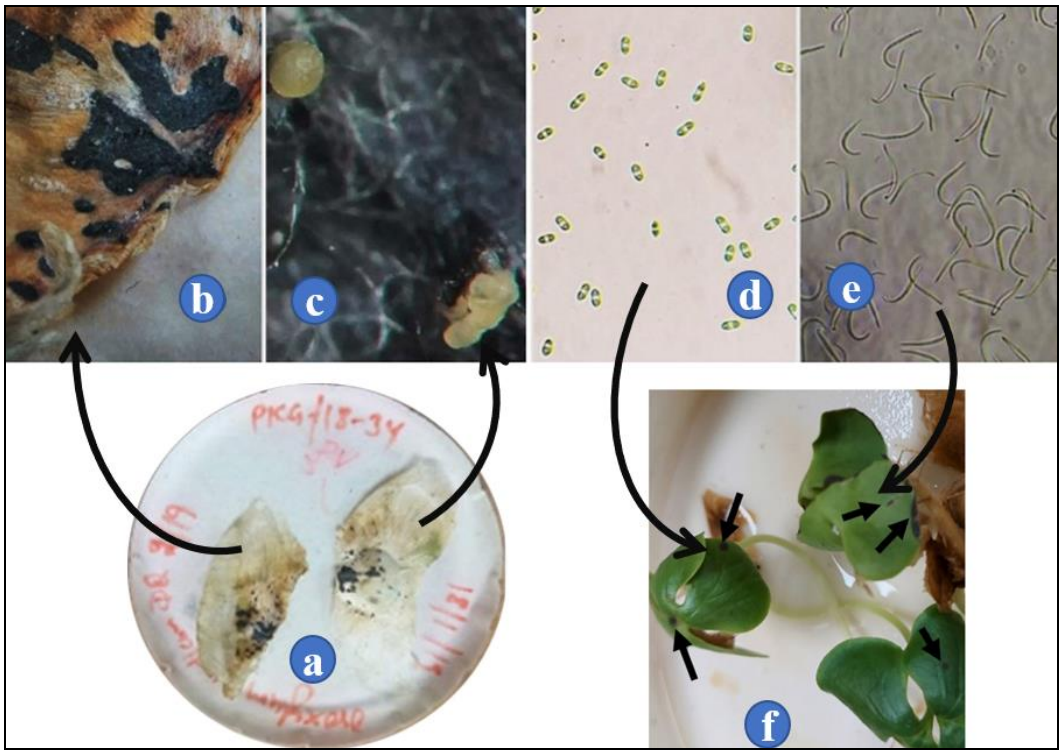

Fig. 1. *Diaporthe phaseolorum* infected seed of broken bones tree showing fungal growth on surface (a), gelatinous cirrus (b and c), α conidia (d), ß conidia (e), and pathogenicity on *O. indicum* seedlings (f)

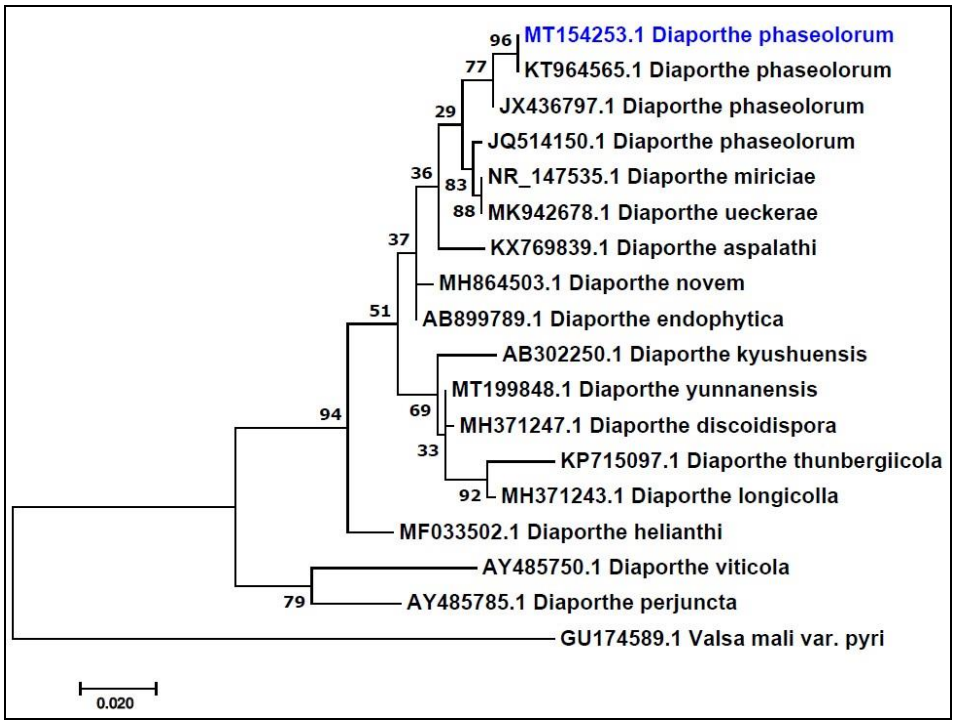

Fig. 2. Maximum likelihood tree based on ITS sequences of *Diaporthe* spp. showing phylogenetic position of strain understudy. *Valsa mali* var. *pyri* was used as an outgroup.

