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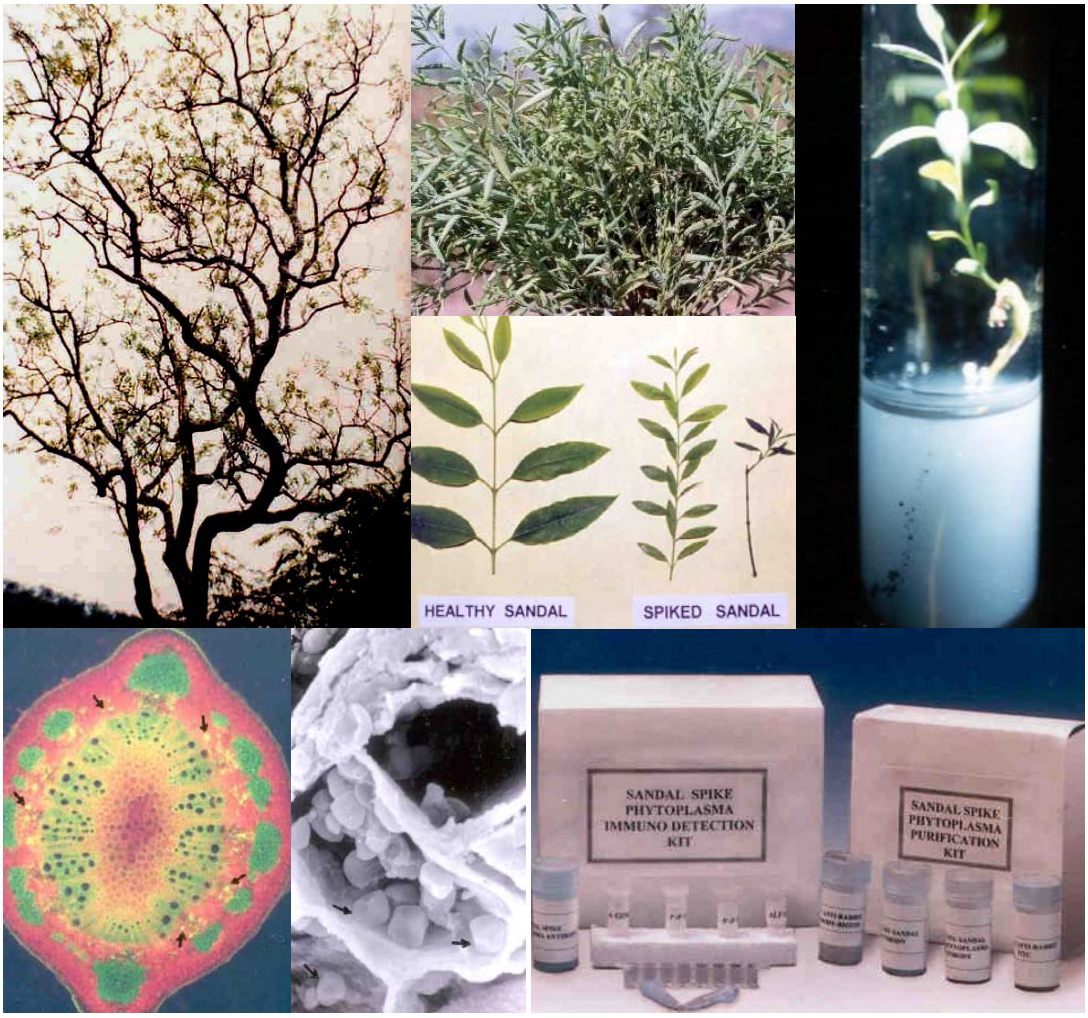

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(Final Report of the Project KFRI 247/96)

Development of Spike Disease Resistant Sandal Seedlings through Biotechnology Involving ELISA Technique and Tissue Culture

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April 2004

CONTENTS

Abstract of the Project Proposal

1. Project No. : KFRI 247/96

2. Title : Development of Spike Disease Resistant Sandal Seedlings through Biotechnology Involving ELISA Technique and Tissue Culture

3. Principal investigator : Dr. M. Balasundaran

4. Associate : Dr. E.M. Muralidharan

5. Research Fellows : T. B. Suma
Sunil Thomas

6. Objectives :

i) Development of ELISA technique for detection of Mycoplasma-like-organisms in tissues/sap of spike diseased sandal, host plants showing phyllody symptoms and in insect vectors.

ii) Identification of disease resistant trees using ELISA technique.

iii) Micro-propagation of disease resistant superior genotypes

iv) Development and standardization of technology for production of ELISA kits for detection of spike disease.

7. Date of commencement : December 1995

8. Duration : 3 Years

9. Funding Agency : Department of Biotechnology

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M. Balasundaran

E. M. Muralidharan

Abstract

Sandal (*Santalum album* L.), the root hemi-parasitic tree is famous for its highly valuable heartwood and oil. Spike disease is the most serious disease of the species; infected trees die within one to two years after the appearance of disease symptoms. The disease is caused by a non-culturable phytoplasma, seen exclusively in the phloem tissues. The present study was undertaken to develop immunological techniques for the detection of sandal spike phytoplasma, identify disease resistant trees among disease-evaded trees and multiply the disease evaded trees through tissue culture via somatic embryogenesis.

Stem and leaf tissues from diseased sandal and its host plants were screened for the presence of phytoplasma *in situ* using fluorescence microscopy employing the fluorochrome, 4'-6- diamidino-2-phenyl indole (DAPI). The fluorescent spots were detected exclusively in the phloem of diseased sandal tissues and not in healthy tissues. No fluorescent spots were seen in the phloem of the host plants of spike disease affected sandal. Ultrastructural studies using scanning electron microscopy confirmed the pleomorphic nature of sandal spike phytoplasma.

Phytoplasma from diseased sandal tissues was purified using a differential filtration technique. The technique takes advantage of the property of phytoplasma to pass through 0.45 μm pore size membrane filters. The purity of phytoplasma pellet obtained after ultracentrifugation was confirmed using transmission and scanning electron microscopy.

The purified phytoplasma was injected to rabbits to raise polyclonal antibody. Precipitin bands were seen against the extract of diseased sandal only and not against healthy sandal when the polyclonal antibody was subjected to Ouchterlony double diffusion test.

Direct and indirect enzyme linked immunosorbent assay (ELISA) was standardised to detect phytoplasma in diseased sandal. Indirect ELISA utilised both HRP conjugated

anti-rabbit antibodies as well as avidin and streptavidin amplification systems. Indirect ELISA was found to be very sensitive for detecting the pathogen compared to direct ELISA. For indirect ELISA, one hour was found to be optimum for both antigen coating and incubation of antibody. Antibodies at a dilution of 1:2000 could be used as a probe in indirect ELISA and could detect a minimum of 25 ng of phytoplasma protein. The techniques could not detect phytoplasma in the host plants of spike disease affected sandal and in witches' broom affected *Zizyphus oenoplia*. The techniques confirmed that sandal spike phytoplasma is specific to sandal and is not transmitted through the host plants. Sandal spike phytoplasma was not detected using ELISA tests in salivary gland and intestine of the insect vector, *Redarator bimaculatus*, collected from diseased trees.

The reagents developed for detecting sandal spike phytoplasma have the potential to be commercially marketed as kits which may be beneficial to the sandalwood industry. The kits, viz., Sandal Spike Phytoplasma Purification Kit and Immuno Detection Kit could be used for purifying sandal spike phytoplasma and detection of the pathogen in sandal.

In sandal forest Reserve 51 of Marayoor Range, 15 mature trees which evaded the disease for more than 20 years were identified in highly diseased area. Six-monthly monitoring of infection in these trees for four years using indirect ELISA tests showed infection in three trees, six months prior to external expression of disease symptoms, confirming the potential of early detection of disease through ELISA tests.

Tissue culture experiments using internodal explants from disease-evaded trees through somatic embryogenesis produced plantlets. But the conversion rate of somatic embryos to plantlets was very low and many of such plantlets were abnormal. When the hardened plantlets were out-planted in sterile potting medium, the percentage of survival was extremely poor. Co-culturing *in vitro* of sandal with *Cajanus cajan* as host plant and subsequent out-planting did not improve the survival of tissue culture-raised plantlets.

1. Development of ELISA Technique for Detection of Sandal Spike Phytoplasma

1.1. Introduction

Sandal (*Santalum album* L.) (Family: Santalaceae), the xylem tapping root hemi-parasitic tree is the source of the aromatic East Indian sandalwood and oil. Sandalwood oil, formed in the heartwood of the tree has a characteristic pleasant, woody odour. The oil is widely employed in the perfume industry, particularly in high-priced perfumes. Both the wood and oil are used in incense and medicine; besides, the wood is used in carving (Srinivasan *et al*., 1992; Coppen, 1995). Sandal is considered as a royal tree and has been rated as the most precious and valuable tree (Fig.1).

1.1.1. Distribution of *Santalum* sp.

The genus *Santalum* consists of 25 species, distributed between India in the West to Juan Fernandez Islands in the East and from Hawaiian Archipelago in the North to New Zealand in the South (George, 1984; Srinivasan *et al*., 1992). They vary greatly in habit, from small shrubs to large trees (Radomiljac, 1994). The commercially valuable sandalwood, *Santalum album* L. occurs naturally in Southern India and in the islands of eastern Indonesia, notably Timor and both the countries are the major producers and exporters of East Indian sandalwood and oil (Fox *et al*., 1994; Coppen, 1995).

In India, sandal is found mainly in the Deccan Plateau and its extension, and in small numbers in almost all regions, except the Himalayas. Large natural stands of sandal occurs in Karnataka (5,245 km^2) and Tamil Nadu (3,040 km^2) accounting for nearly 90% of sandal in India (Venkatesan, 1981). Sandal forests in Kerala are chiefly distributed in the Anjanad Valley in the eastern side of Western Ghats falling in Marayoor forest range of Munnar forest division with an extent of 15.42 km^2 in reserved forests and 47.26 km^2 in revenue lands (Mathew, 1995). In India, production of sandalwood has plummeted

from around 3000 tones per annum during 1985 to around 1000 tones in 1997; similarly oil production also declined from 140 tones in 1985 to 40 tones in 1997 (Jain *et al.*, 1999). The sandal tree grows at altitudes from sea level to about 1200 m above mean sea level. It grows to a height of 10-15 m and a girth of 100 cm and attains full maturity at an age of 60-80 years (Ghosh *et al.*, 1985; Jain *et al.* 1999). India has a monopoly in the world sandalwood market. Most of the existing sandal populations are not dense and are devoid of larger-girth-class trees, due to illegal felling, encroachment of sandal forests and due to spike disease.

1.1. 2. Diseases of sandal

Diseases of sandal include seedling diseases and leaf spot diseases caused by different fungal species, leaf curl disease caused by virus, and the spike disease (Srinivasan *et al.*, 1992). Collar rot caused by *Fusarium* spp. is a serious disease affecting seedlings.

1.1.2.1. Spike disease

Spike disease, the most serious disease of *S. album* is characterised by extreme reduction in the size of leaves and internodes accompanied by stiffening of the leaves. In advanced stage, owing to the progressive reduction in leaf size and internodes, the whole shoot looks like a `spike inflorescence' (Fig.2).

