Purification of sandal spike phytoplasma for the production of polyclonal antibody

Sandal (Santalm album. L), a semi-root parasitic tree is the source of the East Indian sandalwood and oil. Spike disease caused by phytoplasma is the major disease of sandal. The disease is noticed in all major sandal-growing states of India. Spike disease is characterized by extreme reduction in leaf size accompanied by stiffening and reduction of internode length. In advanced stage, the entire shoot gives the appearance of a spike inflorescence. Spiked trees die within 1–2 years after the appearance of visible symptoms. The pathogen, sandal spike phytoplasma, was first detected by electron microscopy in 1969 by three independent groups. Phytoplasmas are pleomorphic and fragile organisms occupying relatively small areas within the sieve tubes of the host plants. The major obstacle limiting research on phytoplasma disease is that the organism has not so far been isolated and cultivated in vitro. To avoid dependency on electron microscopic techniques many workers have used different methods like light microscopic techniques using Giemsa and Dienes’ stain, fluorescent microscopic techniques employing aniline blue and Hoechst 33258 (refs 8, 9), and DAPI stain for detecting the pathogen. But most of these techniques are indirect and non-specific techniques that can detect any other phytoplasma. We had earlier reported employing the polymerase chain reaction technique for detecting the pathogen.

Immunassays are highly specific techniques used in pathology for diagnosis of disease, identification and quantitation of micro-organisms. Highly purified antigens are needed to raise specific polyclonal antibodies for immunassays. Phytoplasma from diseased plants are purified using the techniques – differential centrifugation method, elute pad filtration technique and percoll density gradient centrifugation to raise polyclonal antibodies for the subsequent detection of the pathogen by immunological techniques. Most of these techniques are tedious requiring several cycles of centrifugation that in turn decreases the efficiency of purification due to loss of phytoplasma during centrifugation.

Attempts have been made earlier to purify sandal spike phytoplasma using ammonium sulphate precipitation and differential centrifugation method, but the efficiency of the purification was not tested and the polyclonal antibody generated had poor titre values. In this paper we report a differential filtration technique for the purification of phytoplasma; the purity was assessed using scanning and transmission electron microscopy. Polyclonal antibodies raised against purified phytoplasma were used to detect the spike disease pathogen using double diffusion test, enzyme linked immunosorbent assay (ELISA) and dot immunobinding assay (DIBA). The size of the antigenic protein was revealed by immunoblotting (Western blotting). Further, the amino acid composition of the purified phytoplasma was also studied.

Diseased sandal tissues were collected from Marayoor, Munnar Forest Division, Kerala, India. Inner bark and stem tissues from diseased branches were utilized for the purification of phytoplasma. Tissues were washed in running tap water for 10 min, treated with Ethane (Merck, India) for 3 min and again washed thoroughly with tap water. Subsequent steps were carried out at 4°C. Diced tissues were homogenized in ice cold 0.3 M glucose–sodium hydroxide buffer, pH 8.0 containing 0.02 M magnesium chloride (1 g fresh weight tissue/4 ml buffer). The extract was passed through two layers of cheesecloth followed by filtration through Whatman 1 and 5 filter papers (Whatman, UK). The clear extract was then passed through 0.45 μm pore size Millipore filter (Millipore, USA) and centrifuged (Sorvall OTD 65 B, USA) at 45,000 g (r, s) for 45 min. The resuspended small yellow pellet was incubated with undiluted antisera prepared against extract from healthy sandal for 2 h at room temperature for cross absorption of any plant debris present in the phytoplasma pellet. After low speed centrifugation at 4700 g for 20 min, the pellet was discarded and the supernatant centrifuged at 65,000 g for 45 min. The pale yellow pellet was resuspended in 1.0 ml of the same buffer. The sedimentation constant was calculated using the equation of Payment et al.

For scanning electron microscopy (SEM), the centrifuged pellet was embedded in 3% agarose (Sigma, USA) in 0.2 M phosphate buffer, pH 7.0. The agarose block was cut into pieces of 1 mm and fixed in 2.5% glutaraldehyde for 1 h, washed thrice in the same buffer and post-fixed in 2% potassium permanganate in 0.2 M phosphate buffer, pH 7.0 for 90 min at 4°C. The blocks were then washed in the same buffer and subsequently in distilled water, dried in a desicator followed by gold coating. Specimens were then viewed in Philips 501 B scanning electron microscope. Healthy and diseased sandal stem tissues were also processed similarly, critical-point dried followed by gold coating and viewed in Leo 435 VP scanning electron microscope (LEO, UK).

