Detection of sandal spike phytoplasma by polymerase chain reaction

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Spike disease affected sandal (*Santalum album* L.) tissues were screened for the presence of the pathogen, a non-culturable phytoplasma using polymerase chain reaction (PCR) technique. Oligonucleotide primers specific to the conserved region of 16S rRNA gene were used to amplify a 558 bp sequence of the phytoplasma. Four DNA fragments were obtained when the PCR products after 20 cycles of amplification were subjected to restriction fragment length polymorphism analysis (RFLP) with *Alu*I restriction endonuclease. The technique confirms that sandal spike phytoplasma belongs to group I of the eleven major phytoplasma groups.

SPIKE disease, the major disease in sandal (*Santalum album* L.), is caused by a non-culturable phytoplasma seen exclusively in the phloem tissues[1]. Detection of the pathogen was mainly through electron microscopy[2–4] or indirect methods using different stains like aniline blue[5] and Giemsa[6] stain by light microscopy. Recently, the DNA binding fluorochrome, 4,6 diamidino-2-phenylindole (DAPI) was employed for the detection of the pathogen[7]. Since phytoplasmas are associated with diseases of many plant species[8], neither of these methods allows the differentiation of the organism and its classification[9].

Studies of DNA homology in the highly conserved genes encoding ribosomal RNA and ribosomal protein have shown that phytoplasmas comprise a coherent set distinct from other prokaryotes[10,11]. A system for classification of phytoplasma based on amplification of the 16S rDNA by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) was introduced by Ahrens and Seemuller[9]. Since then, several groups of phytoplasmas have been differentiated on the basis of nucleotide sequence variation in 16S rRNA genes[11–13]. Sinclair *et al.*[14] have classified phytoplasmas into eleven major taxonomic groups based on the 16S RFLP fragments. In this paper we report the detection of phytoplasma from spike disease affected sandal by PCR using group specific primer followed by RFLP analysis.

Spike disease affected sandal was collected from Marayoor, Munnar Forest Division, Kerala and Chamundi Hills, Mysore, Karnataka. Total DNA samples from healthy and diseased plants were extracted following the modified protocol of Doyle and Doyle[15]. One gram tissue of midrib and young stem was ground into fine powder using liquid nitrogen. Two ml of hot (65°C) cetyl trimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris, pH 8.0; 20 mM EDTA, pH 8.0; 1.4M NaCl) was added to the powder and samples incubated at 65°C for 1 h. The samples were extracted with chloroform/isoamyl alcohol (24:1) followed by low speed centrifugation (1600 g) for 5 min and aqueous phase eluted out. To this was added 1/10 volume 3M sodium acetate (pH 5.2) followed by the addition of 2 volumes of cold (–20°C) absolute alcohol. After incubation at –20°C for 12 h, the DNA precipitate was centrifuged for 10 min at low speed. Ethanol (95%) was added to the DNA pellet and recentrifuged at the same speed. The supernatant was discarded, air dried and the pellet dissolved in 100 µl sterile distilled water. The total DNA was estimated at A_{260} (1 O.D = 50 µg ml^{-1}).

The method followed by Ahrens and Seemuller[9] that allowed the amplification of a 558 bp fragment of the 16S rRNA gene of phytoplasma was adopted for PCR with slight modification. The primers were designed from the conserved regions of the 16 S rRNA gene of O-MLO[16] located between 759 and 1359 bp. The sequence of the forward primer is 5′-ACGAAAGCGTG-GGGAGCAAA-3′ and the reverse primer is 5′-GAAGTCGAGTTGCAGACTTC-3′. A total volume mixture of 50 µl contained 1 µl of test DNA (200 ng), 1 µl of each primer (Bangalore Genei, India), 2.5 mM each of four dNTPs, 1 µl (3 units) of *Taq* polymerase (Bangalore Genei, India) or Dynazyme II (Finnzymes Oy, Finland) and 5 µl *Taq* buffer or Dynazyme buffer. The mixture was covered with two drops of mineral oil and subjected to 20 amplification cycles (PTC-150 Minicycler, MJ Research, USA) each of 30 s denaturation (95°C), 30 s annealing (55°C) and 30 s extension (72°C). The final extension step was for 5 min.

The PCR amplification products obtained from DNA of healthy and diseased sandal after 20 cycles were electrophoresed in 1.5% horizontal agarose (Sigma, USA) gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide and bands compared with a 100 bp DNA ladder (Bangalore Genei, India). 15 µl of the reaction mixture obtained using *Taq* polymerase was digested with 1 µl of undiluted *Alu*I (Bangalore Genei, India) following manufacturer's instruction at 37°C for 12 h. 10 µl of the digest was used to resolve the restriction fragments on a 3% horizontal agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), and the bands were visualized after staining with ethidium bromide. The gels were documented using Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak, USA).

