DETECTION OF SANDAL SPIKE PHYTOPLASMA USING IMMUNOLOGICAL AND MOLECULAR TECHNIQUES

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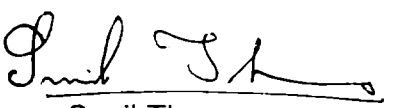
DECLARATION

I hereby declare that this thesis entitled `**DETECTION OF SANDAL SPIKE PHYTOPLASMA USING IMMUNOLOGICAL AND MOLECULAR TECHNIQUES'** submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy under the Faculty of Environmental Studies of Cochin University of Science and Technology is my original work and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.



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CERTIFICATE

This is to certify that the thesis entitled **`DETECTION OF SANDAL SPIKE PHYTOPLASMA USING IMMUNOLOGICAL AND MOLECULAR TECHNIQUES'** embodies the result of original research work conducted by Mr Sunil Thomas (Reg. No. 1837), under my guidance and supervision. No part of this thesis has previously formed the basis for the award of any degree, diploma, associateship or other similar titles of this or other Institute or any University or Society. I further certify that he has passed the Ph.D qualifying examination.

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Sunil Thomas

AD MAJOREM DEI GLORIAM

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER IV

CHAPTER V

ABBREVIATIONS

A	Adenine
Abs	Absorbance
A_{260}	Absorbance at 260 nm
Ab	Antibody
AEC	Amino ethyl carbazole
Ag	Antigen
Alu I	*Arthrobacter luteus* I
bp	base pair
BSA	Bovine serum albumin
C	Celsius/Cytosine
cm	centimetre
C. Ab	Conjugated antibody
cDNA	complimentary deoxyribonucleic acid
CS	Cross section
CTAB	Cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenyl indole
DAS-ELISA	Double antibody sandwich ELISA
DIBA	Dot immunobinding assay
DNA	Deoxyribonucleic acid
16S rDNA	16S ribosomal DNA
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscope
FITC	Fluorescein isothiocyanate
G	Guanine

g	gram/gravity
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IAA	Indole acetic acid
Ig G	Immunoglobulin G
ISEM	Immunosorbent electron microscopy
kba	kilobase
kDa	kiloDalton
km	kilometre
M	Molar
m	metre
mm	millimetre
ml	millilitre
mM	milliMolar
M Da	Million Daltons
MLO	Mycoplasma-like organism
Msl	Mean sea level
Mab	Monoclonal antibody
MOPS	3-(N-morpholino)propane sulfonic acid
µg	microgram
µl	microlitre
µm	micrometre
nm	nanometre
OD	Optical density
ODD	Ouchterlony double diffusion
O-MLO	Oenothera MLO
OPDA	O-phenylene diamine
Pab	Polyclonal antibody

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
$r_{.av}$	average rotor radius
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
16S rRNA	16S ribosomal RNA
$S_{20,w}$	Sedimentation coefficient (Svedberg)
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
Sq.kms	Square kilometres
T	Thymine
TAE	Tris acetic acid EDTA (buffer)
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA (buffer)
TEM	Transmission electron microscopy
UV	Ultra violet

1. INTRODUCTION

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 sweet, woody odour. The oil is widely employed in the fragrance industry, particularly in the higher 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).

1.1. DISTRIBUTION OF SANDAL

The genus, *Santalum* consists of 25 species distributed between 30^0N and 40^0S, from 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). *Santalum* species are characterised by two main features - obligate hemi-parasitism and aromatic heartwood. 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 stand 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). Limited distribution of sandal is also seen in the reserves of Arienkavu and Kasargod forest ranges (Chand Basha, 1977). Production of sandalwood has plummeted from around 3000 tonnes per annum during 1985 to around 1000 tonnes in 1997; similarly oil production also declined from 140 tonnes in 1985 to 40 tonnes in 1997 (Jain *et al.*, 1999).

1.2. DISEASES OF SANDAL

Diseases of sandal include seedling diseases caused by *Phytophthora* sp. and *Fusarium oxysporum* and leaf spot diseases caused by different fungal pathogens like *Ascochyta santali*, *Macrophomina phaseoli*, *Asterina congesta* and *Sphaceloma santali*. *Ganoderma applanatum* causes white mottled rot, whereas, *Ganoderma lucidum* causes white spongy rot, attacking roots and spreading to the basal part of the stem. Another disease, though not of much economical importance is the leaf curl disease caused by a virus. But the major disease of sandal is the spike disease (Mukerji and Bhasin, 1986; Ghosh *et al.*, 1992; Srinivasan *et al.*, 1992).

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' (Figs.1.1, 1.2). Spiked plants do not bear flowers or fruits; occasionally abortive flowers are developed. Spiked trees usually die within one-to-two years after the appearance of the symptoms. In Kerala, although the production of sandalwood has not declined markedly because of the extraction of dead trees (which increased in number as a result of spike disease), the stock in the forest is depleted considerably (Ghosh *et al.*, 1992); whereas, in Karnataka, the growing stock has been reduced to 25 percent of its initial level in the last two decades (Swaminathan *et al.*, 1998). The disease is not known in Timor and does not affect other species of *Santalum* (Fox and Barrett, 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).

Fig.1.1. Sandal tree infected with spike disease.

Fig. 1.2. Comparison of healthy and spike disease affected sandal twig.

1.2.2. The pathogen

Sandal spike disease was thought to be caused by a virus (Coleman, 1923; Parthasarathi *et al.*, 1966) until 1969 when three independent groups by electron microscopy confirmed 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). The pathogens are seen exclusively in the sieve tubes of phloem tissues of leaves, petioles, stem and root causing symptoms such as leaf yellows, little leaf, phyllody, witches' broom, etc. Phytoplasmas has been implicated as pathogens in more than 300 plant diseases world-wide (McCoy *et al.*, 1989). The disease caused by the pathogens include peanut witches' broom, lilac witches' broom, ash yellows, chrysanthemum yellows, lethal yellowing of palms, blue berry stunt, rice yellow dwarf, X disease of *Prunus*, sugarcane white leaf, apple chlorotic leaf roll, etc. (Sinclair *et al.*, 1996).

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 Kirkpatrick, 1993). The change in terminology from mycoplasma-like organism (MLO) to phytoplasma in 1994 reflected new knowledge about the plant inhabiting mollicutes (Sears and Kirkpatrick, 1994).

Phytoplasmas has 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. Until recently, phytoplasmas could be studied only on the basis of biological properties such as disease symptoms, plant host range, and insect vector specificity. Now, the pathogen could be visualised by electron microscopy and their presence in phloem tissues demonstrated by fluorochromic DNA stains; but these methods do not discriminate among different groups of phytoplasmas (Clark *et al.*, 1989; Neimark and Kirkpatrick, 1993).

1.2.3. Disease diagnosis and detection

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 of the study of these diseases. This is principally due to the lack of methodologies for detection of pathogens in the field or in quarantine material. In addition, the relatedness among various phytoplasma groups and the epidemiology of many of the phytoplasma diseases could not be studied until recently. Fortunately, development and exploitation of new approaches to pathogen detection now promise to overcome many of the problems that hinder correct diagnosis and identification (Davis and Lee, 1988).

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. Immunoassays are used in plant pathology for identification, diagnosis and quantitation of plant pathogens (Barbara and Clark, 1986). The development of procedures to obtain phytoplasma enriched preparations from infected plants has permitted the production of phytoplasma specific polyclonal and monoclonal antibodies and has been routinely used to detect the pathogen in a wide variety of plants (Hobbs *et al.*, 1987; Clark *et al.*, 1989; Saeed *et al.*, 1993).

Detection and identification of phytoplasma using molecular techniques like polymerase chain reaction (PCR) has become popular because of the high sensitivity of the test. PCR is preferred in situations where the concentration of phytoplasma may be very low. Thus PCR followed by restriction fragment length polymorphism (RFLP) is employed in the detection and identification of the pathogen (Saeed and Cousin, 1995). Seemuller *et al.* (1998) has classified phytoplasmas into twenty groups based on the RFLP analysis of 16S rDNA (ribosomal DNA).

1.3. OBJECTIVES OF THE WORK

Spike disease in 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) and 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, Rangaswamy, 1995). But most of these techniques are insensitive, indirect detection methods leading to misinterpretation of results. Moreover, to identify disease resistant sandal trees, highly sensitive techniques are needed to detect the presence of the pathogen. In sandal forests, several host plants of sandal like *Zizyphus oenoplea* (Fig. 1.3) also exhibit the yellows type disease symptoms. Immunological and molecular assays have to be developed to confirm the presence of sandal spike phytoplasma in such hosts. The major objectives of the present work includes:

1. *In situ* detection of sandal spike phytoplasma by epifluorescence microscopy and scanning electron microscopy.
2. Purification of sandal spike phytoplasma and production of polyclonal antibodies.
3. Amino acid and total protein estimation of sandal spike phytoplasma.
4. Immunological detection of sandal spike phytoplasma.
5. Molecular detection of sandal spike phytoplasma.
6. Screening for phytoplasma in host plants of spike disease affected sandal using immunological and molecular techniques.

Fig.1.3. Comparison of healthy (left) and witches' broom disease affected (right) *Zizyphus oenoplea.*

1.4. ORGANISATION OF THE THESIS

The thesis is organised into eight chapters. The first chapter introduces the topic of research and the objectives of the work. The second chapter reviews the work done so far in sandal spike disease and also describes the immunological and molecular techniques used to detect phytoplasma affecting other plant species. The third chapter describes several *in situ* detection techniques to detect the pathogen. The method used to purify sandal spike phytoplasma is explained in the fourth chapter. The chapter also brings out the results of protein and amino acid studies of the pathogen. The fifth chapter explains different immunological tests used to detect sandal spike phytoplasma, while the sixth chapter deals with the molecular detection techniques. Commercial exploitation of techniques developed to detect the pathogen is discussed in the seventh chapter and the major results are summarised in the last chapter. There is a common bibliography for all the chapters under the title `References'

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Plants are essential for the survival and well-being of mankind and are the renewable sources of food, fibre and timber. Whether cultivated or naturally seeded, plants are governed in their adaptability and yield by the interaction of biotic and abiotic factors that constitute a given habitat. When one or more of these factors limit plant growth, disease may be manifested as growth retardation or as death of entire plant or their parts (Tainter and Baker, 1996). A plant may be said to be diseased when there is a harmful deviation from normal functioning of the physiological process (Federation of British Plant Pathologists, 1973).

2.1. FOREST PATHOLOGY

Forest Pathology is the branch of plant pathology that deals with diseases of woody plants growing in natural forests, plantations and in urban environment (Manion, 1981). The science of forest pathology began in 1874, when Robert Hartig (1839-1901), the German forester, described the association of fungal hyphae with decaying wood, and he is referred as the 'Father of Forest Pathology' (Tainter and Baker, 1996). Since then various types of tree diseases are well documented in India (Singh, 1975; Mukerji and Bhasin, 1986; Basu and Giri, 1993) and abroad (Westcott, 1971; Brooks, 1981; Manion, 1981; Dickinson and Lucas, 1982).

The yellows-diseases of trees rank among the earliest recognised and most important diseases of plants known to man (Raychaudhuri and Varma, 1980). The disease, caused by phytoplasmas shows the characteristic symptoms of stunting, yellowing or reddening of leaves, proliferation of roots and shoots; abnormal flowers and eventual decline and death of the plant (Agrios, 1997).

2.2. SANDAL

The genus *Santalum* consists of 25 species and is distributed between 30^0N and 40^0 S from India in the west to Juan Fernandez Islands in the East and from Hawaiian Archipelago in the North to New Zealand in the South (Srinivasan *et al.*, 1992; Radomiljac, 1994). Sandal, *Santalum album* L. is the most economical of the species due to large amount of oil in the heartwood. The fragrant heartwood is commercially known as East Indian sandalwood and the heartwood powder upon steam distillation yields the renowned East Indian sandalwood oil. The wood is used for carving and religious purposes and the oil is used in perfumery and cosmetics.

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). In India, it is distributed all over the country and more than 90% lies in Karnataka and Tamil Nadu covering 8300 sq. kms. Other states where

sandal trees found distributed are Kerala, Andhra Pradesh, Maharashtra, Madhya Pradesh, Orissa, Rajasthan, Uttar Pradesh, Bihar and Manipur (Srinivasan *et al.*, 1992). In Kerala the species is mainly distributed in the Anjanad Valley (850-1050 m above msl) in the eastern side of Western Ghats falling in Marayoor range of Munnar forest division (Ghosh *et al.*, 1985).

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. Production of sandalwood has plummeted from around 3000 tonnes per annum during 1985 to around 1000 tonnes in 1997; similarly oil production also declined from 140 tonnes in 1985 to 40 tonnes in 1997 (Jain *et al.*, 1999). In 1996-97, only 303 tonnes of sandalwood was extracted from Karnataka (Swaminathan *et al.*, 1998) and 65 tonnes from Kerala (Prabhu, 1998).

2.3. SANDAL SPIKE DISEASE

Sandal spike is one of the most serious yellows-type disease 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 incidence of spike disease ranged from 1 to 55 percent in Karnataka.

Symptoms of spike disease were first described by McCarthy in 1899 (McCarthy, 1899; Barber, 1903). The disease is characterised by extreme reduction in size of the leaf and internode and in final stages, the twigs looks like a `spike inflorescence' (Ghosh *et al.*, 1992). Infected plants showed extensive deterioration of the root system, particularly the tap root, with reduced number of lateral roots and root hairs (Nayar and Ramanujam, 1985). Spike disease affected trees die within one to two years after the appearance of disease symptoms (Ghosh *et al.*, 1992).

2.3.1. Etiology

Initially the spike disease was suspected to be a root disease, 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 and Coleman (1917, 1923) attributed the disease to a virus.

The discovery of phytoplasmas associated with yellows disease of aster, witches' broom of potato and paulownia, and mulberry dwarf in 1967 by Japanese scientists (Doi *et al.*, 1967) gave impetus to a reconsideration of the causative organisms of plant diseases of unknown and unconfirmed etiology. Two years later the viral theory of sandal spike disease was disproved when three groups of workers by transmission electron microscopy showed phytoplasma in the phloem tissues of spike diseased plants (Dijkstra

and Ie, 1969; Hull *et al.*, 1969; Verma *et al.*, 1969). The pleomorphic bodies of about 40 to 750 nm size were devoid of cell walls and the cytoplasm was bound by a unit membrane, 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 into diseased trees further confirmed the phytoplasmal etiology of the disease (Raychaudhuri *et al.*, 1972).

2.3.2. *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 remains a non-culturable organism.

2.3.3. Disease detection

2.3.3.1. Physical characters

Spike disease is generally diagnosed by the manifestation of external symptoms. Attempts have been made to identify the disease by calculating

the ratio of the length and breadth of mature leaves or the length of internodes. The ratio in healthy plant leaves is usually between 2.0 and 2.5 and in spiked plants between 3.5 and 4.0. The length of internodes in healthy plants is 8 to 30 mm, and in spiked plants 3 to 5 mm (Iyengar, 1931, 1961).

2.3.3.2. Staining techniques

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. Free-hand sections of diseased twigs when stained with Mann's stain gave violet colour to the phloem, whereas sections from healthy twigs stained pink (Parthasarathi *et al.*, 1966). 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). Rangaswamy (1995) found that the intensity of fluorescence was high in the stem and petiole than in the root when spiked tissues were stained with Hoechst 33258. These studies conclude that detection of the pathogen using fluorescent microscopy is superior to light microscopy.

2.3.3.3. Electrical resistance

Ghosh *et al.* (1985) used a shigometer to detect spike diseased sandal. The electrical resistance of the inner bark of diseased trees was correlated with the intensity of visual symptoms. Healthy trees had electrical resistance of 3.0 to 7.0 kilo ohms, whereas the diseased trees had a resistance ranging from 7.1 to 13.1 kilo ohms.

2.3.3.4. Immunological detection

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 poor antibody titre (1:250 dilution). The antibody could detect phytoplasma in spike disease affected sandal and *Catharanthus roseus* plants infected artificially with sandal spike phytoplasma. Rangaswamy (1995) used a differential centrifugation method for purifying the pathogen to raise polyclonal antibody. However, the efficiency of the purification protocol was not tested by electron microscopy in the study. The polyclonal antibody was used to detect phytoplasma by indirect ELISA technique in spike disease affected sandal and *Catharanthus roseus* plants infected artificially with sandal spike phytoplasma. The titre of the antibody was calculated to be 1:1000 dilution and could detect antigen only upto a dilution of 1:200.

2.3.4. Disease transmission

2.3.4.1. Insect vector

The transmission of sandal spike disease in the field was suspected to be caused by several insect vectors like *Moonia albimaculata* (Dover and Appanna, 1933), *Jassus indicus* (Rangaswami and Griffith, 1941) and *Nephotettix virescens* (Sivaramakrishnan 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.

