



ISSN Print: 2664-844X
ISSN Online: 2664-8458
NAAS Rating (2026): 4.97
IJAFS 2026; 8(3): 455-459
www.agriculturaljournals.com
Received: 07-01-2026
Accepted: 10-02-2026

KN Divyashree
Research Scholar,
Postgraduate Department of
Microbiology Maharani's
Science College for Women
(Autonomous) JLB Road,
Mysuru, Karnataka, India

K Girish
Associate Professor,
Department of Microbiology
Government College for
Women (Autonomous)
Mandya, Karnataka, India

Morphological and molecular identification of *Phomopsis azadirachtae* causing die-back of neem from different regions of Tamil Nadu, India

KN Divyashree and K Girish

DOI: <https://www.doi.org/10.33545/2664844X.2026.v8.i3f.1280>

Abstract

Die-back of neem is currently the most serious disease of neem causing considerable damage to the neem trees across India. A fungus *Phomopsis azadirachtae* Sateesh, Bhat and Devaki is the pathogen that causes die-back of neem. At present the disease can be seen in every neem grown areas of India. Die-back diseased neem twigs were collected from different geographical areas of Tamil Nadu. These twigs were used for isolation of the pathogen and its identification was done by observing colony characteristics and spores. Then for precise identification of the fungus PCR amplification with species-specific primers was employed. Fifteen fungal isolates that exhibited significant variations in the colony colour, radial growth and margin, mycelial type, texture were obtained from the sampled twigs. They were identified via morphological characteristics as *Phomopsis* sp. and via molecular identification (PCR) they were confirmed to be *Phomopsis azadirachtae*.

Keywords: *Azadirachta indica*, cultural characteristics, neem, PCR, *Phomopsis azadirachtae*, plant disease

Introduction

Globally phytopathogenic fungi cause severe problems in agriculture and forestry, mainly in the subtropical and tropical regions. Different parts of a plant such as roots, stems, leaves, flowers and fruits are infected by pathogenic fungi causing the symptoms like anthracnose, blights, spots, rots, wilts, etc. (Thilagam *et al.*, 2018) [12].

From ages neem (*Azadirachta indica*) tree has gained worldwide importance due to its diverse medicinal properties. Various parts of the neem tree such as the leaves, bark, fruit, flowers, oil, and gum have been used for the treatment of several medical problems like cancer, hypertension, heart diseases, and diabetes (Girish and Shankara Bhat, 2008a) [4]. A few fungi are known to cause diseases in nurseries and plantations of neem. Presently die-back disease is emerging as a prominent disease affecting neem in majority of the neem growing areas of India. Tamil Nadu ranks second in the number of neem trees and neem is a major economically important tree providing subsidiary income to the farmers. But die-back of neem is drastically reducing the availability of neem seeds and leaves that are the main plant parts of economical value (Girish and Shankara Bhat, 2008b; Girish *et al.*, 2009) [5, 8]. Die-back of neem (Fig. 1) is caused by *Phomopsis azadirachtae* Sateesh, Bhat & Devaki (Sateesh *et al.*, 1997) [9]. *P. azadirachtae* (Fig. 2) infects leaves, twigs and inflorescence of neem trees of all size, height and age (Fig. 1). Owing to quick and wide spread of the disease, there is an immediate need to develop effective controlling measures (Girish and Shankara Bhat, 2008b) [5]. Primarily it is important to isolate and precisely identify the pathogen before undertaking further research.

Corresponding Author:
K Girish
Associate Professor,
Department of Microbiology
Government College for
Women (Autonomous)
Mandya, Karnataka, India



Fig 1: a. Healthy Neem tree with green canopy; b. Die-back affected Neem tree showing twig blight symptom and loss of canopy

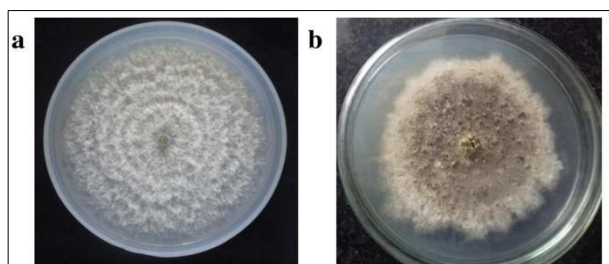


Fig 2: *Phomopsis azadirachtae* culture on potato dextrose agar (10-day-old; a) with pycnidia (15-day-old; b)

Consequently, present study was designed to isolate, characterize and identify the die-back of neem pathogen from several places of Tamil Nadu. Specific objectives include: (1) to isolate *P. azadirachtae* from diseased twigs collected from 15 districts of Tamil Nadu; (2) to characterize the fungus based on morphological characteristics and (3) to do precise identification of all the *P. azadirachtae* isolates using species specific primers.

