KAVAKA 61(1): 27-33 (2025) DOI: 10.36460/Kavaka/61/1/2025/27-33

Molecular Detection and Phyto-pathological Investigation of *Colletotrichum siamense* Associated with Severe Foliar Blight Disease of Teak (*Tectona grandis*) Seedlings in Kerala, India

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(Submitted on January 12, 2025; Accepted on February 13, 2025)

ABSTRACT

Teak (*Tectona grandis*) is one of the most valuable hardwoods in the world. Teak seedlings hold significant importance for various reasons, particularly in the context of forestry, environmental conservation, and economic development. Teak seedlings with a severe foliar blight infection were observed between April 2020-22 in a social forestry nursery in Thrissur, Kerala. Infected leaves displayed brownish, necrotic brightening lesions that grew larger as the disease advanced. The disease prevalence was 100%. An associated fungal pathogen was isolated and examined to determine the causative agent. Based on cultural characteristics, morphology, and multigene sequence analysis and molecular phylogenetic analysis using concatenated sequence alignment of ITS-TUB2-GAPDH, the pathogen was identified as *Colletotrichum siamense*. Further, Koch's postulates were fulfilled by inoculating the healthy seedlings with isolated fungal pathogen and characteristic symptoms were recorded. The identity was confirmed after re-isolation and thus confirming its association. To the best of our knowledge, this is the first report of *C. siamense* causing foliar blight disease in teak seedlings from Kerala, India. This research emphasizes the need for effective disease management strategies to ensure healthy teak seedlings production for conservation programme.

Keywords: Nursery diseases, Foliar disease, Mycopathogens, Glomerellaceae, PCR

INTRODUCTION

Tectona grandis L. f. (Lamiaceae) is one of the most precious timbers in the world. It is native to India and Southeast Asia, and is grown as a major plantation tree species in the tropics. In recent years, several African countries have become increasingly important exporters of teak logs and sawn wood in the world market (Kollert and Walotek, 2015). Teak is a highly durable and much priced major tropical timber yielding species. Natural teak populations harbour a variety of characteristics that determine their economic, ecological and environmental importance. Teak is a precious timber tree that is often over-harvested in its native range. Teak seedlings play a crucial role in reforestation and afforestation efforts. Planting teak trees helps restore degraded forests and establish new forested areas, which is essential for biodiversity conservation and combating deforestation (Warrier *et al.,* 2022; Mankur *et al.,* 2023). In India, teak is used for various afforestation programmes on a large scale. It's important to note that the significance of teak seedlings is closely tied to responsible and sustainable forestry practices. While the country's forest cover increased through different programmes by almost 1% in 2017, the amount of forest cover in some parts of India has decreased (Kumari *et al.,* 2019). In this context, Government of India launched various afforestation schemes like Green India Mission (GIM) to improve quality of degraded forests as well as establishing more tree cover in non-forest areas through social and farm forestry.

Teak plants and their seedlings are vulnerable to a variety of biotic threats, among them fungal diseases, such as root rot, dieback and leaf blight, affect teak seedlings. These diseases can cause wilting, yellowing, of the leaves and necrotic lesions on stems, and in severe infection, they ultimately kill the entire seedlings (Sharma *et al.*, 1985; Pathak *et al.,* 2015; Doilom *et al.*, 2016; Jayawardena *et al.,* 2019; Kiran *et al.*, 2021; Soumyakala *et al.,* 2023; Mushineni *et al.*, 2024). Therefore, early detection and regular monitoring of biotic threats are crucial for sustainable control measures and effective nursery management practices to protect these seedlings.