For transmission electron microscopy (TEM) the technique of Jiang and Chen was followed. Agarose (3%) (Sigma, USA) was used instead of agar for mixing the phytoplasma cells. Dehydration and embedding were done as mentioned elsewhere. Ultra thin sections were stained with 2% lead citrate for 10 min, washed and examined using Philips CM10 transmission electron microscope.

The phytoplasma, purified as described above, was suspended in 1.0 ml of glycine buffer without magnesium chloride and sonicated thrice (Vibracell, USA), 30 s each, with an interval of 2 min in ice. New Zealand White rabbits were injected intramuscularly in the hind legs at two sites with an emulsion of equal volume of sonicated phytoplasma preparation obtained from 6.25 g diseased sandal tissues and Freund’s complete adjuvant (Sigma, USA) in the first week and Freund’s incomplete adjuvant in subsequent injections given at two-week intervals. Blood was collected from the ear vein after 12 weeks of the first injection. Pellet from healthy plant extract,
purified as antigen to raise antibody for cross absorption of partially purified phytoplasma was also injected into another rabbit two months prior to the actual purification of phytoplasma following the same method. Booster injections were given once in a month after bleeding the rabbit to maintain high antibody titre.

The undiluted antiserum was used in double immunodiffusion to detect antigen in healthy and diseased plant extracts following the method of Ball.

An indirect ELISA technique was employed to detect the pathogen. Initial experiments were done to calculate the optimum titre of the antiserum and 1:2000 dilution was found to be the best (data not shown). Different dilutions of the antigen in PBS, pH 7.4 were coated directly onto ELISA strips (Polysorp – Nunc, Denmark) for one hour at 37°C. After washing and blocking the strips they were incubated with phytoplasma specific antibody (1:2000 dilution) in PBS for 1 h at 37°C. Later they were incubated with biotinylated goat anti-rabbit IgG (B. Genei, India) (1:2000 dilution), followed by incubation in streptavidin–HRP conjugate (1:2000 dilution). Finally the strips were treated with the substrate O-phenylene diamine (OPDA) and hydrogen peroxide in citrate buffer, pH 5.0, incubated in dark at room temperature for 1 h (ref. 22). The reaction was stopped by the addition of 2 M sulphuric acid (25 µL) and the absorbance read at 490 nm (Span Autoreader, India). Each strip had 8 wells, of which 3 were coated with healthy antigens and 5 with diseased antigen. Both purified and unpurified antigens (crude plant extract) were tested. Values greater than the threshold value (mean of healthy plant antigen + twice the standard deviation) were considered positive.

For DIBA, the method of Lazarovits was followed with slight modifications. Pencil-marked nitrocellulose membrane (Schleicher and Schuell, Germany) was washed with deionized water thrice, followed by air drying. Different quantities of the crude extract (1 g/4 ml buffer) of healthy and diseased trees diluted in PBS, pH 7.4 was spotted on the membrane. After washing thrice in PBS-tween, the membrane was blocked in PBS-tween–BSA (1%) for 30 min followed by incubation in phytoplasma-specific antibody (1:500, 1:1000 and

Figure 1. Scanning electron micrograph of purified phytoplasma. Note the absence of plant debris on phytoplasma cells (× 2500).

Figure 2. Scanning electron micrograph of a, Diseased sandal tissue. Note the phytoplasma cells in phloem; b, Healthy sandal tissue. Note the absence of phytoplasma cells in phloem.
Electrophoresis was done following the protocol of Saeed et al.26 and the method of Hammond27 was employed for immunoblotting. The immunoblotted membrane was probed with phytoplasma-specific antibody as in DIBA and the position of the band calculated with respect to the marker proteins.

Phytoplasma cells obtained after centrifugation (from 6.25 g tissue in 25 ml buffer) were resuspended in 1.0 ml 0.1 M phosphate buffer, pH 8.0. The cells after acid hydrolysis were analysed for amino acid composition using high performance liquid chromatography (Shimadzu LC-10 A, Japan)28. Tryptophan were estimated as described by Sastry and Tammuru29.

Phytoplasma pellet was not obtained after passing the diseased extract through 0.2 µm membrane or centrifugation below 45,000 g. Optimum amount of pellet was collected from the 0.45 µm membrane-filtered diseased sap after 45 min of centrifugation. Increasing the quantity of plant tissues (>1 g/4 ml buffer) blocked the 0.45 µm membrane.

