Efficacy of nested-PCR for the detection of phytoplasma causing spike disease of sandal

J. A. Khan*, P. Srivastava and S. K. Singh

Virology Lab, National Botanical Research Institute, Lucknow 226 001, India

Sandal (*Santalum album* L.) is a valuable tree of southern India. It is severely affected by sandal spike (SAS) disease, which is characterized by witches' broom symptoms. SAS is caused by a phytoplasma with its uneven distribution in the phloem tissues. A sensitive nested-PCR (polymerase chain reaction) was developed for the detection of phytoplasma in diseased sandal trees. Two sets of universal primer pairs based on 16S rDNA sequences were employed in direct/nested-PCR assays. In direct-PCR, templates consisting of SAS phytoplasma total crude DNA extracts in dilutions up to 1 : 90,000 were primed. The direct-PCR products thus obtained were subsequently used in nested-PCR. The nested-PCR was sensitive enough to reamplify the direct-PCR product (obtained from 90,000 times diluted crude DNA extract), resulting in a DNA fragment of 1.2 kb. The efficacy of nested-PCR showed that it could reamplify the direct-PCR product in dilutions of 1 : 60,000. The specificity of nested-PCR fragments was confirmed by Southern hybridization. The availability of a sensitive nested-PCR-based system should facilitate detection of the phytoplasma in potential vector insects and identification of other host plants of SAS.

SANDAL (*Santalum album* L.) is a hemi-root parasitic tree commonly found in the dry regions of peninsular India. Karnataka and Tamil Nadu are its natural habitats. The tree parasitises the roots of many plant species through haustorial connections. The scented heartwood and oil are most valuable products of sandal trees, as they have great industrial utility[1]. India has been the main exporting country for products of both sandalwood and its oil.

The sandal spike disease (SAS) is incited by a phytoplasma restricted to phloem tissues[2–4]. The witches' broom symptoms consist of reduction in the size of the leaves which become very narrow. The affected pale-green or yellow leaves stand out stiffly from the branch which acquires a spike-like appearance. Moreover, the leaves become crowded on the branch due to shortening of the internodes. Other characteristics of the disease are abnormally erect growth and sprouting of normally dormant buds. An early symptom of the disease consists of phyllody of flowers on an otherwise healthy-looking branch[5,6]. The tree dies-off within a couple of years after visible symptoms have appeared[7,8].

*For correspondence. (e-mail: jawaidkhan14@yahoo.co.in)

Phytoplasmas are cell-wall-less prokaryotic pathogens and cause devastating diseases in a wide range of plant hosts. They are present in the phloem of infected plants, not culturable on artificial media and non-mechanically transmissible. In phytoplasmas, detection has been hampered due to lack of their *in vitro* culture and low concentration in host plants. Detection of phytoplasma has mainly relied upon electron microscopy, an expensive technique which is beyond the means of many laboratories. Various other techniques have been applied for the detection of SAS phytoplasma. In light microscopy, it was indirectly visualized by staining the callose, developed in sieve elements of phloem tissues, with aniline blue[9] or Giemsa stain[10]. DNA-binding fluorochrome, 4,6, diamidino-2 phenylindole (DAP1) has been used for *in situ* localization of SAS-associated phytoplasma[11]. This technique is not sensitive enough to detect SAS phytoplasma in other natural hosts, e.g. *Ziziphus oenoplia* or *Lantana camara*, though Hull *et al.*[12] were able to detect it in the latter by electron microscopy. Moreover, electron microscopic and DAP1 staining techniques are suitable when the titre of phytoplasmas is relatively high in the host tissues. Thomas and Balasundaran[13] could target it by indirect ELISA. It is tedious and cumbersome to isolate phytoplasma in pure form and raise antiserum. In another example, dot hybridization and DAP1 were applied to monitor apple proliferation phytoplasma and the sensitivity of the two was compared. In this case, hybridization was shown to be more sensitive[14].