The genus *Colletotrichum* belongs to family Glomerellaceae is globally distributed, and is one

of the top ten phytopathogens (Dean *et al.,* 2012). *Colletotrichum* includes many important plant pathogens that cause significant losses across diverse crops globally (Agrios, 2004; Hyde *et al.,* 2009, 2014; Cannon *et al.*, 2012; Bhunjun *et al.,* 2021; Cui *et al.,* 2025, Megha *et al.,* 2025). *Colletotrichum siamense* affects a wide range of hosts, including fruit crops (mango, coffee, banana, avocado), forestry species (teak, eucalyptus), and non-timber forest products (NTFPs). It causes anthracnose, leading to defoliation, fruit rot, and seedling mortality, significantly impacting crop yield, plantation health, and biodiversity. In forestry, it affects timber quality and seedling establishment. In NTFPs, it threatens medicinal and economically valuable plant resources. Its rising significance in plant pathology necessitates molecular diagnostics (Cai *et al.*, 2009; Weir *et al.,* 2012; Jayawardena *et al.,* 2021) for detection and diagnosis that helps to develop integrated disease management, and conservation strategies to mitigate negative impacts and economic losses.

The aim of the present study was to detect and authentically identify the phytopathogen associated with the foliar blight disease of teak seedlings using morpho-cultural studies, pathogenicity and multigene molecular phylogenetic analysis.

MATERIAL AND METHODS

Sample collection

During the years 2020-22, incidence of foliar blight disease was noticed in teak seedlings of social forestry nursery in Thrissur, Kerala. In total, around 1000 teak saplings assessed and all the saplings were found associated with foliar blight disease. The severity ranged from severe blight infection (80 – 100 % leaf lamina were infected) to death of infected saplings. The representative infected leaf samples were collected and brought to the laboratory for detail phytopathological investigation. Diseased samples were examined visually and under a binocular stereo zoom microscope to record the symptoms.

Isolation of phytopathogen

The infected leaf sample was cut into small pieces (5×5mm), washed under running tap water thoroughly to remove any dirt and then surface disinfected with alcohol followed by 1% sodium hypochlorite for 30 seconds. Then washed three times with sterilized distilled water and cut into small bits and then placed on a Potato Dextrose Agar (PDA) medium, incubated at 27±2°C for 5-7 days under 12/12 hours light and dark conditions (Sreelakshmi *et al.,* 2021). After 7 days of incubation, the hyphal tips from the margin of each developing colony were sub-cultured and maintained on PDA (Kripa *et al.,* 2024). Isolated fungal culture and fungal structure were observed under Leica DM200 LED stereomicroscope and measurements were taken using LAZ software. Colony morphology including colour and shape were determined after 7 days of incubation. Morphological description was based on slide preparation according to Maharachchikumbura *et al.* (2012) and Senanayake *et al.* (2020).

Molecular characterization and phylogenetic analysis

To confirm the accurate identity of isolated fungal pathogen, molecular characterisation was performed. Briefly, the genomic DNA was extracted from the seven days old actively growing plates of the isolated fungal culture by using the CTAB method with slight modifications in the protocol of Kim *et al.,* (2010) and Kumar *et al.,* (2025). The quality of the extracted DNA was analyzed on a 0.8% agarose gel. The PCR reaction was carried out with thermal cycler (Bio-Rad T100, India). The internal transcribed spacer (ITS), beta-tubulin (*TUB2*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were amplified using the primer pairs ITS1/ITS4 (White *et al.,* 1990), Bt2a/Bt2b (Glass and Donaldson, 1995), and GDF/GDR (Templeton *et al.,* 1992) respectively. For PCR amplification, a 25 µl reaction mixture prepared for the PCR amplification contained 1.5 µl of template DNA (~100ηg), 12.5 µl of Taq DNA polymerase Master mix (Hi-Media), 1 µl each of forward and reverse primers (10 pM; Eurofins, Bangalore, India), and the remaining 9 µl of nuclease-free water. PCR conditions employed for ITS: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 50 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes; for *TUB2:* denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes; and for *GAPDH*: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. The amplicons were visualised in gel documentation system on a 1.2% agarose gel (Bio-Rad, model number 1708270). The amplified PCR products were sequenced by using DNA sequence analyzer (ABI3730I DNA analyzer Applied Biosystems, Foster City, California) at Genespec Pvt. Ltd., Kochi, Kerala. The obtained DNA sequences were compared to nucleotide sequences in the NCBI-nBLAST database, and the closest matches were considered for inclusion in phylogenetic tree construction alongside reference sequences. Further, reference sequences were retrieved from GenBank and phylogenetic tree was

constructed using MEGA X software (Kumar *et al.,* 2018). The evolutionary distances were computed using the Kimura 2-parameter method.