Materials and Methods

Sites sampled

Twig samples from severely infected neem trees showing typical die-back symptoms were collected from 15 different agro-climatic regions of Tamil Nadu, South India like Dharmapuri, Salem, Namakkal, Tiruchchirappalli, Tanjavur, Thiruvavur, Ramanathapuram, Rameshwaram, Thiruchendhur, Kanniyakumari, Tirunelveli, Virudhunagar, Madurai, Palani, Coimbatore. The twigs were brought to the lab in polythene covers and used immediately or stored in refrigerator at 4°C until further studies.

Isolation of *Phomopsis* from infected neem twigs

Using sterile blades the diseased neem twigs were cut into 2 - 3 cm pieces having middle transition zone of healthy and infected portion and washed separately with running tap water. Then they were cut into 1 - 1.5 cm segments, with transition zone in the middle. Healthy neem twigs served as control. After removing the bark of twig segments, they were washed and surface-sterilized with sodium hypochlorite solution (with 5% available chlorine), followed by rinsing with sterile distilled water for five times. Four sterilized segments were then placed aseptically on each of the Petri plates with potato dextrose agar (PDA, 20 ml/plate) amended with 100 ppm of chloramphenicol. After inoculation plates were incubated at room temperature (RT,

26 ± 2°C) for 10 d and observed for the fungal growth from the plated twig segments. The incubation was extended up to 15 d and plates were observed for sporulation. The fungal spores were observed under microscope and based on their descriptions reported by Sateesh *et al.* (1997), the fungus was confirmed as *Phomopsis*. All the confirmed isolates were then sub-cultured on to fresh PDA plates by plating 5-mm mycelial-agar disc taken from the mycelial mat margin and were incubated similarly as above. Each isolate was maintained in triplicates (Girish and Shankara Bhat, 2011) [7].

Cultural characteristics

Each isolate was cultured on PDA plates by inoculating mycelial plugs from the growing margins of 7-day-old cultures and incubated for 10 d at RT (26 ± 2°C). The isolates were observed for their cultural characteristics including colony colour, its diameter, radial growth and margin, mycelial type and texture after incubation. After 15 d of incubation the cultures were observed for pycnidial production and the pycnidial ooze was stained and observed under microscope for the presence of alpha (α) and beta (β) - conidia (Fig. 3).

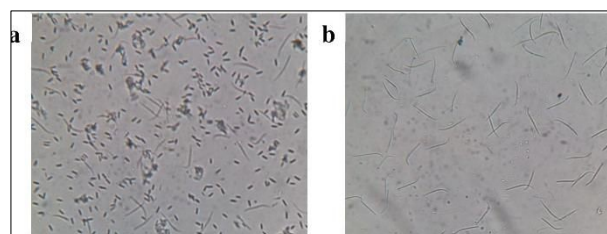


Fig 3: (a) Alpha (α) and (b) Beta (β) conidia of *Phomopsis azadirachtae*

Molecular identification of *P. azadirachtae*

Molecular identification was carried out at GeneSpy Research Services, Mysuru, India. Primers were procured from Sakhala enterprises, Bengaluru, India; Taq DNA polymerase from Sigma Aldrich, Bengaluru, India and all other chemicals from Merck BioSciences, Bengaluru, India. BioRad thermal cycler was used for PCR.

DNA isolation

5.0 mm diameter mycelial plug was inoculated in conical flasks (250 ml) having 100 ml of potato dextrose broth (PDB) and incubated at RT (26 ± 2°C) for 10 d. For DNA extraction, 100 mg of the dried mycelium was ground in mortar and pestle using liquid nitrogen following the procedure of Vedashree *et al.* (2015) [13]. DNA concentration, yield and quality based on absorbance at 260 and 280 nm (A260/280 ratio) were determined. Isolated DNA was stored at -20°C until further studies.

PCR amplification

DNA extracted from 13 Tamil Nadu isolates was used as template in separate tubes. A tube containing DNA isolated from Mysuru *P. azadirachtae* isolate served as a positive control and DNA from *Aspergillus niger* served as a negative control. The *P. azadirachtae* species-specific primer pair named pa-EF1 αF / pa-EF1 αR (5'-TATACTGCACCTCAAATCAT-3', 5'-GTTGTTTCACATGCATTGATC-3') reported to amplify a 240-bp product (Shirahatti *et al.*, 2015) [10] was used. PCR

was carried out using 50 µl reaction mixture containing: 50 ng DNA, 20 µM each dNTP, 2.5 U Taq DNA polymerase, 25 pMoles each of primers pa-EF1 αF / pa-EF1 αR and 1X PCR buffer with 1.5 mM MgCl₂. The thermal conditions were 95°C for 8 min as the initial step followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 30 s and a final extension at 72°C for 60 s (Shirahatti *et al.*, 2015) [10].