Since phytoplasmas are unevenly distributed and present in low titre in the phloem tissues of infected hosts, it is of utmost importance to have a highly sensitive detection system. Current classification of phytoplasmas is based on nucleotide sequence and RFLP (restriction fragment length polymorphism) of the 16S rRNA gene. This gene is present in all the prokaryotes and its conserved and variable regions make it ideal for phylogenetic classification[15–17]. PCR amplification of 16S rDNA of phytoplasmas has significantly contributed to the identification and characterization of unidentified phytoplasmas[18–21]. It is considered to be more sensitive than microscopic, serological and hybridization methods. The objective of this study was to detect SAS phytoplasma using nested-PCR. This communication describes the presence of a phytoplasma in crude DNA samples prepared from SAS-infected leaf tissues and reports the sensitivity of this technique.

Leaves of sandal trees showing characteristic symptoms of SAS disease and those of healthy trees were collected from Chamundi Hills, Mysore. One gram of each leaf collected from the diseased and healthy trees of sandal was ground in liquid nitrogen. Total DNA was isolated from infected and healthy tissues following the procedure essentially described by Ahrens and Seemüller[22]. The resulting DNA extracts were quantified by a spectrophotometer (Model CINTRA-20) at A_{260} (1 O.D. = 50 $\mu g\ ml^{-1}$).

Two sets of universal primers were used to amplify phytoplasma DNA in PCR that comprised of two steps, i.e. direct-PCR followed by nested-PCR. A primer pair, viz. P1/P7, located in the 16S rDNA, intergenic spacer and 23S rDNA region of the phytoplasma[23,24], was employed in direct-PCR to prime a DNA fragment of 1.8 kb expected size. The second set of primers, viz. R16mF2/R16mR1 was nested within the positions of annealing of primers P1 and P7 along the 16S rDNA of the phytoplasma[25]. The sequences of the primers and their positions are given in Table 1 and Figure 1.

In direct-PCR, the template consisted of total DNA extracted from SAS-infected (2.7 µg/µl) or healthy leaves. Different dilutions (samples containing a ratio of infected sap to sterile deionized water) of total DNA, i.e. 1 : 50,000, 1 : 60,000, 1 : 70,000, 1 : 80,000, 1 : 90,000, 1 : 100,000 were prepared before using them as templates in direct-PCR. A total of 50 µl PCR mixture contained 75 µM of dNTPs, 30 pmol of P1 and 40 pmol of P7 primers, 10 × PCR reaction buffers, 1.5 mM $MgCl_2$ and 1.5U *Taq* DNA polymerase. Amplifications were carried out in Perkin Elmer DNA thermocycler 9700. The PCR parameters consisted of 25 cycles of denaturation at 94°C for 1 min (4 min for the first cycle), annealing at 48°C for 1 min, and extension at 72°C for 2 min. The last cycle was extended for 5 min. An aliquot of 5 µl of each direct-PCR product was analysed by electrophoresis in a 1% agarose gel containing ethidium bromide and visualized on an UV transilluminator. All PCR reagents and oligo primers were procured from Bangalore Genei, India.

Table 1. Oligonucleotide primer sequences used for PCR amplification

Primer	Location	Oligonucleotide sequence
P1	16S	5′ AAGAGTTTGATCCTGGCTCAGGATT 3′
P7	23S	5′ CGTCCTTCATCGGCTCTT 3′
R16mF2	16S	5′ CATGCAAGTCGAACGA 3′
R16mR1	16S	5′ CTTAACCCCAATCATCGAC 3′

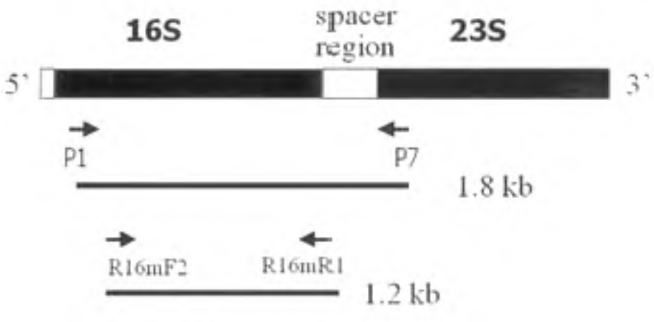

Figure 1. Schematic diagram of a phytoplasma rRNA operon showing the 16S and 23S genes and intergenic spacer region. Positions of oligonucleotide primers are shown by arrows.