Spiked plants do not bear flowers or fruits; occasionally phyllody or abortive flowers are developed. Spiked trees usually die within 1 – 2 years after the appearance of the symptoms. Although, in Kerala, the production of sandalwood has not declined markedly because of the extraction of dead trees (dead trees increased in number as a result of spike disease), the stock in the forest is depleted considerably (Ghosh *et al.*, 1992). In Karnataka, the growing stock has been reduced to 25 per cent of its initial level in the last two decades (Swaminathan *et al.*, 1998). The disease is not known in Timor and does not affect the other species of *Santalum* (Fox *et al.*, 1994).

Although, spike disease was first observed in Coorg by McCarthy in 1899 (McCarthy, 1899; Barber, 1903) subsequent investigations showed that the disease had made its appearance in Coorg several years before McCarthy noticed it. The disease was observed in North Coimbatore in 1903, in Salem in 1913, and in Tirupathur Javadis in 1917 (Srinivasan *et al*., 1992). In Kerala, the disease was first noticed at Marayoor in 1980 (Ghosh *et al*., 1985).

1.1.2.2. The pathogen

Sandal spike disease was thought to be caused by a virus until 1969, when three independent groups confirmed through electron microscopic studies that the disease was caused by a phytoplasma (Dijkstra and Ie, 1969; Hull *et al*., 1969; Verma *et al*., 1969). Phytoplasmas were first reported by Japanese workers in 1967 (Doi *et al*., 1967). These pathogens are seen exclusively in the sieve tubes of phloem tissues of leaves, petioles, stem (Fig.3) and root, causing symptoms such as yellowing of leaf, little leaf, phyllody, witches' broom, etc. Phytoplasmas have been implicated as pathogens in more than 300 plant diseases worldwide (McCoy *et al*., 1989).

Morphologically, phytoplasmas resemble animal or human mycoplasmas (Class: Mollicutes) and share several characteristics with mycoplasmas. These include unicellular and pleomorphic nature and absence of cell wall, the cells being delimited only by a membrane, passage through bacteriological filters and resistance to antibiotics that interfere with cell wall formation (Neimark and Kirckpatrick, 1993). Phytoplasmas have remained uncultured despite extensive efforts over many years. The inability to grow these agents *in vitro* has severely hindered their study. As a result, phytoplasmas are among the most poorly characterised groups of plant pathogens. The pathogen could be visualized by electron microscopy, and their presence in phloem tissues demonstrated by fluorochromic DNA stains, but these methods cannot discriminate among different groups of phytoplasmas (Clark *et al*., 1989; Neimark and Kirckpatrick, 1993).

1.1.3. Diagnosis of phytoplasma diseases

Accurate diagnosis is a necessary prelude to any successful disease control. However diagnosis of plant mollicute diseases has often been one of the difficult aspects in the study of these diseases. It has long been known that most plant pathogens possess, as part of their structure, specific antigenic determinant in the form of proteins or other antigenic moieties. Recognition of the diagnostic potential of such determinants for both experimental and applied investigations in plant pathology has resulted in an array of techniques, collectively referred to as immunoassays. However, for this technique, extremely pure preparations of the organisms are required.

Initial efforts of a few laboratories to purify phytoplasma for the production of polyclonal antisera (Sinha, 1979; Caudwell *et al*., 1982; Sinha and Chiykowski, 1984) were discouraging due to high contamination with plant proteins which resulted in inferior quality and non-specific antisera. Hobbs *et al*. (1987) used antibody raised against healthy plant to remove plant specific proteins from the semi-pure phytoplasma pellet by cross absorption. Since then, several modifications of the protocol have been used in the purification of different phytoplasmas (Jiang and Chen, 1987; Jiang *et al*., 1988; Clark *et al*., 1989). All these techniques use several centrifugation steps (differential centrifugation) for the purification of phytoplasma which invariably leads to the loss of phytoplasma cells during successive centrifugation.

1.1.4. Diagnosis of spike disease of sandal

Spike disease of sandal is generally diagnosed by the manifestation of external symptoms. Attempts have been made to detect the diseased plants by determining the length/breadth ratio of leaves (Iyengar, 1961), histochemical tests using Mann's stain (Parthasarathi *et al*., 1966), Dienes' stain (Ananthapadmanabha *et al*., 1973) aniline blue and Hoechst 33258 (Ghosh *et al.,* 1985, Rangaswami, 1995). But most of these

techniques are insensitive indirect detection methods leading to misinterpretation of results. Highly sensitive techniques are needed to detect the presence of the pathogen.

This chapter reports detection of sandal spike phytoplasma through immunological methods such as double immunodiffusion test and direct and indirect enzyme linked immunosorbent assay (ELISA).

1.2. Review of literature

1.2.1. Sandal spike disease

Sandal spike is one of the most serious yellows-type diseases of forest trees known in the world. The disease has spread progressively over the years, devastating large forest tracts and threatening the entire sandal industry in India (Raychaudhuri and Varma, 1980). According to Subba Rao (1980) the percentage incidence of spike disease ranged from 1 to 55 in Karnataka.

1.2.2. Etiology

Initially the spike disease was suspected to be a root disease and a physiological disorder caused by unbalanced sap circulation brought about by adverse factors such as forest fires (Hole, 1917). Latham (1918) was of the opinion that the disease was due to a fungus, whereas Fischer (1918) thought that the disease was caused by some ultramicroscopic bacteria. Coleman (1917, 1923) attributed the disease to a virus.

The association of phytoplasmas with yellows diseases by Japanese scientists (Doi *et al*., 1967) gave impetus to a reconsideration of the causative organisms of plant diseases of

unknown and unconfirmed etiology. The viral theory of sandal spike disease was disproved when three groups of workers showed phytoplasma in the phloem tissues of spike diseased plants through transmission electron microscopy studies (Dijkstra and Ie, 1969; Hull *et al*., 1969; Verma *et al*., 1969). The pleomorphic bodies with 40 to 750 nm size were devoid of cell walls; the cytoplasm was bound by a unit membrane of 10 to 12 nm thick. The organism contained a fibrillar network of DNA and ribosomal bodies. The remission of spike disease symptoms after the infusion of tetracycline antibiotics further confirmed the phytoplasmal etiology of the disease (Raychaudhuri *et al*., 1972).

1.2.3. *In vitro* culture of the pathogen

Nayar and Ananthapadmanabha (1970) reported successful culturing of sandal spike phytoplasma *in vitro* using PPLO broth. They claimed to have reproduced spike symptoms on sandal and *Stachytarpheta* inoculated with the culture. However Muniyappa *et al*. (1980), Subba Rao (1980) and Ghosh *et al*. (1985) could not reproduce the results and the pathogen still is considered as a non-culturable organism.

1.2.4. Disease detection

Spike disease is generally diagnosed by the manifestation of external symptoms. Diseased plants can be detected by light and fluorescent microscopic techniques. The detection of abnormal levels of wound callose produced in response to injury to phloem cells had been suggested as an indirect method of diagnosis of phytoplasma. Ananthapadmanabha *et al*. (1973) employed Giemsa and Dienes' stain for the detection of the pathogen. In spike diseased sandal, aniline blue stained sections showed large number of fluorescent spots throughout the phloem tissue; a DNA binding fluorochrome, Hoechst 33258, has been used to detect the pathogen (Ghosh *et al*., 1985; Rangaswamy, 1995). Ghosh *et al*. (1985) used shigometer to detect spike diseased sandal. The electrical resistance of the inner bark of diseased trees was correlated with the intensity of visual symptoms.

1.2.4.1. Immunological techniques

According to Brock and Madigan (1988) antigens are substances that can be bound specifically by antibodies of the immune system of vertebrates. Injected into animals in the appropriate manner, antigens elicit an immune response, thus initiating the synthesis of specific antibodies. Antibodies are directed towards restricted parts of a macro-molecule, namely the antigenic determinants or epitopes (Roitt *et al*., 1993). Antibodies are found in blood serum. They are referred to as immunoglobulin molecules (Ig) and can be divided into five classes. Every warm blooded animal is capable of producing antibodies. Rabbits (Saeed *et al*., 1992a, 1993) and mice (Jiang and Chen, 1987; Jiang *et al*., 1988) are generally used to raise antiserum against phytoplasma. Two types of antibodies can be distinguished - polyclonal and monoclonal antibodies. Polyclonal antibodies are purified from the raw serum fraction of the blood of an immunised animal. Heterogeneity, is the outstanding feature of polyclonal antisera. Whereas homogeneity is the important feature of monoclonal antibodies since they are produced from a single antibody producing B-lymphocyte and multiplied as clones.

Immunological methods are among the simplest to use and interpret and are most valuable for diagnosis for diseases with inconsistent and undeveloped symptoms (Fox, 1998). Particularly, since the report of Engvall and Perlmann (1971) on the use of antibody-enzyme conjugate, the technique has become foundational to some of the most sensitive immunoassays in use today, including enzyme linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA), immuno microscopy and immunoblotting (Western blotting). Immunoassays are used in plant pathology for diagnosis of disease, and identification and quantitation of microorganisms (Barbara and Clark, 1986).

Immunological methods of detection of plant mollicutes have been used depending on the availability of specific antiserum. Various laboratories have reported the production of polyclonal antibodies to selected phytoplasma derived from plant tissue extracts (Sinha, 1979; Hobbs *et al*., 1987; Clark *et al*., 1989; Saeed *et al*., 1993). Such polyclonal antibodies (Pab) are capable of distinguishing the phytoplasma affected diseased plants

from healthy ones. But some of the antisera obtained were highly contaminated with anti-plant antibodies and were of poor quality. The use of monoclonal antibodies (Mab) (Lin and Chen, 1986) circumvents many of the problems encountered with polyclonal antibodies, but the production of suitable Mabs require specialised laboratory, considerable time, effort and a degree of luck (Clark *et al*., 1989). Nayar and Ananthapadmanabha (1975) purified sandal spike phytoplasma by ammonium sulphate precipitation method and used the same to raise polyclonal antibodies in rabbit. The polyclonal antibody was used in gel diffusion and agglutination tests.

1.2.4.2. Disease transmission

The transmission of sandal spike disease in the field was suspected to be caused by several insect vectors such as *Moonia albimaculata* (Dover and Appanna, 1933), *Jassus indicus* (Rangaswami and Griffith, 1941) and *Nephotettix virescens* (Shivaramakrishnan and Sen-Sarma, 1978). But subsequent studies could not confirm the findings (Lasrado, 1955; Subba Rao, 1980; Muniyappa *et al*., 1980). Ghosh *et al*., (1985) reported *Redarator bimaculatus* as the insect vector. Most of the earlier workers suspected host plants as the agents transmitting the spike disease. Nayar and Srimathi (1968) were of the opinion that *Lantana* acted as a symptomless carrier. It was also felt that sandal, in association with certain hosts was more susceptible to disease than others. Studies conducted by Subba Rao (1980) proved that phytoplasma could not be transmitted from diseased sandal to healthy sandal through haustorial connection. Laboratory transmission of the disease is achieved mainly through grafting and dodder. Coleman (1923) was the first to demonstrate the graft transmissibility of the disease to healthy trees. The establishment of the scion was found to be a prerequisite for disease transmission (Ghosh *et al*., 1992).

