

First record of Leaf Spot of *Ficus religiosa* Caused by *Alternaria alternata* in Pakistan

HaoLan Du^{1†}, Tanvir Ahmad^{1,2,3†}, Mingjun He², Anam Moosa³, Yonguan Zheng⁴, Chengrong Nie², Fuguo Xing^{1*}, Yang Liu^{1,2*}

¹Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences / Key Laboratory of Agro-products Quality and Safety Control in Storage and Transport Process, Ministry of Agriculture and Rural Affairs, Beijing 100193, P. R. China.

²School of Food Science and Engineering, Foshan University/South China Food Safety Research Center, Foshan 528231, Guangdong, P. R. China.

³Department of Plant Pathology, University of Agriculture Faisalabad P.O. Box 38040, Pakistan.

⁴State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, P.R. China

*Corresponding authors: Yang Liu (liuyang@fosu.edu.cn) and Fuguo Xing (xingfuguo@caas.cn)

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Ficus religiosa (L.) belongs to the family *Moraceae*, native to India and commonly known as ‘Peepal’. It has high medicinal value due to its antibacterial, antiviral and antioxidant properties (Singh et al., 2015; Kalpana et al., 2009). In August 2021, leaf spots were observed on *F. religiosa* trees in Pabbi forest park Kharian (32°50'01.4"N 73°50'17.7"E), District Gujrat, Pakistan. The disease incidence was recorded approximately 30%. The leaf spots were irregular in shape, brown in colour, 3-9 mm in size and encircled by yellowish halo. In severe condition, the spots coalesced and produced necrotic areas on leaf surface (Figure 1). The samples (n=21) were collected based on symptoms and infected leaf segments were excised into small pieces, surface disinfected with 1% NaClO for 20s and rinsed 3 times with sterilized distilled water. The pieces were plated on Potato Dextrose Agar (PDA) medium and incubated at 28°C for 7 days. All the pure cultures were obtained through single spore method on PDA and preserved in 30% glycerol at -80°C. The colonies were olive green to dark brown with white margin and later turned dark olive or black with enormous sporulation. Conidia (n=24) were obclavate, ovoid, brown in colour and measuring 10.2 to 34.1 µm long x 5.9 to 12.3 µm wide with 1 to 6 transverse and 1 to 3 longitudinal septa (Figure 2). Based on these characteristics, the pathogen was identified as *Alternaria alternata* (Gilardi, G., et al. 2019). For molecular identification, the Internal Transcribed Spacers (ITS) region, endopolygalacturonase (*endoPG*) gene and major allergen (Alt a1) gene were amplified using ITS1/4 (White et al. 1990), PG3/PG2b (Andrew et al. 2009) and Alt-4for/Alt-4rev (Lawrence et al. 2013) primers respectively. Based on molecular characteristics, all isolates were identified as *A. alternata*. The sequences of the representative isolate FLB-1 were submitted in the GenBank with the accession numbers

OL514181 for ITS, OK315658 for *endoPG* and OK315659 for *Alt* al showing 100% similarity with ITS accession KP124298, and *endoPG* accession AY205020 and 99.7% with *Alt* al accession KP123847 sequences of CBS106.24 *A. alternata* after BLASTn queries. The Phylogenetic reconstruction based on maximum likelihood, using Mega X (Kumar et al. 2018) and FLB-1 grouped with *A. alternata* (Figure 3). Pathogenicity test was performed on nine months old healthy *F. religiosa* (L.) seedlings (n=12) to fulfil the Koch's postulates. The leaves were pinpricked and sprayed with FLB-1 conidial suspension (10^7 spores/ml) by using atomizer (Bajwa et al., 2010). The leaves of *F. religiosa* (L.) seedlings (n=12) sprayed with sterilized distilled water served as control. All the seedlings were incubated at $25 \pm 3^\circ\text{C}$ in the glasshouse. The experiment was performed three times under the same conditions. The typical symptoms appeared on inoculated leaves after 7-14 days that were similar to the symptoms observed on original infected *F. religiosa* (L.) trees. In the control treatment leaves remained asymptomatic (Figure 4). The pathogen from the artificial infected leaves was re-isolated and identified as *A. alternata* based on morphological and molecular characteristics. To our knowledge, this is the first report of leaf spot of *F. religiosa* (L.) caused by *A. alternata* in Pakistan. The leaves of *F. religiosa* (L.) are commonly used in Asia for different purposes and this leaf spot disease may represent a significant threat to *F. religiosa* (L.) tree health.

