Box 1. *Melia dubia* is a money-spinning tree in a short span: a case study

A retired teacher of higher secondary school planted *Melia dubia* as a shade tree at 5 × 5 m in dragon fruit on five acres in 2017 at Tembhurni, Solapur districts of Maharashtra. During harvesting, a team of ICAR-NIASM, Baramati visited the field and recorded observations on component-wise fresh biomass. The biomass of trees ranged from 97.5 to 405.4 kg per tree with an average of 259.34 kg per tree after 4.5 years. The farmer sold it to the plywood industry in Gujarat at an average rate of Rs 6700 per tonne (on a fresh weight basis and above 12-inch girth). The average commercial bole biomass was 83 tonnes per ha and earned a gross income of Rs 556,100 per ha. Nowadays, for quick return, farmers usually cut trees at an early age, but if trees can be cut after 6 to 8 years will give higher biomass and profitability.

an average yield of 75 t/ha. Those linked with paper industry get assured gross income of Rs 337,500 per ha by selling @ Rs 4500 per tonne. As per the study carried out by AICRP on Agroforestry at FC&RI, Mettupalayam, *M. dubia* based agroforestry system provides a net benefit of Rs 1,193,060 per ha with benefit : cost ratio of 1 : 3.94 @ 15% discounting rate, net present value of Rs 484,083 per ha and an internal rate of return of 79%[6]. Table 3 shows the economic feasibility of *M. dubia*-based agroforestry system in India (Box 1).

Melia-based agroforestry system has greater potential to meet the ever-rising demand of wood-based industries. Moreover, it has the potential to reverse land degradation, sequester carbon and recover site productivity. Emphasis on systematic production for its multivarious uses and proper marketing chain in *M. dubia* will ensure income and livelihood opportunities in a sustainable way.

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Development of a sensitive and single-step PCR-based assay for detection of sandal spike phytoplasma†

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***Santalum album* commonly known as Indian sandalwood, is extensively exploited due to its demand in the international market for essential oil. In the Indian subcontinent, the species is approaching commercial extinction due to overexploitation and sandal spike disease caused by phytoplasma. Molecular detection**

†The sequence data have been submitted to GenBank (accession number MT745881).

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using PCR and RFLP is reported for disease diagnosis. In the present study, a single-step, stringent PCR-based assay was developed to detect the sandal spike phytoplasma. The titre estimation revealed that the marker maintained detection sensitivity up to 0.8 ng of template DNA. Subsequent to validation of the marker in a larger number of symptomatic individuals, this diagnostic assay can be routinely used to detect the pathogen. It will facilitate early detection and enable plantation managers to take adequate measures to control the spread of the disease.

Keywords: Control measures, diagnostic markers, nested PCR, pathogen, phytoplasma, sandal spike disease.

SANTALUM album L., commonly known as Indian or white sandalwood, is a slow-growing, hemi-root parasitic tree belonging to the family Santalaceae. Sandalwood is one of the major commodities in international trade for its use in fragrance and essential oil industries. The essential oil content in the heartwood of *S. album* is the highest (6–7%) compared to other species, demonstrating its global annual demand of 100–120 million tonnes of oil. The threat to this species in India has reached a critical level due to overexploitation, illegal harvesting, monopoly in trade, poor germination, lack of established plantations, habitat loss and incidence of spike disease[1]. The species is considered to be approaching commercial extinction, widening the gap between supply and demand of essential oil. Several diseases are reported to affect sandal populations. However, the most devastating disease causing extensive economic loss is the sandal spike disease (SSD) caused by phytoplasma belonging to the class Mollicutes[2]. In southern India, the disease was first reported in Coorg, Karnataka, and subsequently from other states of peninsular India[3]. It is transmitted through phloem-feeding insect vectors, including plant hoppers, leaf hoppers and psyllids. Several control measures have been undertaken with limited success. The most effective strategy reported was the deployment of insect vector-resistant trees through breeding and genetic manipulations[4]. Considering the economic loss of sandalwood caused by the phytoplasma, microscopy, ELISA and conventional two-step nested PCR-based early detection methods have been reported[5–8]. In the present study, a single-step, stringent PCR method is presented for detection of SSD. The efficacy of the protocol was compared with the conventional two-step nested, PCR recommended by International Standard for Phytosanitary Measures (ISPMs).