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After 20 amplification cycles, the DNA fragment of 558 bp was amplified from samples which contained DNA extracted from diseased sandal but not from healthy samples or water control. Bands were visualized for both *Taq* polymerase and Dynazyme II polymerase (Figure 1). On comparing the spike diseased tissues from Kerala and Karnataka, the bands appeared to be identical (Figure 2). Restriction analysis of the amplified fragment with *Alu*I revealed the presence of a pattern characterized by restriction sites at position a, b, and c with fragments of 240, 191, 71 and 56 bp (Figure 3 *a*, *b*), as described by Ahrens and Seemuller[9] for the first group of phytoplasmas.

Since phytoplasma cannot be cultured under axenic conditions, their identities and taxonomic position were unclear or uncertain until recently, when methods of molecular genetics were introduced into plant mycoplasmology[17]. The 16S rRNA gene, is a universal character which provides valuable phylogenetic and taxonomic information on culturable mollicutes and other prokaryotes[9,10,13]. Hence, this gene has widely been used to obtain phylogenetic information on non-culturable phytoplasmas. RFLP analysis of the amplified PCR product (rDNA) could distinguish various groups and subgroups of phytoplasma[17].

PCR method of detecting phytoplasmas gained popularity after the classical work of Ahrens and Seemuller[9]. The primers used were designed from the conserved regions of the 16S rRNA gene of the *Oenothera* phytoplasma (O-MLO). Phytoplasmas infecting several plants collected from different continents maintained in *Catheranthus roseus* for several years were used for their study. The mollicutes have a high mutation rate resulting in many totally unique oligonucleotides[18]. In our study, we used young stem and leaf midrib tissues from diseased and healthy sandal for isolating DNA, since the greatest yields of DNA will always be obtained using the youngest, freshest tissue. The liquid nitrogen

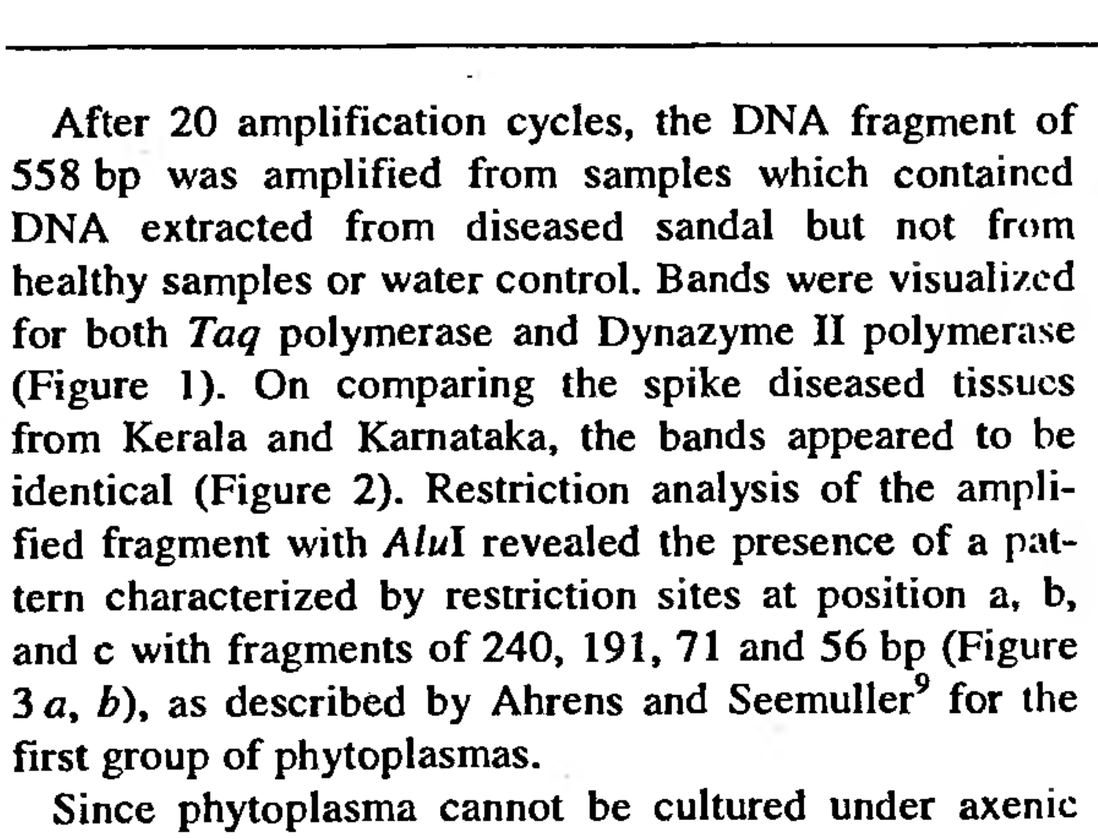

Figure 1. Agarose gel electrophoresis of polymerase chain reaction product (16S rDNA fragment –558 bp) of sandal spike phytoplasma after 20 cycles of amplification using both *Taq* polymerase (lanes 2–4) and Dynazyme II (lanes 5–7). Lane 1, 100 bp ladder; lanes 2 and 5, healthy sandal; lanes 3, 4, 6 and 7, diseased sandal; lane 8, water control.