Table 2. Details of the Isolates Used in the Phylogenetic Analysis Using Large Subunit of rRNA Sequences

Sr No.	Taxon Name	Isolate/Collection No.	GenBank Accession
1.	*Diaporthe phaseolorum*	DpO1	OL798081.1
2.	*D. phaseolorum*	FAU458	AY346279.1
3.	*Diaporthe eres*	Z15	MW474945.1
4.	*Diaporthe helianthi*	CBS_592.81	MT378370.1
5.	*Diaporthe acaciarum*	CBS 138862	MH878645.1
6.	*Diaporthe phragmitis*	CBS 138897	MH878644.1
7.	*Diaporthe musigena*	CBS 129519	MH876824.1
8.	*Diaporthe novem*	CBS 127271	MH875941.1
9.	*Diaporthe lusitanicae*	CBS 123213	MH874804.1
10.	*Diaporthe cynaroidis*	CBS 122676	MH874757.1
11.	*Diaporthe vaccinii*	CBS 160.32	MH866710.1
12.	*Diaporthe foeniculina*	CBS 187.27	MH866420.1
13.	*Diaporthe hickoriae*	CBS 145.26	MH866365.1
14.	*Diaporthe clematidina*	MFLUCC 17-2060	MT214613.1
15.	*Diaporthe caatingaensis*	URM7486_CBS141542	KY085930.1
16.	*Diaporthe rudis*	CBS 113201	MH874489.1
17.	*Diaporthe ambigua*	CBS 114015	MH874517.1
18.	*Alternaria tropica*	CBS 631.93	MH874097.1

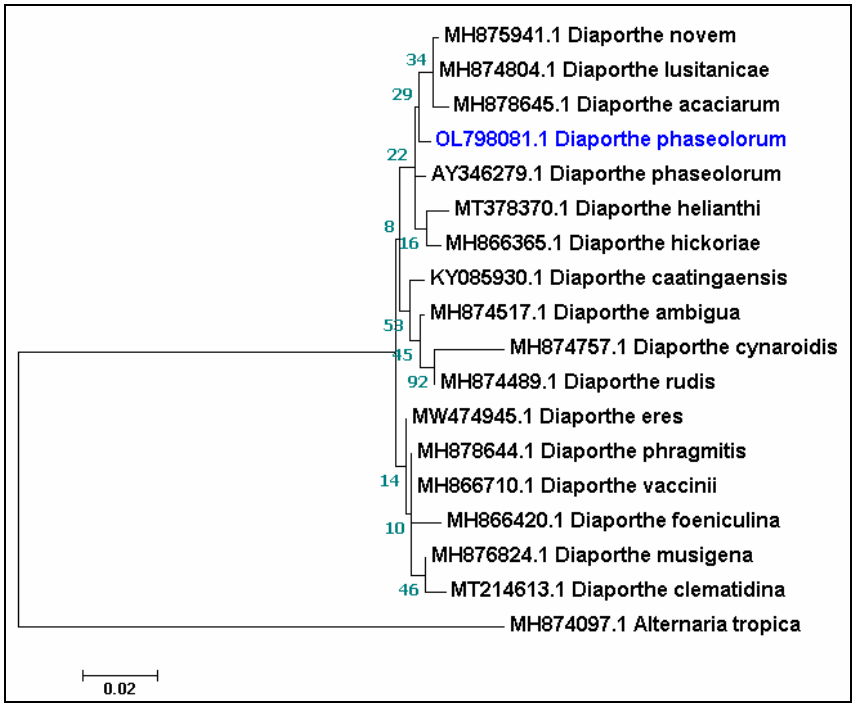

Fig. 3. Maximum likelihood tree based on large subunit of rRNA gene sequences of *Diaporthe* spp. showing phylogenetic position of strain understudy. *Alternaria tropica* was used as an outgroup.

Diaporthe phaseolorum is a destructive pathogen causing severe yield losses in various host crops. Based on the working experience of *Diaporthe* spp., it is difficult to identify a species isolated from any host for which the species is not previously described. This is because many of the species have a wide host range and there are few characteristics that can differentiate them (Uecker 1988), whereas some species are

thought to be host-specific. Therefore, caution is needed when confirming species of the genus in an individual host (Santos and Phillips 2009).

If such infected seeds are conserved for a long period at the National Genebank, there is a possibility that the fungus can survive in seeds for more than a decade (Akhtar *et al.* 2016). Such seeds may prove to be the hidden carrier of the pathogen and thereby spread the pathogen from one place to another.

CONCLUSIONS

The observation on infection of *D. phaseolorum* in *O. indicum* seed is a new record from India and anywhere else. This can be helpful in highlighting the threat at an early stage, thereby minimizing the damage of this medicinally important potential crop. The important and general results of the current study are as follows:

1. *Diaporthe phaseolorum* was reported for the first time on Indian trumpet flower seed.
2. A complete loss of seed germination was reported due to infection of *D. phaseolorum*.
3. Research reveals that *D. phaseolorum* may pose a serious threat to the cultivation of the Indian trumpet flower.

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