One µl each of direct-PCR product (obtained from total DNA of various dilutions) was reamplified by nested-PCR. Those components of nested-PCR mixtures were the same as those described for direct-PCR, except the primers. The nested-PCRs were primed using the second set of primers, R16mF2/R16mR1. A total of 30 thermal cycles were performed with denaturation at 94°C for 1 min (5 min for the first cycle), annealing at 50°C for 2 min, extension at 72°C for 3 min, which was extended for 5 min in the last cycle. An aliquot of 5 µl was analysed in 1% agarose gel as mentioned in the direct-PCR and photographed on gel documentation system (Nighthawk, Pdi, USA).

To determine the efficacy of nested-PCR, 1 µl of direct-PCR product (resulting from total DNA in dilution of 1 : 90,000) was diluted with sterile deionized water in various dilutions, i.e. 1 : 10,000, 1 : 20,000, 1 : 30,000, 1 : 40,000, 1 : 50,000 and 1 : 60,000. One µl of each sample was reamplified in nested-PCR. PCR conditions and parameters remained the same as described above.

The authenticity of DNA fragments obtained from direct-PCR or reamplified in nested-PCR was checked by Southern hybridization using a cloned DNA (designated as pCR16S-5) representing the 16S rDNA of phytoplasma infecting *Catharanthus roseus* (periwinkle).

The DNA clone (pCR16S-5) was prepared from total DNA isolated from leaf tissues of *C. roseus* infected with phytoplasma at Lucknow. Employing universal pairs of primers for detection of phytoplasma[25], nested-PCR was performed on total DNA as described above. It yielded a DNA fragment of 1.2 kb, which was cloned into pUC19 vector at *Sma*I site using SureClone Ligation kit according to manufacturer's recommendations (Amersham Biosciences). The identity of the DNA clone was confirmed after nucleotide sequence determination and its alignment with sequences of 16S rDNA of other phytoplasmas which is available in GenBank (unpublished results). The sequences were analysed using BLAST searches[26].

The hybridization probe representing the 16S rDNA of *C. roseus* was prepared by performing nested-PCR with universal pairs of primers on the DNA clone pCR16S-5, as described above. The nested-PCR product was run on 0.7% agarose gel and the desired DNA fragment of 1.2 kb was cut-off from the gel and purified using Sephaglas™ Band Prep kit (Amersham Biosciences). The eluted DNA was used to prepare DNA probe labelled with α-^{32}P dCTP following primer extension method[27].

Nested-PCR-amplified DNA fragments obtained from SAS phytoplasma were transferred to Hybond N membrane (Amersham Biosciences) following the capillary method[28]. The membranes with transferred DNA fragments obtained from SAS phytoplasma in nested-PCR were subjected to prehybridization at 42°C for 1 h following hybridization with the radiolabelled probe at 65°C (overnight) in a hybridization oven. They were washed twice each in 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 5 and 15 min respectively, followed by another

wash in 1 × SSC, 0.1% SDS for 15 min. All the washing steps were carried out at room temperature. The blots were exposed overnight to Kodak diagnostic film for autoradiography.

In the case of direct-PCR, use of primers P1/P7 failed to yield visible amplified DNA fragments either from infected or healthy leaf tissues of sandal. Lack of visible PCR products from crude nucleic acid samples prepared from symptomatic leaves could be due to the presence of DNA concentration below the detection limit in ethidium bromide-stained agarose gel. Other factors, such as insufficient phytoplasma concentration in DNA template or the presence of *Taq* polymerase inhibitors in the PCR mixture could not be ruled out.

Direct-PCR products (obtained from total sandal DNA templates that had been diluted up to 1 : 100,000 times) were reamplified in the nested-PCR. This yielded a DNA fragment of 1.2 kb, when the direct-PCR products resulting from crude DNA extract diluted up to 1 : 90,000 were used as templates (Figure 2 *a*, lanes 1–5). There was no reamplification of direct-PCR product obtained from 1 : 100,000 diluted crude DNA (Figure 2 *a*, lane 6) or from healthy sandal leaf tissues (results not shown).