2.3.4.2. Haustoria

Most of the earlier workers suspected host plants as the agents transmitting the spike disease. Norris (1930) and Venkata Rao (1935) suspected that the associated rank vegetation of sandal plays a decisive role in the spread of disease in nature. Nayar and Srimathi (1968) were of the opinion that *Lantana* acts as a symptomless carrier It was also felt that sandal, in association with certain hosts was more susceptible to disease than others. These inferences led Kristensen (1960) and Bhatnagar (1965) to classify the host plants according to susceptibility to disease infestation. Nayar and Ananthapadmanabha (1977) had reported several herbs, shrubs, and trees such as *Stachytarpheta indica, Zizyphus oenoplea*, etc., growing along with sandal showing the typical yellows type disease, but immunological tests

could not confirm sandal spike phytoplasma in these plants (Nayar, 1981; Rangaswamy, 1995). Studies conducted by Subba Rao (1980) proved that phytoplasma could not be transmitted from diseased sandal to healthy sandal by haustorial connection.

2.3.4.3. Grafting

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 methods generally adopted are the grafting of diseased twigs to healthy plants by wedge grafting and covering the scion with polyethylene bags until the establishment of the scion. The establishment of the scion is found to be a prerequisite for disease transmission (Ghosh *et al.*, 1992).

2.3.4.4. Dodder

Cuscuta sp. is generally used in experimental transmission of spike disease. Through this parasite, transmission is achieved not only from sandal to sandal but also from sandal to periwinkle (*Catharanthus roseus)*, an indicator plant and vice versa (Dijkstra and Lee, 1973). Subba Rao (1980) could not transmit phytoplasma to healthy sandal from witches' broom affected *Stachytarpheta*, little leaf affected periwinkle, brinjal and sesamum phyllody

using dodder. Attempts to transmit sandal spike disease to *Zizyphus* using dodder by Rangaswamy (1995) was unsuccessful.

2.4. PHYSIOLOGICAL CHANGES IN SPIKED SANDAL

The onset of spike disease in sandal results in a number of physiological changes. Some of the prominent changes are: increase in levels of carbohydrate, particularly starch (Iyengar, 1928), total nitrogen, phosphorus (Rama Rao and Sreenivasaya, 1928), nitrate content (Parthasarathi *et al.*, 1962), phenolic bodies (Parthasarathi and Ramaswamy, 1961), ascorbic acid (Parthasarathi *et al.*, 1963), IAA (Sowbhagyam *et al.*, 1977) and protein (Rangaswamy, 1995). Iron content (Parthasarathi and Rao, 1962) and Gibberellic acid (Gowda and Narayana, 1998) were found to be less in tissues affected by spike disease. Angadi and Ananthapadmanabha (1988) studied the peroxidase, malate dehydrogenase and esterase isozyme pattern of healthy and spiked leaves. It was observed that the esterase isozyme pattern changed from young leaf stage to mature leaf stage in the diseased, whereas, in healthy, the pattern remained the same at any stage irrespective of its morphological condition. Studies by Rangaswamy (1995) confirmed that the diseased leaves exhibited high esterase and peroxidase activity compared to the healthy leaves.

2.5. DISEASE CONTROL

2.5.1. Chemotherapy

With the discovery of phytoplasma in sandal, attempts were made to control the disease using antibiotics. Raychaudhuri (1977) used chlorotetracycline (aureomycin), dimethyl chlorotetracycline hydrochloride (ledermycin), tetracycline hydrochloride (achromycin) and benlate (methyl-1-butylcarbamoyl)-2-benzimidazole carbamate), a systemic fungicide to treat the disease by girdling and spraying. Though they were effective initially, disease symptoms reappered after sometime. Nayar *et al.* (1973) and Nayar and Ananthapadmanabha (1974) also reported similar results while using tetracycline antibiotics. Ghosh *et al.* (1985) and 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. The disease reappeared within 3-7 months. Infusion of digitonin also gave the same results.

2.5.2. Heat therapy

Nayar (1977) used boiling water in the treatment of the disease, but mortality of sandal was high. Srinivasan *et al.* (1992) were of the view that frequent forest fires in sandal growing areas reduced incidence of spike disease.

2.6. PLANT DISEASES CAUSED BY PHYTOPLASMAS

In 1967, wall-less microorganisms resembling mycoplasmas were seen with the aid of electron microscope in the phloem of plants infected with several yellows-type diseases (Doi *et al.*, 1967). Such diseases were until then thought to be caused by viruses. Furthermore, it was shown that these microorganisms were susceptible to tetracycline but not to penicillin antibiotics and that the symptoms of infected plants could be suppressed at least temporarily by treatment with antibiotics (Ishiie *et al.*, 1967).

Since then, more than 300 plant species affected by phytoplasma has been reported (McCoy *et al.*, 1989). Generally, yellows-disease caused by phytoplasmas are most serious in perennial crops particularly woody plants, which once infected remains infected for life. Phytoplasma diseases are lethal, although death may be slow (Seliskar and Wilson, 1981). Among these are some very destructive diseases of trees such as pear decline, elm phloem necrosis, coconut lethal yellowing, X-disease of peach, apricot chlorotic leaf roll, cherry moliere disease, grapevine flavescence doree, and apple proliferation; but the pathogen also causes diseases in herbaceous plants such as aster yellows in ornamentals and potato witches' broom, and strawberry witches' broom. Furthermore several diseases, such as citrus stubborn and corn stunt were shown to be caused by helical mycoplasmas known as spiroplasmas. The main characteristics of yellows type diseases

include more or less gradual, uniform yellowing or reddening of the leaves, smaller leaves, shortening of the internodes and stunting of the plant, excessive proliferation of shoots and formation of witches' broom, greening or sterility of flowers and reduced yield, and finally a more or less rapid dieback, decline and death of the plant (Smith *et al.*, 1988; Agrios, 1997).

Although phytoplasmas have been observed in the phloem of diseased plants, in sap extracted from such plants and in the insect vectors of some of them, the true nature of phytoplasmas and their taxonomic position among the lower organisms is still uncertain. Morphologically, the organisms observed in plants resemble the typical mycoplasmas found in animals and humans and those living saprophytically, but phytoplasmas cannot be grown in artificial media. Also, so far, no plant disease has been reproduced on healthy plants inoculated directly with phytoplasma from diseased plants (Agrios, 1997).

2.7. CHARACTERISTICS OF MYCOPLASMAS

The trivial name mycoplasmas (myco: fungus; plasma: form) is used for organisms comprising a very large group of prokaryotes distinguished phenotypically from other bacteria by their minute dimensions and total lack of cell walls (Razin and Freundt, 1984). Morphologically, mycoplasmas vary in shape from spherical or pear-shaped structures (0.3-0.8 μm in diameter) to

branched or helical filaments. The size of fully developed coccoid mycoplasmas may vary from one to a few micrometers, while slender branched filamentous forms may range in length from a few to about 150 μm. The total lack of a cell wall explains many of the unique properties of the mycoplasmas, such as sensitivity to osmotic shock and detergents, resistance to penicillin, and formation of peculiar fried-egg shape colonies. Thin sections of mycoplasmas reveal that the cells contain three organelles- the cell membrane, ribosomes and the characteristic prokaryotic genome. Mycoplasmas are devoid of cell wall and are Gram-negative when stained by Gram-stain. The mycoplasma membrane resembles plasma membrane of other prokaryotes in being composed of approximately two third proteins and one-third lipids. They are the smallest self-replicating prokaryotes. Reproduction is by budding and by binary transverse fission. Though mycoplasmas are resistant to penicillin, they are sensitive to tetracycline and to certain other antibiotics (Freundt and Edward, 1979; Razin, 1983, 1992).

Taxonomically, the lack of cell wall is used to separate the mycoplasmas from other bacteria in a class named Mollicutes (mollis: soft; cutis: skin) which has three orders Mycoplasmatales, Acholeplasmatales and Anaeroplasmatales (Razin, 1978, 1992). The classification of Mollicutes is shown in table 2.1.

Table.2.1. Taxonomy and properties of mycoplasmas (Class Mollicutes) (Razin, 1978, 1992).

Classification	Genome size (MDa)	GC content (mol%)	Cholesterol requirement	Distinctive property	Habitat
Order I: Mycoplasmatales Family I: Mycoplasmataceae Genus I: Mycoplasma	400-800	23-41	+		Humans, animals, plants, insects.
Genus II: Ureaplasma	500-700	27-30	+	Urease Positive	Humans, animals
Family II: Spiroplasmataceae Genus I: Spiroplasma	1000	25-31	+	Helical Filament	Insects, plants
Order II: Acholeplasmatales Family: Acholeplasmataceae Genus I: Acholeplasma	1000	27-36			Animals, plants, insects
Order III: Anaeroplasmatales Family: Anaeroplasmataceae Genus I: Anaeroplasma	1000	29-33	+	Obligate Anaerobes	Bovine-ovine rumen
Genus II: Asteroleplasma	1000	40		Obligate Anaerobes	Bovine-ovine rumen

The mycoplasmas have the smallest recorded genomes among self-replicating organisms. The reported genome sizes for members of the Mollicutes appear to fall into two clusters: one composed of *Mycoplasma* and *Ureaplasma* species with a genome size of about 500 MDa, or approximately

750 kb, and the other of *Acholeplasma*, *Spiroplasma*, *Anaeroplasma* and *Asteroleplasma* species, with a genome of about twice the size (Razin, 1985). The minute size of the *Mycoplasma* and *Ureaplasma* genome implies a minimal number of genes. The estimated number of genes in species of these genera does not exceed 500 (Muto, 1987). This is expressed by a small number of cell proteins and by the lack of many enzymatic activities and metabolic pathways, which is in accordance with the parasitic mode of life and fastidious nature of mycoplasmas. The base composition is characterised by a low GC content of 20 mol% and a high AT content of about 80 mol% (Razin, 1985). The mycoplasmas are usually facultative anaerobes but *Anaeroplasma* and *Asteroleplasma* species are obligate anaerobes and are very sensitive to oxygen (Robinson and Freundt, 1987). Mycoplasma identification has been based of the classical bacteriological tests, including morphology, cultural characteristics, physiological and antigenic properties. A variety of tests based on molecular analysis of genomic DNA, ribosomal RNAs, cell proteins and lipids has been introduced (Razin, 1989).

Most of the human and animal mycoplasmas are pathogenic and cause several diseases. They adhere tenaciously to the epithelial linings of the respiratory or urogenital tract and rarely invade the tissues and blood stream (Razin and Yogev, 1989). The limited biosynthetic abilities of

mycoplasmas make them dependent on their hosts for the supply of many nutrients (Razin, 1992).

2.8. PROPERTIES OF PHYTOPLASMAS

The phytopathogenic mycoplasmas include species of the genus *Spiroplasma* and phytoplasmas closely related to mycoplasmas of the genus *Acholeplasma* as revealed by phylogenetic studies (Lee and Davis, 1992). They lack cell wall, are bounded by a triple-layered 'unit' membrane and have cytoplasm, ribosomes and strands of nuclear material. Their shape is usually spheroid to ovoid or irregular tubular to filamentous and their sizes comparable to those of the typical mycoplasmas (i.e. around 1 μm) (Agrios, 1997).

Phytoplasmas are seen in the sap of phloem sieve tubes. Ivanov *et al.* (1992) was of the opinion that some of the phytoplasmas could penetrate into plant cells directly through an intact root system. However, most phytoplasmas are transmitted into plants by insects (Skripal, 1983; Lee and Davis, 1992). Because they lack rigid cell wall, phytoplasmas can become thread like, enabling them to penetrate from cell to cell through sieve tube pores and plasmodesmata (Bilai *et al.*, 1988). Chernov *et al.* (1996) was of the opinion that the pathogens reproduce by binary fission or gemmation in infected plants. Phloem sieve tubes usually contain phytoplasma cells at various stages of development - young, mature and old (600-800, 800-1200

and over 1200 nm in diameter respectively). Young cells have a clearly visible 12-14 nm thick membrane and dense cytoplasm filled with ribosomes (17 nm in diameter). Mature cells possess a 7 to 9 nm thick membrane and their cytoplasm is more transparent due to fewer ribosomes. In old phytoplasma cells, the plasma membrane becomes very thin and eventually ruptures, releasing the contents of the cell (Bilai *et al.*, 1988).

2.8.1. Amino acid composition

In clover phyllody and aster yellows phytoplasma (Sinha and Madhosingh, 1980), the concentration of glycine was the highest, followed by alanine, whereas phenyl alanine and tyrosine concentration was found to be low. Studies conducted by Bove and Saillard (1979) in *Spiroplasma citri*, the mollicute infecting citrus, found that the amino acid composition of protein lacked methionine, histidine and tryptophan. Among the animal mycoplasmas, the composition of amino acid in *Mycoplasma mycoides* and *Mycoplasma laidlawii*, the concentration of aspartic acid and glutamic acid was the highest and both lacked cysteine (Chelton *et al.*, 1968).

2.8.2. Host-pathogen interaction

While molecular methods have improved the knowledge with respect to the identity of phytoplasmas and their phylogenetic and taxonomic relationships, their mechanisms of inducing disease are still not well understood (Lepka *et*

al., 1999). Histological studies on several tree species revealed the first detectable anatomical aberration as an abnormal deposition of callose on the sieve areas of the sieve tubes, followed by the collapse of these elements and the companion cells. Depending on the susceptibility of the host, a smaller or larger proportion of the sieve tubes necrotize (Batjer and Schneider, 1960; Braun and Sinclair, 1976; Kartte and Seemuller, 1991). Analysis of pear decline affected pear trees showed that the starch content in the roots of diseased trees is only about one half to one third of that of healthy trees (Batjer and Schneider, 1960). In proliferation-diseased apple trees, a strong reduction or virtually complete depletion of starch was observed in the roots, whereas in woody stem tissue the starch content was similar to or higher than that of healthy trees (Kartte and Seemuller, 1991). Catlin *et al.* (1975) detected considerable accumulation of both soluble carbohydrates and starch in leaves of diseased pear trees. Soluble carbohydrates and starch was found to be higher in source leaves of diseased periwinkle and tobacco than in the source leaves of healthy plants, but the same was found to be less in diseased roots compared to healthy roots. The amino acid concentration also increased in diseased leaves (Lepka *et al.*, 1999).

According to Bilai *et al.* (1988) the interaction between mycoplasmas and host cells is usually divided into three stages. In the first stage, to penetrate young phloem parenchyma cells, phytoplasmas interact with the

plasmalemma. The plasmalemma forms protuberances directed towards the phytoplasma cell, resulting in fusion of the plasma membranes of both cells and the exchange of contents (Borkhsenius and Chernova, 1989). By attaching to the plasmalemma of the plant, the pathogen is able to obtain the necessary nutrients from the host cell and affect its genome. In the second stage, the ultrastructure of plant cells is disturbed; three or four days after infection, the cytoplasm of young phloem parenchyma cells becomes dense due to the appearance of numerous ribosomes and polysomes located near the plasmalemma or other cell membranes. The number of ribosomes significantly increases compared to adjacent uninfected cells, probably reflecting enhanced protein synthesis. During the final stage, parenchyma cells, cell membranes, mitochondria and chloroplasts are destroyed resulting in the yellowing and chlorosis of plant leaves. In this stage, the nuclei of the infected cells mostly contain condensed chromatin, and several chloroplasts escaping destruction have poorly developed granal and stromal thylakoids; the structure of mitochondria varies from normal to extremely dense, with blisterlike cristae and other symptoms of destruction (Midyannik, 1995; Chernov *et al.*, 1996).

2.9. IMMUNOLOGY IN PHYTOPLASMOLOGY

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. They are then referred to as immunogens. The majority of proteins, lipoproteins and polysaccharides are antigens with immunogenic properties. Antibodies are found in serum of the blood. They are referred to as immunoglobulin molecules (Ig) and can be divided into five classes - IgA, IgD, IgE, IgG and IgM based on their physical, chemical and immunological properties (Brock and Madigan, 1988; Roitt *et al.*, 1993). Every warm blooded animal is capable of producing antibodies, but 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. They represent a complex mixture of antibodies directed towards different antigenic determinants and with varying affinities (Neumeier, 1984). Sera of this type contain antibodies produced from diverse B-cell clones (=polyclonal) against several epitopes of target antigen, in addition to many antibodies present before immunisation. Heterogeneity, therefore 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-lymphocytes and multiplied as clones. By this procedure all cells of one clone synthesise exactly the same (=monoclonal) antibodies, which are directed towards a single epitope (Brock and Madigan, 1988, Jordan, 1990).

2.10. PURIFICATION OF PHYTOPLASMA

The most important part dealing with the production of polyclonal antibodies is purification of phytoplasma with minimal plant contamination. Sinha and Chiykowski (1984) were the first to purify phytoplasma from a tree affected by Peach Eastern X-disease. Generally, glycine-NaOH buffer, pH 8.0 containing 0.1 M Magnesium chloride is used for the purification of phytoplasma (Sinha, 1979; Sinha and Chiykowski, 1984; Clark *et al.*, 1989). Sinha (1974, 1979) used a celite-pad adsorption technique in association with column chromatography for the purification of phytoplasma. Seventy five percent of phytoplasma cells were lost during this purification procedure (Sinha, 1979). Later on different workers employed the differential centrifugation technique for the purification of phytoplasma (Caudwell *et al.*, 1982; Clark *et al.*, 1983; Sinha and Benhamou, 1983). The main drawback of the technique was the presence of plant debris among the phytoplasma pellet leading to the production of inferior quality phytoplasma specific antibody.