Pathogenicity assay

For the pathogenicity assay, an *in vitro* spore suspension method was employed using detached leaf method. A spore suspension (10^6conidia/mL) of the isolated pathogen was prepared in sterile distilled water. Healthy leaves of teak were randomly selected, pin-pricked, and inoculated with the spore suspension at three equidistant points. Leaves were incubated in a moist chamber at 25±2°C for 5–7 days to observe the symptoms development. Control leaves were inoculated with sterile distilled water (Jose *et al.,* 2023; Kumar *et al.*, 2025a). In vivo pathogenicity tests were not conducted due to a lack of healthy seedlings

RESULTS AND DISCUSSION

Teak seedlings exhibiting foliar blight disease in a social forestry nursery in Thrissur showed 100% disease incidence. Infection on leaves was amphigenous, circular, sub-circular to irregular, necrotic to blight, initially dark brown, but later turned blackish. The infected plants showed water-soaked greyish brown patches that enlarged rapidly and covered a large part or the entire leaf lamina. The blighted leaves often showed holes in the infected portion as a result of shedding of infected tissues. The infected leaves shrivelled up and eventually shed. The disease spreads laterally in the nursery through overlapping foliage of the adjoining seedlings often resulting in group blighting of seedlings. In each case of severe infection, seedlings led to the defoliation and group blighting through lateral spread.

A total of 20 infected leaves were collected, surface disinfected, and cultured on PDA for fungal isolation. A total of six isolates were obtained which were culturally identical. Therefore, one representative isolate (KFRIMCC 047) was used and slides were prepared and observed under microscope. The fungus showed following characteristics; On PDA colony of pathogen showed whitish coloured 40mm growth on the 7th day at 25°C; mycelia were septate, olivaceous brown; stromata or acervulii were 40-75 µm in dia.; setae were absent. Conidiophores (in fascicles), were cylindrical, erect to procumbent, straight to flexuous, unbranched, smooth, thin-walled, hyaline, 25–65 × 1.5–2 µm (n=20). Conidiogenous cells were terminal, monoblastic, hyaline, smooth walled. Conidia were solitary, unbranched, simple, thin-walled, smooth, hyaline, cylindrical, slightly constricted at the middle, apex rounded, base obtuse, subtruncate, 14–22 × 3.5–5.5 µm (n=20), 0-septate **(Figure 1)**. All the cultural appearance and micro morphological characters were found similar to *Colletotrichum* sp. (Sutton, 1980; Hyde *et al.,* 2009).

Spore suspension inoculation methods were used for *in vitro* pathogenicity assay. For this, nine healthy leaves were inoculated, resulting in 100% typical symptom development. Necrotic lesions appeared within 5 days of inoculation, expanding to form characteristic blight symptoms after 7–10 days. By 10–15 days post-inoculation, slimy conidial spore masses were observed, resembling original host symptoms. Control leaves showed no symptoms and remained healthy even after 15 days of incubation. Consistent results were obtained with a 90% isolation frequency. The fungal pathogen was consistently re-isolated on PDA and identified based on cultural and morphological characteristics. Koch's postulates confirmed *Colletotrichum* sp. as the causal agent of teak seedling blight.