Another *P. azadirachtae* species-specific primer pair named, the forward primer PAF (5'- CCCTAGGGGGCCCCCTCGG-3') and the reverse primer PAR (5'-AGGGCCTGCTTTTGGGT-30') reported to amplify 313 bp product (Vedashree *et al.*, 2015) [13] was also employed for identification. PCR was performed in 50 µl reaction volume consisting of 1 µl of template DNA, 5 µl 10⁹ PCR buffer, 1 µl 2.0 mM dNTPs, 1 µl each of 50 pM primers PAF and PAR, and 0.25 µl (1 U) Taq DNA polymerase and 40.75 µl sterile distilled water. The amplification regime was initial denaturation at 95°C for 3 min, followed by 35 cycles consisting of denaturation at 95°C for 30s, annealing at 60°C for 45s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min (Vedashree *et al.*, 2015) [13].

Results

Isolation of *Phomopsis* from infected neem twigs

Fungal pathogen causing die-back of neem was isolated from all the infected neem twig samples gathered from 15 different places of Tamil Nadu. All the isolates were labelled as in table 1. The isolates differed significantly in cultural characteristics such as mycelial growth, colony colour, colony diameter, radial growth and margin, colony texture (Fig. 4). All the isolates produced characteristic alpha (α) and beta (β) - conidia (Table 1).

Molecular identification of *P. azadirachtae*:

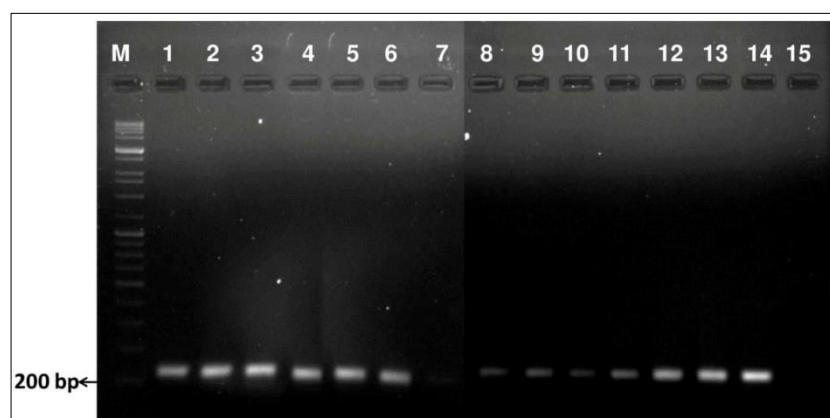
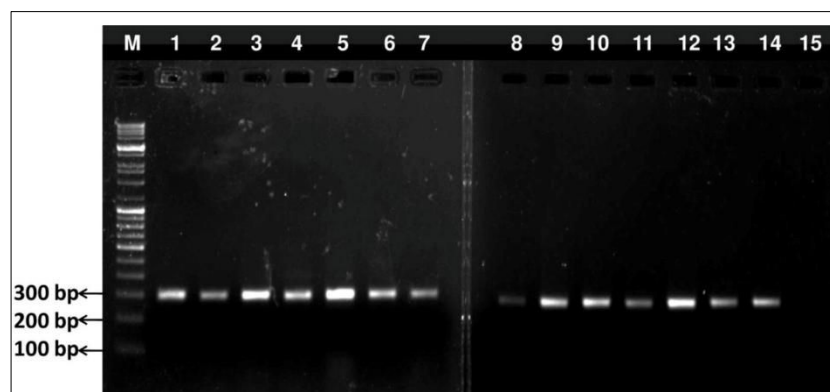
The primer pairs pa-EF1 αF / pa-EF1 αR amplified DNA obtained from the mycelia of all 13 Tamil Nadu isolates and also Mysuru isolate (positive control), generating a expected size PCR product of 240 bp. DNA of *Aspergillus niger* (negative control) was not amplified (Fig. 5). The PCR assay using PAF / PAR primers produced an expected sized band of 313 bp in all the 13 Tamil Nadu isolates and Mysuru isolate but not in the *A. niger* (Fig. 6). The results confirmed that all the fungal isolates obtained from die-back infected neem twigs sampled from different districts of Tamil Nadu were *Phomopsis azadirachtae*.