Hobbs *et al.* (1987) incubated the partially purified phytoplasma pellet with antibody produced against healthy plant extract. The technique was found to be highly superior since the final pellet was rich in phytoplasma cells and contamination with plant material was found to be minimum. For the purification of aster yellows phytoplasma from lettuce, Jiang and Chen (1987)

employed percoll density gradient centrifugation technique. The technique was further improved using affinity chromatography (Jiang *et al.*, 1988). Seddas *et al.* (1993, 1995) employed the affinity chromatography for the purification of flavescence doree phytoplasmas from leafhoppers and broadbean. Clark *et al.* (1989) and Sarindu and Clark (1993) used PEG 6000 to precipitate phytoplasma cells after chromatography, which was subsequently used in both antibody production and antigen characterisation. However the method of Hobbs *et al.* (1987) has been generally adopted since the technique has been found to be rapid with minimum plant contamination (Saeed *et al.*, 1992a,b; 1993; 1994).

2.11. DIAGNOSIS OF DISEASES CAUSED BY PHYTOPLASMAS

Diagnosis is the process of identifying disease. The most important facet of this is identification of the pathogen (Fry, 1982). Correct diagnosis is a necessary prelude to any successful disease control. However diagnosis of plant mollicute diseases have often been one of the more difficult aspects in their study. This is due to the general lack of methodologies for detection of the pathogens. Development and exploitation of new approaches to pathogen detection now promise to overcome many of these problems that hinder correct diagnosis and control (Davis and Lee, 1988).

Earlier workers employed electron microscopy and light microscopic techniques for detecting phytoplasma in plant tissues. In light microscopy, Dienes' stain, aniline blue and Hoechst 33258 were routinely employed for detecting the pathogen (Deeley *et al.*, 1979; Raju *et al.*, 1981; Ghosh *et al.*, 1985; Rangaswamy, 1995). But the sensitivity and specificity of these stains were poor (Sinclair *et al.*, 1989). Sinclair *et al.* (1996) has described different options for the detection of phytoplasma. These options include both non-specific methods wherein any phytoplasma could be detected and specific methods which is species (group) specific. The non-specific detection techniques include fluorescent microscopy employing DAPI stain and the use of electron microscopy; whereas, specific detection assays include the use of both immunological and molecular techniques.

2.11.1. DAPI test

DAPI (4',6-diamidino-2-phenyl indole) is a DNA specific fluorochrome which forms a complex by attaching to the minor groove of A-T rich sequences of DNA (Kapuscinski, 1995). Russel *et al.* (1975) for the first time used the fluorochrome to detect mycoplasma contamination in cultured HeLa cells and subsequently the stain was used by Seemuller (1976) to detect phytoplasma. Since then it has been widely used in the detection of phytoplasma in several plants like peach and chokecherry (Douglas, 1986), ash (Sinclair *et al.*, 1989,

1996), alder (Marcone *et al.*, 1994a), wild blackberry (Marcone *et al.*, 1994b), wild radish (Marcone and Ragozzino, 1995a), *Brassica* spp. (Marcone and Ragozzino, 1995b), periwinkle (Marcone and Ragozzino, 1995c), apricot (Marcone *et al.*, 1995a), lettuce (Marcone *et al.*, 1995b), sweet potato (Gibb *et al.*, 1995), buckthorn (Maurer and Seemuller, 1996) and apple (Jarausch *et al.*, 1996).

2.11.2. Electron microscopy

Electron microscopy is the only method by which the ultra structural characteristics of phytoplasmas can be studied (Marcone and Ragozzino, 1996). Transmission electron microscopy (TEM) provided the first evidence that many yellows diseases of plants were caused by wall-less prokaryotes (Doi *et al.*, 1967). Since then TEM has been routinely employed in the detection of phytoplasma. TEM acts as a confirmatory technique since the anatomical structure like the absence of a cell wall and the presence of a cell membrane, the characters specific to phytoplasmas can be revealed in detail. The technique has been used to detect phytoplasmas affecting plants like sandal (Dijkstra and le, 1969; Hull *et al.*, 1969; Verma *et al.*, 1969; Ghosh *et al.*, 1985), *Eucalyptus* (Dafalla *et al.*, 1986, Balasundaran *et al.*, 1988), *Melia* (Munoz *et al.*, 1987), *Alnus*, *Populus* and *Crataegus* (Seemuller and Lederer, 1988), paulownia (Jin, 1988), pinus and cypress (Gopo *et al.*, 1989), ash (French *et al.*, 1989), periwinkle (Marcone and Ragozzino, 1995c), sweet

potato (Gibb *et al.*, 1995), wild radish (Marcone and Ragozzino, 1995a), *Withania* (Zaim and Samad, 1995), papaya (Siddique *et al.*, 1998), parsley (Khadhair *et al.*, 1998), and strawberry (Andersen *et al.*, 1998b).

Scanning Electron Microscopy (SEM) allows rapid examination of large areas, such as entire cross or longitudinal sections of petioles and stem at high magnifications and with great depth of field, compared with small volume sampling of thin sections employed in TEM (Marcone and Ragozzino, 1996). The technique was introduced for studying mollicutes by Biberfield and Biberfield (1970) and subsequently was widely used to observe phytoplasmas infecting several plant species (Haggis and Sinha, 1978; Marcone *et al.*, 1994b; Marcone and Ragozzino, 1995a; Marcone and Ragozzino, 1996).

2.11.3. Immunological techniques

Immunological techniques are subdivided into liquid and solid phase tests. In the first case, both antigen and antibody are in solution until they react. These tests include various agar diffusion tests, ring precipitation and immuno electrophoresis. The solid phase assays have two common basic principles. One is a solid carrier to which antigen or antibody is linked. This can be microtitre plates or membranes. Secondly, there is a system for detection of the antigen/antibody reaction, in which the antibody is primarily

or secondarily marked with a substance such as an enzyme, fluorochrome or radioactive substance (Hampton *et al.*, 1990).

Immunological techniques originated in human biology, where they were initially used in clinical microbiology (Voller *et al.*, 1974; Feldmann *et al.*, 1976; Brock and Madigan, 1988). Since Clark and Adams (1977) introduced the enzyme linked immunosorbent assay (ELISA) for assaying plant viruses, immunological methods have been used increasingly in agricultural research and practice (Werres and Steffens, 1994).

2.11.3.1. Ouchterlony double diffusion (ODD) test

Agar diffusion in plates represents an ideal medium for studying strain relationships and are used frequently in testing materials from field surveys (Ball, 1990). Dickey *et al.* (1987) investigated the use of ODD for the diagnosis and identification of strains of *Erwinia chrysanthemi* isolated from corn and other hosts. Iannelli *et al.* (1982) used the test to differentiate strains of *Fusarium*. The major antigens of four pathovars of *Pseudomonas syringae* were studied using the test (Guillorit and Samson, 1993). Jan and Yeh (1995) used the test to study serological relationship of passion fruit woodiness virus with other viruses of potyvirus group. Relationship studies have been done in diffusion plates with ten strains of cucumber mosaic virus and two of peanut stunt virus (Ahmad and Scott, 1985), with 17 isolates of

bean yellow mosaic (Barnett *et al.*, 1987) and with 70 isolates of prunus necrotic ringspot virus (Mirik *et al.*, 1987). Though there are large number of papers in double diffusion test on other microorganisms, very few papers have been published on the same in phytoplasmology probably since the test was found to be less sensitive compared to the other tests.

2.11.3.2. Enzyme linked immunosorbent assay (ELISA)

The term enzyme linked immunosorbent assay (ELISA) was introduced by Engvall and Perlmann (1971). Since the work of Clark and Adams (1977) on ELISA protocols for use with plant viruses, ELISA has been used for the detection and quantitative estimation of a large number of antigens from diseased plants. Sinha and Benhamou (1983) employed the technique to detect aster yellows phytoplasma. ELISA was effective in detecting peach eastern X-disease phytoplasma in purified and partially purified preparations, but not from crude plant extracts (Sinha and Chiykowski, 1984). Jiang and Chen (1987) detected aster yellows phytoplasma in lettuce using indirect ELISA. Jiang *et al.* (1988) and Clark *et al.* (1989) used the indirect ELISA technique to compare the efficiency of polyclonal and monoclonal antibody in detecting the aster yellows phytoplasma and primula yellows phytoplasma respectively. Whereas Chen *et al.* (1993) used the monoclonal antibody against grapevine yellows phytoplasma to study strain relationship of phytoplasmas causing apple proliferation, ash yellows, aster yellows, peanut witches' broom, eastern X-disease and sweet potato witches' broom using indirect ELISA. Hobbs *et al.* (1987) employed the protein-A indirect ELISA for

detecting phytoplasma causing peanut witches' broom disease. They used phytoplasma specific antiserum cross-absorbed to healthy plant protein to detect the pathogen. Cross-absorbed antiserum was also employed by Saeed *et al.* (1992a) to detect phytoplasma in *Catharanthus roseus* and Faba bean phyllody (Saeed *et al.*, 1993) by different types of indirect ELISAs. Sarindu and Clark (1993) also used the indirect ELISA format to detect phytoplasma in sugarcane whiteleaf disease and bermuda-grass white leaf disease.

The ELISA technique has also been used to detect *Spiroplasma citri* in leaves of sweet orange and periwinkle, in which *Spiroplasma* antigens were detectable at concentrations as little as 5 to 10 ng (Saillard *et al.*, 1978). Archer and Best (1980) and Clark *et al.* (1983) also used the technique to detect *Spiroplasma*. Linfield (1993) used an indirect ELISA technique to detect *Fusarium* in the ornamental, *Narcissus*. Spiegel and Martin (1993) employed the triple antibody-sandwich ELISA to detect potato leaf roll virus; but the technique could not detect the virus from crude samples. Whereas Skaf and Carroll (1995) used DAS-ELISA and indirect ELISA technique to detect barley yellow streak mosaic virus in leaf extracts of barley and *Nicotiana benthamiana*.

2.11.3.3. Dot immunobinding assay (DIBA)

DIBA differs from conventional ELISA primarily in that the solid phase matrix used to adsorb the test material is a nitrocellulose membrane or paper sheet rather than a well in a polystyrene microtiter plate (Lazarovits *et al.*, 1987).

According to Lazarovits (1990) DIBA is the most simple, rapid and sensitive assay for immunological characterization of phytopathogenic bacteria and fastidious prokaryotes, obtained either from cultures or from diseased tissues. When whole tissues are employed in blotting, the technique is called tissue-blotting or tissue printing. Tissue-blotting has been used to detect different viruses in passionfruit, papaya, bamboo, barley (Lin *et al.*, 1990), onion (Helguera *et al.*, 1997), pineapple (Hu *et al.*, 1997) and phytoplasma causing big-bud disease of tomato (Lin *et al.*, 1990).

Eventhough DIBA has been extensively used in the detection of bacteria (Malin *et al.*, 1985; Lopez-Vidal and Svennerholm, 1986), fungus (Mitchell, 1988; Kimishima and Kobayashi, 1990), viruses (Graddon and Randles, 1986; Powell, 1987; Mahmood *et al.*, 1997) and mycoplasma (Kotani and McGarrity, 1985), there are very few reports which employ this method in phytoplasmology. However, Saeed *et al.* (1993) used the dot-blot technique to detect phytoplasma infecting faba bean and Lee *et al.* (1993) used the technique to differentiate aster yellows phytoplasma strains. As in ELISA, plant constituents in the sample solution can cause non-specific interactions. Purification and clarification of the plant extracts may reduce background values and increase sensitivity of the dot blot (Hammond and Jordan, 1990).

2.11.3.4. Immunomicroscopy

Immunomicroscopy (immunohistochemistry) in plant pathology is a highly sensitive and specific technique utilising the specificity of antibodies and the

sensitivity of microscopy for detection of the pathogen. Immunomicroscopy includes, immunolight microscopy, immunofluorescence microscopy and immunoelectron microscopy. Ever since immunofluorescence was introduced by Coons and Kaplan (1950) as a technique for the localization of cellular constituents, considerable progress in immunocytological procedures has been achieved. The common feature of all these procedures is a marker substance that is attached to an antibody, visible at the light or electron microscope levels or both. The labelled antibody preparation is then used for the detection of particular cellular constituents. Fluorescent chemicals (Coons, 1956), ferritin (Singer, 1959), heavy metals (Pepe, 1961) and enzymes (Nakane and Pierce, 1966) have been used as the markers.

Horseradish peroxidase is the most popular enzyme used in immuno histochemistry since it permits a more precise localization and identification of ultrastructures (Avrameas, 1973). As in other immunoassays, avidin-biotin system can be employed for amplification of the signal and 4-Chloro-1-naphthol or 3-Amino-9-ethylcarbazole (AEC) is used as the insoluble substrate for the test. Although immunolight microscopy is popular in medical and animal pathology the technique is rarely employed in plant pathology (Finan, 1984; Bullock and Petrusz, 1985-1989). Gerik *et al.* (1987) and Gerik and Huisman (1988) used the technique to study the colonisation of cotton roots by *Verticillum dahliae*; whereas Benhamou *et al.* (1985) utilised the technique in the detection of a toxic glycopeptide of *Ophiostoma ulmi*.

Immunofluorescence microscopy is a highly sensitive technique to detect the presence of antigen. FITC having an excitation wavelength of 490 nm and emission wavelength of 520 nm is coupled to the antibody of interest and is used in the test (Keren and Warren, 1992). Fluorescent spots are visible against a dark background facilitating detection of the pathogen of interest. Da Rocha *et al.* (1986) used the technique to detect phytoplasma in periwinkle. Cousin *et al.* (1989) employed the technique to detect phytoplasma of faba bean phyllody and stolbur disease of tomato, whereas Guo *et al.* (1996) used the technique to detect phytoplasma in chokecherry and Guo-Zhong *et al.* (1996) used the same in the detection of the pathogen in paulownia. Shen and Lin (1993) used monoclonal antibody as the probe to detect the pathogenic phytoplasma causing sweet potato witches' broom by immunofluorescence microscopy. The technique is also highly popular in the *in situ* detection of bacteria (De Boer and McNaughton, 1986; Abmus *et al.*, 1997), fungus (Fitzell *et al.*, 1980; Kumar *et al.*, 1986) and virus (Hosokawa and Mori, 1982; Mahmood *et al.*, 1997), affecting different plant species.

A few workers employed the immunosorbent electron microscopy (ISEM) technique for detecting phytoplasma (Caudwell *et al.*, 1982, 1983; Sinha and Benhamou, 1983; Sinha and Chiykowski, 1984, 1986; Sinha, 1988). The main drawback of ISEM was that the recognition of the phytoplasmas as such rested on comparison with controls and with ELISA results, as no labelling was attempted using protein-A gold and individual

phytoplasma bodies were not identified among the background of host-derived membrane debris (Vera and Milne, 1994).

Colloidal gold has become one of the most popular methods for labelling at the ultrastructural level (Roth, 1983). The gold particles can be stabilised by various protein solutions and can be used as labels. Among the most commonly used proteins is Staphylococcal protein-A, which bind specifically to the Fc fragment of IgG (Romano and Romano, 1977). Seddas *et al.* (1993) employed the immunoelectron microscopic technique using protein-A gold label to detect flavescence doree phytoplasma, whereas Vera and Milne (1994) used the technique to detect the pathogen in infected periwinkle and the leaf hopper vectors. Lherminier *et al.* (1990) and Milne *et al.* (1995) also used the technique to detect several phytoplasmas. The technique has also been employed for screening viruses (Milne, 1992; Jan and Yeh, 1995) and in the localisation of variable proteins on the surface of *Mycoplasma bovis* (Behrens *et al.*, 1996). The main advantage of the technique was that the specific pathogens could be easily identified and distinguished from other strains (Vera and Milne, 1994).

2.11.3.5. Immunoblotting

Protein blotting techniques are popularly known as 'Western blotting' (Burnette, 1981) or immunoblotting, a name derived from the previously

coined `Northern' blots (RNA bound to the support) versus Southern blots (DNA bound to the support). Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. The technique generally detects the presence of the specific antigenic protein from the electrophoretically separated proteins by incubating the membrane with specific antibodies directed against them (Renart *et al.*, 1996). The technique was first demonstrated by Towbin *et al.* (1979).

Immunoblotting can be used to determine a number of important characteristics of protein antigens - the presence and quantity of an antigen, the relative molecular weight of the polypeptide chain, and the efficiency of extraction of the antigen (Harlow and Lane, 1988). The technique had been employed by Jiang *et al.* (1988) in the detection of the antigenic protein of aster yellows phytoplasma that had a molecular weight of about 18.5 kDa. Studies by Clark *et al.* (1989) on phytoplasmas of primula yellows found the antigenic protein to be about 22.4 kDa. Saeed *et al.* (1992) found that phytoplasma causing faba bean phyllody had two antigenic proteins of 18 kDa and 36 kDa. Similarly, the flavescence doree phytoplasma revealed two antigenic proteins of 19 and 55 kDa by immunoblotting (Seddas *et al.*, 1993, 1995). Whereas the antiserum recognised only a 23 kDa antigenic protein in aster yellows infected plants (Errampalli and Fletcher, 1993).

