3. **In situ** Detection of Sandal Spike Phytoplasma
3. IN SITU DETECTION OF SANDAL SPIKE PHYTOPLASMA

Free hand sections of spike disease affected sandal (Santalum album L.) and its hosts were stained with the DNA binding fluorochrome, 4',6-diamidino-2-phenyl indole (DAPI), to detect the presence of phytoplasma. Yellow-green fluorescence was detected in the phloem of diseased sandal, while the phloem tissue of healthy sandal and host plants of the spike disease affected sandal in the field as well as in glass house did not show fluorescence. The intensity of fluorescence was high in the young stem and inner bark compared to the root, petiole and leaf. When the efficiency of the DAPI stain was compared with Dienes' stain to detect phytoplasma, the latter was found to be less efficient. Ultrastructural studies of the pathogen using scanning electron microscopy revealed the pleomorphic morphology of phytoplasma.

3.1. INTRODUCTION

The causative organism of spike disease affected sandal was first detected by transmission electron microscopy in 1969 (Dijkstra and le, 1969; Hull et al., 1969; Varma et al., 1969). Since then, a number of staining techniques has been tested to improve the efficiency of detection of phytoplasma in plants and
to reduce dependency on electron microscope for identification of yellows
diseases (Douglas, 1986). Light microscopic detection of phytoplasma involves
indirect detection whereby callose formed in sieve elements of phloem tissue as
a response to wounding is visualized by staining with either aniline blue (Ghosh
et al., 1985), Manns' stain or Giemsa stain (Parthasarathi et al., 1966). A DNA
binding fluorochrome, Hoechst 33258 has been used to detect phytoplasma in
spike disease affected sandal (Ghosh et al., 1985; Rangaswamy, 1995). DAPI
(4',6-diamidino-2-phenyl indole), a DNA binding fluorochrome provides a quick
non-specific microscopic detection of phytoplasma in plant tissues (Sinclair et
al., 1996) and has been used in the detection of phytoplasma in several plants
like sweet potato (Gibb et al., 1995), peach, chokecherry (Douglas, 1986), white
ash, and lilac (Sinclair et al., 1996).

Electron microscopy is still the only method by which the ultrastructural
characteristics of phytoplasma can be studied. In particular, scanning electron
microscopy (SEM) is unrivalled for the demonstration of topographical forms in
three dimensions. SEM allows rapid examinations of large areas, such as entire
cross or longitudinal sections of petioles and stems at high magnifications and
with great depth of field, compared with the small volume sampling of thin
sections employed in TEM (Marcone and Ragozzino, 1996).

This section reports in situ detection of phytoplasma in spike disease
affected sandal and its host plants using DAPI stain. The efficiency of DAPI
stain was compared with Dienes' stain. Morphology of the sandal spike phytoplasma in the phloem of diseased sandal tissues was studied using scanning electron microscopy.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Spike disease affected sandal and the host plants, Lantana camara and witches' broom affected Zizyphus oenoplea were collected from Marayoor, Munnar Forest Division, Kerala. The tissues were transported either in ice or the excised branches dipped vertically in water and covered with polyethylene bags. The plant materials were stored at 4°C.

3.2.2. Disease transmission

To make diseased plant material available in glass house for experiments, the spike disease affected sandal twigs (scion), were wedge grafted to one-to two-year-old healthy sandal seedlings grown in glass house. Pongamia glabra and Pterocarpus marsupium were provided as the host species.
3.2.3. DAPI staining

Healthy and spike disease affected sandal and host plants in the field and glass house were screened for the presence of phytoplasma using DAPI stain (Seemuller, 1976). Tissues were fixed in 5% formaldehyde in 0.1 M phosphate buffer, pH 7.0 for 30 minutes. They were then washed in phosphate buffer, pH 7.0, for 3 minutes. Free-hand sections of 20 μm thickness were stained with 0.001% DAPI (Sigma, USA) in 0.01 M phosphate buffered saline, pH 7.4, for one hour, mounted in water or glycerin and viewed under Leitz Dialux fluorescence microscope using HBO 50W bulb.

3.2.4. Dienes' staining

Dienes' stain was prepared by dissolving 2.5 g of methylene blue, 1.25 g azure II, 10.0 g maltose and 0.25 g sodium carbonate in 100 ml distilled water (Deeley et al., 1979). Free-hand sections of 20 μm thickness were stained with 0.2% of Whatman-1 filtered Dienes' stain for 10 minutes. They were then washed in distilled water, mounted in water or glycerin and observed under light microscope.
3.2.5. Scanning electron microscopy

Healthy and diseased sandal stem tissues were cut into pieces of 1 mm³ and fixed in 2.5% glutaraldehyde for 1 hour, washed thrice in the same buffer and post fixed in 2% potassium permanganate in 0.2 M phosphate buffer, pH 7.0, for 90 minutes at 4°C. The blocks were then washed in the same buffer, critical point dried, followed by gold coating and viewed in Leo 435 VP scanning electron microscope (LEO, UK).