The efficacy of nested-PCR was determined by reamplifying, in serial dilutions, the direct-PCR product developed from 1 : 90,000 diluted crude DNA. Nested-PCR was sensitive enough to reamplify the template diluted up to 50,000 times (Figure 3 *a*, lanes 1–5).

The presence and specificity of PCR-amplified DNA fragments from direct/nested-PCR were checked by Southern hybridization using radiolabelled DNA probes prepared from homologous nested-PCR-amplified DNA fragments or 16S rDNA sequence of related phytoplasma harbouring *C. roseus*.

The hybridization results were in line with those of direct/nested-PCR. There was no hybridization when the direct-PCR products were probed with either homologous or heterologous labelled DNA.

It also confirmed the presence of phytoplasma DNA reamplified in nested-PCR using direct-PCR products obtained from total DNA diluted up to 1 : 90,000 (Figure 2 *b*, lanes 1–5). However, direct-PCR product (obtained from 100,000 diluted total DNA) could not be reamplified in nested-PCR and it did not reveal any positive signal (Figure 2 *b*, lane 6).

Notably, Southern hybridization gave positive but faint signals with the reamplified nested-PCR products, when the direct-PCR product (obtained from 90,000 times diluted DNA template) was diluted to 1 : 60,000 (Figure 3 *b*).

SAS disease has been diagnosed by means of light and electron microscopy, serological assays and PCR. These techniques, however, have limitations due to the presence of phytoplasmas in low concentrations in phloem tissues and their uneven distribution in infected plants. Sensitive

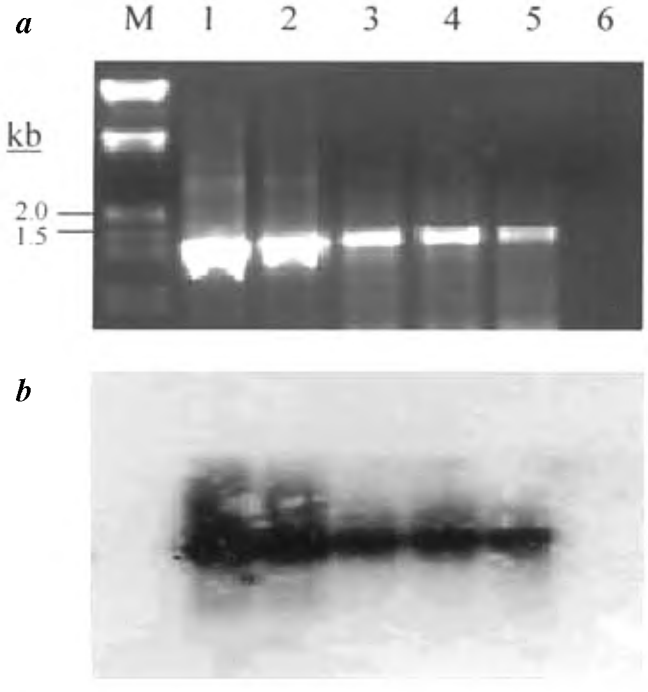

Figure 2. *a*, Agarose gel showing nested-PCR amplification of 16S rDNA of SAS phytoplasma of 1.2 kb. The template consisted of the product of direct-PCR obtained from crude DNA extracts of SAS phytoplasma in dilution of 1 : 50,000 (lane 1); 1 : 60,000 (lane 2); 1 : 70,000 (lane 3); 1 : 80,000 (lane 4); 1 : 90,000 (lane 5); 1 : 100,000 (lane 6). M, λ-DNA digested with *Eco*RI and *Hin*dIII. *b*, Southern hybridization of nested-PCR reamplified DNA fragments with α-^{32}P-labelled DNA probe prepared from 16S rDNA of a periwinkle (*Catharanthus roseus*) phytoplasma.