2.11.4. Molecular techniques

When molecular cloning came of age around 1970, it became a routine affair to synthesise desired amounts of any DNA molecule. This innovation was followed in 1985 by a second breakthrough: the discovery of the polymerase chain reaction (PCR) for DNA amplification (Saiki *et al.*, 1985; Landegren, 1996). Application of molecular techniques has facilitated and improved detection, identification and classification of phytoplasmas (Gundersen *et al.*, 1994; Smart *et al.*, 1996).

2.11.4.1. Polymerase chain reaction (PCR)

The technique of polymerase chain reaction (PCR) was conceived by Kary Mullis in mid 1980's (Saiki *et al.*, 1985; Mullis and Faloona, 1987). Since its introduction, this technology has revolutionised the way in which DNA analysis and manipulation has been carried out in research and diagnostics. The PCR technology has become essential to the clinical diagnostic and forensic laboratories as well as to basic research in molecular biology and evolution. PCR is an *in vitro* method in which DNA sequences or transcripts are amplified rapidly with very high specificity and fidelity using oligonucleotide primers and *Taq* DNA polymerase in a simple automated reaction system (Mullis and Faloona, 1987). The importance of PCR lies in its ability to amplify a specific DNA or cDNA transcript *in vitro* from trace amounts of a complex template. It is possible to amplify a single copy of

target sequence in the presence of surrounding DNA from 1.5 x 10^6 cells that does not contain the gene of interest (Sorscher, 1997). In comparison with serological reagents, PCR primers with any desired degree of selectivity can be synthesised, at a much lower comparable cost than that associated with the development of monoclonal or polyclonal antibodies. Because of its great sensitivity, the PCR provides a good alternative to other diagnostic methods and can speed diagnosis, reduce sample size required and often eliminate the need for radioactive probes. These facts, coupled with the vast increase in nucleotide sequence data available for plant pathogens have enhanced the attractiveness of the PCR technique as a viable diagnostic tool (MacKenzie *et al.*, 1997). Application of PCR in plant pathology includes the detection and diagnosis of plant pathogens, screening for transgenic plants, molecular cloning of viral and viroid genomes and in PCR-mediated nucleotide sequence analysis of viroids (Hadidi *et al.*, 1995).

The use of PCR for the detection of phytoplasmas was first reported by Deng and Hiruki (1991), who designed a series of primers from comparisons of available 16S rRNA gene sequences. Ahrens and Seemuller (1992) also reported successful amplification of segments of the 16S rRNA gene. In several studies, nested PCRs have been used to distinguish strains of phytoplasmas and for increased sensitivity of detection. Nested PCRs involve re-amplification of a primary PCR product with internal primers (Lee *et al.*, 1995, Gibb *et al.*, 1995, Andersen *et al.*, 1998a). Some of the most important plant diseases caused by phytoplasmas and detected by amplifying the 16S rRNA gene by PCR are shown in table 2.2.

Table 2.2. Plant diseases caused by phytoplasmas and detected by amplifying the 16S rRNA gene by PCR.

Disease	Reference
Clover phyllody	Firrao *et al.*(1993)
Mulberry dwarf	Namba *et al.*(1993)
Apple proliferation	Firrao *et al.* (1994)
Decline of Nashi pears	Pollini *et al.* (1994)
Declining in alder	Marcone *et al.* (1994a)
Faba bean phyllody	Saeed *et al.* (1994)
Lethal yellowing of windmill palm	Harrison *et al.* (1994)
Paulownia witches' broom	Yoshikawa *et al.* (1994)
Phyllody in Sesamum	Nakashima *et al.* (1995)
Pear decline	Lorenz *et al.* (1995)
Stunting in lettuce	Marcone *et al.* (1995b)
Sweet potato little leaf	Gibb *et al.* (1995)
Maize bushy stunt	Harrison *et al.* (1996)
Yellows disease in olive	Pollini *et al.* (1996)
Papaya dieback	Gibb *et al.* (1996)
Grapevine yellows disease	Marcone *et al.* (1996a)
Little leaf in eucalyptus	Marcone *et al.* (1996b)
Peach vein enlargement	Marcone *et al.* (1996c)
Spartium witches' broom	Marcone *et al.* (1996d)
Virescence of Papaver	Marcone *et al.* (1996e)
Elm yellows	Marcone *et al.* (1997a)
Witches' broom of Sarothamnus	Marcone *et al.* (1997b)
Ranunculus phyllody	Kanehira *et al.* (1997)
Garlic decline	Conci *et al.* (1998)
Yellows disease in New Zealand flax	Andersen *et al.* (1998a)
Yellows disease in strawberry	Andersen *et al.* (1998b)
Yellows disease in parsley	Khadhair *et al.* (1998)
Lethal yellowing of coconut palm	Mpunami *et al.* (1999)
Hydrangea phyllody	Sawayanagi *et al.* (1999)
Sugarcane white leaf	Nakashima *et al.* (1999a)
Sesamum phyllody	Nakashima *et al.* (1999b)
Black wood in grapes	Osti and Triolo (1999)
Ash yellows	Sinclair and Griffith (2000)

2.11.4.2. Restriction fragment length polymorphism (RFLP)

Over the past few years, the use of modern molecular biological techniques to determine the degree of sequence conservation between microbial genomes has led to the development of methods based solely on the detection of naturally occuring DNA polymorphisms. These polymorphisms are a result of point mutations or rearrangements in the DNA and can be detected by scoring band presence versus absence in banding patterns that are generated by restriction endonucleases and have been termed restriction fragment length polymorphism (RFLP) (Beckmann and Soller, 1986, Janssen *et al.*, 1996). Because of their universal distribution, high conservation and apparent absence of interspecific transfer, 16S rRNA genes are commonly subjected to RFLP analysis after PCR amplification (Moyer *et al.*, 1994). The resulting RFLP patterns served to establish the phylogenetic groups of phytoplasmas (Gundersen *et al.*, 1994; Seemuller *et al.*, 1994,1998; Lee *et al.*, 1998a).

2.11.5. Phylogenetic classification of phytoplasma based on PCR and RFLP studies

Initially MLOs were thought to be related to animal mycoplasmas, but preliminary phylogenetic studies based on 16S rRNA and ribosomal protein gene sequences by Lim and Sears (1989, 1992) was the first to propose that a mycoplasma-like organism, the aster yellows phytoplasma represented a

new member of the class Mollicutes. Based on comprehensive phylogenetic analyses of 16S rRNA or both 16S RNA and ribosomal protein gene sequences, the phylogenetic position of this plant pathogen was clearly established (Seemuller *et al.*, 1994). The trivial name `phytoplasma' was officially adopted in 1994 to replace `mycoplasma-like organism' The established phylogenies formed a basis for classification of these uncultured plant pathogens. The closest identified relatives of the phytoplasmas are *Acholeplasma palmae* and *A. modicum* (Lee *et al.*, 1998a,b) in the *Anaeroplasma* clade defined by Weisburg *et al.* (1989).

Attempts were made to identify and classify unknown phytoplasmas based on direct sequencing and analysis of phytoplasma 16S rDNA or the 16S to 23S intergenic spacer region (Kuske and Kirkpatrick, 1992; Namba *et al.*, 1993). This approach was not always found to be practical when a large number of unknown phytoplasma are to be analysed. Ahrens and Seemuller (1992) maintained phytoplasmas inflicting diseases in 17 plant species, including sandal spike phytoplasma, in periwinkle and used the PCR technique to detect the pathogens. RFLP analysis of the amplified DNA product (16S rDNA sequences) with *Alu* I restriction endonuclease differentiated the phytoplasmas into four distinct groups, with sandal spike phytoplasma being classified in the first group. RFLP analysis of PCR amplified 16S rDNA sequences with a number of restriction enzymes was subsequently used to differentiate various phytoplasmas on the basis of distinct RFLP patterns (Schneider *et al.*, 1993). This procedure proved to be

simple, reliable and practical. The authors examined 52 geographically and pathologically diverse phytoplasmas, most of them maintained in periwinkle, and divided into seven major taxonomic groups (16S RFLP groups). They classified the sandal spike phytoplasma to group I (Aster yellows group, sub group A). Schneider and Seemuller (1994) studied the taxonomic relationships of several phytoplasmas maintained in periwinkle using RFLP and Southern blot analysis; the study also classified the sandal spike phytoplasma to group I, subgroup A. The review paper by Sinclair *et al.* (1996) classified phytoplasmas into eleven groups; placing sandal spike phytoplasma in the aster yellows group (Group I). Lee *et al.* (1998a) used the primer pairs, R16mF2/R16m R1 and R16F2n/R16R2, rpF1/rpR1 rp(V)F1/rpR1 and rp(V)F2/rpR1 for detecting phytoplasmas infecting a wide variety of plants. This was followed by treating the PCR products with 17 restriction enzymes; based on similarity coefficients derived from RFLP analysis the different phytoplasma strains were divided into 14 groups and 32 subgroups. During the same period Seemuller *et al.* (1998) classified phytoplasmas into 20 groups and placed sandal spike phytoplasma in the Aster yellows group (Group I), subgroup B.

2.12. GENOME SIZE OF PHYTOPLASMAS

Another important characteristic of these unculturable plant pathogens is their genome size. For the culturable mollicutes, the genome size range from more than 2,200 to less than 600 kb, with overlapping values between

the various mollicute genera. The *Mycoplasma* spp., has a genome size ranging from 1,380 down to 580 kb, and *Spiroplasma* spp., which has an even greater span, from 2,220 to 780 kb. In the genus *Acholeplasma*, the size range is narrower, varying between 1,500 and 1,650 kb (Razin *et al.*, 1998). The genome size range of phytoplasma varies from 1,350 to 530 kb (Zreik *et al.*, 1995; Marcone *et al.*, 1999). Marcone *et al.* (1999) by pulsed-field gel electrophoresis calculated the genome size of sandal spike phytoplasma maintained in periwinkle to be about 690 kb. The smallest chromosome, of about 530 kb, was found in two Bermuda grass white leaf phytoplasma isolates, which also appears to be the smallest chromosome known for any cell.

3. *IN SITU* DETECTION OF SANDAL SPIKE PHYTOPLASMA

3. *IN SITU* DETECTION OF SANDAL SPIKE PHYTOPLASMA

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

3.1. INTRODUCTION

The causative organism of spike disease affected sandal was first detected by transmission electron microscopy in 1969 (Dijkstra and Ie, 1969; Hull *et al.*, 1969; Varma *et al.*, 1969). Since then, a number of staining techniques has been tested to improve the efficiency of detection of phytoplasma in plants and

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

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

This section reports *in situ* detection of phytoplasma in spike disease affected sandal and its host plants using DAPI stain. The efficiency of DAPI

stain was compared with Dienes' stain. Morphology of the sandal spike phytoplasma in the phloem of diseased sandal tissues was studied using scanning electron microscopy.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Spike disease affected sandal and the host plants, *Lantana camara* and witches' broom affected *Zizyphus oenoplea* were collected from Marayoor, Munnar Forest Division, Kerala. The tissues were transported either in ice or the excised branches dipped vertically in water and covered with polyethylene bags. The plant materials were stored at 4^0C.

3.2.2. Disease transmission

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

3.2.3. DAPI staining

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

3.2.4. Dienes' staining

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

3.2.5. Scanning electron microscopy

Healthy and diseased sandal stem tissues were cut into pieces of 1 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).

3.3. RESULTS

3.3.1. Plant material

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

3.3.2. Disease transmission

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

3.3.3. DAPI staining

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

3.3.4. Dienes' staining

On staining the tissues with Dienes' stain, the phloem of diseased tissues showed discrete dark blue stained cells, but the phloem of healthy plants

remained unstained. The xylem and stone cells of both the healthy and diseased tissues were also coloured blue (Fig. 3.7)

3.3.5. Scanning electron microscopy

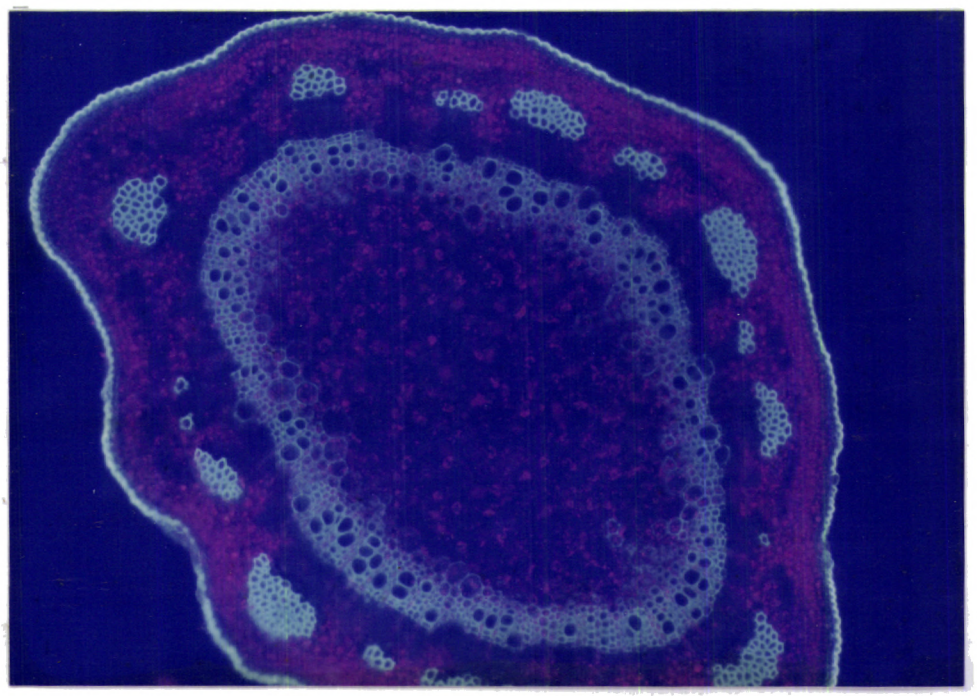
With this technique, it could be confirmed that phytoplasmas in the infected samples examined, were similar in size, shape and colonization pattern to those observed by transmission electron microscopy (Ghosh *et al.*, 1985). The microorganisms were present only in the sieve tube elements of diseased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

3.4. DISCUSSION

As the spike disease affected sandal is confined to Marayoor, which is approximately 200 km from the laboratory, experiments were done initially to increase the shelf life of the stored samples. Samples transported in ice, though stored in air-tight containers in the refrigerator at 4^0C, were damaged within three weeks. When the samples were vertically placed, with their base in contact with water and covered with polyethylene bags, the tissues remained fresh. As the base of the sample was in contact with water the upper part was dry and on subsequent storage at 4^0C had a higher shelf life.