Reference

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Figure 1. Leaf spot disease symptoms on *F. religiosa*

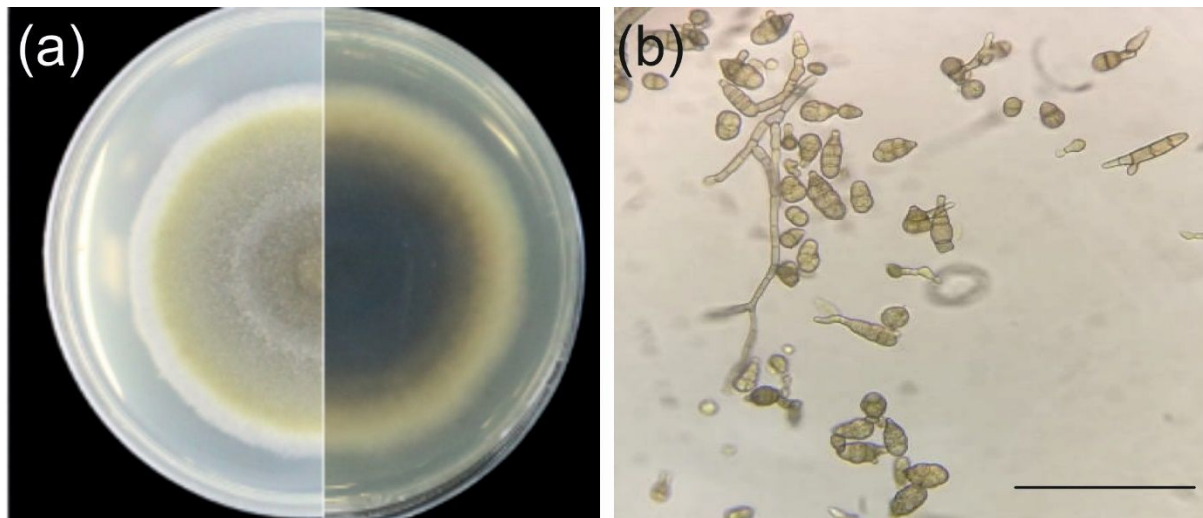


Figure 2. Colony morphology and microscopic features of *A. alternata*. (Scale bar = 20 μ m).

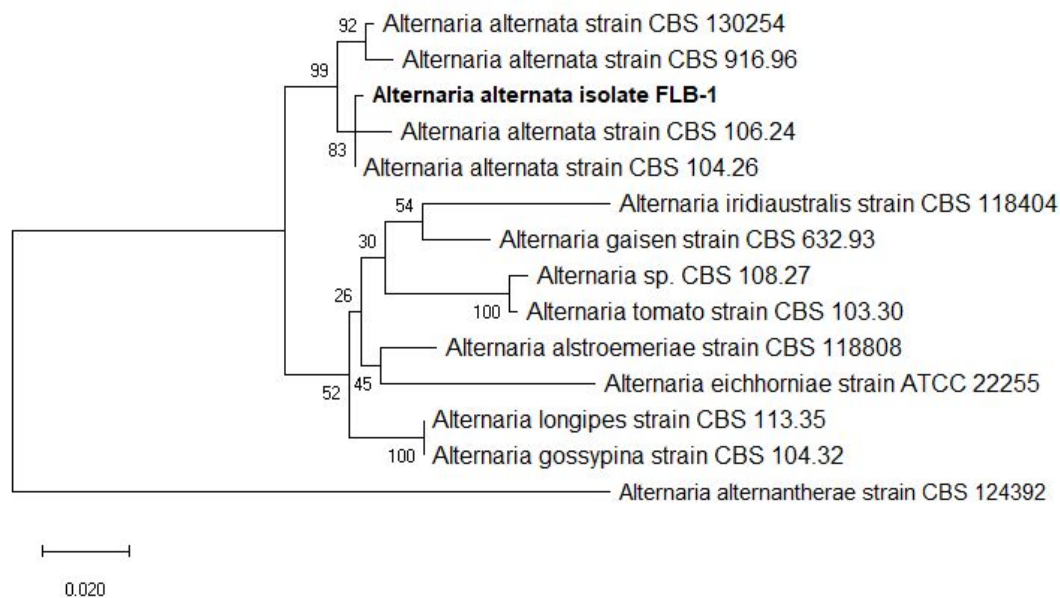


Figure 3. Phylogeny of the representative isolate and other *Alternaria* species. Phylogenetic tree was constructed based on concatenated sequences of Internal Transcribed Spacers (ITS) region, endopolygalacturonase (*endoPG*) gene and major allergen (Alt a1) gene. Numbers in the branch were calculated from the bootstrap test of 1000 replicates.