Scanning electron micrographs of partially purified pellets of phytoplasma prior to cross absorption with antisera against healthy plant extract showed plant debris among phytoplasma cells, whereas impurities were sparse when the pellets were subjected to cross absorption (Figure 1). No phytoplasma cells were observed in pellets obtained from extract of healthy sandal (figure not shown). SEM studies of spike disease-affected sandal showed phytoplasma cells, spherical in appearance with an average size of 1 µm diameter (Figure 2 a), whereas no mollicutes were present in the phloem of healthy sandal (Figure 2 b). Passing the diseased plant extract through 0.45 µm membrane filter followed by centrifugation might have slightly altered the morphology of the mollicutes giving them an elliptical structure as seen in the electron micrograph.

Transmission electron micrographs confirmed the presence of purified phytoplasma cells (Figure 3), whereas no such structures were present in healthy control (figure not shown). The sedimentation coefficient (S20, w) of phytoplasma was calculated to be 466.0 Svedbergs.

Precipitin bands were not observed against healthy plant antigen (Figure 4), whereas clear precipitin bands appeared against crude diseased plant extract and 0.45 µm membrane filter-purified diseased plant extract.

The indirect ELISA techniques employed immunogenic probes at 1:2000 dilution; they were highly sensitive in that there was a wide difference (> 20-fold) in values between the purified healthy and diseased antigens. When crude healthy and diseased plant extracts were used for the test, the sensitivity was reduced (< 4-fold) probably due to the contaminating plant debris which interfered with the sensitivity of the test (Table 1). During the final test when 24 individual plants were tested to determine whether phytoplasmas could be detected in plants with spike disease symptoms, all the diseased samples showed higher values than the healthy control (Table 2).

DIBA was highly specific and rapid in that red-coloured dots were visualized on the membrane within three hours (Figure 5), but the sensitivity of the test was very less compared to ELISA. Use of immunogenic probes at 1:500, 1:1000 and 1:2000 dilutions could help to detect the presence of the antigen, even though 1:500 dilution was observed to be more sensitive to detect antigen even at a low dilution of 1:25.

Silver staining of SDS–PAGE gels of total protein of purified or semipurified phytoplasma could not reveal the antigenic protein associated with the micro-
organism. Only a few high molecular weight proteins were visible as bands while most of the low molecular weight proteins were not clearly visible (figure not shown). Immunoblotting of the gel revealed the presence of a low molecular weight protein of 14,000 daltons in both semi-pure and purified phytoplasma (Figure 6); the same was absent in healthy sandal.

The amino acid composition of sandal spike phytoplasma is shown in Table 3. The sulphur-containing amino acids—cysteine and methionine were totally absent and so was tryptophan. The total protein content of phytoplasma obtained from 6.25 g tissues was found to be 256.54 µg ml⁻¹ using HPLC.

A reliable and accurate detection of plant pathogen is a pre-requisite to develop disease management strategies. Highly purified phytoplasma is needed for immunological studies like ELISA and biochemical studies of the organism.

Phytoplasma found exclusively in the phloem tissues was first purified from a tree species (Peach) by Sinha and Chikwowski, using the cellite pad filtration technique. Most of the studies on phytoplasma purification have used differential centrifugation technique, which takes advantage of differences in sedimentation velocity that result from variation in physiological properties. This method involves a series of centrifugations wherein at the end of each centrifugation, the particles (phytoplasma) remaining in suspension are separated from the pellet (plant debris) by decantation and subjected to further centrifugation. A large amount of phytoplasma is lost when it sinks along with the plant debris and only a small amount of phytoplasma will be left behind in the final step. Sinha and Chikwowski reported that about 75% of phytoplasma was found to be lost during the purification procedure of diseased aster.

Detection of sandal spike phytoplasma using PCR and RFLP studies has revealed that sandal spike phytoplasma obtained from diseased sandal of both Marayoor and Mysore were of the same group. Preliminary studies on phytoplasma from these two sandal populations showed that they are of the same antigenic group (data not shown).

This paper reports a simple and efficient method for purification of phytoplasma using differential filtration technique which takes advantage of the filterable property of phytoplasma. Inner bark and stem tissues of diseased plants were utilized for purifying the phytoplasma, since in our earlier studies, using the fluorescent stain DAPI, it was found that these tissues harbour large quantity of phytoplasma compared to leaf and root tissues. Whatman 1 (11 µm pore size) and Whatman 5 (2.5 µm pore size) were used to clarify the plant sap. Phytoplasma could easily pass through these filters, whereas the filters retained much of the plant debris. The pleomorphic morphology of phytoplasma enabled it to pass through the 0.45 µm Millipore membrane filter which in turn slightly altered the morphology of the organism as seen in the scanning electron micrograph.

