Molecular detection of *Phomopsis azadirachtae*, the causative agent of dieback disease of neem by polymerase chain reaction

Neem (*Azadirachta indica* A. Juss.), commonly called ‘Indian lilac’, has a history of its own regarding its diversified and unique advantages to mankind. These include excellent insect-control activities and medicinal properties1-3. Intensive search during the past decade for a safer insecticide has resulted in the identification of neem as a better alternative to toxic pesticides due to its biodegradability and low toxicity. Neem showed control activities against as many as 250 insect species5.

Neem trees are now under great threat due to destructive dieback disease caused by a mitosporic fungus, *Phomopsis azadirachtae* (Figure 1)4-6. The disease is spreading at an alarming rate in different parts of southern India. Isolation and identification of the causative agent by conventional methods require at least 2-3 weeks. Sensitive techniques efficient in detecting low number of pathogen propagules are required. Molecular diagnostic tools such as nucleic acid probes are useful for such tasks5,6. Isolation of nucleic acids and construction of alien nucleic acid probes, help detect the disease-causing agent quickly and more precisely. Here we report a method to detect *P. azadirachtae* using polymerase chain reaction (PCR).

Five isolates of *P. azadirachtae* (Figure 2a) and one isolate of *F. moniliforme* grown on malt extract agar (MEA) were used for the study. *Phomopsis* isolates (1-5) were obtained from diseased neem trees of Krishnaraja Nagur (Karnataka), Mysore (Karnataka), Amravati (Maharashtra), and Virudhunagar (Tamil Nadu) respectively. DNA preparations were made from the isolates following the procedures of Zhang et al.5 with a slight modification. Pure cultures of fungi (about 200 mg) were grown in microfuge tubes with blunt end of disposable pipette tips and 700 μl of extraction buffer (0.13 M Tris-HCl, pH 8.9; 0.017 M EDTA pH 7.0; 0.83% SDS, 5% PVP and 1 M LiCl) and incubated at 65°C (15 min) followed by vortexing several times. The supernatant was treated with 700 μl mixture of phenol:chloroform (1:1) and vortexed (1 min) and centrifuged (2000 rpm, 8 min). DNA was re-extracted with an equal volume of ice-cold 99.5% isopropanol, incubated at −20°C (60 min), centrifuged (8000 rpm, 8 min) and the pellet was rinsed with 80% ethanol, air-dried, resuspended in 40 μl of nucleic acid-free water and used directly for PCR. Similarly, DNA was isolated from *F. moniliforme* and used as control. Primers were designed by multiple sequence alignment of 5.8S rDNA of *Phomopsis* spp. using CLUSTAL program. Conserved sequences were selected and the forward and reverse primers were designed to get a 141-bp DNA as amplified product. Primers were synthesized and obtained from Bangalore Genei, India. The two primers used were Phf-5’ CGGATCTCTTGGTCTTGCA-3’ and Phr-5’ GACGCTCGACACCGCAGTGC-3’

PCR was performed using Advanced Thermus 25 thermocycler (Peqlab, Germany). PCR mixture (50 μl) was prepared with 50 ng of DNA, 0.2 μM of each primer, 200 μM of each dNTP, 1× PCR buffer, 10 mM MgCl₂, 0.5 unit of Taq polymerase and distilled water to a final volume of 50 μl. The program consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The 141-bp band was visualized on a 1% agarose gel.

**Figure 1.** a. Dieback disease of neem showing dying of twigs from the top. b. Symptoms on newly emerged twigs.

**Figure 2.** a. *Phomopsis azadirachtae* pure culture showing mycelia with dark-coloured fruit bodies at the centre. b. Agarose gel showing amplified products of 141 bp of isolates *P. azadirachtae*. Lane M, 100 bp DNA ladder; lanes 1–5 *Phomopsis azadirachtae* isolates; lane 6, *F. moniliforme*. 
pared with 2 μl of DNA sample with 10X PCR buffer, 2.5 mM MgCl2, 200 μM dNTPs, 20 pmol of each forward and reverse primer and 1.5 U of Taq DNA polymerase (all reagents were from Fermentas, Canada). The PCR conditions include: 95°C (5 min) for initial denaturation, followed by 30 cycles of denaturation at 94°C (1 min), and primer annealing at 59°C (1 min), primer extension at 72°C (1 min). The amplified PCR products were separated on 1.5% agarose containing 4 μl of ethidium bromide, visualized and documented in a Biorad UV Transilluminator.

Results of the PCR analysis are presented in Figure 2b. The expected 141-bp amplified DNA product was detected in all the isolates of P. azadirachtae irrespective of geographic location (lanes 1–5). On the other hand, F. moniliforme did not show any amplified product, indicating that primers used in the study were genus-specific and could amplify only 5.8S rDNA of Phomopsis. Irrespective of the differences in cultural characteristics, different isolates of P. azadirachtae are similar in 5.8S rDNA. Similar studies have been conducted based on transcri-