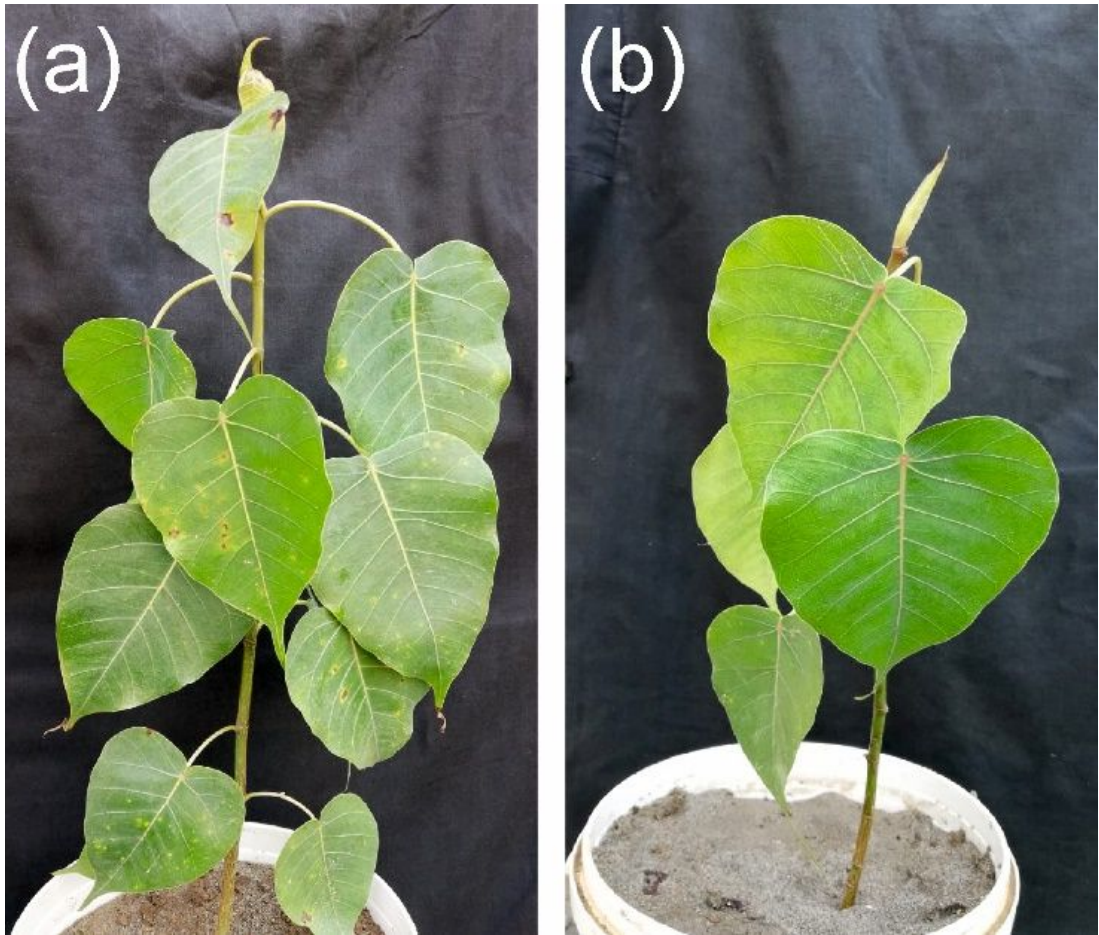


Figure 4. Pathogenicity of *A. alternata* on healthy *F. religiosa* seedlings (a) Inoculated *F. religiosa* seedlings with FLB-1 (10^7 spores/mL) suspension (b) Control treatment.