1.2.5. Disease control

With the discovery of phytoplasma in sandal, attempts were made to control the disease using antibiotics. Raychaudhuri *et al*. (1972) used dimethyl chlorotetracycline

hydrochloride, tetracycline hydrochloride and benlate (methyl 1-(butylcarbaucyl)-2-bensimidazole carbamate, a systemic fungicide to treat the disease by girdling and spraying, but appreciable recovery of diseased plants were not observed. The antibiotics achromycin, aureomycin, and ledermycin also did not show positive response (Nayar *et al*., 1973; Nayar and Ananthapadmanabha, 1974). But Ali *et al*. (1987) reported temporary remission of spike disease in various degrees by injection method using five tetracycline antibiotics - tetracycline hydrochloride, oxytetracycline HCl, ledermycin, aureomycin and doxycycline. However, the disease reappeared within 3-7 months. Infusion of digitonin also gave the same results.

1.3. Materials and Methods

1.3.1. Plant material

Spike disease-affected sandal, and the host plants, *Lantana camara* and witches' broom-affected *Zizyphus oenoplia* 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 using polythene bags. The plant materials were stored at 4^0C.

1.3.2. 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.

1.3.3. Disease detection

DAPI staining*:* 4',6-diamidino-2-phenyl indole, a DNA binding fluorochrome specific for staining phytoplasma was used to detect sandal spike phytoplasma in apparently diseased sandal plants. 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 0.1M phosphate buffer, pH 7.0 for 30 minutes, then washed in phosphate buffer, pH 7.0, for 3 minutes. Free-hand sections of 20 μm thickness (approx.) were stained with 0.001% DAPI (Sigma, USA) in 0.01M phosphate buffered saline, pH 7.4, for one hour, mounted in water or glycerine and viewed under Leitz Dialux fluorescence microscope using HBO 50 W bulb.

Scanning electron microscopy: Healthy and diseased sandal stem tissues were cut into pieces of 1 mm^3 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 4^0C. 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).

1.3.4. Purification of phytoplasma

Inner bark and stem tissues from diseased branches, collected from Marayoor were utilized for the isolation and purification of phytoplasma. Tissues were washed in running tap water for 10 minutes, treated with Extran (Merck, India) for 3 minutes and again washed thoroughly with tap water. Subsequent steps were carried out at 4^0 C. Diced tissues were homogenised in ice-cold 0.3 M glycine-sodium hydroxide buffer, pH 8.0 containing 0.02M magnesium chloride (Clark *et al*., 1989) (1g fresh weight tissue/4 ml buffer). The extract was passed through two layers of cheese cloth, 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 45,000g ($r._{av}$) for 45 minutes. The re-suspended pellet was incubated with undiluted antiserum prepared against extract from healthy sandal for 2 hours at room temperature for cross absorption of any plant debris present in the

phytoplasma pellet. After low speed centrifugation at 4700g for 20 minutes the pellet was discarded and the supernatant centrifuged at 65,000g for 45 minutes. The pale yellow pellet was re-suspended in 1.0 ml of the same buffer. The sedimentation constant (Payment *et al.*, 1991) was calculated using the equation: $t_{(hours)} = K/S_{20w}$, where K is a factor relative to the specific rotor provided by the manufacturer and S_{20w}, the sedimentation constant.

Electron microscopy*:* For scanning electron microscopy (SEM) of the purified phytoplasma, 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 $1mm^3$ 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 4^0C. The blocks were then washed in the same buffer and subsequently in distilled water, dried in a desiccator followed by gold coating. Specimens were then viewed in Philips 501 B scanning electron microscope.

For transmission electron microscopy (TEM), the technique of Jiang and Chen (1987) was followed with slight modification. The pellet obtained after centrifugation was fixed in 2.0% glutaraldehyde in 0.3 M mannitol-20mM MOPS buffer, pH 7.0 for 1 hour at 4^0C. The suspension was centrifuged at 45,000g for 30 minutes. The supernatant was aspirated and the pellet rinsed twice with the same buffer and post fixed with 1% osmium tetroxide at 4^0C for 6 hours, followed by washing in buffer. The pellet was mixed with 3% agarose and the solidified block cut into pieces of 1 mm^3, and suspended in 0.5% uranyl acetate for 12 hours at 4^0C. Dehydration and embedding were done as described by Cole (1983). Ultra thin sections were stained with 2% lead citrate for 10 minutes, washed and examined using Philips CM10 transmission electron microscope.

1.3.5. Total protein estimation

For total protein estimation, the phytoplasma was pelleted as stated above, but during the second centrifugation step, instead of glycine buffer, 0.1M phosphate buffer, pH 8.0 was

added followed by centrifugation at 4700g for 20 minutes. The pellet was discarded and the supernatant centrifuged at 65,000g for 45 minutes. The pale yellow pellet was re-suspended in 1.0 ml of 0.1M phosphate buffer, pH 8.0. Total protein of phytoplasma cells obtained from 6.25 g tissue in 25 ml buffer was estimated at A_{205} and A_{280} (Simonian, 1996) in 0.025 M phosphate buffer, pH 7.0 using a spectrophotometer (Unicam, U.K), by Lowry method (Lowry *et al*., 1951), and silver binding method (Krystal *et al*., 1985).

1.3.6. Production of polyclonal antibody

For polyclonal antibody production the method of Saeed *et al*. (1993) was followed with modifications. Sandal spike phytoplasma purified by the differential filtration method was suspended in 1.0 ml of glycine buffer without magnesium chloride and sonicated thrice (Vibracell, USA), 30 seconds each with an interval of two minutes at ice-cold temperature. New Zealand White rabbits were injected intramuscularly in the hind legs at two sites with an emulsion of equal volume of sonicated phytoplasma preparation and Freund's complete adjuvant (Sigma, USA) in the first week and Freund's incomplete adjuvant in the subsequent injections given at two-week interval. 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.

1.3.7. Purification of polyclonal antibody

Serum was processed from the blood according to the method of Ball *et al*. (1990). IgG was purified using protein-A affinity chromatography using an IgG purification kit (Bangalore Genei, India) following manufacturers' instructions.

1.3.8. Double immunodiffusion

The Ouchterlony method of double immunodiffusion as described by Ball (1990) was employed for preliminary detection of the presence of the antigen in the test plants - healthy and diseased sandal and the host plants viz., *Lantana* and witches' broom-affected *Zizyphus.*

1.3.9. Enzyme linked immunosorbent assay (ELISA)

Both the direct and indirect methods of ELISA techniques were used to detect the pathogen.

1.3.10. Direct ELISA

IgG was conjugated to horseradish peroxidase (HRP) (Sigma, USA) following the method of Mackenzie (1990). For direct ELISA, the method of Saeed *et al*. (1993) was followed with modifications. Polystyrene ELISA strips (Polysorp-Nunc, Denmark) were used to coat antigen. The ELISA strip wells were coated with purified antigen or crude extract (100 µl) at different dilutions in PBS, pH 7.4, at 37^0C for one hour followed by washing thrice with PBS buffer containing 0.025% Tween 20 (wash buffer). Each strip had 8 wells, of which 3 were coated with purified healthy sandal pellet or extract of healthy sandal (crude extract – without purification) and 5 with purified phytoplasma or the extract of diseased sandal (crude extract – without purification). Coating was followed by blocking the strips in phosphate buffered saline-tween (PBS-T) containing 0.2% BSA for 30 minutes and again washing thrice. The strips were then incubated with diluted HRP-IgG conjugate in conjugate buffer (PBS, pH 7.4 containing 0.025% tween and 0.20% BSA) at 37^0C for one hour. After washing, the substrate, O-phenylene diamine (OPDA) (Sigma, USA) and hydrogen peroxide in citrate buffer, pH 5.0, was added and incubated in dark at room temperature for one hour. The reaction was stopped by the addition of 2M sulphuric acid (25 µl) and the absorbance read at 490 nm (Span

Autoreader, India). Values greater than the threshold value (mean of healthy plant antigen ± twice the standard deviation) were considered positive (Sutula *et al*., 1986).

Initial experiments were conducted to assess the optimum time for coating antigen and incubation with polyclonal antibody. In these experiments, the ELISA strips were coated with purified antigen (1:100 dilution) followed by incubation with conjugated antibody (1:250 dilution). In another experiment, different dilutions of conjugated antibody (1:250 and 1:500 dilutions) was tested to detect the presence of phytoplasma in purified antigen. In the final test, the crude extract (obtained by pooling healthy or diseased sandal from three different trees and homogenised in glycine buffer at the ratio of 1g sample : 4 ml buffer- without further purification) was used to detect the presence of phytoplasma using conjugated antibody of dilutions- 1:100, 1:250 and 1:500.

1.3.11. Indirect ELISA

Indirect ELISA techniques consisted of using HRP-conjugated anti-rabbit antibody, biotin-avidin and biotin-streptavidin systems. As in direct ELISA, polystyrene strips were used to coat antigen in PBS, pH 7.4 for one hour at 37^0C. After washing thrice, the strips were blocked using PBS-tween-BSA (0.2%) for 30 minutes, washed thrice and the strips incubated with phytoplasma specific antibody in PBS for one hour at 37^0C. Subsequently, they were incubated with either goat anti-rabbit IgG-HRP conjugate (Sigma, USA) or biotinylated goat anti-rabbit IgG (B. Genei, India) for one hour. This was followed by incubation in either avidin or streptavidin conjugated HRP (B. Genei, India). Finally the HRP coated strips were treated with the substrate OPDA and hydrogen peroxide in citrate buffer, pH 5.0, and kept in dark at room temperature for 1 hour. The reaction was stopped by the addition of 2M sulphuric acid (25µl) and the absorbance read at 490 nm. Each strip had 8 wells, of which 3 were coated with purified healthy sandal pellet or extract of healthy sandal (crude extract – without purification) or its hosts and 5 with purified phytoplasma or extract of diseased sandal (crude extract – without purification) or the host plants. Values greater than the threshold value (mean of healthy plant antigen ± twice the standard deviation) were considered positive (Sutula *et al*., 1986).

Initial tests were conducted to assess the optimum concentration of the specific polyclonal antibody (1:500 to 1:10,000 dilution) required for indirect ELISA using a constant amount of purified phytoplasma (1:1000 dilution). An experiment was conducted to probe for different dilutions of purified antigen (1:50-1:10000 dilution) using polyclonal antibody at dilutions 1:500, 1:1000 and 1:2000. In the test, HRP conjugated to streptavidin (1:2000 dilution) was used as the secondary probe. The efficiency of goat anti-rabbit antibody HRP conjugate and avidin -HRP was also tested using the purified phytoplasma. For the final test involving detection of phytoplasma in crude extract of sandal (obtained by pooling samples from three different trees and homogenised in glycine buffer at the ratio of 1g sample : 4 ml buffer) polyclonal antibody at a dilution of 1:2000 was used as the primary probe and goat anti-rabbit antibody conjugated to HRP (1:2000 dilution) or streptavidin-HRP (1:2000 dilution) was used as the secondary probe. For confirmation of the efficiency of the polyclonal antibody, indirect ELISA was used to test 24 individual plant samples (crude extract – 1:1000 dilution) selected at random from Marayoor using streptavidin amplification system (1:2000 dilution).