Fig. 3.1. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal stem (CS) stained with DAPI (X70). Note the fluorescent spots in the phloem of diseased tissue.

a)



b)

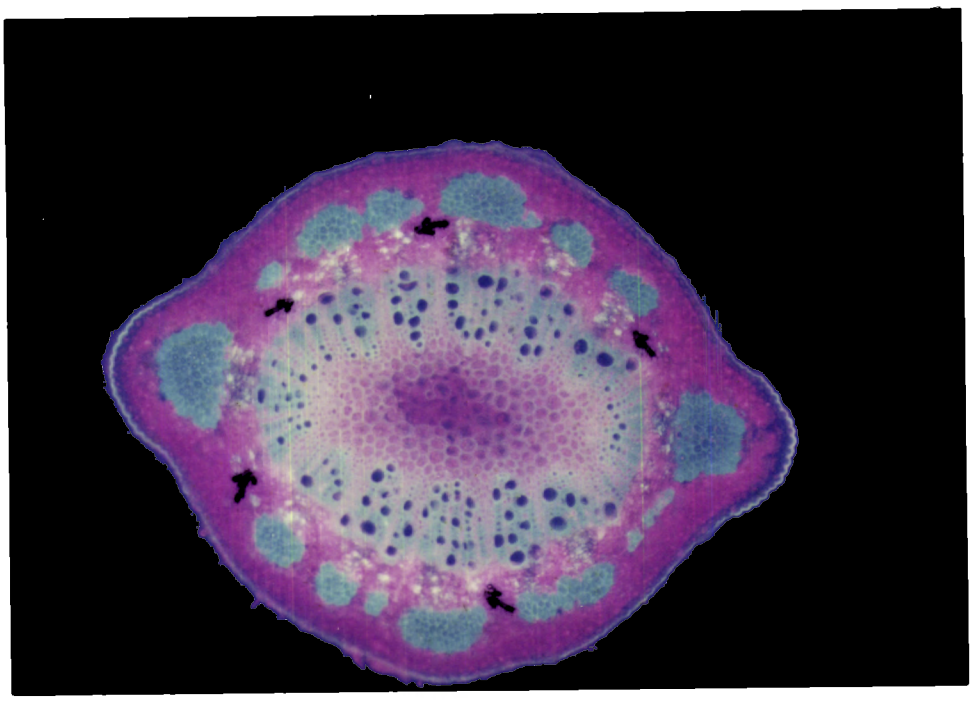
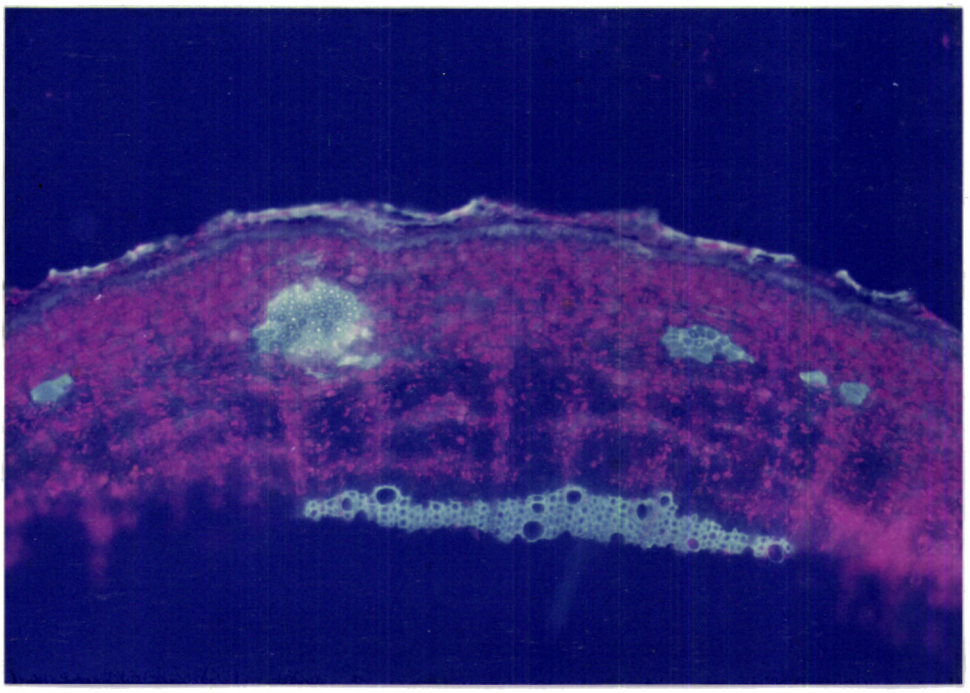


Fig. 3.2. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal inner bark (CS) stained with DAPI (X70). Note the fluorescent spots in the phloem of diseased tissue.

a)



b)

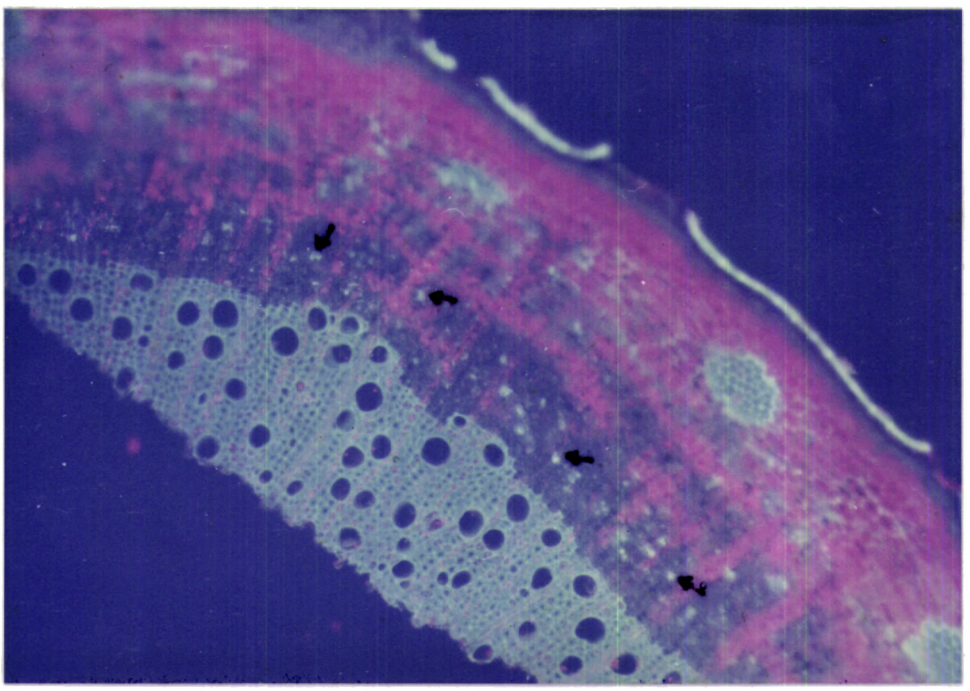
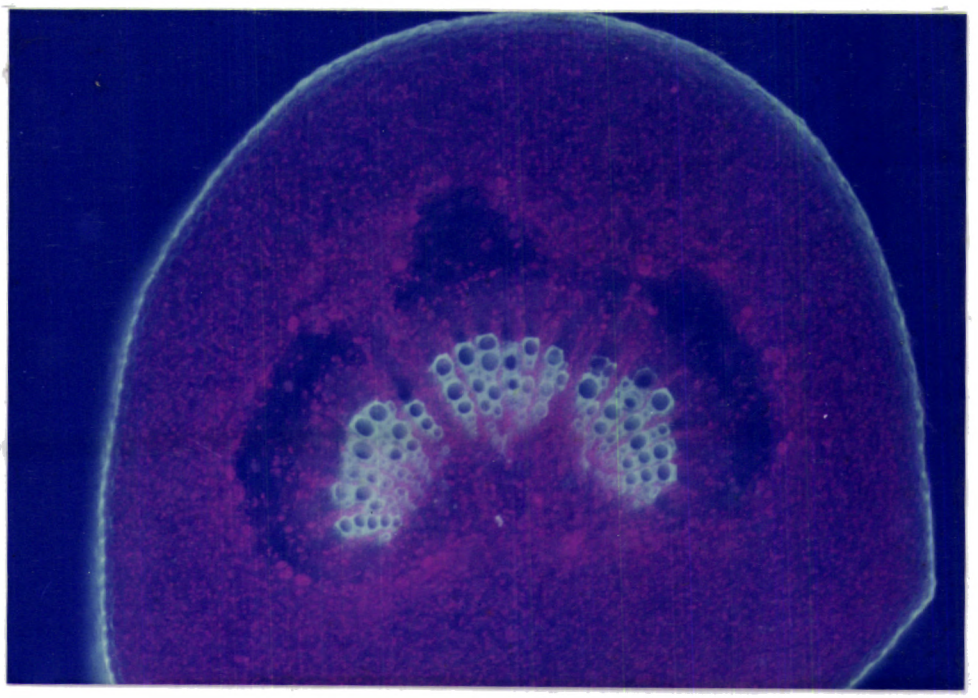


Fig. 3.3. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal petiole (CS) stained with DAPI (X70).

a)



b)

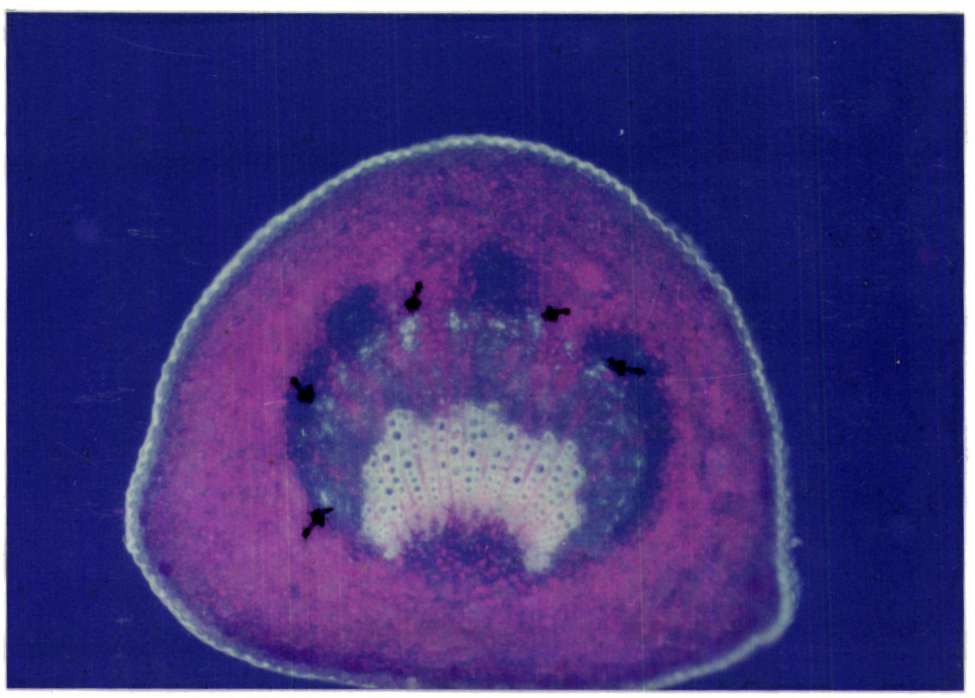
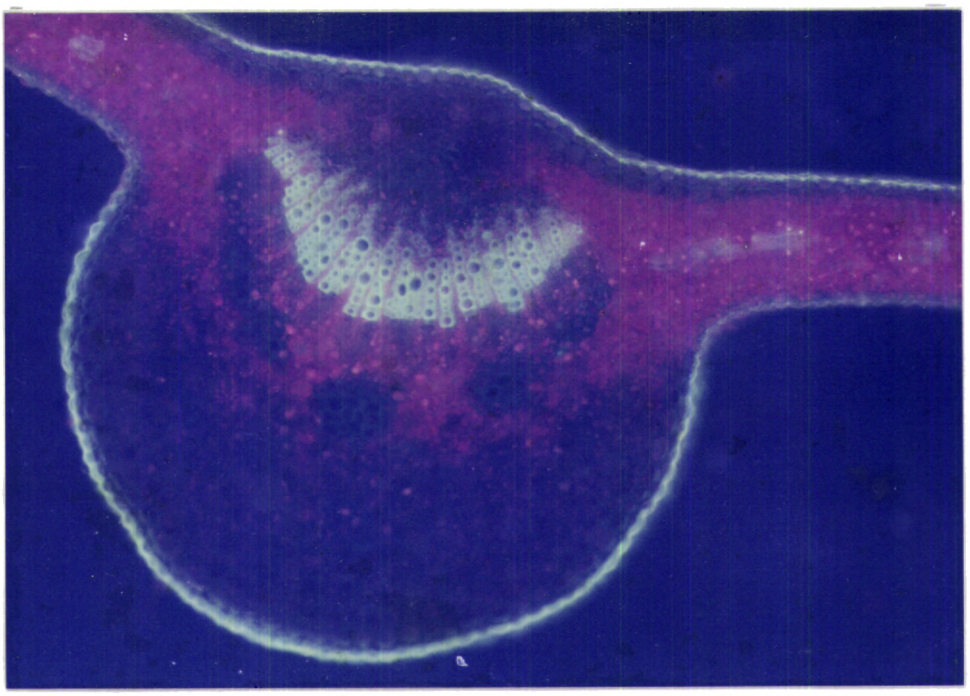


Fig. 3.4. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal leaf (CS) stained with DAPI (X70).

a)



b)

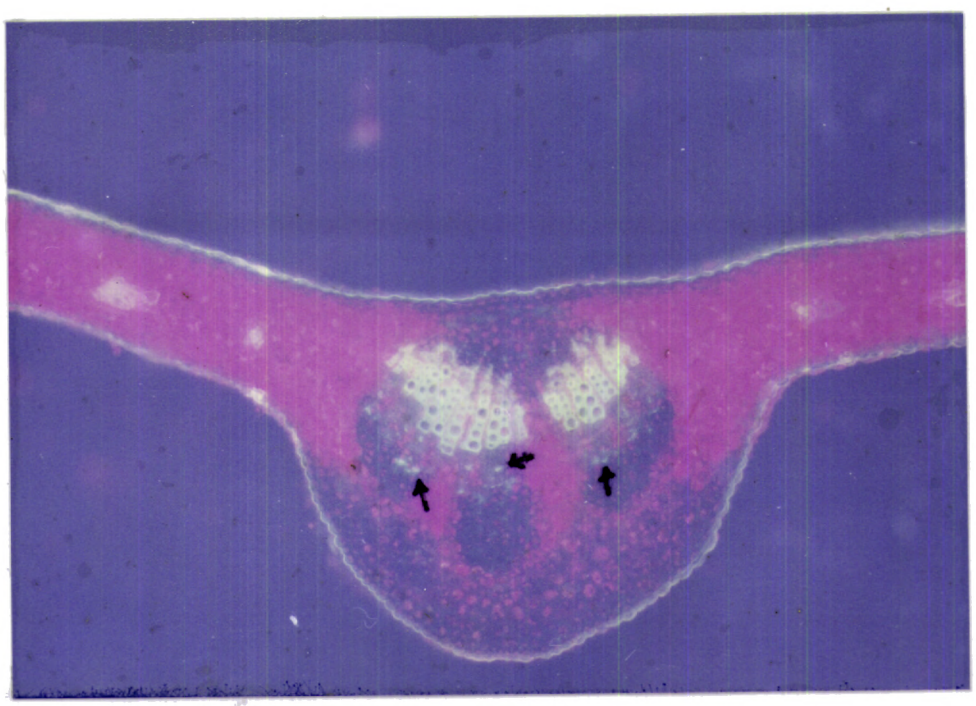
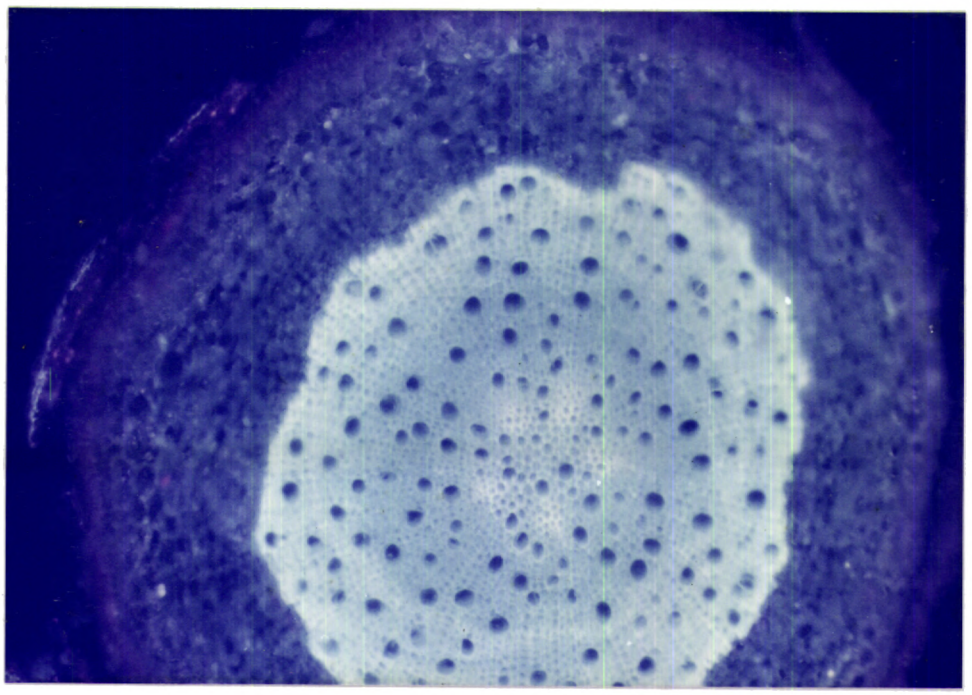


Fig. 3.5. Fluorescent photomicrograph of (a) healthy and (b) diseased sandal root (CS) stained with DAPI (X70).

a)



b)

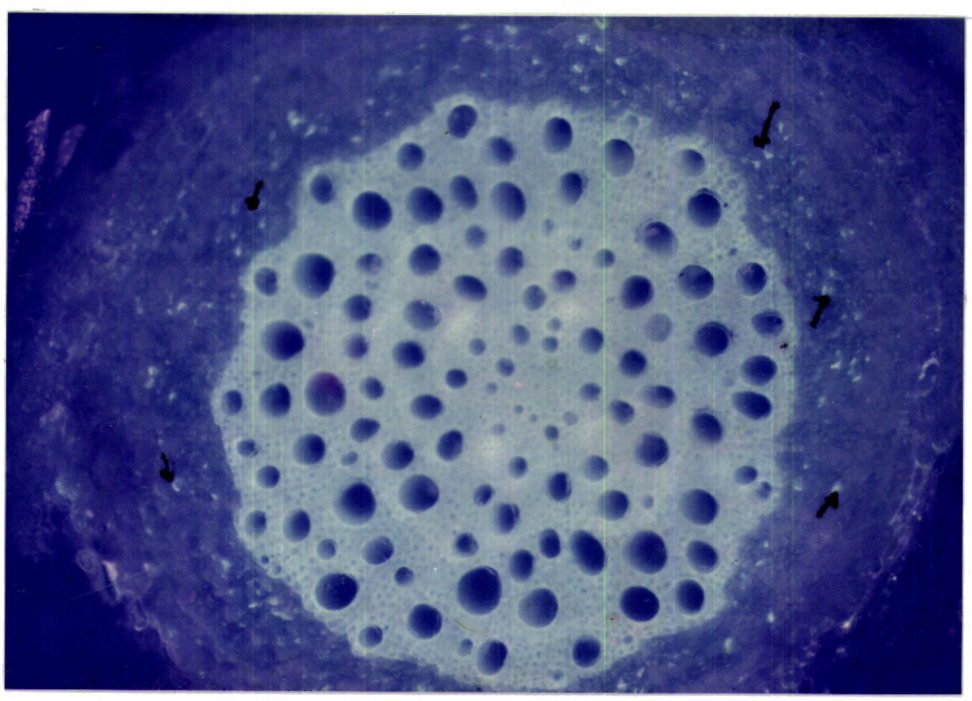


Fig. 3.6. Fluorescent photomicrograph of *Pongamia glabra* stem (CS) grown as host of diseased sandal stained with DAPI (X70).