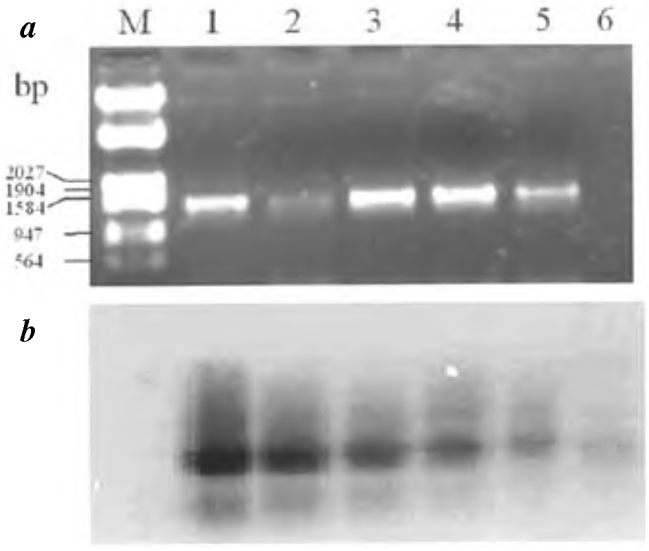

Figure 3. *a*, Agarose gel showing nested-PCR amplification of 16S rDNA of SAS phytoplasma of 1.2 kb. The template consisted of the product of direct-PCR (obtained from 90,000 diluted crude DNA extracts of SAS phytoplasma) in dilutions of 1 : 10,000 (lane 1); 1 : 20,000 (lane 2); 1 : 30,000 (lane 3); 1 : 40,000 (lane 4); 1 : 50,000 (lane 5); 1 : 60,000 (lane 6). M, λ-DNA digested with *Eco*RI and *Hin*dIII. *b*, Southern hybridization of nested-PCR reamplified DNA fragments with α-^{32}P-labelled DNA probe prepared from 16S rDNA of a periwinkle (*C. roseus*) phytoplasma.

detection of phytoplasma is important for identification of the hosts and potential vector insects.

In the present study, we have developed a sensitive nested-PCR for the detection of SAS phytoplasma. It was demonstrated that primer pairs designed from 16S rDNA sequences of SAS phytoplasma could effectively be employed to detect the associated phytoplasma. Although PCR has been applied to detect the presence of SAS phytoplasma, no sensitivity limit was reported[29]. With the procedure described here, phytoplasma DNA could be detected from direct-PCR products obtained from 90,000 times diluted crude DNA extracts. Further, direct-PCR product diluted to 60,000 times, could effectively be reamplified. Thus, the development of this highly sensitive nested-PCR-based system will greatly facilitate detection of the phytoplasma in potential vector insects and identification of other host plants of SAS.

Direct-PCR did not result in apparent band. Southern hybridization did not reveal any positive signals when direct-PCR products were probed with labelled DNA probes prepared either from the homologous DNA or heterologous DNA of a closely related phytoplasma (results not shown). Specific reamplification of direct-PCR products in nested-PCR, however, clearly demonstrates that the concentration of the SAS DNA in direct-PCR amplified product was below the detection limit in ethidium bromide-stained gels or Southern hybridization assay. These studies also show that the visualization of PCR-amplified SAS DNA products by ethidium bromide staining of agarose gels is almost comparable to molecular hybridization.