For molecular identification, the genomic DNA was extracted from 7day pure culture of representative isolate (KFRIMCC 047) and the PCR were amplified for the internal transcribed spacer (ITS), beta-tubulin (*TUB2*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes. The amplified PCR products were purified and sequenced. The obtained DNA sequences in FASTA format were searched to NCBI-nBLAST data base (https://blast.ncbi.nlm.nih.gov) and the closest match was considered and included in phylogenetic tree constructions with reference sequences. The sequences were aligned and the phylogenetic trees were constructed using sequence alignment in MEGA-X. The concatenated sequence alignment of *ITS-TUB2-GAPDH* sequence data were used for phylogenetic tree construction. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 23 nucleotide sequences including present isolate and outgroups sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 424 positions in the final dataset (Kimura, 1980). The evolutionary history was inferred using the Maximum Likelihood method and the optimal tree with the sum of branch length = 0.2981 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches **(Figure 2)**.

The NCBI-nBLAST search analysis for the ITS sequence of the isolate KFRIMCC 047 shared 99.56% similarity (448/450, 0 gap) with *Colletotrichum* sp. strain LWYF53 (MT570090), followed by *C. siamense* strain ZS-ZJ-J-36 (MW653778), isolate GZYD_X80 (OQ652497) and *C. aenigma* isolate JZ1071 (MT570086)

respectively. The *TUB2* gene sequence data shared 100% (418/418, 0gap) sequence similarity with *Colletotrichum siamense* isolate B52F (ON932370) followed by *C. fructicola* strain CNUCC 916-1-2-1 (PP870990), *C. siamense* strain TQ01 (MN418898) and *C. siamense* isolate DF-32 (OP660851) respectively.