1.3.12. Testing host plants of spike diseased sandal and insect vector

Since indirect ELISA was found to be superior to direct ELISA, the host plants of spike disease-affected sandal viz., *Lantana* and witches' broom affected *Zizyphus* were used to detect the presence of phytoplasma using the technique. For the preliminary tests three host plants were pooled and the extract was used for the test. The final test involved detecting the presence of pathogen in 10 individual host samples.

Redarator bimaculatus, supposed to be the insect vector of sandal spike disease (Ghosh et al., 1985) were collected from spike diseased sandal. Twelve adult insects were collected from diseased sandal of Reserve 54, where large number of trees were infected. The insects were brought to the laboratory, keeping them in cages provided with moistened diseased twigs covered with fine plastic nets. The insects were homogenized

in the extraction buffer, centrifuged at 5000 rpm and the supernatant diluted to 1:100 to 1:1000. The solutions were used for indirect ELISA test.

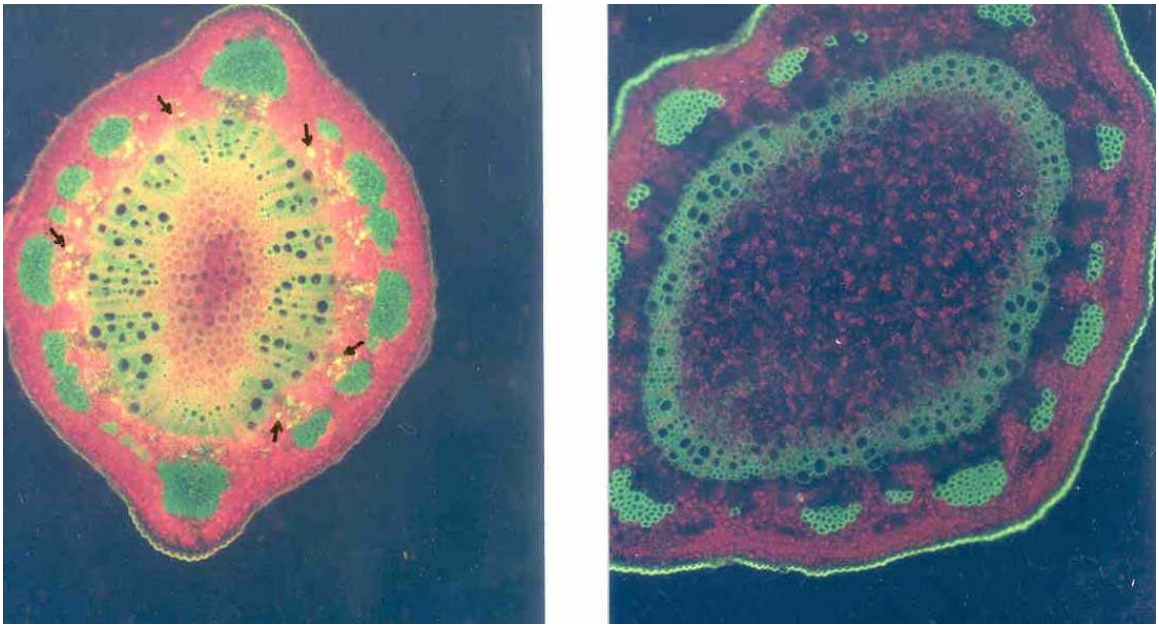
1.4. Results

1.4.1. Infected plant material

Spike disease-affected tissues transported in ice had a shelf life of only three weeks when stored at 4^0C; the tissues got damaged after three weeks. Whereas, the samples which were transported vertically by placing the base of the stem in water and thereafter stored at 4^0C, remained fresh even after four weeks.

1.4.2. 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 (Fig.4.) in the



(a) (b)

Fig. 4. Fluorescent photomicrograph of (a) diseased and (b) healthy sandal stem (cross section) stained with DAPI (X70). Note the fluorescent spots in the phloem of diseased tissue.

phloem region after being stained with DAPI, the intensity of fluorescence was high in the stem and inner bark compared to petiole, leaf and root.

1.4.3. Scanning electron microscopy

(a) (b)

Fig. 5. Scanning electron micrograph of (a) heathy and (b) diseased sandal stem. The phytoplasma cells are seen exclusively in the phloem tissues of diseased sandal.

With this technique, it could be confirmed that phytoplasmas in the infected samples 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. 5). The average size of the organism was found to be around 1 μm; the pathogens were generally pleomorphic, with some cells taking the shape of the cell wall of the phloem cells.

1.4.4. Disease transmission

Grafts established on 75% of healthy plants and the spike disease symptoms appeared within 60 days after grafting (Fig.6). Generally, grafts were found to establish readily during the monsoon months rather than the summer months.

1.4.5. Purification of phytoplasma

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

1.4.6. Electron microscopy

Scanning electron micrographs (Fig. 7) of partially purified pellets of phytoplasma prior

(a) (b)

to cross absorption with antiserum against healthy plant extract showed plant debris among phytoplasma cells whereas impurities were sparse when the pellets were subjected to cross absorption with antiserum raised against healthy plant extract. The mollicutes showed an elliptical structure as seen in the electron micrograph. Passing the diseased plant extract through 0.45 μm membrane filter followed by centrifugation had

slightly altered the phytoplasma morphology due to the pleomorphic property of the pathogen (Fig.7 (b). No phytoplasma cell was observed in pellets obtained from extract of healthy sandal.

Transmission electron micrographs confirmed the presence of purified phytoplasma cells (Fig. 8), whereas no such structure was present in healthy control. The sedimentation constant ($S_{20,w}$) of phytoplasma was calculated to be 777.0 svedbergs.

Fig. 8. Transmission electron micrograph of purified phytoplasma (x 29,000)

1.4.7.Total protein content

The total protein content was calculated to be 256.54 μg mL^{-1} using HPLC. The concentration of protein estimated using spectrophotometer at A_{205} and A_{280} in 0.025 M phosphate buffer, Lowry and silver binding method is shown in table 1.

Table 1. Total protein estimation of sandal spike phytoplasma (obtained by purifying 6.25g plant tissue in 25 ml buffer) using different methods (total protein estimation of phytoplasma using HPLC = 256.54 μg ml^{-1}). Phytoplasma cells in 0.025 M phosphate buffer was used to estimate the total protein at A_{205} and A_{280} by U.V. spectrophotometer.

Protein estimation method	A_{205}	A_{280}	Lowry	Silver binding
Protein conc. (μg ml^{-1})	300*	400	440	230

* Mean of 3 replicates

1.4.8. Double immunodiffusion

Precipitin bands were not observed against healthy plant extract (Fig. 9), whereas clearly visible precipitin bands appeared against crude diseased plant extract and 0.45 μm pore size membrane-purified diseased plant extract. When the membrane filter-purified

diseased plant extract was diluted, precipitin bands appeared only against concentrated and 1:2 diluted plant extracts whereas no precipitin band was observed at higher dilutions. Clear bands were also visible against phytoplasma of diseased sandal collected

from Mysore. No band was visible against the extract of the host plants of spike disease affected sandal - *Lantana* and witches' broom-affected *Zizyphus.*

1.4.9. Enzyme linked immunosorbent assay (ELISA)

1.4.10. Direct ELISA

Initial experiments on the effect of time on coating the antigen in wells of polystyrene strips showed that one hour of coating was optimum (Fig. 10). Also, one hour duration was found to be ideal for incubating the conjugated antibody (Fig. 11). The results

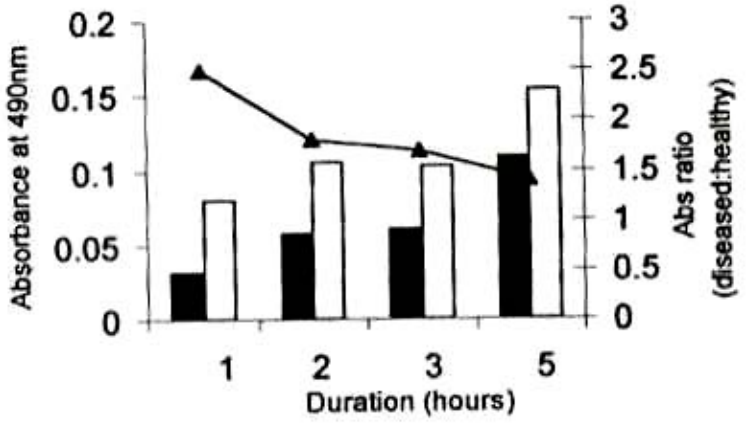


Fig. 10. Effect of time on efficiency of antigen coating in direct ELISA. Plates were coated with antigen (1:100 dilution-1 hour incubation) followed by incubation with conjugated antibody (1:250 dilution). Each value represents the mean of 5 replicates. : Healthy plant antigen, : diseased plant antigen, : ratio of absorbency values of diseased and healthy plants.

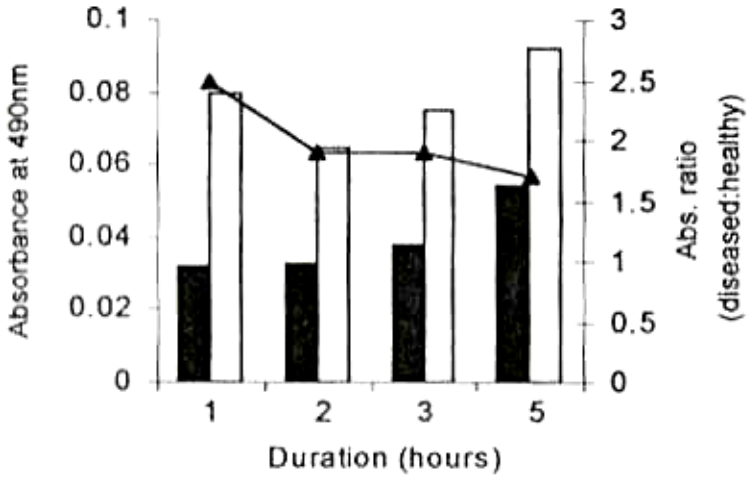


Fig. 11. Effect of time on efficiency of incubation of the conjugated antibody in direct ELISA. Plates were coated with antigen (1:100 dilution) followed by incubation with conjugated antibody (1:250 dilution-1hour incubation). Each value represents the mean of 5 replicates. : Healthy plant antigen, : diseased plant antigen, : ratio of absorbance values of diseased and healthy plants.

obtained when the purified samples were probed for the presence of phytoplasma using the conjugated antibody (1:250 and 1:500 dilutions) are shown in table 2.

Table 2. Direct ELISA values of different dilutions of purified diseased antigen. The antigens were probed with HRP conjugated phytoplasma specific antibodies (C.Ab). (Value for buffer=0.010).