Leaf samples from four asymptomatic/uninfected (10°14′55.8″N, 77°07′0.5″E) and three symptomatic/infected (10°16′19.1″N, 77°09′16″E) individuals of *S. album* were collected from Marayoor, Kerala, India. Additionally, genomic DNA was isolated from 35 uninfected/asymptomatic individuals collected from seven natural populations of sandals to test for the presence of phytoplasma (Table 1). Scanning electron microscopic (SEM) study was conducted using the method of Poghosyan and Rosales[9] for each symptomatic/infected and asymptomatic/uninfected leaf sample. The processed samples were coated with gold in an ion sputter and viewed under SEM (FEI, Quanta 200, Thermo Scientific, USA) at an accelerating voltage of 15 kV. Genomic DNA was isolated using Arbor Easy® DNA isolation kit (Institute of Forest Genetics and Tree Breeding, Coimbatore, India) and quantified using Nanodrop ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, USA). Three primer pairs, viz. SSDI: forward – 5′AAACTCTAGTGGCGAACGGG3′, reverse 5′ATTTAACCGCCTACGCACCC3’, SSDII: forward – 5′GAAACTCTGACCGAGCAACG3′, reverse – 5′CCTGGTGAGGTTTTTCGGGT3′, and SSDIII: forward – 5′GCTGGGTCTTTACTGACGCT3′, reverse –5′AGACGGTTCCCTCTTCTTGC3′ were designed from the 16S rDNA region of sandal spike phytoplasma (accession number EF050071.1)[10]. PCR was conducted in DNA isolated from an infected sample with all three primer pairs. 10 µl of PCR reaction contained 100 ng DNA, 10× *Taq* buffer with 3 mM $MgCl_2$, 400 µM dNTPs, 200 nM of each primer and 1 U *Taq* DNA polymerase (Thermo Fisher Scientific, USA). The PCR reaction consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2 min, with final extension for 10 min. The diagnostic assay was designed with SSDI (selected as the marker to detect SSD) and an internal control SSES2: forward – 5′CAGGATCATGCGAGAAAACG3′, reverse – 5′TCCTCACCTTCCCAGCCTAA3′, designed from sesquisabinene B synthase 2 (ref. 11). The assay was conducted in three symptomatic/infected and asymptomatic/uninfected samples using the above-mentioned PCR conditions. This single-step diagnostic assay was compared for its efficacy with the two-step conventional nested PCR recommended by the International Plant Protection Convention (IPCC). The assay was conducted at annealing temperatures of 53°C (P1/P7 primers) and 50°C (R16F2n/R16R2 primers) with product sizes of 1800 and 1250 bp respectively[8]. For the estimation of detection sensitivity, PCR reactions were performed with internal control and diagnostic marker at different concentrations (40–0.1 ng) of DNA from the infected sample following the same PCR conditions described above. The amplified products were analysed in 1% agarose gel and documented.

The phytoplasma infection elicited an array of symptoms in the infected leaves like short internodes, reduction in leaf size, thickening of petioles and bunchy, spike-like appearance due to stiffness of leaves (Figure 1 *a*). The infected and uninfected leaves were processed for SEM analysis. The electron photomicrographs of the infected leaves documented the presence of phytoplasma in clusters, while these structures were absent in healthy, uninfected tissues (Figure 1 *b*). Genomic DNA isolated from the infected leaf tissues showed amplification in all the

Table 1. Natural populations of sandalwood used for the diagnostic assay

Disease status of the tree	Number of individuals collected	Location	State	Latitude	Longitude
Asymptomatic	5	Bargur	Tamil Nadu	11°46′33.5	77°33′32
Asymptomatic	5	Chitteri	Tamil Nadu	11°45′26.5	78°13′36.9
Asymptomatic	5	Agali	Kerala	11°10′17.47	76°37′39.92
Asymptomatic	5	Mysore	Karnataka	12°20′59.9	76°32′03.7
Asymptomatic	5	Shivmogga	Karnataka	13°43′4.2	75°27′15.5
Asymptomatic	5	Koraput	Odisha	18°49′26.7	82°42′34.3
Asymptomatic	5	Seoni	Madhya Pradesh	22°52′5.9	79°40′21.3