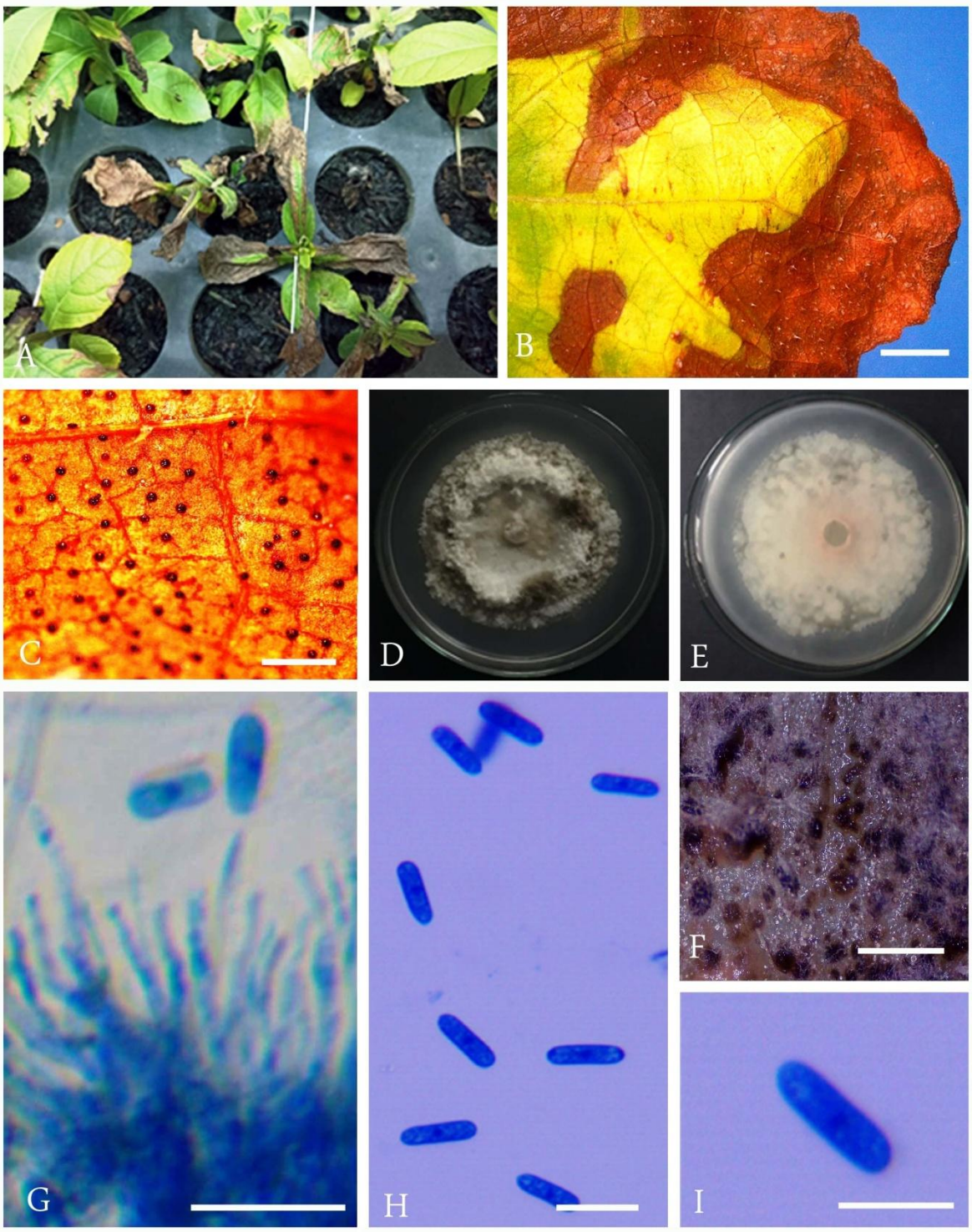

Figure 1: Symptomatological, morpho-cultural and microscopic characteristics of foliar blight disease caused by *Colletotrichum siamense* on teak seedlings in Kerala, India. A, Teak seedlings showing leaf blight in nursery beds; B-C, Enlarged view of symptoms showing fruiting bodies; D-E, Isolated fungus on PDA (upper and reverse view); F, Enlarged view of culture showing fruiting bodies; G, Conidiophores with attached conidia; H, Conidia; I, Single conidium. Scale bars B=50 µm; C=500 µm; F=500 µm; G=50 µm; H-I=20 µm.