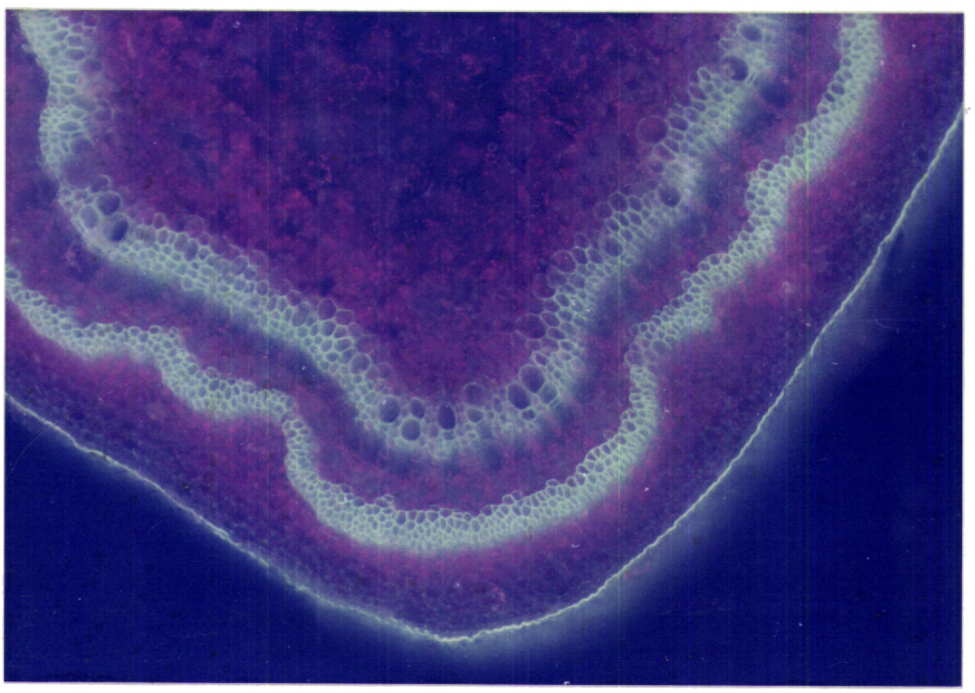
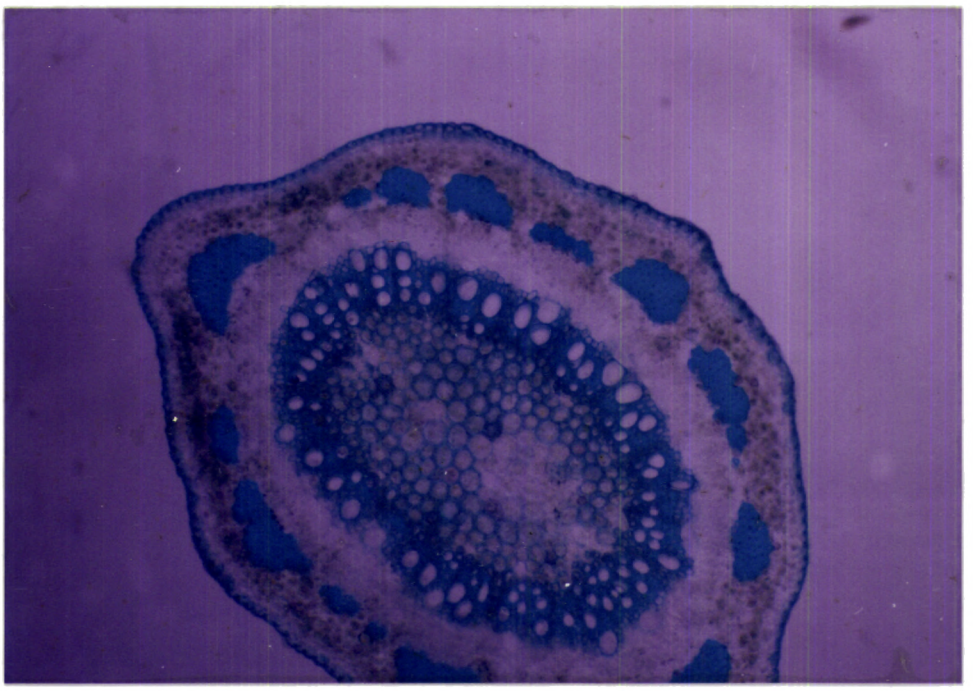


Fig. 3.7. Photomicrograph of Dienes' stained (a) healthy and (b) diseased sandal stem (CS). Note the blue spots in the phloem region of diseased sandal.

a)



b)

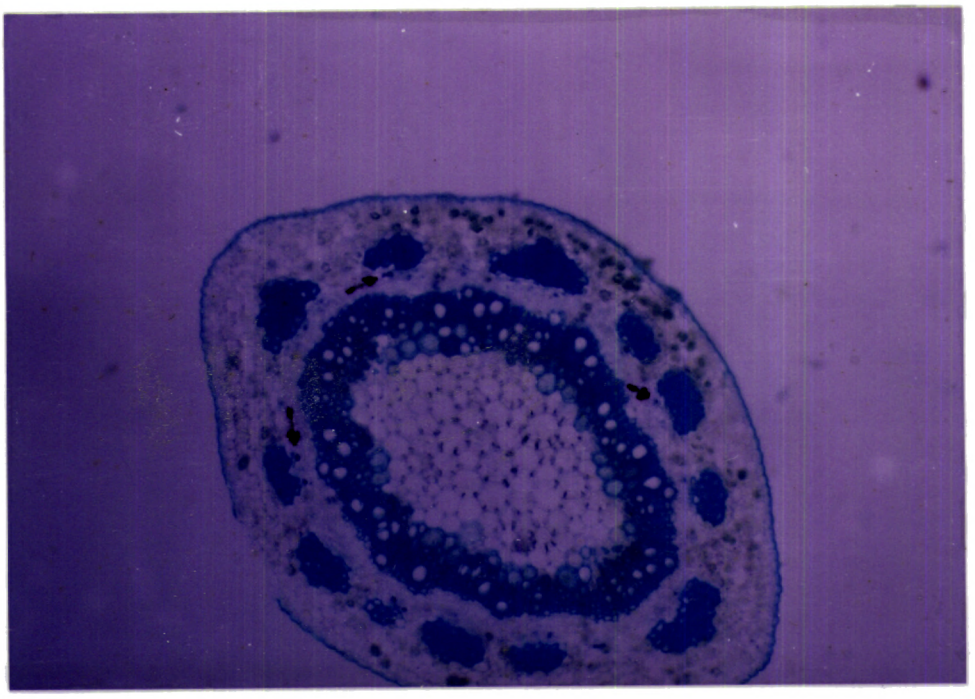
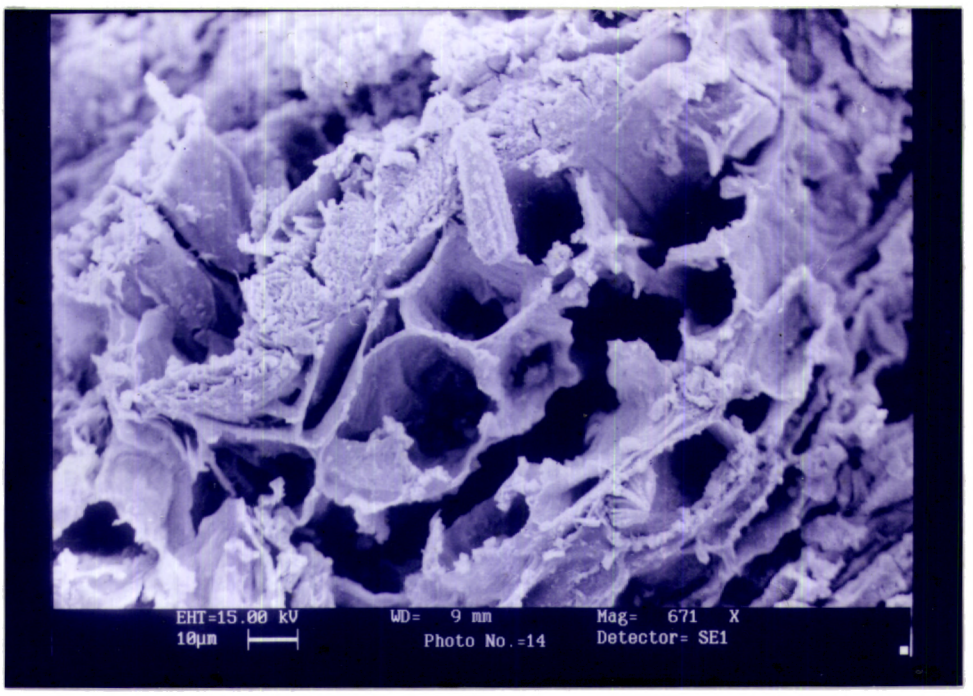


Fig. 3.8. Scanning electron micrograph of (a) healthy and (b) diseased sandal stem. The phytoplasma cells are seen exclusively in the phloem tissues of diseased sandal.

a)



b)

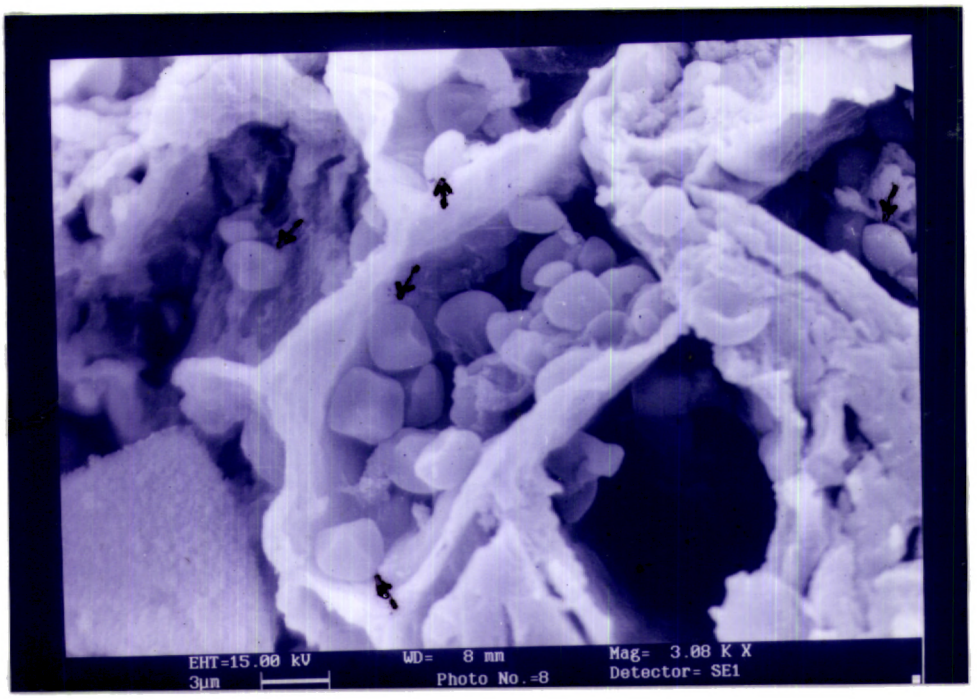
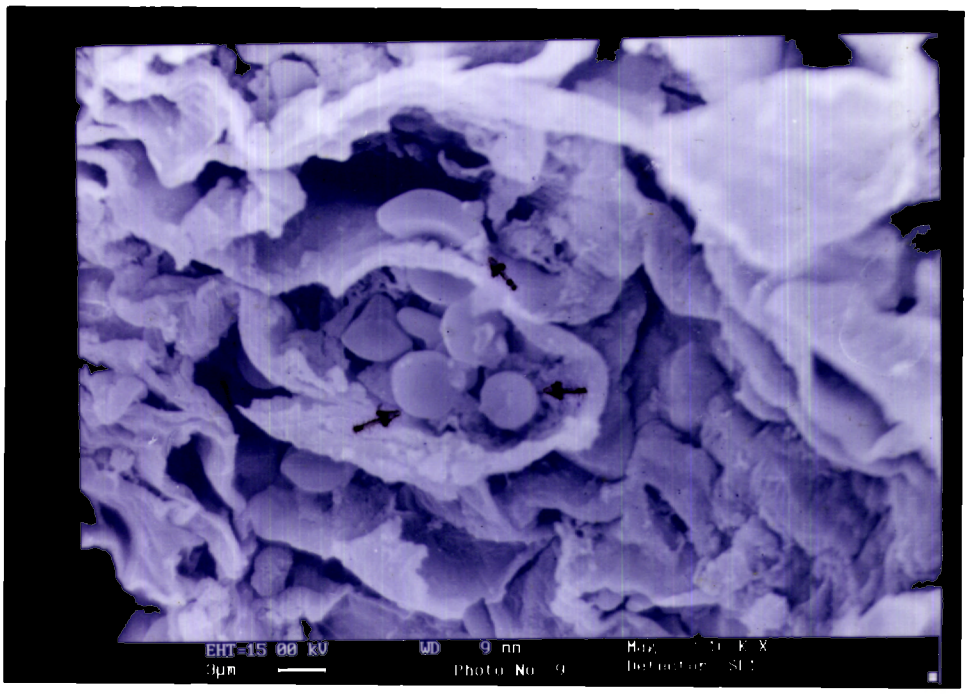
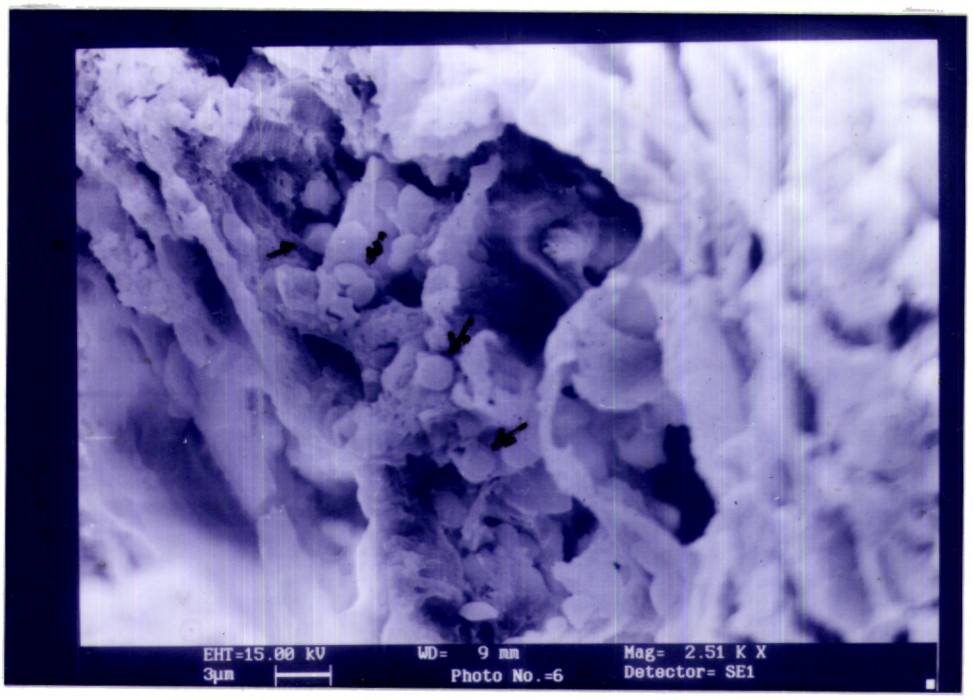


Fig. 3.8. Scanning electron micrograph of (c,d) diseased sandal stem. Note the pleomorphic phytoplasma cells seen exclusively in the phloem tissues of diseased sandal.

c)



d)



Fluorescence is a form of luminescence, which occurs after photons of light are absorbed by a fluorochrome at the ground electronic state. Most of the fluorochromes require excitation in the long wave UV to produce fluorescence in the visible range. Some of the biochemical substances present in the cells have the property to autofluoresce under UV light (Kasten, 1993). The present study also showed that the xylem and sclerenchymatous stone cells exhibited the characteristic autofluorescence in both healthy and diseased unstained tissues under UV light.