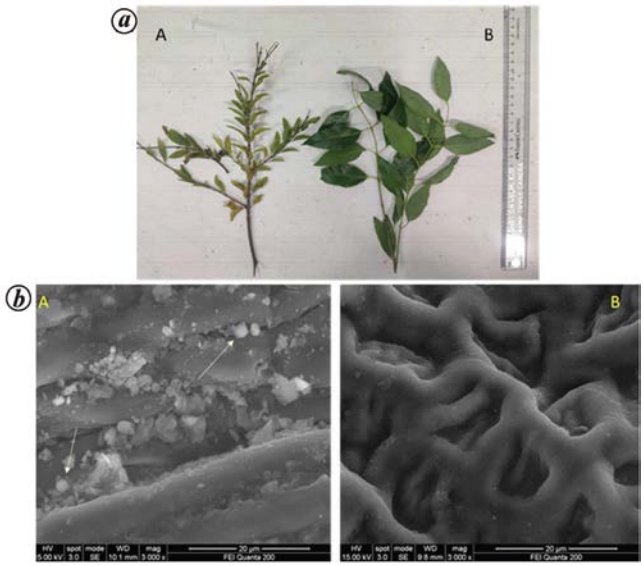

Figure 1. ***a***, Symptoms of sandal spike disease observed in the sample collected from Marayoor, Kerala, India. A, Phytoplasma infected leaves. B, Uninfected leaves. ***b***, Scanning electron photomicrographs of (A) infected and (B) uninfected leaves at 3000× magnification. Arrows indicate presence of phytoplasma.

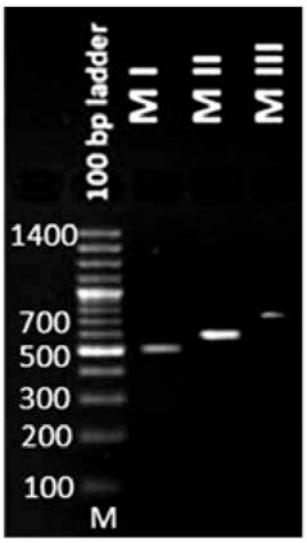

Figure 2. Amplification of primer pairs (SSD I, SSD II and SSD III) in symptomatic/infected leaves collected from Marayoor. Table 1 gives details of the primer sequences. M, 100 bp ladder.

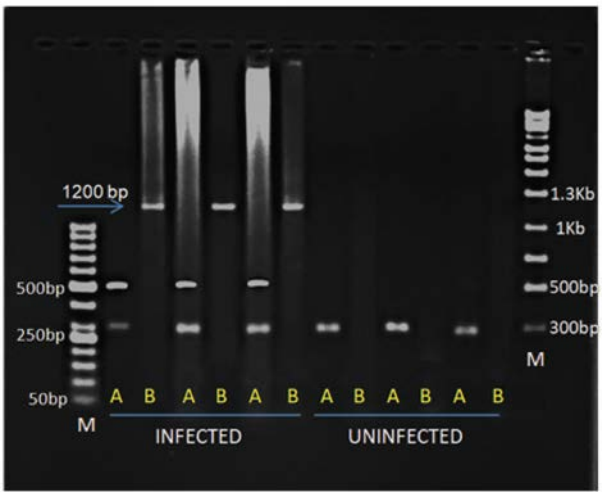

Figure 3. Single-step PCR of the diagnostic marker SSDI and internal control SSES 2 and its comparison with the conventional two-step nested PCR recommended by International Plant Protection Convention (IPPC) in three symptomatic/infected samples. (A) Diagnostic assay developed in the present study. (B) Two-step nested PCR recommended by IPPC. M, 50 bp and 1 kb ladder.

three primer pairs, viz. SSD I, SSD II, SSD III at 500, 600 and 700 bp respectively (Figure 2). The products were sequenced and the concatenated sequence of 1.3 kb was submitted to NCBI with GenBank accession number MT745881. In the uninfected samples, no amplification was observed in SSDI, while SSD II and SSD III showed amplification at 600 and 700 bp respectively (data not shown), indicating the specificity of SSD I to sandal spike phytoplasma. Hence SSD I was used for subsequent studies.

Single-step PCR (SSD I and SSES2) was conducted along with conventional PCR (P1/P7 and R16F2n/R16R2) in three samples, each sourced from infected and uninfected individuals. In the infected samples, both primer pairs showed amplification at 300 and 500 bp respectively, while in samples from uninfected trees amplification of the internal control at 300 bp was documented. In conventional PCR, amplification was recorded only in infected individuals at 1.25 kb (Figure 3). Additionally, the single-step PCR assay along with conventional nested PCR was conducted on five individuals, each representing seven natural populations of sandalwood. Amplification

of internal control was documented in all samples, while no amplification was recorded using conventional PCR, indicating the absence of phytoplasma in these samples (Figure 4). The detection sensitivity of the marker was estimated at different concentrations of template DNA. Amplification of the diagnostic marker SSD I was detected up to 0.8 ng DNA concentration without any decrease in detection sensitivity (Figure 5). This indicated that the diagnostic assay could be used to screen the phytoplasma at a very low titre.