Dilution	1:10	1:50	1:100	1:250	1:500	1:750
C.Ab-1:250	0.281* (0.243)**	0.276 (0.097)	0.172 (0.075)	0.088 (0.042)	0.062 (0.038)	0.055 (0.034)
C.Ab- 1:500	0.188 (0.212)	0.173 (0.075)	0.122 (0.059)	0.056 (0.035)	0.041 (0.038)	0.034 (0.027)

- Mean of 5 replicates.

****** Threshold value which represents the mean of 3 healthy sandal (purified antigen) ± twice the standard deviation.

The sensitivity of 1:250 dilution of conjugated antibody was higher compared to the dilutions at 1:500. When the crude sample was used to detect the presence of phytoplasma at different dilutions of conjugated antibody (1:100, 1:250; and 1:500), the dilution at 1:250 was found to be better compared to the other dilutions (Table 3).

Table 3. Direct ELISA values of different dilutions of unpurified diseased plant extract. The antigens were probed with HRP conjugated phytoplasma specific antibodies (C.Ab). (Value for buffer=0.010).

Dilution	1:10	1:100	1:250	1:500	1:750	1:1000
C.Ab-1:100	0.054* (0.065)**	0.044 (0.038)	0.043 (0.028)	0.039 (0.022)	0.037 (0.020)	0.039 (0.020)
C.Ab-1:250	0.070 (0.072)	0.052 (0.037)	0.040 (0.027)	0.041 (0.024)	0.038 (0.020)	0.033 (0.021)
C.Ab-1:500	0.059 (0.063)	0.041 (0.039)	0.034 (0.027)	0.029 (0.024)	0.028 (0.018)	0.025 (0.017)

***** Mean of 5 replicates.

****** Threshold value which represents the mean of 3 healthy sandal ± twice the standard deviation.

None of the tests could detect the presence of phytoplasma in the plant sample dilution of 1:10 probably due to the high content of plant proteins competing with phytoplasma cells for adsorption on to the plate.

Since the protein content of phytoplasma was about 257 $\mu g\ ml^{-1}$, substituting the values in direct ELISA shows that the test could detect up to 340ng (1:750 dilution) of phytoplasma protein.

1.4.11. Indirect ELISA

Indirect ELISA was found to be very sensitive compared to its direct counterpart. Since amplification using streptavidin was considered to be very sensitive, it was used in the initial experiments for different studies. When the sensitivity of different dilutions of the

polyclonal antibody was tested using a constant dilution (1:1000) of purified antigen, antibody dilution at 1: 2000 was found to be the most sensitive for the test (Fig. 12).

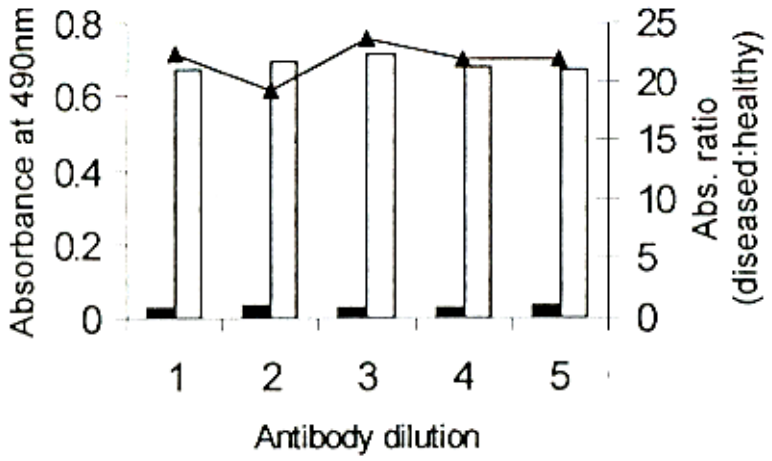


Fig. 12. Comparison of different antibody titre (dilution) in indirect ELISA. Plates were coated with antigen (1:1000 dilution) followed by incubation with antibody at different dilutions(**1**. 1:500, **2**. 1:1000, **3**.1:2000, **4**.1:5000, **5**.1:10000 dilution-1hour). This was followed by incubation in biotinylated goat anti-rabbit antibody (1:2000 dilution-1 hour incubation) and finally in HRP-streptavidin conjugate (1:2000 dilution-1 hour). The results were read at 490nm. Each value represents the mean of 5 replicates. : Healthy plant antigen, : diseased plant antigen, : ratio of absorbance values of diseased and healthy plants.

The purified antigen at different dilutions (1:50-1:10,000) were probed with different dilutions of polyclonal antibody (1:500, 1:1000 and 1:2000) using the streptavidin amplification system (Table 4). The results confirm that polyclonal antibody at a dilution of 1:2000 was the most sensitive. From the result it is inferred that a minimum of 25ng phytoplasma protein could be detected by indirect ELISA, since purified phytoplasma of 1:10,000 dilution could be detected.

The results obtained using the avidin system and goat anti-rabbit HRP conjugate for detection of the presence of phytoplasma in purified sample is shown in table 5. The indirect test employing goat anti-rabbit HRP was found to be more sensitive compared to the avidin-biotin system. Hence, to detect the presence of phytoplasma in crude extract, only goat anti-rabbit HRP and the biotin-streptavidin systems were considered. The

results are shown in table 6. From the results it is confirmed that the test employing biotin-streptavidin system is the most sensitive of the tests. This system was utilised to detect the pathogen in 24 individual sandal plants. The polyclonal antibody could detect the pathogen in diseased plants and the result is shown in table 7.

Table 4. Indirect ELISA values of different dilutions of purified diseased antigen using streptavidin amplification system. The antigens were probed with different dilutions of sandal spike phytoplasma specific antibody, biotinylated antirabbit antibody (1:2000 dilution) followed by HRP conjugated streptavidin (1:2000 dilution). (Buffer=0.010).

Dilution	1:50	1:100	1:250	1:500	1:1000	1:2000	1:5000	1:10000
Ab- 1:500	2.671* (0.180)**	2.468 (0.111)	1.472 (0.072)	1.009 (0.056)	0.649 (0.044)	0.317 (0.024)	0.147 (0.037)	0.080 (0.031)
Ab-1:1000	2.780 (0.148)	2.609 (0.102)	1.370 (0.063)	0.997 (0.053)	0.602 (0.038)	0.298 (0.027)	0.137 (0.038)	0.072 (0.036)
Ab- 1:2000	2.554 (0.153)	2.447 (0.087)	1.861 (0.066)	1.321 (0.055)	0.746 (0.053)	0.404 (0.044)	0.198 (0.040)	0.118 (0.044)

***** Mean of 5 replicates.

** Threshold value which represents the mean of 3 healthy sandal (purified antigen) ± twice the standard deviation.

Table 5. Indirect ELISA values of different dilutions of purified diseased antigen. The antigens were probed with sandal spike phytoplasma specific antibody (Ab) (1:2000 dilution) followed by either **(A)** goat anti-rabbit antibody conjugated to HRP (1:2000 dilution) or **(B)** biotinylated anti-rabbit antibody (1:2000 dilution). Avidin conjugated to HRP (1:2000 dilution) was used to probe the biotinylated anti-rabbit antibody. (Value for buffer=0.010).

Antibody systems	**Dilution of purified diseased antigen**							
	1:50	1:100	1:250	1:500	1:1000	1:2000	1:5000	1:10000
A	1.738* (0.058)**	1.763 (0.041)	1.359 (0.028)	0.977 (0.022)	0.586 (0.022)	0.274 (0.023)	0.121 (0.021)	0.061 (0.020)
B	2.213 (1.326)	2.044 (0.924)	1.922 (0.792)	1.716 (0.663)	1.415 (0.564)	1.219 (0.523)	0.807 (0.499)	0.635 (0.437)

***** Mean of 5 replicates.

****** Threshold value which represents the mean of 3 healthy sandal (purified antigen) ± twice the standard deviation.

Table 6. Indirect ELISA values of different dilutions of unpurified diseased plant extract. The antigens were probed with sandal spike phytoplasma specific antibody (Ab) (1:2000 dilution) followed by either **(A)** goat anti-rabbit antibody conjugated to HRP (1:2000 dilution) and **(B)** biotinylated anti-rabbit antibody (1:2000 dilution). Streptavidin conjugated to HRP (1:2000 dilution) was used to probe the biotinylated anti-rabbit antibody. (Value for buffer=0.010).

Antibody systems	**Dilution of unpurified diseased antigen**							
	1:10	1:100	1:250	1:500	1:1000	1:2000	1:5000	1:10000
A	0.564* (0.268)**	0.481 (0.241)	0.408 (0.188)	0.280 (0.162)	0.209 (0.131)	0.140 (0.114)	0.114 (0.097)	0.116 (0.088)
B	0.571 (0.177)	0.371 (0.139)	0.304 (0.071)	0.253 (0.055)	0.190 (0.057)	0.142 (0.042)	0.084 (0.032)	0.054 (0.025)

***** Mean of 5 replicates.

** Threshold value which represents the mean of 3 healthy sandal ± twice the standard deviation.

Table 7. Detection of sandal spike phytoplasma in individual plant samples using indirect ELISA.

Sample No.	ELISA Value	Sample No.	ELISA Value
1	1.235*	13	1.286
2	1.205	14	1.350
3	1.595	15	1.582
4	1.483	16	1.669
5	1.724	17	1.498
6	1.962	18	1.749
7	1.589	19	2.143
8	1.828	20	1.581
9	1.479	21	1.934
10	1.637	22	1.523
11	1.357	23	1.265
12	0.324**	24	0.415**

* Mean of 3 replicates

** Healthy trees

Since the indirect ELISA employing biotin-streptavidin system was found to be very sensitive, the same was utilised in the detection of phytoplasma in the host plants of spike disease affected sandal. The results are shown in table 8. The host plants of diseased

Table 8. Indirect ELISA values of different dilutions of extract of host plants of spike disease affected sandal using streptavidin system. (Value for buffer=0.010).

Host plant	**Dilution of host plant extract**			
	1:10	1:100	1:500	1:1000
Lantana	0.157* (0.140)**	0.060 (0.058)	0.033 (0.029)	0.027 (0.033)
Zizyphus	0.134 (0.203)	0.136 (0.149)	0.086 (0.080)	0.052 (0.054)

* Mean of 5 replicates.

** Threshold values which represents the mean of 3 host plants of healthy sandal ± twice the standard deviation.

sandal did not show variation in results compared to the host plants of healthy sandal. When individual host plants were screened for the presence of phytoplasma, the test could not detect the presence of the pathogen in any of the hosts (Table 9). The results indicated that sandal spike phytoplasma is confined to sandal. The ELISA test carried out using insect tissues did not show positive result in all the dilutions.

Table 9. Indirect ELISA values of host plants (*Lantana* and witches' broom affected *Zizyphus*) of spike disease affected sandal.

Lantana		*Zizyphus*	
Sample No.	ELISA Values	Sample No.	ELISA Values
1	0.066*	1	0.077
2	0.067	2	0.077
3	0.065	3	0.081
4	0.079	4	0.077
5	0.072	5	0.079
6	0.074	6	0.073
7	0.067	7	0.082
8	0.108	8	0.083
9	0.065	9	0.079
10*	0.161	10*	0.091

- Mean of 4 replicates.
- Host plants of healthy sandal

1.5. Discussion

1.5.1 Indirect evidence of phytoplasma in spike diseased sandal through DAPI staining

Mycoplasmas are wall-less prokaryotes with a genome size of $5x10^8$ daltons. They have a low G+C content (23 to 30 moles percent G+C) and a high A+T content. The affinity of the fluorochrome, DAPI to double stranded DNA is very high. DAPI binds specifically to the minor groove of A-T rich sequences (Kapuscinski, 1995). 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).