Fig 4: 1-15 10-day-old *Phomopsis azadirachtae* isolates from different places of Tamil Nadu on potato dextrose agar plates.

Table 1: Isolate numbers used for various Tamil Nadu isolates of *Phomopsis azadirachtae* with place of isolation

Isolate numbers	Geographical location of isolation	Production of alpha (α) and beta (β) - conidia
TN-Pa01	Dharmapuri	+
TN-Pa02	Namakal	+
TN-Pa03	Salem	+
TN-Pa04	Thiruchirapalli	+
TN-Pa05	Thanjavur	+
TN-Pa06	Thiruvavur	+
TN-Pa07	Ramanathapuram	+
TN-Pa08	Rameshwaram	+
TN-Pa09	Thiruchendur	+
TN-Pa10	Kanniyakumari	+
TN-Pa11	Tirunelveli	+
TN-Pa12	Virudhunagar	+
TN-Pa13	Madurai	+
TN-Pa14	Palani	+
TN-Pa15	Coimbatore	+

**Fig 5:** Amplification of *Phomopsis azadirachtae* isolates using the pa-EF1 aF /pa-EF1 aR primers yielding 240 bp band. Lane M: 10kb DNA ladder; 1. Dharmapuri (TN-Pa01); 2. Namakal (TN-Pa02); 3. Salem (TN-Pa03); 4. Thiruchirapalli (TN-Pa04); 5. Thanjavur (TN-Pa05); 6. Ramanathapuram (TN-Pa07); 8. Rameshwaram (TN-Pa08); 9. Thiruchendur (TN-Pa09); 10. Kanniyakumari (TN-Pa10); 11. Virudhunagar (TN-Pa12); 12. Madurai (TN-Pa13); 13. Palani (TN-Pa14); 14. Coimbatore (TN-Pa015); 7 & 15. *Aspergillus flavus***Fig 6:** Amplification of *Phomopsis azadirachtae* isolates using the PAF / PAR primers yielding 313 bp band. Lane M: 10kb DNA ladder; 1. Mysuru isolate; 2. Dharmapuri (TN-Pa01); 3. Namakal (TN-Pa02); 4. Salem (TN-Pa03); 5. Thiruchirapalli (TN-Pa04); 6. Thanjavur (TN-Pa05); 7. Ramanathapuram (TN-Pa07); 8. Rameshwaram (TN-Pa08); 9. Thiruchendur (TN-Pa09); 10. Kanniyakumari (TN-Pa10); 11. Virudhunagar (TN-Pa12); 12. Madurai (TN-Pa13); 13. Palani (TN-Pa14); 14. Coimbatore (TN-Pa015); 15. *Aspergillus flavus*

Discussion

In the present investigation the die-back causing fungi has been isolated from diseased twigs collected from die-back infected neem trees from all the sampled districts of Tamil Nadu. Isolation of the pathogen was done using tissue from the diseased neem twigs and its identification was done by studying colony and microscopic morphology characteristics. Based on mycelial morphology, pycnidia production and production of conidia (alpha and beta), all

the isolates were identified as *Phomopsis* spp. Studying morphology is a primary step in identification and classification of pathogenic fungi at the genus level (Sunpapao, 2022) ^[11]. There are limitations for both morphological and biological species concepts as alpha and beta conidia are produced by all the species of *Phomopsis*. Isolates of the *Phomopsis* spp. obtained from different districts of Tamil Nadu displayed significant morphological variability. *Phomopsis* spp., including *P. azadirachtae* are

reported to have morphological variability (Fathima *et al.*, 2004; Girish and Shankara Bhat, 2010) [3, 6]. For the species level identification of plant pathogenic fungi, the DNA sequences data have been used in addition to morphology. The polymerase chain reaction (PCR) is the most important and sensitive technique presently available for the identification of plant pathogens up to species level. Initially primers PF1 and PF2 (Girish *et al.*, 2009) [8] were employed for amplification that produced the desired 154 bp product in PCR. However, Vedashree *et al.* (2015) [13] have shown that the above primers though amplified *P. azadirachtae* DNA, they also amplified DNA from other species of *Phomopsis* i.e. these primers are not species specific.