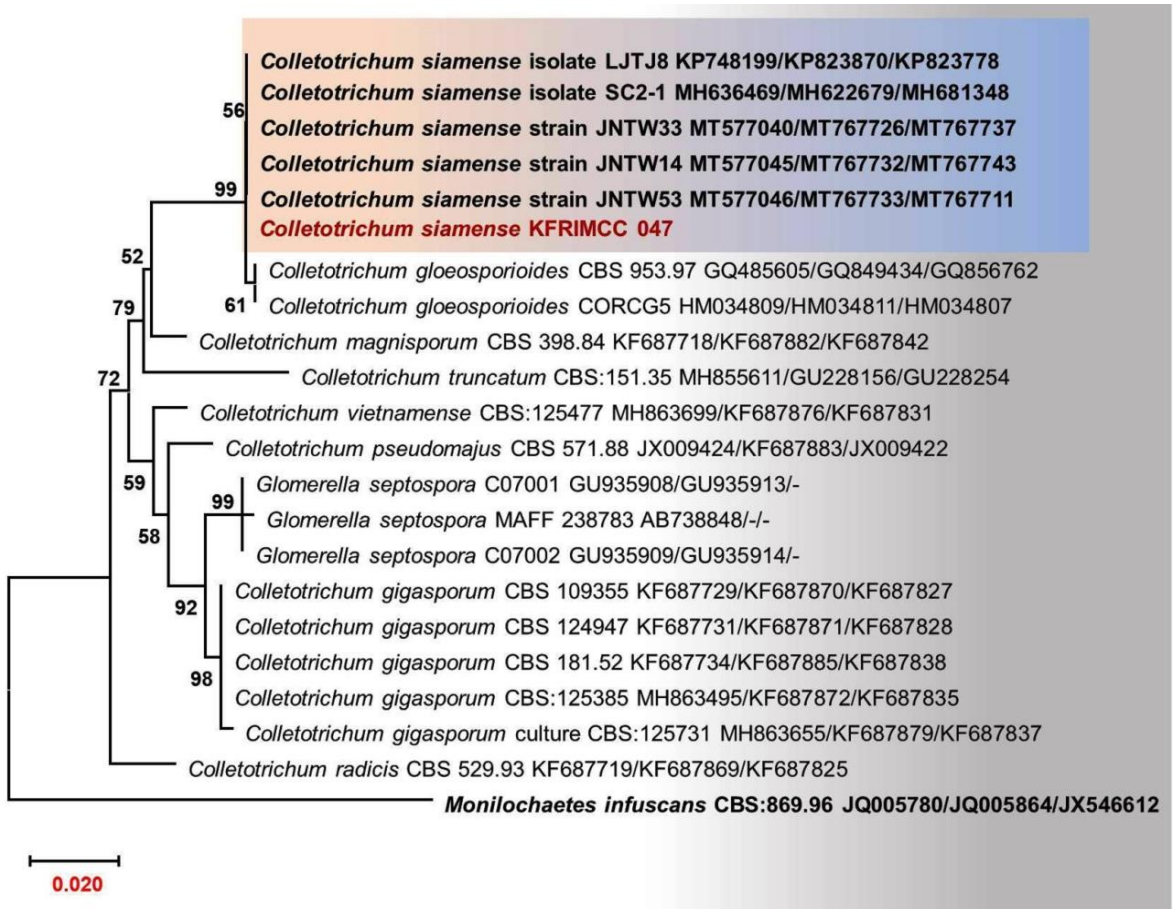

Figure 2: Concatenated phylogenetic tree generated from Neighbor-Joining method (NJ) analysis for the analyzed *Colletotrichum sp.* isolates using *ITS-TUB2-GAPDH* genes (Kimura 2-parameter method and nearest neighbour-interchange search options with 1000 bootstrap replicates were used). The sequence from the present study is indicated in bold and in red color. The scale bar represents the expected number of changes per site. The tree is rooted with *Monilochaetes infuscans* (CBS: 869.96).

Further, *GAPDH* sequence data also showed 100 % (279/279 bp, 0gap) sequence similarities with *C. siamense* strain SA3 (PQ586290), followed by strain ZJLA-W061 (OP611071), isolate PCZJXS7 (OP750551), strain JZB330170 (MT210250) and strain JZB330172 (MT210252) respectively. The DNA sequences of the present isolate, *C. siamense* (KFRIMCC 047) were deposited in GenBank and accession numbers were obtained (ITS: OQ681955, *TUB2:* PV033443 and *GAPDH:* PV033444).

The present study established the identity of the isolated fungal pathogen as *C. siamense* (Prihastuti *et al.,* 2009), based on morphological features, cultural and multi gene sequence analysis and phylogenetic analysis. Perusal of literature indicates that there is no record of *C. siamense* on this host from all over the world (Farr and Rossman, 2024). From India *C. gloesporioides* has been reported as an endophyte on this host (Murali *et al.,* 2007). Hence, this is the first report of *C. siamense* causing foliar blight on teak seedlings in Kerala, India (Florence, 2004).

CONCLUSION

On the basis of combination of culture characteristics, morpho-molecular phylogenetic analyses and pathogenicity, the pathogen was identified as *C. siamense*. It is a significant phytopathogen which causes great economic loss across the globe, by infecting the various medicinal, agricultural, forest and horticultural plants, leading to yield losses, reduced quality, and economic impact. Hence, early detection and identification of the pathogen during the initial stages of infection are crucial for mitigating its virulence and severity. Given the high demand of teak seedlings in plantations, it is essential to protect nursery stocks from this severe fungal infection. Therefore, the implementation of effective management strategies and control measures is imperative to ensure the propagation and supply of high-quality, disease-free seedlings.

ACKNOWLEDGMENTS

The authors are grateful to the Director, Kerala Forest Research Institute, Kerala for providing necessary support. We would like to acknowledge

the Science & Engineering Research Board (SERB), Department of Science & Technology (DST), Govt. of India for financial support (CRG/2019/005014).

CONFLICT OF INTERESTS

We declare there is no conflict of interests reported by the authors.

DATA AVAILABILITY STATEMENT

All data are incorporated into the article and its online supplementary material.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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