1.5.2. Absence of phytoplasma in host tissues

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 as hosts of diseased sandal but detected the same in witches' broom-affected *Zizyphus* growing in sandal forests using transmission electron microscopy.

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.

1.5.3. Immunological detection of Phytoplasma

A reliable and accurate detection of plant pathogen is a pre-requisite to develop disease management strategies (Khan *et al*., 1998). Highly purified phytoplasma is needed for

immunological studies such as ELISA (Hobbs *et al*., 1987; Saeed *et al*., 1992a) and biochemical studies of the organism (Sinha and Madhosingh, 1980).

Phytoplasma, was first purified from a tree species (Peach) by Sinha and Chiykowski (1984), using the celite pad filtration technique. Most of the studies on phytoplasma purification have used differential centrifugation technique (Clark *et al*., 1989; Saeed *et al.*, 1992b, 1993) 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 (Deter, 1973). A large amount of phytoplasma is lost when it sinks along with the plant debris during the low speed centrifugation. Hence, in the final centrifugation step, the net yield of phytoplasma will be less. Sinha (1979) reported that about 78% of phytoplasma was found to be lost during purification procedure of aster yellows phytoplasma.

1.5.3.1. Purification of Phytoplasma

The method of purification of phytoplasma using differential filtration technique takes advantage of the filterable property of phytoplasma. Whatman 1 (11 μm pore size) and Whatman 5 (2.5 μm pore size) were used to clarify the plant sap. Phytoplasma could pass through both these filters whereas the filters retained much of the plant debris. The 0.45 μm Millipore filter prevented plant debris whereas the pleomorphic morphology of phytoplasma enabled it to pass through the membrane which in turn slightly altered the morphology of the organism as seen in the scanning electron micrograph.

Centrifugation at 4700g enabled the plant protein coupled with anti-plant antibody to settle down; the low speed prevented phytoplasma sedimentation. When speed was increased to 65,000g in the final step, it enabled maximum amount of phytoplasma sedimentation, since no plant debris was present in the supernatant.

Since phytoplasmas are non-culturable microorganisms, the only method available for quantitation of the pathogen is by total protein estimation. On comparing the total protein content of sandal spike phytoplasma by using the HPLC derived result as standard, the silver binding method was found to be the second sensitive assay. The assay could detect protein with an accuracy of 90%. The sensitivity of the method may be due to the property of silver to bind to sulphydryl and carboxyl moieties in proteins (Sasse and Gallagher, 1996). Spectrophotometric assays at A_{280} and Lowry method was least sensitive probably due to the low concentration of aromatic amino acids, thereby giving false results. Spectrophotometric assay at A_{205}, was more sensitive than A_{280} since the quantitation is based on the absorbance by the peptide bond (Stoscheck, 1990). Since buffers of higher or lower ionic strength was found to be insensitive for quantitation of total protein, 0.025 M phosphate buffer was used in direct estimations (Simonian, 1996).

Ever since the non-culturable mollicutes, the phytoplasmas, were first discovered in 1967, they have been implicated as pathogens in more than 300 plant diseases worldwide (McCoy *et al*., 1989). Although phytoplasmas can be visualised by electron microscopy and their presence in phloem tissues demonstrated by fluorochromic DNA stains, these methods cannot discriminate among phytoplasma groups (Clark *et al.,* 1989). Immunological assays are one among the most important methods for disease diagnosis and pathogen detection. Several polyclonal and monoclonal antibodies have been prepared against plant pathogenic phytoplasmas (Sinha and Benhamou, 1983; Sinha and Chiykowski, 1984; Clark *et al*., 1989; Saeed *et al*., 1993) for different immuno assays like double diffusion test, ELISA, dot-blot, and immunoblotting.

1.5.3.2. Ouchterlony double diffusion test

Nayar and Ananthapadmanabha (1975) purified sandal spike phytoplasma by ammonium sulphate precipitation method and used the same to raise polyclonal antibodies in rabbit. The polyclonal antibody was used in gel diffusion and agglutination tests. They reported a very poor titre of 1:250 dilution probably due to presence of plant debris along with the phytoplasma cells which might have decreased the sensitivity of polyclonal antibodies.

The antibody could detect phytoplasma in spike disease affected sandal and *Catharanthus roseus* plants infected artificially with sandal spike phytoplasma.

Double diffusion tests are used frequently for testing material from field surveys. The test is widely used for screening viruses (Ahmad and Scott 1985; Barnett *et al*., 1987) and bacteria (Bouzar and Moore 1987; Azad and Schaad 1988). In the present study, even though precipitin bands could be observed against diseased sandal extract, no band was observed against the hosts, *Lantana* or witches' broom-affected *Zizyphus*. The result suggests that considerable amount of phytoplasma was present in the extract of diseased sandal and probably none in the extract of the host plants. Nayar (1981) also reported absence of precipitin bands against witches' broom affected *Zizyphus* when treated with sandal spike phytoplasma specific antibody. Since precipitin bands were observed in both Mysore and Marayoor phytoplasma populations, the pathogens must have been of the same antigenic group. However the sensitivity of the test was found to be lower compared to other techniques as it could only detect phytoplasma in concentrated and 1:2 dilution samples.

1.5.3.3. ELISA tests

ELISA is a quantitative method of immunological detection. The technique is highly sensitive to detect the presence of pathogens. Polysorp plates were used for the present study since they preferentially adsorb lipoproteins (Nunc, 1997) which are the major antigenic determinants of the mollicutes. According to Kemeny (1992) one hour duration was found to be satisfactory for coating antigen to the plate and for subsequent incubation steps with the immunogenic probes. Initial experiments showed that increasing the duration of incubation with immunogenic reagents decreased the sensitivity of the test probably due to unspecific binding. In the present study also the absorbance ratio was found to be higher for one hour, whereas it decreased with time (Fig.10, 11). Therefore, one hour was kept constant for both the coating and incubation steps. Direct ELISA is seldom employed in immuno-diagnostic tests due to its low sensitivity (Crowther, 1995), but, from the present study it was found that though, the technique was less sensitive

compared to indirect ELISA, it could, nevertheless detect the presence of phytoplasma in both purified and crude sample.

For indirect ELISA, polyclonal antibody at a dilution of 1:2000 was found to be optimum as indicated by the absorbance ratio (Fig.12). Though, the indirect method employed three types of systems, the biotin-streptavidin system was found to be the most sensitive of the assay, probably due to the high amplification property of the system compared to avidin and anti-rabbit HRP systems. When biotin-streptavidin and anti-rabbit HRP systems were compared for detection of the presence of phytoplasma in crude samples, the former was again found to be more sensitive. However, the tests could detect the presence of pathogen even at dilutions up to 1:10,000 probably due to the high sensitivity of the antibody generated using the purified phytoplasma. Correlating the phytoplasma protein values with antigen dilution suggests that indirect ELISA could detect a minimum of 25ng antigenic protein. When the efficiency of the polyclonal antibody was tested by screening large number of diseased sandal plants, only the diseased plants showed high values compared to the healthy plant values (Table 7). Rangaswamy (1995) produced polyclonal antibody to detect phytoplasma in spike disease affected sandal and *Catharanthus roseus* plants infected artificially with sandal spike phytoplasma by indirect ELISA. The titre of the antibody was calculated to be 1:1000 dilution and could detect antigen upto a dilution of 1:200.

Though, most of the workers employed indirect ELISA, they could not detect the pathogen at higher dilutions of plant extract, probably due to contamination of plant proteins in the purified phytoplasma pellet which might have generated antibodies against healthy plant proteins thereby decreasing the efficiency of ELISA. Hobbs *et al*. (1987) could detect phytoplasma in peanut witches' broom only up to a dilution of 1:400, whereas Clark *et al*. (1989) could detect dilutions up to 1:600 while detecting tomato big bud phytoplasma. Saeed *et al*. (1993) detected phytoplasma in faba bean phyllody up to a dilution of 1:300.

Even though, the highly sensitive indirect ELISA technique using biotin-streptavidin system was employed, phytoplasma could not be detected in the host plants of spike

disease affected sandal. The hosts of diseased sandal did not show much variation in the result compared to the control plants (Table 8). The results were identical when large number of samples were screened for the detection of sandal spike phytoplasma (Table 9). Thus both the immunological techniques- Ouchterlony double diffusion test and indirect ELISA could not detect phytoplasma in the hosts of diseased sandal confirming that sandal spike phytoplasma is specific to sandal. Studies by Rangaswamy (1995) using indirect ELISA also could not detect sandal spike phytoplasma in diseased *Zizyphus*. The negative reaction obtained when insect tissues were ELISA tested can not be taken as conclusive. The experiment has to be repeated systematically by rearing the insect vector on diseased plants or testing the vector after proper acquisition feeding.

In spite of using a highly sensitive protein detection technique using the silver nitrate method it was not possible to locate the antigenic protein in the SDS-PAGE gel. Jiang *et al*. (1988) and Saeed *et al*. (1992) also could not identify phytoplasma specific proteins among the contaminating plant proteins using SDS-PAGE. Bloom *et al*. (1987) were of the opinion that silver staining was far more sensitive than coomassie blue to detect the presence of protein in the nanogram range. From the present study it is confirmed that sandal spike phytoplasma antigenic protein has a molecular weight of 14,000 daltons and could be detected in both purified and semi-purified phytoplasma but the same was absent in healthy sandal. Clark *et al*. (1989) reported that primula yellow phytoplasma and European aster yellow phytoplasma had a single major antigen of 22,400 daltons while aster yellow phytoplasma affecting lettuce had an antigenic protein of 18,500 daltons (Jiang *et al*., 1988). Saeed *et al*. (1992b) reported that faba bean phyllody phytoplasma had two antigenic proteins of 18,000 and 36,000 daltons.

According to Clark *et al*. (1983), the identification of infection by phytoplasmas may be difficult when symptoms are absent or indistinct. Rapid immunological techniques like ELISA, circumvents most of these problems. From the present study it is inferred that polyclonal antibody raised against sandal spike phytoplasma was found to be efficient for detection of the pathogen through immunological techniques. The same antibody can be used as the primary probe for other immunological tests such as Dot immuno-binding

assay, immuno microscopy, etc. Another positive factor of the technique is that the pathogen could be detected in the crude sample itself. Thus immuno diagnostics could be used as a tool for early detection of the pathogen in plant samples.

2. Commercial exploitation of ELISA technique for detecting sandal spike phytoplasma

2.1. Introduction

Immunological techniques have been developed to detect sandal spike phytoplasma. The reagents developed for diagnosis of sandal spike phytoplasma and early detection of spike disease can be marketed as kits which may be useful for the sandalwood industry. The commercial exploitation of the reagents developed is explained in this chapter.