1. Jain, J. C. and Rao, P. S., *Indian For.*, 1966, **92**, 16–18.
2. Dijkstra, J. and Ie, T. S., *Neth. J. Plant. Pathol.*, 1969, **75**, 374–378.
3. Hull, R., Horne, R. W. and Nayar, R. M., *Nature*, 1969, **224**, 1121–1122.
4. Varma, A., Chenulu, V. V., Ray Chaudhuri, S. P., Prakash, N. and Rao, P. S., *Indian Phytopathol.*, 1969, **22**, 289–291.
5. Basu Choudhuri, J. C., *Indian For.*, 1963, **89**, 456.
6. Rao, P. S., Ramaiah, P. K., Nanjundappa, D. and Wesley, D. G., *Proc. Indian Acad. Sci., Sect. B*, 1965, **LX1**, 121–123.
7. Ghosh, S. K., Balasundran, M. and Ali, M. I. M., In *Plant Diseases of International Importance* (eds Mukhopadhyay, A. N., Kumar, J. and Chaube, H. S. J.), Prentice Hall, USA, 1992, vol. IV, pp. 296–310.
8. Srinivasan, V. V., Sivaramakrishnan, V. R., Rangaswamy, C. R., Ananthapadmanabha, H. S. and Sankarnarayana, K. H., Indian Council of Forestry Research and Education, 1992 (cited in ref. 13).
9. Hiruki, C., Giannotti, J. and Dijkstra, J., *Neth. J. Plant Pathol.*, 1974, **80**, 145–153.
10. Parthasarthy, K., Gupta, S. K. and Rao, P. S., *Proc. Indian Acad. Sci., Sect. B*, 1966, **64**, 152–156.
11. Thomas, S. and Balasundaran, M., *Curr. Sci.*, 1998, **74**, 989–993.
12. Hull, R., Plaskitt, A., Nayar, R. M. and Ananthapadmanabha, H. S. J., *Indian Acad. Wood Sci.*, 1970, **1**, 632–634.
13. Thomas, S. and Balasundaran, M., *Curr. Sci.*, 2001, **80**, 1489–1494.
14. Bonnet, F., Saillard, C., Kollar, A., Seemüller, E. and Bové, J. M., *Mol. Plant–Microb. Interact.*, 1990, **3**, 438–443.
15. Lee, L-M., Hammond, R. W., Davis, R. E. and Gundersen, D. E., *Phytopathology*, 1993, **83**, 834–842.
16. Lee, L-M., Gundersen-Rindall, D. E., Davis, R. E. and Bartoszyk, I. M., *Int. J. Syst. Bacteriol.*, 1998, **48**, 1153–1169.
17. Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A. and Göschl, M., *J. Plant Pathol.*, 1998, **80**, 3–26.
18. Montano, M. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P. and Brioso, P. S. T., *Int. J. Syst. Evol. Microbiol.*, 2001, **51**, 1109–1118.
19. Harrison, N. A., Womack, M. and Caprio, M. L., *Plant Dis.*, 2002, **86**, 676–681.
20. Cardova, I., Jones, P., Harrison, N. A. and Oropeza, C., *Mol. Plant Pathol.*, 2003, **4**, 99–108.
21. Harrison, N. A., Boa, E. and Carpio, M. L., *Plant Pathol.*, 2003, **52**, 147–157.
22. Ahrens, U. and Seemüller, E., *Phytopathology*, 1992, **82**, 828–832.
23. Deng, S. and Hiruki, D., *J. Microbiol. Methods*, 1991, **14**, 53–61.
24. Schneider, B., Seemüller, E., Smart, C. D. and Kirkpatrick, B. C., In *Molecular and Diagnostic Procedures in Mycoplasmology* (eds Razin, S. and Tully, J. G.), Academic Press, San Diego, USA, 1995, vol. 1, pp. 369–380.
25. Gundersen, D. E. and Lee, I.-M., *Phytopathol. Mediterr.*, 1996, **35**, 144–151.
26. Altschul, S. F., Thomas, L. M., Alejandro, A. S., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J., *Nucleic Acids Res.*, 1997, **25**, 3389–3402.
27. Feinberg, A. P. and Vogelstein, B., *Ann. Biochem.*, 1983, **150**, 6–12.
28. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, 2nd edn.
29. Thomas, S. and Balasundaran, M., *Curr. Sci.*, 1999, **76**, 1574–1576.

ACKNOWLEDGEMENTS. We thank Dr Jeanne Dijkstra, Wageningen University, The Netherlands for helping with the identification and collection of SAS phytoplasma samples and reviewing the manuscript with valuable suggestions. Dr P. Pushpangadan, Director, NBRI, Lucknow is acknowledged for providing necessary research facilities.

Received 7 October 2003; revised accepted 9 December 2003