Eventhough, there is only scant information on the biochemical characters of phytoplasma, there is much knowledge about its animal counterpart, the mycoplasma. Generally, mycoplasmas are wall-less prokaryotes with a genome size of 5×10^8 daltons. They have a low G+C content (23 to 30 moles percent G+C), i.e., a high A+T content. The affinity of the fluorochrome, DAPI to double stranded DNA is very high, the apparent association constant (K_{app}) is in the range of 10^5-10^7 M^{-1} DAPI binds specifically to the minor groove of A-T rich sequences (Kapuscinski, 1995). In aster plants with yellows disease, it was found that the immature sieve elements close to the mature sieve elements degenerated (Raza and Esau, 1961). In the present study also it was found that in severely diseased trees, the fluorescent spots were less in number towards terminal portion of the stem and root but high intensity of fluorescence was detected in several rows of secondary phloem in

the inner bark. This may be due to degeneracy of phloem in the newly formed tissues. Rangaswamy (1995) found that the intensity of fluorescence was high in the stem and petiole than in the root when spiked tissues were stained with Hoechst 33258. Detection of phytoplasma using DAPI stain has gained popularity since the test is a highly rapid method. The technique has been used in the rapid diagnosis of phytoplasma in blueberry (Schaper and Converse, 1985), alder (Lederer and Seemuller, 1991), lettuce (Marcone *et al.*, 1995b) and periwinkle (Marcone and Ragozzino, 1995c).

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

Lantana, periwinkle and *Zizyphus*, when planted along with spike disease affected sandal. Similarly, when healthy sandal was provided with the hosts - witches' broom affected *Zizyphus* and little leaf affected periwinkle there was no change in the status of the healthy plant.

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

Scanning electron microscopy has been employed mainly to detect the presence of fastidious prokaryotes in plants infected with the yellows type disease (Bove, 1984). The phytoplasmas occur in the phloem elements, which are usually devoid of interfering cytoplasmic material, thus making their detection by SEM possible. According to Marcone and Ragozzino, (1996) the

two-dimensional structure of these microorganisms, as observed by TEM, can resemble some cell organelles; but their three dimensional structure observed by SEM are unique in that the cell surface and ultrastructural features of phytoplasmas as well as their interaction with surfaces of host cells can be studied in detail.

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

Attachment to the host membrane is a characteristic feature of many human and animal pathogenic mollicutes and is considered to be an important requirement for pathogenecity (Kahane and Horowitz, 1993). Some of the

phytoplasma cells were found to adhere to the inner surface of the phloem in different plant species like blackberry (Marcone, *et al.*, 1994b), wild radish (Marcone and Ragozzino, 1995a) and *Brassica* spp. (Marcone and Ragozzino, 1995b). The scanning electron micrograph from this study also provided evidence that some of the parasiting phytoplasmas are attached to the inner surface of the sieve tubes.

4. PURIFICATION OF SANDAL SPIKE PHYTOPLASMA

4. PURIFICATION OF SANDAL SPIKE PHYTOPLASMA

A method was developed to purify sandal spike phytoplasma using differential filtration technique. The technique takes advantage of the property of phytoplasma to pass through 0.45 μm pore size membrane filter. The purity of the phytoplasma pellet obtained after ultracentrifugation was assessed using scanning and transmission electron microscopy. The amino acid composition and total protein content of the microorganism was estimated. The pathogen was found to have only 6.23 percent of aromatic amino acids. The silver binding method was found to be a highly sensitive method for phytoplasma protein quantitation.

4.1. INTRODUCTION

Phytoplasmas comprise non-culturable group of plant pathogens. Although phytoplasmas can be visualised by electron microscopy and their presence in phloem tissues demonstrated by fluorochromic DNA stains, these methods cannot discriminate among various groups of phytoplasmas (Clark *et al.*, 1989). 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) resulted in partial success only due to high contamination with plant

proteins which resulted in poor quality antisera. Hobbs *et al.* (1987) used antibody raised against healthy plant extract to remove plant specific proteins from the semi-pure phytoplasma pellet by cross absorption. Since then several modifications of the protocol has 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 lead to the loss of phytoplasma cells during successive centrifugation.

Eventhough, phytoplasmas were purified by many workers, there are very few reports on the biochemical characterization of the microorganism (Sinha and Madhosingh, 1980). This chapter reports a differential filtration technique followed by centrifugation for the purification of sandal spike phytoplasma; the purity of phytoplasma pellet was assessed using scanning and transmission electron microscopy. The amino acid composition and total protein estimation of the pathogen is also reported.

4.2. MATERIALS AND METHODS

4.2.1. Purification of phytoplasma

Diseased sandal tissues were collected from Marayoor, Munnar Forest Division, Kerala, India. Inner bark and stem tissues from diseased branches were utilized

for the purification of phytoplasma. Tissues were washed in running tap water for 10 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 0.02 M 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 resuspended 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 resuspended 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 and provided by the manufacturer and S_{20w}, the sedimentation coefficient.

4.2.2. Electron microscopy

For scanning electron microscopy (SEM) the centrifuged pellet was embedded in 3% agarose (Sigma, USA) in 0.2 M phosphate buffer, pH 7.0. The agarose block was cut into pieces of 1 mm^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 dessicator 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 was fixed in 2.0% glutaraldehyde in 0.3 M mannitol-20 mM 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.

4.2.3. Total protein estimation

For total protein estimation, the phytoplasma was pelleted as stated above, but during the second centrifugation step, instead of glycine buffer, 0.1 M 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 resuspended in 1.0 ml of 0.1 M 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), Lowry method (Lowry *et al.*, 1951), and silver binding method (Krystal *et al.*, 1985).

4.2.4. Amino acid analysis

For amino acid analysis also, the phytoplasma was pelleted as stated above. Phytoplasma cells (obtained from 6.25 g tissue in 25 ml buffer) after acid hydrolysis was analysed for amino acid composition using high performance liquid chromatography (Shimadzu LC-10 A, Japan) following the procedure of Ishida *et al.* (1981). Tryptophan was estimated as described by Sastry and Tammuru (1985).

4.3. RESULTS

4.3.1. 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 at 45,000g. Increasing the quantity of plant tissues (>1g/4 ml buffer) blocked the 0.45 μm membrane.

4.3.2. Electron microscopy

Scanning electron micrographs of partially purified pellets of phytoplasma prior to cross absorption with antiserum against healthy plant extract showed plant debris among phytoplasma cells (Fig. 4.1), whereas impurities were sparse when the pellets were subjected to cross absorption with antiserum raised against healthy plant extract (Fig. 4.2). 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. No phytoplasma cell was observed in pellets obtained from extract of healthy sandal (Fig. 4.3).

Transmission electron micrographs confirmed the presence of purified phytoplasma cells (Fig. 4.4), whereas no such structure was present in healthy control (figure not shown). The sedimentation coefficient ($S_{20,w}$) of phytoplasma was calculated to be 466.0 Svedbergs.

Fig. 4.1. Scanning electron micrograph of partially purified phytoplasma before cross absorption with healthy plant antiserum. Note the plant debris covering phytoplasma cells (X1250).

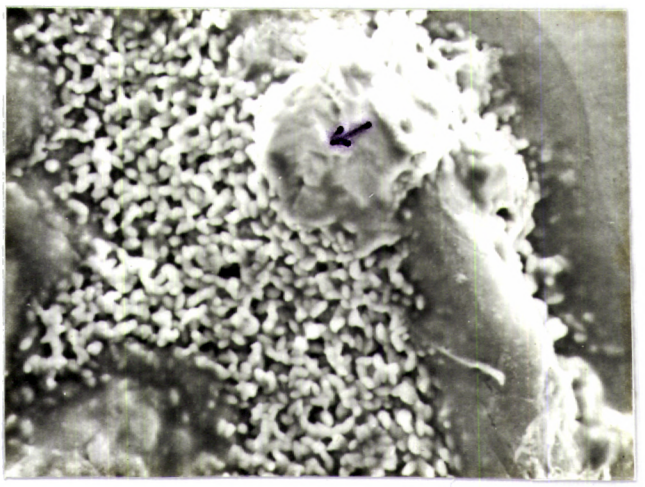


Fig. 4.2. Scanning electron micrograph of purified phytoplasma. Note the absence of plant debris on phytoplasma cells (X2500).

Fig. 4.3. Scanning electron micrograph of purified healthy sandal pellet. Note the absence of phytoplasma cells.

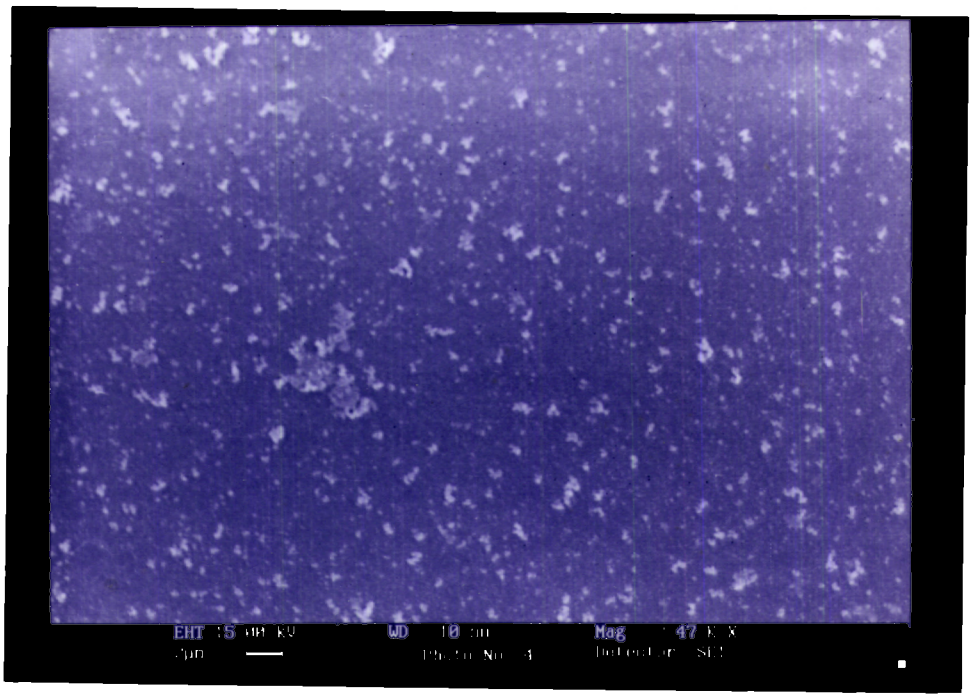
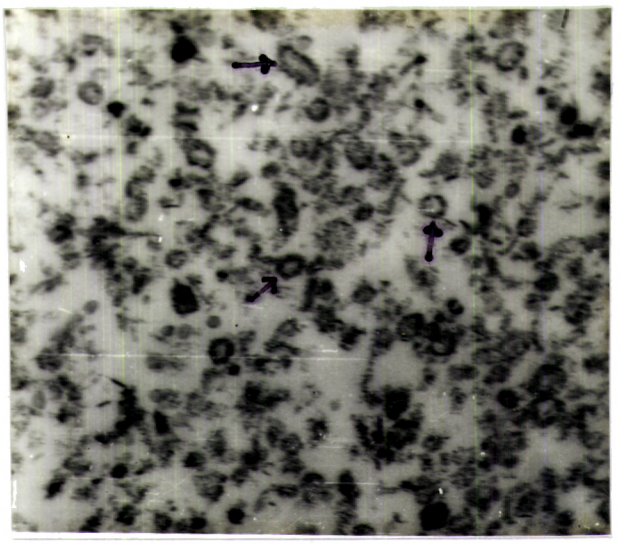


Fig. 4.4. Transmission electron micrograph of purified phytoplasma (X29,000).



4.3.3. 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 methods are shown in table 4.1.

Table 4.1. Total protein estimation of sandal spike phytoplasma (obtained by purifying 6.25g plant tissue in 25 ml buffer) using different methods.

Protein estimation method	HPLC	A_{205}	A_{280}	Lowry	Silver binding
Protein conc.* (μg ml^{-1})	256.54	300	400	440	230

* Mean of 3 replicates

4.3.4. Amino acid composition

The amino acid composition of sandal spike phytoplasma is provided in table 4.2. The concentration of glutamic acid was the highest followed by aspartic acid. The sulphur containing amino acids - cysteine and methionine were totally absent and so was tryptophan. The concentration of aromatic amino acids - phenyl alanine, tyrosine and tryptophan, were found to be 6.23% of the total amino acids.

Table 4.2. Amino acid composition* of sandal spike phytoplasma (cells extracted from 6.25g plant tissue in 25 ml buffer).

Amino acid	Concentration ($\mu g\ ml^{-1}$)	Percentage of total amino acid
Glycine	17.01	6.63%
Alanine	21.64	8.43%
Valine	14.38	5.60%
Leucine	24.62	9.59%
Isoleucine	9.24	3.60%
Serine	16.57	6.45%
Threonine	13.96	5.44%
Aspartic acid	25.87	10.08%
Glutamic acid	38.83	15.13%
Lysine	14.98	5.83%
Arginine	12.80	4.98%
Histidine	17.68	6.89%
Phenyl alanine	12.39	4.82%
Tyrosine	3.62	1.41%
Tryptophan	0.00	0.00%
Cysteine	0.00	0.00%
Methionine	0.00	0.00%
Proline	12.95	5.04%

*Mean of two replicates

4.4. DISCUSSION

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 like ELISA (Hobbs *et al.*, 1987; Saeed *et al.*, 1992a) and biochemical studies of the organisms (Sinha and Madhosingh, 1980).

Phytoplasma, found exclusively in the phloem tissues 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.*, 1992, 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, so in the final centrifugation step the net yield of phytoplasma would be less. Sinha (1979) reported that about 75 percent of phytoplasma was found to be lost during purification procedure of aster yellows 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.

Agar is commonly used to trap phytoplasma cells in electron microscopy (Jiang and Chen, 1987; Cole, 1983). In this study 3% agarose was employed since agarose was found to be more pure than agar. Examination of agarose by SEM without embedding healthy or diseased pellet did not show any artifacts, which proved that agarose could be used as an "embedding medium" for phytoplasma in electron microscopy. Glutaraldehyde was used as a fixative since it not only stabilised the fine structure and prevented gross distortion of cell membrane during embedding but also increased the permeability of the

tissues to the embedding medium. In SEM studies, $KMnO_4$ was used as it was found to be a strong oxidising agent and acted as both fixative and stain (Hayat, 1970). OsO_4 was used in TEM since it acted as both stain and fixative and penetrated hydrophilic and hydrophobic lipids (Adams, 1958). When OsO_4 was treated with phytoplasma embedded in agarose, no black precipitate was formed probably because OsO_4 does not react with most of hexoses and pentoses or their polymers (Hayat, 1970).

The amino acid composition of very few phytoplasmas has been reported earlier. In clover phyllody and aster yellows phytoplasma (Sinha and Madhosingh, 1980), the concentration of glycine was the highest, followed by alanine, whereas phenyl alanine and tyrosine concentration was found to be low. Since glycine buffer was suspected to interfere with estimation of glycine, in the present study, glycine buffer was substituted with phosphate buffer. In *Spiroplasma citri*, the mollicute causing citrus greening, the amino acid composition of protein lacked methionine, histidine and tryptophan (Bove and Saillard, 1979). Among the animal mycoplasmas, the composition of amino acid in *Mycoplasma mycoides* and *Mycoplasma laidlawii*, has some similarity with that of sandal spike phytoplasma to the extent that the concentration of aspartic acid and glutamic acid was the highest and both lacked cysteine (Chelton *et al.*, 1968).

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).

5. DETECTION OF SANDAL SPIKE PHYTOPLASMA USING IMMUNOLOGICAL TECHNIQUES

5. DETECTION OF SANDAL SPIKE PHYTOPLASMA USING IMMUNOLOGICAL TECHNIQUES

Sandal spike phytoplasma purified by differential filtration method was injected to rabbit to raise polyclonal antibody. The polyclonal antibody was used to detect sandal spike phytoplasma in diseased sandal and the host plants using different immunological techniques. In Ouchterlony double diffusion test, precipitin bands were visible in the extract of diseased sandal but not in healthy sandal or in the host plants. Both direct ELISA and indirect ELISA could detect the presence of phytoplasma in diseased sandal but not in healthy sandal or in the host plants of diseased sandal. A rapid and cheaper method - dot immunobinding assay (DIBA) was standardised for detecting the pathogen. Immuno microscopic techniques - immunolight microscopy, immunofluorescence microscopy and immunoelectron microscopy were employed in the *in situ* detection of the pathogen. An *ex situ* immunoelectron microscopic method was also standardised for detecting the pathogen. When protein profiles from SDS-PAGE gel was transferred to nitrocellulose membrane and probed with the polyclonal antibody (immunoblotting), a single major antigenic protein of approximately 14,000 daltons was observed in gel profiles of purified and semi-purified phytoplasma.

5.1. INTRODUCTION

Immunological methods are among the simplest to use and interpret and are invaluable 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, 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 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).

This chapter reports production of sandal spike phytoplasma specific polyclonal antibody and detection of the phytoplasma using the immunological techniques - double immunodiffusion test, direct and indirect enzyme linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA), immuno microscopic techniques (immunohistology) – immunolight microscopy, immunofluorescence microscopy and immunoelectron microscopy. The size of the antigenic protein of the pathogen was estimated using immunoblotting (Western blotting).