Owing to this, to identify the *Phomopsis* isolates from Tamil Nadu precisely at species level, species specific primers reported such as pa-EF1 α F / pa-EF1 α R (Shirahatti *et al.*, 2015) [10] and PAF / PAR (Vedashree *et al.*, 2015) [13] were employed. The PCR assay produced a 240-bp PCR product and 313-bp PCR product respectively from all the Tamil Nadu isolates of *Phomopsis* confirming them as *P. azadirachtae*. PCR has now become a highly dependable molecular technique for precise identification of fungal pathogens by amplification of conserved regions of their genome (Chakraborty and Chakraborty, 2021) [2]. Identification based on morphology can be useful for genus level identification, but accurate species identification requires molecular data (Bhunjun *et al.*, 2021) [1]. Precise identification of fungal pathogens is crucial to understand the distribution of the pathogen and its potential risk as well as for effective management of the pathogen (Bhunjun *et al.*, 2021) [1].

Conclusion

The results of present study indicates that die-back of neem caused by *Phomopsis azadirachtae* is disseminating at very fast rate and is present in all the neem growing areas of Tamil Nadu, India.

Acknowledgements

The authors sincerely thank Science and Engineering Research Board (SERB), New Delhi for their financial support to carry out the research work (SERB No. SB/FT/LS-416/2012; OYS scheme) and Postgraduate Department of Microbiology, Maharani's Science College for Women, Mysuru for providing laboratory facility.

References

1. Bhunjun CS, Phillips AJL, Jayawardena RS, Promptutha I, Hyde KD. Importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's postulates. *Pathogens*. 2021;10(9):1096. doi:10.3390/pathogens10091096.
2. Chakraborty BN, Chakraborty U. Molecular detection of fungal pathogens and induction of phytoimmunity using bioinoculants. *Indian Phytopathol*. 2021;74:307-322. doi:10.1007/s42360-021-00351-1.
3. Chowdappa P, Brayford D, Smith J, Flood J. Molecular discrimination of *Phytophthora* isolates on cocoa and their relationship with coconut, black pepper and bell pepper isolates based on rDNA repeat and AFLP fingerprints. *Curr Sci*. 2003;84:1235-1238.
4. Fathima SK, Shankara Bhat S, Girish K. Variation in *Phomopsis azadirachtae*, the incitant of die-back of neem. *Indian Phytopathol*. 2004;51:30-33.
5. Girish K, Shankara Bhat S. Neem - a green treasure. *Electron J Biol*. 2008;4(3):102-111.
6. Girish K, Shankara Bhat S. *Phomopsis azadirachtae* - the die-back of neem pathogen. *Electron J Biol*. 2008;4(3):112-119.
7. Girish K, Shankara Bhat S. Cultural, morphological and biochemical variability among isolates of *Phomopsis azadirachtae* from Tamil Nadu. *J Mycol Plant Pathol*. 2010;40:395-401.
8. Girish K, Shankara Bhat S. Physiological variability among isolates of *Phomopsis azadirachtae* from Tamil Nadu. *J Yeast Fungal Res*. 2011;2:65-74.
9. Girish K, Shankara Bhat S, Raveesha KA. PCR-based detection of *Phomopsis azadirachtae* in die-back affected neem seeds. *Arch Phytopathol Plant Prot*. 2009;42:626-632.
10. Raeder U, Broda P. Rapid preparation of DNA from filamentous fungi. *Lett Appl Microbiol*. 1985;1:17-20.
11. Sateesh MK, Shankara Bhat S, Devaki NS. *Phomopsis azadirachtae* sp. nov. from India. *Mycotaxon*. 1997;65:517-520.
12. Shirahatti PS, Ramu R, Purushothama CRA, Prasad MNN. Development of a simple and reliable species-specific detection of *Phomopsis azadirachtae* using the translation elongation factor 1-alpha gene. *Eur J Plant Pathol*. 2015;141(4):769-778. doi:10.1007/s10658-014-0577-4.
13. Sunpapao A, Suwannarach N, Kumla J, Dumhai R, Riangwong K, Sanguansub S, *et al.* Morphological and molecular identification of plant pathogenic fungi associated with dirty panicle disease in coconuts (*Cocos nucifera*) in Thailand. *J Fungi (Basel)*. 2022;8(4):335. doi:10.3390/jof8040335.
14. Thilagam R, Kalaivani G, Hemalatha N. Isolation and identification of phytopathogenic fungi from infected plant parts. *Int J Curr Pharm Res*. 2018;10:26. doi:10.22159/ijcpr.2018v10i1.24404.
15. Vedashree S, Sateesh MK, Chowdappa P, Nirmalkumar BJ. Species-specific PCR-based assay for identification and detection of *Phomopsis* (*Diaporthe*) *azadirachtae* causing die-back disease in *Azadirachta indica*. *J Phytopathol*. 2015;163:818-828.