2.2. Diagnostics

Diagnostics can be viewed as a discipline in its own right, combining a wide range of techniques in developing simple, fast and reproducible (SFR) procedures that measure a feature of the biological material in hand in a way that is easily interpretable. Improved diagnostics could be useful in epidemiological studies to determine the distribution and abundance of pests and pathogens (Skerritt and Appels, 1995).

During the past few years, progress in molecular biology, biochemistry and immunology has promoted the development of many new methods of pathogen detection and disease diagnosis (Miller and Martin, 1988). Immunoassays have been developed and are commercially available for the identification of plant pathogens, mycotoxins, pesticides and plant hormones. Polyclonal antibodies are extremely useful since their broader spectrum can sometimes be more useful than the highly specific monoclonal antibodies (Miller and Williams, 1990).

2.3. Commercial exploitation of the techniques based on the present work

The techniques used in the present study for the purification of sandal spike phytoplasma using the differential filtration method was rapid and economical. The purity of the phytoplasma cells thus generated was found to be very high. The purified phytoplasmas elicited immune response in rabbits to produce highly sensitive polyclonal antibodies, which could be used in different immunological techniques. Thus, the immunological tests like Ouchterlony double diffusion test, and direct and indirect ELISA could be standardised for pathogen detection. Since, one hour of duration was found to be optimum for both coating and incubation, direct ELISA could be completed within three hours and indirect ELISA within seven hours. Even though, the number of steps was more in indirect ELISA, the sensitivity of the test was high when biotin-streptavidin system was adopted.

Thus, two major kits could be developed based on the present studies viz., sandal spike phytoplasma purification kit and immuno-detection kit (Fig. 13). The reagents that can be supplied is listed in the table (Table 10).

In India, marketing of sandal is restricted exclusively to government agencies leaving very little scope for private agencies. So the kits developed *per se* has limited chance for commercial exploitation in the open market in the present scenario. But, since, the

techniques developed are highly specific and sensitive, the same could be used in other tree improvement projects.

Most of the companies supply antibodies either as freeze dried powder or with preservatives which has a shelf life of about 12 months at 40C or for longer period at -20^0C (Adgen, 1998). The positive and negative control samples are supplied as freeze-dried sap/extract which should be reconstituted with distilled water or buffer before use. Adgen sells most phytoplasma kits of 1000u for around US $600 (Rs.27,500). These kits contain reagents for ELISA only. The reagents of sandal spike phytoplasma, when produced in large scale, could be supplied at one–fourth the price of Adgen due to low labour and other input costs.

Table 10. Kits and reagents developed to detect sandal spike phytoplasma.

KIT	REAGENTS
SANDAL SPIKE PHYTOPLASMA PURIFICATION KIT	Healthy sandal antibody, Glycine buffer.
IMMUNO DETECTION KITS 1. Double diffusion kit 2. ELISA kit	Phytoplasma specific antibody, Phytoplasma specific antibody-HRP, Anti-rabbit antibody-HRP, Anti-rabbit antibody biotin, Streptavidin-HRP, Phosphate buffered saline, Positive control (phytoplasma).

3. Identification of 'disease resistant' trees using ELISA technique

3.1. Introduction

In Marayoor Range, nine natural sandal reserves, spread in an area of 15.5 km^2 in reserve forests and 47 km^2 in the adjoining revenue lands constitute the main sandal tract. The sandal reserve forests of Marayoor is considered to be the best in India in respect of sandal trees per unit area and oil yield (5%). Extraction of sandal is limited to dead and wind fallen trees not only from reserve forests but also from revenue lands. Only the forest department is authorised to extract such trees.

Though, spike disease was reported first in 1980 only from the Sandal Reserve 51 (382 ha in area), the disease might have started a few years before (Ghosh *et al*., 1985). Enumeration of sandal trees in 1971 in the reserve recorded a population of more than 23,000 trees (Varghese, 1976). In 1985, more than 50 per cent of the trees in the reserve were found affected by the disease and by 2000, more than 80 per cent of the trees had perished.

Ghosh *et al*. (1985) reported the apparent infection rate 0.066 - 0.086 per unit per month in disease monitoring plots. Disease spread is radial and infected trees die within 2 years on an average. In diseased tracts, usually, almost all the trees get infected and die. However, in Reserve 51, about 45 trees of various girth classes were observed as disease free during 1996 in about 100 ha area surrounding the epicenter of the disease. These trees have either evaded the infection or may be resistant against spike disease.

In this chapter, result of monitoring the health of 15 such apparently more than 25-year-old trees are provided. The monitoring involved periodical ELISA tests of leaf extracts for the early detection of sandal spike phytoplasma.

3.2. Materials and methods

The girth of the selected trees ranged from 32 cm to 55cm. Apparently, all these trees had existed when the spike disease was reported first in 1980. The 15 trees, located in about one km^2 area, were protected from human interference by providing 3 m wide chain-linked fence around each tree (Fig. 14).

3.3. Results

All the 15 trees were healthy till March 1998 and none of the trees showed any doubtful external symptom of infection. ELISA tests were conducted on leaf extracts of all the trees in March 1998 and none of the trees were positive. But in September 1998 two adjacent trees (tree No. 8 and 9, standing 15 feet apart) were ELISA positive (Table 11) indicating that the trees were already infected. But, typical symptom of spike disease was not expressed on the leaves or branches. However, emergence of new sprouts from branches were absent. In March 1999, these two trees showed severe defoliation and typical spike disease symptoms almost on all branches. Both the trees dried partially by September 1999 and completely by March 2000. Likewise, tree No. 4 was ELISA positive from March 1999 onwards, but externally healthy during September 1999. It dried partially by March 2000. Remaining trees were free of phytoplasma and were apparently healthy till March 2000.

Table 11. Result of ELISA test on three 'disease evaded' trees showing ELISA positive reaction before external symptom expression.

Tree No.	GBH (cm)	Expression of disease symptom				ELISA test result (Presence of phytoplasma)	
		1996	1997	1998	1999	1998	1999

		Mar	Sep	Mar	Sept	Mar	Sept	Mar	Sept	Mar	Sept	Mar	Sept
4	32	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Neg.	Neg.	Pos.	Pos.
8	44	Nil	Nil	Nil	Nil	Nil	Nil	Yes	Dry-ing	Neg.	Pos.	Pos.	Pos.
9	41	Nil	Nil	Nil	Nil	Nil	Nil	Yes	Dry-ing	Neg.	Pos.	Pos.	Pos.

Nil : No disease symptom; Pos. : ELISA Positive; Neg. ELISA Negative

3.4. Discussion

Reports of phytoplasma disease resistance in tree crops are very rare. Expression of disease symptoms is always associated with presence of phytoplasma. In this experiment, it was possible to detect the pathogen in the leaf extract of tree No. 4, 8 and 9, six months before the expression of the disease symptoms. Even though, the remaining trees were pathogen free till September 2000, it is not possible to conclude that these trees will evade the disease in future also or they are actually disease resistant trees. Considering the fact that those sandal trees which remained uninfected amid severely infected trees for more than 15 years became vulnerable to infection subsequently, prevents us from concluding that resistance to spike disease exist in sandal population in Marayoor.

Since, all the attempts to culture the pathogen *in vitro* has failed, it is not possible to inoculate the disease-evaded trees with phytoplasma to prove their field resistance. Tests of susceptibility through graft transmission or through parasitic flowering plants seems artificial, not known to occur in the reserve forest. Hence, inoculation with infective insect vector is the only logical method to test the resistance of a tree.

However, the success achieved in the early detection of spike disease through ELISA technique may be useful to control the pathogen through infusion of tetracycline group of antibiotics before the full multiplication of the phytoplasma in phloem tissues.

4. Micropropagation of disease evaded genotypes

4.1. Introduction

Selection and planting of disease resistant genetic material is the primary approach to improve productivity of a plant species. In Marayoor forest range of Munnar Forest Division in Kerala, spike disease has caused serious destruction to the sandal trees (Ghosh *et al.,* 1985). Death of almost the entire population existing in an infected patch is characteristic of the disease. However, a few trees, which have remained free from the disease for the past 20 years, are considered either resistant or capable of evading the disease. One of the objectives of this project is the clonal multiplication of a few such disease-evading trees located in the disease ravaged sandal forests in Marayoor, through tissue culture via somatic embryogenesis.

In India, extensive work on tissue culture of sandalwood was carried out mainly in two laboratories – BARC, Bombay (Bapat and Rao, 1979, 1984; Rao and Raghavaram, 1983) and Indian Institute of Science, Bangalore (Lakshmi Sita 1986, 1992; Lakshmi Sita *et al*., 1994). Although multiple shoot formation was reported (Rao *et al*., 1984; Sanjaya *et al*., 1998; Pradhan and Saiju, 1999), regeneration of plantlets through somatic embryogenesis was suggested to be the most efficient method of mass propagation in *Santalum album* (Bapat *et al*., 1990). Even though, sandalwood has got a very high frequency of somatic embryogenesis, the successful utilization of the technique is hampered due to the high percentage of abnormal embryogenic plantlets and poor rate of survival (Rao and Bapat, 1995). Hence, only very few plantlets have been reported to be established outside the laboratory after out planting.

In the present study, an attempt has been made to multiply a few spike disease-evading sandal trees identified in a heavily diseased area in Marayoor Forest Range of Munnar Forest Division through tissue culture via somatic embryogenesis.

4.2. Materials and Methods

4.2.1. Explants

The internodes, nodes, petioles, leaf segments and root suckers collected from trees that were apparently more than 20-year-old were used as explants for the present study. The explants, were washed in tap water with Extran (Merck), kept for 15 minutes in running tap water, sterilized with 0.1% $HgCl_2$ for 8 minutes and washed in sterile water four times, before inoculation onto appropriate culture medium.

4.2.2. Culture media

The minerals and vitamins of the MS Medium (Murashige and Skoog, 1962), White's medium (White, 1963), Woody plant medium (WPM) (Lloyd and McCown, 1981) and B_5 Medium (Gamborg *et al*., 1968) were tried at various stages of culture. The pH of the media was adjusted to 5.7 using 0.1N NaOH and 0.1 N HCl prior to autoclaving at 120^oC at $15lbs/in^2$ pressure for 20 minutes.

4.2.3. Growth regulators

Various auxins (2,4-D, 2,4,5-T, NAA, IAA, Dicamba and Picloram) and cytokinins (BAP and Kinetin) were supplemented in the media during the different stages of culture viz. induction, maintenance and conversion of embryogenic callus to plantlets.

4.2.4. Additives

To compare the improvement in the rate of proliferation of soft friable calli prior to embryogenesis, the following additives were used.

i. Malt extract
ii. Yeast extract
iii. Casein hydrolysate
iv. Coconut water

4.2.5. Growth adjuvants

In order to improve the efficiency of embryogenesis, the following growth adjuvants were used.

i. Abscisic acid (ABA)

ii. Polyethylene glycol (PEG)

iii. Casein hydrolysate (CH)

iv. Activated charcoal (AC)

4.2.6. Culture conditions

All cultures on semi-solid media were routinely incubated at 25±1^{o}C under diffused fluorescent light with 15-h photoperiod. Subculturing was carried out usually at 4-week intervals.