3.3. RESULTS

3.3.1. Plant material

Spike disease affected tissues transported in ice had a shelf life of only three weeks when stored at 4°C, after which they were damaged. Whereas, the samples which were transported vertically by placing the base of the stem in water and thereafter stored at 4°C, remained fresh even after four weeks.

3.3.2. Disease transmission

Grafts were established on 75% of healthy plants and the spike symptoms appeared within 60 days after grafting. Generally, grafts were found to establish readily in the monsoon months rather than the summer months.
3.3.3. DAPI staining

The xylem and sclerenchymatous stone cells of both the healthy and diseased sections showed green autofluorescence under UV light. While all the tissues of spike disease affected sandal showed the characteristic yellow-green fluorescent spots in the phloem region after being stained with DAPI, the intensity of fluorescence was high in the stem (Fig. 3.1) and inner bark (Fig. 3.2) compared to petiole (Fig. 3.3), leaf (Fig. 3.4) and root (Fig. 3.5). In highly diseased sandal trees growing in the field, the sections of inner bark showed intense fluorescence compared to sections from young stem tissues. Longitudinal section of the inner bark showed continuous fluorescence in the phloem region. No fluorescence was observed either in the sections of diseased root tips or healthy sandal tissues. Further, sections from the host plants of spike disease affected sandal growing in the field like *Lantana* and *Zizyphus* as well as hosts of glass house raised diseased sandal seedlings, *Pongamia* (Fig. 3.6) and *Pterocarpus* did not show the characteristic fluorescence in their phloem.

3.3.4. Dienes' staining

On staining the tissues with Dienes' stain, the phloem of diseased tissues showed discrete dark blue stained cells, but the phloem of healthy plants
remained unstained. The xylem and stone cells of both the healthy and diseased tissues were also coloured blue (Fig. 3.7)

3.3.5. Scanning electron microscopy

With this technique, it could be confirmed that phytoplasmas in the infected samples examined, were similar in size, shape and colonization pattern to those observed by transmission electron microscopy (Ghosh et al., 1985). The microorganisms were present only in the sieve tube elements of diseased plants but not in healthy plants (Fig. 3.8). The average size of the organism was found to be >1 μm; the pathogens were generally pleomorphic, with some cells taking the shape of the cell wall of the phloem cells (Figs. 3.8 b,c,d).

3.4. DISCUSSION

As the spike disease affected sandal is confined to Marayoor, which is approximately 200 km from the laboratory, experiments were done initially to increase the shelf life of the stored samples. Samples transported in ice, though stored in air-tight containers in the refrigerator at 4°C, were damaged within three weeks. When the samples were vertically placed, with their base in contact with water and covered with polyethylene bags, the tissues remained fresh. As the base of the sample was in contact with water the upper part was dry and on subsequent storage at 4°C had a higher shelf life.
Fig. 3.1. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal stem (CS) stained with DAPI (×70). Note the fluorescent spots in the phloem of diseased tissue.
Fig. 3.2. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal inner bark (CS) stained with DAPI (X70). Note the fluorescent spots in the phloem of diseased tissue.

![Image of healthy sandal inner bark](a)

![Image of diseased sandal inner bark](b)
Fig. 3.3. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal petiole (CS) stained with DAPI (X70).
Fig. 3.4. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal leaf (CS) stained with DAPI (X70).
Fig. 3.5. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal root (CS) stained with DAPI (X70).
Fig. 3.6. Fluorescent photomicrograph of *Pongamia glabra* stem (CS) grown as host of diseased sandal stained with DAPI (X70).
Fig. 3.7. Photomicrograph of Dienes' stained (a) healthy and (b) diseased sandal stem (CS). Note the blue spots in the phloem region of diseased sandal.
Fig. 3.8. Scanning electron micrograph of (a) healthy and (b) diseased sandal stem. The phytoplasma cells are seen exclusively in the phloem tissues of diseased sandal.
Fig. 3.8. Scanning electron micrograph of (c,d) diseased sandal stem. Note the pleomorphic phytoplasma cells seen exclusively in the phloem tissues of diseased sandal.
Fluorescence is a form of luminescence, which occurs after photons of light are absorbed by a fluorochrome at the ground electronic state. Most of the fluorochromes require excitation in the long wave UV to produce fluorescence in the visible range. Some of the biochemical substances present in the cells have the property to autofluoresce under UV light (Kasten, 1993). The present study also showed that the xylem and sclerenchymatous stone cells exhibited the characteristic autofluorescence in both healthy and diseased unstained tissues under UV light.