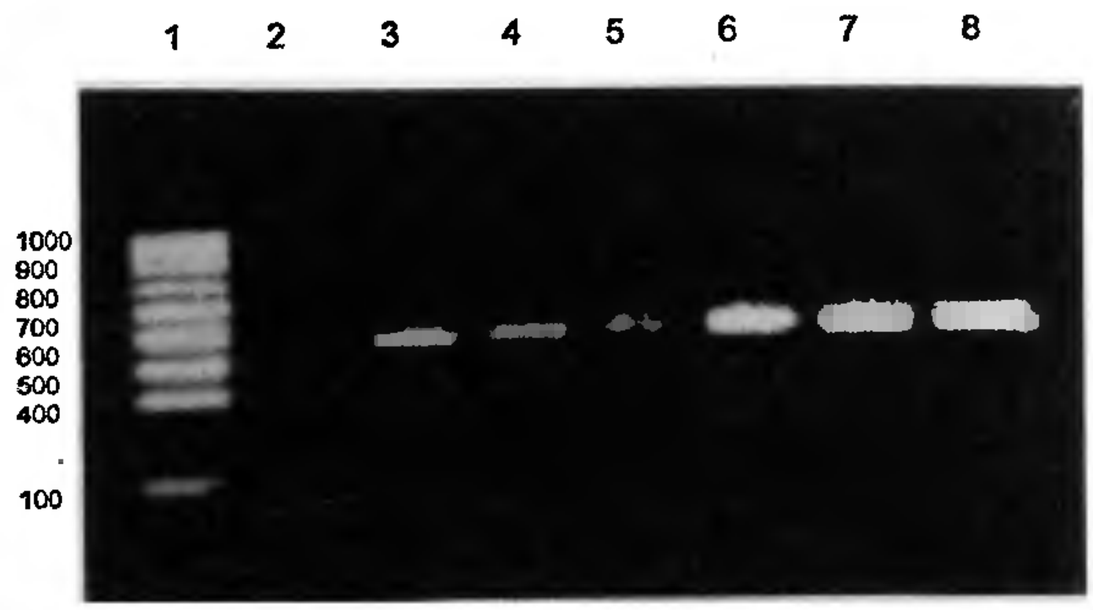

Figure 2. Agarose gel electrophoresis of polymerase chain reaction product (16S rDNA fragment –558 bp) of sandal spike phytoplasma after 20 cycles of amplification using Kerala (lanes 3–5) and Karnataka (lanes 6–8) sandal spike infected tissues. Lane 1, 100 bp ladder; lane 2, healthy sandal.