Centrifugation at 4700 g enabled the plant protein coupled with anti-plant antibody to settle down; the low speed prevented phytoplasma sedimentation. When speed was increased to 65,000 g in the final step, it enabled maximum amount of phytoplasma sedimentation, since no plant debris was present in the supernatant. 0.02 M magnesium chloride was used in the isolation buffer to maintain membrane integrity of the phytoplasma cells during extraction and was avoided during sonication.

In our studies we employed 3% agarose since it was found to be more pure than agar. SEM studies on agarose without embedding healthy or diseased pellet did not show any artifacts, which proved that agarose could be used for electron microscopy (figure not shown). Even though TEM has been earlier employed by different workers for in situ detection of the pathogen, in this report the morphology of the pathogen is studied by SEM.

Polyisorp plates were used for the present study since they preferentially adsorb lipoproteins which are the
Table 2. Detection of phytoplasma in individual plant samples using indirect ELISA. The samples were diluted to 1:1000 and probed with phytoplasma-specific antibody (1:2000 dilution), followed by biotinylated goat anti-rabbit antibody (1:2000 dilution) and HRP conjugated to streptavidin (1:2000 dilution). Absorbance values were read at 490 nm. Each value represents the mean of 3 replicates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Value</th>
<th>Sample</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.235</td>
<td>13</td>
<td>1.286</td>
</tr>
<tr>
<td>2</td>
<td>1.205</td>
<td>14</td>
<td>1.350</td>
</tr>
<tr>
<td>3</td>
<td>1.595</td>
<td>15</td>
<td>1.582</td>
</tr>
<tr>
<td>4</td>
<td>1.483</td>
<td>16</td>
<td>1.669</td>
</tr>
<tr>
<td>5</td>
<td>1.724</td>
<td>17</td>
<td>1.498</td>
</tr>
<tr>
<td>6</td>
<td>1.952</td>
<td>18</td>
<td>1.749</td>
</tr>
<tr>
<td>7</td>
<td>1.589</td>
<td>19</td>
<td>2.143</td>
</tr>
<tr>
<td>8</td>
<td>1.828</td>
<td>20</td>
<td>1.581</td>
</tr>
<tr>
<td>9</td>
<td>1.479</td>
<td>21</td>
<td>1.934</td>
</tr>
<tr>
<td>10</td>
<td>1.637</td>
<td>22</td>
<td>1.523</td>
</tr>
<tr>
<td>11</td>
<td>1.357</td>
<td>23</td>
<td>1.265</td>
</tr>
<tr>
<td>12 (healthy)</td>
<td>0.324</td>
<td>24 (healthy)</td>
<td>0.415</td>
</tr>
</tbody>
</table>

Table 3. Amino acid composition of sandal spike phytoplasma (cells extracted from 6.25 g plant tissue in 25 ml buffer). Each value represents the mean of two replicates.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (µg ml⁻¹)</th>
<th>Percentage of total amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>21.64</td>
<td>8.43</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.80</td>
<td>4.98</td>
</tr>
<tr>
<td>Asp*</td>
<td>25.87</td>
<td>10.08</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Glu**</td>
<td>38.83</td>
<td>18.13</td>
</tr>
<tr>
<td>Glycine</td>
<td>17.01</td>
<td>6.63</td>
</tr>
<tr>
<td>Histidine</td>
<td>17.68</td>
<td>6.89</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>9.24</td>
<td>3.60</td>
</tr>
<tr>
<td>Leucine</td>
<td>24.62</td>
<td>9.59</td>
</tr>
<tr>
<td>Lysine</td>
<td>14.98</td>
<td>5.83</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>12.39</td>
<td>4.82</td>
</tr>
<tr>
<td>Proline</td>
<td>12.95</td>
<td>5.04</td>
</tr>
<tr>
<td>Serine</td>
<td>16.57</td>
<td>6.45</td>
</tr>
<tr>
<td>Threonine</td>
<td>13.96</td>
<td>5.44</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.62</td>
<td>1.41</td>
</tr>
<tr>
<td>Valine</td>
<td>14.38</td>
<td>5.60</td>
</tr>
</tbody>
</table>

*Asp, Asparagine + aspartic acid. **Glu, glutamine + glutamic acid.


ACKNOWLEDGEMENTS. We thank the Department of Biotechnology, Government of India for financial support. S.T. acknowledges CSIR, Government of India for senior research fellowship. The ultrastructural studies were carried out at the Sophisticated Instruments Facility for Electron Microscopy (supported by DST, Govt. of India) at the All India Institute of Medical Sciences, New Delhi.

Received 21 September 2000; revised accepted 1 March 2001