4.2.7. Suspension cultures

Cell suspensions were initiated from friable callus grown on MS medium supplemented with 2,4-D. Suspension cultures were induced in 250 ml conical flasks, shaken on rotary shakers at 125 rpm. Under agitation, the cultures produced a fine suspension of single cells and a few cell aggregates. Subculturing to fresh medium was carried out at 15 days interval after allowing the cells to settle down, decanting the medium and replacing it with an equal amount of fresh medium.

4.3. Results

4.3.1. Callus initiation

Initiation of callus from the exposed cut surface of internode segments took place 3 to 4 weeks after inoculation. Further growth was very slow and it took 2 to 3 months for initiation of a proliferating light yellow, compact callus (Fig.15). Of the different explants tried for callusing, internode explants from mature plants and root suckers gave a good proliferating callus. The nodes, leaves and petioles showed no sign of callusing.

Of the four basal media tried, callus initiation took place only in MS medium. Among the different hormonal combinations tried, MS medium fortified with 2,4-D (0.1 mg/l) and Kinetin (1mg/l) showed callus initiation in 90 per cent of the cultures. No sign of callusing was noticed in all the other combinations tried.

4.3.2. Initiation of the friable soft calli

The compact proliferating callus was routinely subcultured on fresh medium of the same composition at 30 days interval. After 5-6 subcultures, white friable soft calli appeared (Fig. 16) from the side of the brown compact callus. Of the different types of basal media used for proliferation of friable callus, WPM and half strength MS medium gave almost the same proliferation rate, while B_5 medium gave the least amount of proliferation. The friable soft calli could be maintained indefinitely for a long period without losing its embryogenic capacity. Among the different auxins, the rate of proliferation was highest for 2,4,5-T followed by 2,4-D while NAA and IAA gave almost the same amount proliferation and picloram and dicamba gave the least amount of proliferation. Coconut water at 20 per cent level gave the highest level of proliferation and Casein hydrolysate (0.5g/litre) yielded the least amount of proliferation among the additives tried for friable calli proliferation.

The basal MS medium supplemented with 2,4-D was also experimented with different solidifying agents. Almost all the concentrations of agar and phytagel gave rise to almost the same rate of proliferation except agar (0.5 %) in which the callus proliferation was very low.

4.3.3. Induction of embryogenic calli

When the friable soft calli was subcultured on to MS basal medium supplemented with IAA/BAP alone or in combinations gave rise to white friable, granular embryogenic callus (Fig.17).

Of the different combinations tried, IAA (0.5mg/l), and BAP (0.5mg/l) gave the highest amount of embryogenic callus. This embryogenic tissue, after two weeks gave rise to somatic embryos, which followed the typical stages of embryogenesis. Abnormal embryos occurred in large number along with embryos of normal morphology. Somatic embryos of all stages of development were found in the same culture vessel and adventitious embryo formation (repetitive embryogenesis) was also observed.

Of the four different types of basal media combinations tried for embryogenic callus initiation, B5 medium gave rise to embryogenic callus seven days after culture while in the rest of the media, embryogenic callus induction was noticed after three days. Among the four media tried, WPM gave the highest amount of embryogenic callus.

4.3.4. Embryo maturation and conversion

Many of the somatic embryos developed asynchronously and germinated precociously, and some had atypical morphology. This type of precocious germination without passing through characteristic stages of development in sandal wood somatic embryos was found to be controlled to some extent by adding abscisic acid at concentrations ranging from 0.1 to 5 μM. Abscisic acid at 3 μM concentration gave rise to high percentage of embryos normal in appearance. Embryo abnormalities increased greatly above 5 per cent sugar level. Maltose at 0.1 M level was found to be optimum than sucrose at 5 per cent level which was previously reported in sandal. In presence of maltose, the embryos grew larger, morphologically more typical and viable (Fig.18).

The highest percent of normal embryos was obtained when PEG, a non-permeating osmoticum was used in combination with abscisic acid (3μM). Addition of PEG (at 2.5 %) to maturation medium improved the number of embryos and enhanced uniformity.

4.3.5. Cell suspension culture

The effect of initial culture density and effect of total volume of the media on rate of proliferation were tested. In order to ascertain the embryogenic potentiality of suspension culture, after one month of culturing in liquid medium, cells were filtered and washed with MS basal medium and plated on to semi-solid embryo induction medium. MS supplemented with IAA (0.5mg/l) and BAP (0.5mg/l) resulted in the formation of pro-embryogenic masses followed by the differentiation of somatic embryos. After embryo induction, the effective separation of the embryos for further conversion was brought about by transferring the embryogenic masses again to liquid maturation medium. If they were kept on the same semi-solid medium, instead of developing into mature embryos, many of them dedifferentiated again and produced a callus mass. After one month of culture in liquid medium, the cells were filtered and plated on solid media, after which the number of embryos formed were found to be higher. The fully developed embryos were transferred to semisolid conversion medium.

4.3.6. Embryo conversion to plantlets

Even though, improvement in somatic embryo development was achieved, only 20 per cent of the normal embryos were converted into plantlets in the final stage. The development of sandal plants from somatic embryos was attempted by transferring single embryos at the cotyledonary stage to a medium with lower concentration of sucrose and a low concentration of growth regulators. The isolation was found to have stimulating effect on their germination. In many cultures, the embryo instead of developing into plantlets, dedifferentiated again and produced a callus mass at the base which again showed regeneration of somatic embryos. Among the hormonal combinations tried, Whites medium or quarter strength MS medium containing IAA (0.5mg/l), IBA (0.5mg/l) and GA_3 (1mg/l) gave a moderate rate of conversion to plantlets. Along with normal plantlets with well developed tap root and shoot (Fig.19), abnormal plantlets either with prominent shoot and no root or with well developed tap root and no development of shoot were also observed. Since sandal is a semi-root parasite, which draws part of its nutrients from host through haustorial connection, sandal plantlets during the hardening stage were co-cultivated with *Cajanus* seedlings. After 4-6 weeks the root region of sandal plantlets established haustorial connection with the *Cajanus* seedlings (Fig.20). The somatic plantlets with well-developed root and shoot were transferred to sterile vermiculite and soil in plastic cups (Fig. 21). No plantlet survived beyond three months even if the humidity was maintained and plantlets were nourished with nutrient solution.

4.4. Discussion

The production of a large number of embryos from stem-derived callus of superior, disease resistant/evaded mother plant and their successful conversion to plantlets makes a significant step on sandal wood micropropagation. The induction of somatic embryogenesis in *S. album* is one of the earliest reports on somatic embryogenesis in forest trees (Lakshmisita *et al.*, 1979, 1982; Bapat *et al*., 1995). But so far no reported work has claimed satisfactory survival of embryogenic plantlets. All the reported hormonal combinations 2,4-D (0.1mg/l), Kinetin (0.2mg/l), BAP (0.2mg/l) (Rao and Bapat, 1978; Lakshmi Sita *et al*., 1979) failed to give rise to callus from mature explants in the present study. Aberrant/abnormal embryo phenotype and subsequent low

conversion rate of somatic embryos into normal plantlets have been reported (Bapat and Rao, 1984; Rao and Bapat, 1995). The morphogenetic differentiation of a somatic cell to whole plant is a complex developmental process, that involves morphogenetic, biochemical, physiological and molecular changes (Thorpe, 1995). The physiological processes leading to efficient germination of somatic embryos has to be elucidated to improve the conversion rate of somatic embryos and survival rate of embryogenic plantlets (Rao and Bapat, 1995). Though, the culture conditions required for somatic embryogenesis are well defined in *S.album* (Rao and Bapat, 1995), the molecular and biochemical events underlying the phenomenon are ill defined. The semi-parasitic nature of sandal seedlings points to specific nutritional requirements, which has to be studied so that the probability of establishing micropropagated plantlets in large numbers can be explored.

5. Conclusions

1. The study has resulted in the development of a technique for purified preparation of sandal spike phytoplasma for production of polyclonal antibodies. The polyclonal antibodies can be utilized for development of ELISA techniques for early detection of phytoplasma infection. The ability of phytoplasma to pass through 0.45μm millipore filter is exploited to remove host debris larger than their size. Removal of host protein and tiny debris are made possible through cross absorption of semi-purified phytoplasma preparation with antibodies produced against extract from healthy plant leaves. The filtration is followed by centrifugation at 65,000g to obtain purified phytoplasma pellet. This new procedure is an improvement over the earlier methods of cross absorption and repeated low speed centrifugation to obtain phytoplasma pellets. In previous methods, large quantity of phytoplasma are lost along with the host debris during the repeated low speed centrifugation.

2. The purified polyclonal antibodies can be utilized for development of techniques such as Dot Immunobinding Assay (DIBA), Immuno microscopy, Immuno electron microscopy and fluorescent antibody techniques. The DIBA technique can be adopted for field detection of sandal spike phytoplasma.

3. The absence of sandal spike phytoplasma in host plants of spike disease affected sandal and in witches' broom affected *Zizyphus oenoplaea* confirms that sandal spike phytoplasma is confined to sandal only and the disease is not transmitted through host plants. It is also concluded that witches' broom disease of *Z. oenoplaea* is not caused by sandal spike phytoplasma.

4. Incidence of disease in trees which evaded infection for more than 20 years creates the doubt whether resistance against sandal spike phytoplasma exists at all even in disease evaded trees, presumed to be resistant. Absence of resistant trees among disease evaded trees in the field necessitates screening sandal provenances and

populations from all over India and overseas in order to identify spike disease resistant varieties. Resistant genes can also be detected in other species of *Santalum* which can be used for distant hybridization.

5. Considering the economic importance of the species, modern biotechnological tools such as introduction of resistant genes against spike disease through genetic engineering can be attempted.

6. Mass multiplication of disease evaded trees and superior candidate plus trees (CPTs) through somatic embryogenesis is only partially successful. Conversion of somatic embryos to normal plantlets and proper hardening for establishment of outplanted plantlets are yet to be achieved. Hence, more efficient protocol has to be developed for somatic embryogenesis and hardening of plantlets. The survival of the plantlets is probably complicated due to the parasitic nature of the species.

7. It was not possible to detect the phytoplasma in the salivary gland and intestinal tissues of *Redarator bimaculatus*, the insect vector through ELISA techniques. This may be because the vector might not have acquired the pathogen even though they were collected from diseased trees. Further studies are needed to confirm the insect vector through proper acquisition feeding studies and detection of the pathogen through immunological and molecular techniques (RFLP).

6. Research Results of Practical/Field Application

1. New techniques have been developed for purified preparation of sandal spike phytoplasma. Protocols have been standardized for production of polyclonal antibodies against the phytoplasma. Early detection of spike disease, at least six months before the external expression of disease symptoms was possible through application of direct and indirect ELISA technique developed through the present study.

2. The protocols developed for purification of phytoplasmal and standardization of ELISA techniques can be utilized for development for such techniques for early detection of the pathogen in other crop plants.

3. Absence of sandal spike phytoplasma in witches' broom affected-*Zizyphus oenoplaea* and host plants of spike disease affected sandal indicated that host plants of sandal have probably no role in spike disease spread in the forest.

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