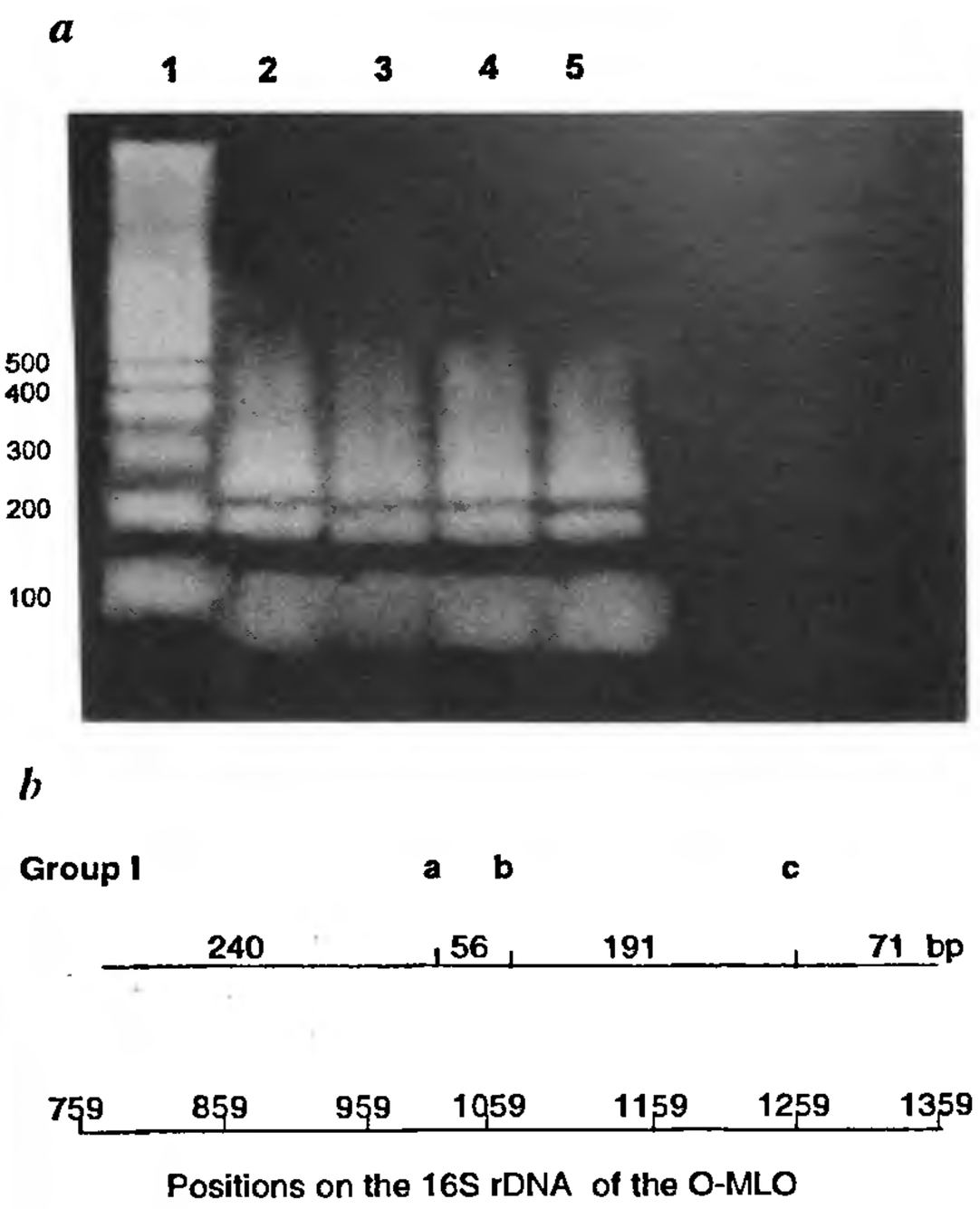

Figure 3. *a*, Agarose gel electrophoresis of *Alu*I digests of PCR products of sandal spike phytoplasma. Lane 1, 100 bp ladder; lanes 2 and 3, sandal spike phytoplasma of Kerala; lanes 4 and 5, sandal spike phytoplasma of Karnataka; *b*, *Alu*I restriction maps of the 558 bp 16S rDNA fragments of group I phytoplasma (Ahrens and Seemuller[9]).

powdered tissues were incubated at 65°C for 1 h for larger DNA yields[19]. 3M sodium acetate was used to induce DNA precipitation[20]. The yield of DNA pellet improved after incubating overnight at –20°C. The DNA pellet was dissolved in sterile distilled water and not in Tris–EDTA buffer since the same can interfere in PCR amplification, as EDTA was found to effectively reduce the magnesium concentration[21].

In our experiment we used both *Taq* polymerase and a DNA polymerase from *Thermus brockianus* (Dynazyme II) for PCR amplification. Both the polymerases amplified the phytoplasma DNA (Figure 1). In the subsequent studies only *Taq* polymerase was used and a 558 bp fragment of DNA was amplified from both the Kerala and Karnataka sandal spike populations (Figure 2). The last extension step was prolonged to 5 min to ensure that all the PCR products are of full length[22].

The restriction enzyme *Alu*I has the recognition sequence 5′-AG/CT-3′. The restriction enzyme was incubated with the PCR product overnight at 37°C rather than for 2 h to increase the efficiency during further detection by electrophoresis. As the PCR product in the study was 558 bp, TAE buffer and 1.5% agarose gel were used since the gel had an effective resolution in the range 3–0.2 kb (ref. 23). TBE buffer and 3% agarose gel were employed to visualize smaller DNA fragments less than 0.2 kb, while TBE buffer provided good resolution than TAE buffer[24].

The RFLP analysis with *Alu*I restriction endonuclease confirms that sandal phytoplasma could be attributed to the first group of phytoplasmas as proposed by Ahrens and Seemuller[9]. Different workers have identified phytoplasmas of the first group associated with diseases in lettuce[25], declining apricot[26], pear decline[27], dieback in papaya[28], periwinkle[29], corn poppy[30], etc. The same technique could be used to detect and classify phytoplasmas affecting different plants in India.

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