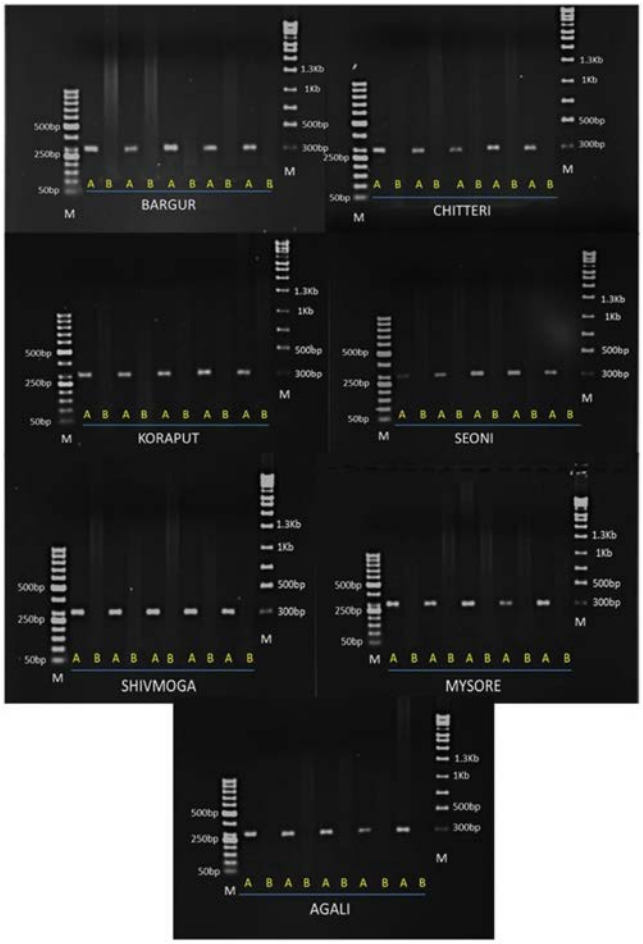

Figure 4. Single-step PCR and conventional nested PCR in seven natural populations of sandalwood. Lane 1, 50 bp ladder; lane 12, 1 kb ladder; lane A, Single-step PCR with SSD 1 (diagnostic marker) and SSES 2 (internal control) in uninfected samples. Amplification documented at 300 bp and lane B, Conventional nested PCR in uninfected samples. No amplification was recorded in the uninfected samples. Table 1 gives details of the populations.

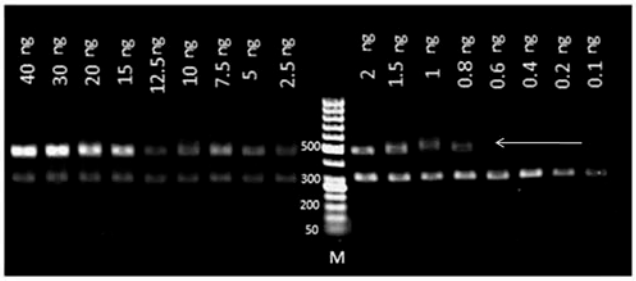

Figure 5. Sensitivity assay of the diagnostic marker at different concentrations of DNA isolated from infected host tissues. Arrow indicates amplification of the diagnostic marker at 500 bp. M, 50 bp ladder.

Phytoplasmas are non-helical prokaryotes associated with several diseases in plant species, including economically important crops and forest trees[12–14]. These prokaryotes are generally not amenable to *in vitro* culture, and hence molecular methods are used to diagnose and differentiate different sub-groups, which include PCR, real-time PCR and loop-mediated isothermal amplification[8,15–17]. Detection of SSD in sandal tissues was also done using the above-mentioned two-step nested PCR at low annealing temperatures of 48°C, 50°C and 55°C, increasing the chances of cross-amplification and false positives[2,18–20]. In the present study, a single-step PCR is proposed with a higher annealing temperature (62°C) to increase the stringency of the assay. An internal control has been included in the assay to ascertain the DNA quality and successful PCR amplification. Further, the primer pair (SSD I) designed to diagnose the presence of the phytoplasma spanned the region of the 16S rDNA with the least sequence similarity to rRNA of viridiplantae, reducing the probability of cross-amplification of sandal rRNA regions. Comparative analysis of the present protocol with conventional nested PCR reiterates its efficacy in detecting phytoplasma at a very low titre. Hence the diagnostic assay proposed in the present study is comparable to the conventional two-step nested PCR recommended by IPPC. This study reports a stringent, single-step, highly sensitive PCR assay which can accurately detect the presence of sandal spike phytoplasma. Detection of the pathogen and diseased tree at an early stage will facilitate plantation managers and State Forest Departments to undertake adequate measures to control the spread of the disease.

Conflicts of interest: The authors declare that they have no conflict of interest.

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