Even though there is only scant information on the biochemical characters of phytoplasma, there is much knowledge about its animal counterpart, the mycoplasma. Generally, mycoplasmas are wall-less prokaryotes with a genome size of $5 \times 10^8$ daltons. They have a low G+C content (23 to 30 moles percent G+C), i.e., a high A+T content. The affinity of the fluorochrome, DAPI to double stranded DNA is very high, the apparent association constant ($K_{app}$) is in the range of $10^5$-$10^7 \text{M}^{-1}$. DAPI binds specifically to the minor groove of A-T rich sequences (Kapuscinski, 1995). In aster plants with yellows disease, it was found that the immature sieve elements close to the mature sieve elements degenerated (Raza and Esau, 1961). In the present study also it was found that in severely diseased trees, the fluorescent spots were less in number towards terminal portion of the stem and root but high intensity of fluorescence was detected in several rows of secondary phloem in
the inner bark. This may be due to degeneracy of phloem in the newly formed tissues. Rangaswamy (1995) found that the intensity of fluorescence was high in the stem and petiole than in the root when spiked tissues were stained with Hoechst 33258. Detection of phytoplasma using DAPI stain has gained popularity since the test is a highly rapid method. The technique has been used in the rapid diagnosis of phytoplasma in blueberry (Schaper and Converse, 1985), alder (Lederer and Seemuller, 1991), lettuce (Marcone et al., 1995b) and periwinkle (Marcone and Ragozzino, 1995c).

Coleman (1923) reported transmission of sandal spike disease from host of sandal through haustorial connection. *Lantana*, a host plant of sandal has been considered as a carrier plant of phytoplasma which spreads the spike-disease in sandal forests (Nayar and Srimathi, 1968), without itself manifesting any morphological changes. In the present study, DAPI staining could not detect phytoplasma in the phloem of the host plants of diseased sandal growing in the field as well as glass house. Hull *et al.* (1970), also could not detect phytoplasma in *Lantana* growing near diseased sandal but detected the same in witches’ broom affected *Zizyphus* growing in sandal forests using transmission electron microscopy. *Pongamia glabra* is also reported to be a symptomless carrier of phytoplasma of sandal (Kristensen, 1960). Subba Rao (1980) and Muniyappa *et al.* (1980) had reported the role of host plants in the transmission of sandal spike disease. No spike symptom was visualised in
*Lantana*, periwinkle and *Zizyphus*, when planted along with spike disease affected sandal. Similarly, when healthy sandal was provided with the hosts - witches' broom affected *Zizyphus* and little leaf affected periwinkle there was no change in the status of the healthy plant.

Earlier studies had shown that staining of tissues with Dienes' stain showed discrete blue coloured cells in the phloem region of phytoplasma-infected plants (Deeley et al., 1979; Raju et al., 1981). In the present study also discrete-blue coloured cells were found in the phloem region of diseased sandal when stained with Dienes' stain. In some of the sieve cells, the stain was poorly visible under low magnification, whereas DAPI-stained sieve elements showed specific fluorescent spots clearly visible even under low magnification. The poor clarity of Dienes’ stained cells may be due to the masking of colour by bright light whereas DAPI detects phytoplasma as bright fluorescent spots against a dark background. This difference indicates that DAPI stain is superior to Dienes’ stain in detecting phytoplasma.

Scanning electron microscopy has been employed mainly to detect the presence of fastidious prokaryotes in plants infected with the yellows type disease (Bove, 1984). The phytoplasmas occur in the phloem elements, which are usually devoid of interfering cytoplasmic material, thus making their detection by SEM possible. According to Marcone and Ragozzino, (1996) the
two-dimensional structure of these microorganisms, as observed by TEM, can resemble some cell organelles; but their three dimensional structure observed by SEM are unique in that the cell surface and ultrastructural features of phytoplasmas as well as their interaction with surfaces of host cells can be studied in detail.

SEM observations disclosed that in the material examined, the pleomorphic phytoplasma cells were present only in the phloem tissues of diseased plants, whereas the healthy phloem cells were devoid of any pathogen (Fig. 3.8). Potassium permanganate was used in SEM as it acts as both a fixative and stain. The rate of penetration of potassium permanganate is slightly greater than that of osmium tetroxide and the former is also considered to be a stronger oxidant; and during the oxidation-reduction reaction, it is reduced mainly to manganese dioxide, a solid brownish-black precipitate (Hayat, 1975). High contrast of the cytoplasmic membrane system is probably due to the "unmasking" of the protein and lipid components of the phospholipoprotein, for this protein reduces the permanganate and thus increases the electron density (Hayat, 1975).

Attachment to the host membrane is a characteristic feature of many human and animal pathogenic mollicutes and is considered to be an important requirement for pathogenecity (Kahane and Horowitz, 1993). Some of the
Phytoplasma cells were found to adhere to the inner surface of the phloem in different plant species like blackberry (Marcone, et al., 1994b), wild radish (Marcone and Ragozzino, 1995a) and Brassica spp. (Marcone and Ragozzino, 1995b). The scanning electron micrograph from this study also provided evidence that some of the parasiting phytoplasmas are attached to the inner surface of the sieve tubes.