5.2. MATERIALS AND METHODS

5.2.1. Test plant material

Spike disease affected sandal twigs were collected from Marayoor, Kerala and Mysore, Karnataka. The host plants of diseased sandal, *Lantana camara* and witches' broom affected *Zizyphus oenoplea* were collected from Marayoor.

5.2.2. 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 (Chapter 4) 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 obtained after centrifugation of 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.

5.2.3. 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 instruction.

5.2.4. 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.*

5.2.5. Enzyme linked immunosorbent assay (ELISA)

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

5.2.5.1. 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 purified antigen or crude extract (100 µl) was coated at different dilutions in PBS, pH 7.4, at 37^{0}C 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^{0}C 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 2 M 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).

Initially, 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) were 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.

5.2.5.2. 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^{0}C. 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^{0}C. 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 2 M sulphuric acid (25 µl) 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 wells 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).

Tests were also 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). 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.

5.2.6. Dot immunobinding assay (DIBA)

The method of Lazarovits (1990) was followed with slight modifications. As in ELISA, both direct and indirect methods were tested for the assay. In direct method, pencil marked nitrocellulose membrane (Schleicher and Schuell, Germany) was washed with deionized water thrice, followed by air-drying. Different quantity of the purified antigen diluted in PBS, pH 7.4 was spotted on the membrane. After washing thrice in PBS-tween, the membrane was blocked with PBS-tween-BSA (1%) for 30 minutes followed by incubation in

phytoplasma specific HRP conjugated antibody (1:250 dilution) for one hour. Subsequently, the membrane was washed thrice in PBS-tween and the final wash in substrate buffer. The membrane was incubated in the substrate, aminoethyl carbazole (AEC) (Sigma, USA) following the protocol of Harlow and Lane (1988) and the dots visualised after 30 minutes of slow agitation.

For indirect method, the washed membrane was spotted with different dilutions of purified antigen. Blocking in PBS-tween-BSA (1%) for 30 minutes and incubation in phytoplasma specific antibody (1:500 dilution) followed washing the membrane (thrice) in PBS-tween. After incubation for an hour at room temperature, the membrane was washed and blocked as in the previous step. This was followed by incubation in biotinylated goat anti-rabbit IgG (1:500 dilution) for one hour at room temperature. After washing, the membrane was incubated in streptavidin-HRP (1:500 dilution) for one hour. The final wash in substrate buffer was followed by incubation in the substrate, AEC and the dots visualised after 30 minutes of slow agitation.

An experiment was conducted to test the efficiency of different dilutions of the polyclonal antibody (Pab). In the experiment, different dilutions of the crude sandal extract (healthy and diseased) was spotted on to separate membranes and later on incubated in three dilutions of phytoplasma specific antibody (1:500, 1:1000 and 1:2000) for one hour. After washing, they were blocked in block buffer and later on probed with

biotinylated goat anti-rabbit IgG (1:500 dilution) for one hour and finally with streptavidin-HRP (1:500 dilution) for one hour. The dots were visualised after treating with the substrate.

5.2.7. Immunolight microscopy

Healthy and diseased sandal stem and inner bark tissues were fixed in 5% formaldehyde for 30 minutes. After washing thrice in PBS, pH 7.4, free hand sections (CS) of the tissues were incubated in hydrogen peroxide (0.1%) for 10 minutes, washed and incubated in phytoplasma specific antibody (1:50 dilution in PBS-tween-0.5% BSA) for 2 hours. The tissues were washed in PBS-tween (PBS-T) followed by blocking in PBS-tween-BSA for 30 minutes. This was followed by incubating the tissues in biotinylated anti-rabbit antibody for 1 hour; the washing and blocking steps were repeated and the tissues were subjected to incubation in streptavidin-HRP conjugate (1:50 dilution) for one hour. After the final three washes, the last wash in the substrate buffer, the tissues were incubated in the substrate, amino ethyl carbazole (AEC) (Sigma, USA) following the protocol of Harlow and Lane (1988). The tissues were then viewed under light microscope (Leitz, Germany).

5.2.8. Immunofluorescence microscopy

The method of Cousin *et al.* (1989) was employed with modifications. Healthy and diseased sandal and the host plants, *Lantana* and witches' broom affected *Zizyphus* tissues were initially processed as required for immunolight microscopy. After blocking with PBS-tween-BSA for 30 minutes, the tissues were incubated in FITC-goat anti-rabbit antibody (1:50 dilution) for 2 hours at room temperature. The specimens were washed thrice and observed under fluorescence microscope (Nikon, Japan) or (Leitz, Germany) provided with HBO 50W bulb.

5.2.9. Immunoelectron microscopy

The healthy and spike disease affected tissues were fixed in 1% glutaraldehyde (Sigma, USA) for 2 hours; fixation and embedding in LR White (London Resin Co., UK) was done according to the procedure of Hayat (1989). The specimens were blocked in 1%BSA for 30 minutes followed by overnight incubation in specific antibody (1:50 dilution) at 4^0C. The washed specimens were blocked in BSA for 30 minutes; rinsed and incubated in protein-A gold (B.Genei, India) (1:100 dilution) for 2 hours. The specimens were stained in saturated uranyl acetate for 7 minutes after washing in PBS and viewed under electron microscope (Philips CM 10). Alternately, the uranyl acetate stained specimens were washed and stained with 0.5% lead citrate for 3 minutes followed by observation under the electron microscope.

For *ex situ* detection of sandal spike phytoplasma, the tissues (both healthy and diseased) were extracted in glycine buffer (1g tissue/4 ml buffer) and clarified using Whatman 1 filter paper. The clarified crude extract was incubated overnight at 4^0 C or for 2 hours at room temperature on a formvar coated grid. The grid was washed in PBS, blocked in 1% BSA for 30 minutes followed by incubation in the phytoplasma specific antibody (1:50 dilution) for 2 hours. The washed grids were incubated in protein-A gold (B.Genei, India) (1:100 dilution) for 2 hours followed by washing and post fixation in 1% osmium tetroxide for 10 minutes. The washed grids were further stained with 2% phosphotungstic acid for 10 seconds and viewed under transmission electron microscope (Philips CM 10).

5.2.10. Immunoblotting

For precipitation of phytoplasma protein and electrophoresis, the method of Saeed *et al.* (1992b) was followed with slight modification. The purified phytoplasma pellet was passed through a protein-A sepharose column (Pharmacia Biotech, Sweden) following manufacturer's instruction. The first passed molecules (fraction collected first), which contained the phytoplasma proteins, were centrifuged at low speed (700g) and the supernatant collected. To this was added 10% trichloroacetic acid (TCA), followed by incubation at -20^0C for 12 hours. Simultaneously, partially purified pellets from healthy and diseased plant extract were prepared by centrifuging both the extract at 45,000g for 45 minutes and the pellet treated with TCA followed by storage at -20^0C. The precipitate formed was later centrifuged at

30,000g for 45 minutes and the pellet dissolved in 100 µl PBS, pH 7.4. The total proteins thus obtained was subjected to electrophoresis (SDS-PAGE) (Laemmli, 1970) using 12.5% acrylamide gel (Mini Protean II, Bio Rad, USA). Protein molecular weight marker (Bangalore Genei, India) was used as standard in the first lane of the gel to facilitate protein size determination. Since two gels could be run simultaneously, one of the gel was silver stained following the protocol of Bloom *et al.* (1987) and the other gel subjected to immunoblotting (Hammond, 1990). The immunoblotted membrane was probed with phytoplasma specific antibody as in DIBA and the position of the band calculated with respect to the marker proteins.

5.3. RESULTS

5.3.1. Double immunodiffusion

Precipitin bands were not observed against healthy plant extract (Fig. 5.1), 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 bands were observed at higher dilutions. Clear bands were also visible against phytoplasma of diseased sandal of Mysore (Figure not shown). No bands were visible against the extract of the host plants of spike disease affected sandal - *Lantana* and witches' broom-affected *Zizyphus* (Fig. 5.2).

Fig. 5.1. Ouchterlony double diffusion test against different antigens. **A**: Antibody raised against sandal spike phytoplasma, **1** and **2**: healthy plant extract; **3**: crude diseased plant extract **4**: purified phytoplasma from diseased extract. Note the clear precipitin bands formed against diseased plant extract and purified phytoplasma.

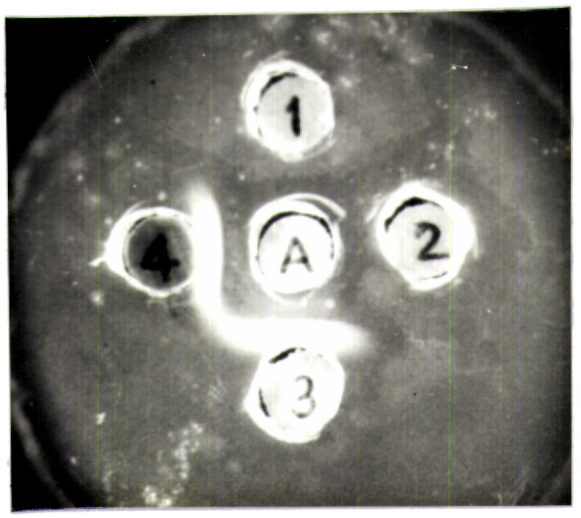


Fig. 5.2. Ouchterlony double diffusion test against extract of different host plants of spike disease affected sandal. **A**: Antibody raised against purified sandal spike phytoplasma, **C**: healthy sandal extract; **S**: diseased sandal extract, **L**. extract of *Lantana*, **Z**: extract of witches' broom affected *Zizyphus*. Note the precipitin band formed against diseased sandal extract.

5.3.2. Enzyme linked immunosorbent assay

5.3.2.1. 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. 5.3). Also, one hour duration was found to be ideal for incubating the conjugated antibody (Fig. 5.4). The results 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 5.1. 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 5.2). None of the test 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 $\mu g\ ml^{-1}$ (Chapter 4), substituting the values in direct ELISA shows that the test could detect up to 340 ng (1:750 dilution) of phytoplasma protein.

Fig. 5.3. 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 absorbance values of diseased and healthy plants.

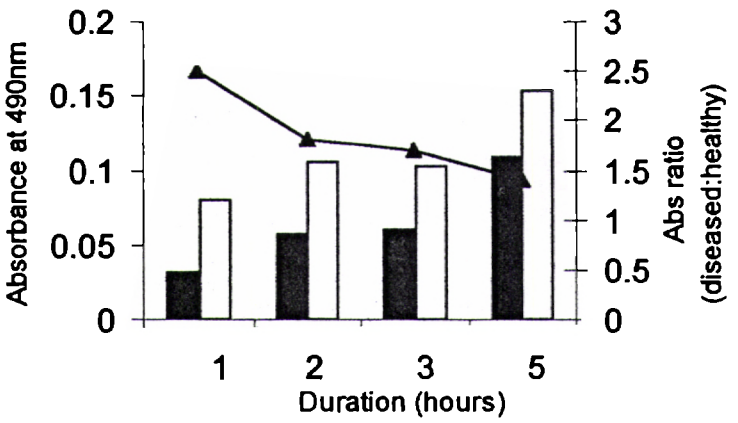


Fig. 5.4. Effect of time on efficiency of incubation of the conjugated antibody in direct ELISA. Plates were coated with antigen (1:100 dilution) followed by incubation with conjugated antibody (1:250 dilution-1hour incubation). Each value represents the mean of 5 replicates. ■ Healthy plant antigen, □ diseased plant antigen, ▲ ratio of absorbance values of diseased and healthy plants.

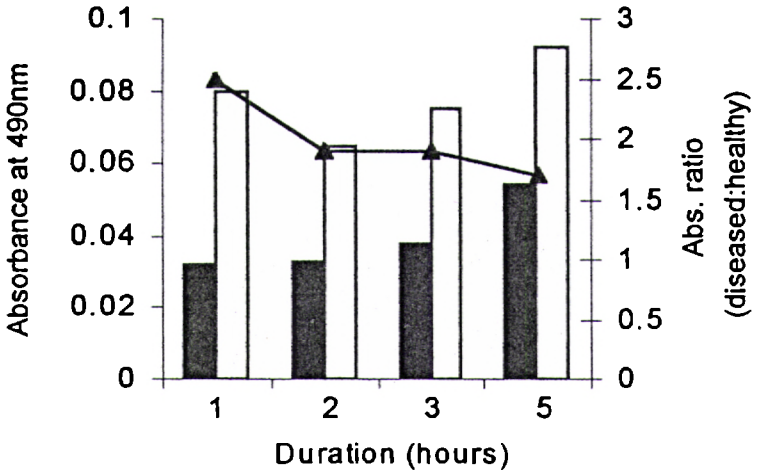


Table 5.1. Direct ELISA values of different dilutions of purified diseased antigen. The antigens were probed with HRP conjugated phytoplasma specific antibodies (C.Ab). (Value of buffer=0.010).

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.

Table 5.2. Direct ELISA values of different dilutions of unpurified diseased plant extract. The antigens were probed with HRP conjugated phytoplasma specific antibodies (C.Ab). (Value of 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.

5.3.2.2. 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. 5.5). The purified antigen at different of dilutions 1:50 1:10,000 were probed with three different dilutions of polyclonal antibody (1:500, 1:1000 and 1:2000) using the streptavidin amplification system (Table 5.3). The results confirm that polyclonal antibody at a dilution of 1:2000 was the most sensitive. From the result it is inferred that indirect ELISA could detect a minimum of 25ng phytoplasma protein, as purified phytoplasma of 1:10,000 dilution could be detected.

Fig. 5.5. 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.

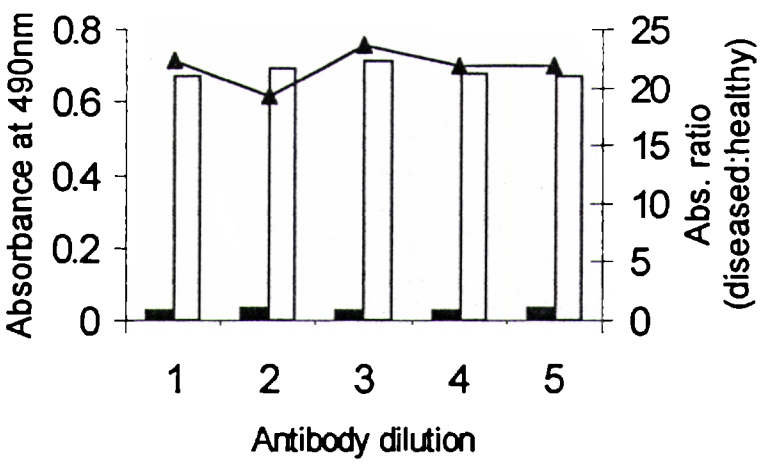


Table 5.3. Indirect ELISA values of different dilutions of purified diseased antigen using streptavidin amplification system. The antigens were probed with different dilutions of sandal spike phytoplasma-specific antibody, biotinylated anti-rabbit antibody (1:2000 dilution) followed by HRP conjugated streptavidin (1:2000 dilution). (Value of 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.

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 5.4. 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 system were considered. The results are shown in table 5.5. From the results it is confirmed that the test employing biotin-streptavidin system is the most sensitive of the tests. This system was further 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 5.6.

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 5.7. The host plants of diseased 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 5.8). The results indicated that sandal spike phytoplasma was confined to sandal.

Table 5.4. 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 of 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 5.5. Indirect ELISA values of different dilutions of unpurified diseased antigen (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) or **(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 of 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 5.6. 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 (Healthy)	0.324	24 (Healthy)	0.415

* Mean of 3 replicates

Table 5.7. Indirect ELISA values of different dilutions of extract of host plants of spike disease affected sandal using streptavidin system. (Value of 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.

Table 5.8. 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 (Healthy)	0.161	10 (Healthy)	0.091

* Mean of 4 replicates.

5.3.3. Dot immunobinding assay

Direct DIBA was less sensitive compared to the indirect counterpart; so the purified phytoplasma was used as antigen in direct DIBA, whereas both purified as well as crude samples were used to detect the presence of sandal spike phytoplasma in indirect DIBA. When direct DIBA was used to detect the presence of the pathogen, the technique could detect dilutions up to 1:25 only (Fig. 5.6), whereas indirect DIBA could detect samples upto a dilution of 1:100 (Fig. 5.7). When crude samples were used to detect the presence of phytoplasma by indirect DIBA, three different dilutions of the polyclonal antibody 1:500, 1:1000 and 1:2000 were used. The 1:500 dilution was observed to be more sensitive to detect antigen even at a low dilution of 1:25 (Fig. 5.8).

Fig. 5.6. Direct dot immunobinding assay (DIBA) of purified sandal spike phytoplasma. The antigen (Dilutions **A**- 1, **B**-1:2, **C**- 1:5, **D**-1:10, **E**- 1:15, **F**-1:25, **G**- 1:50, **H**- 1:100) was probed with C.Ab of dilution 1: 250. (h=healthy sandal).

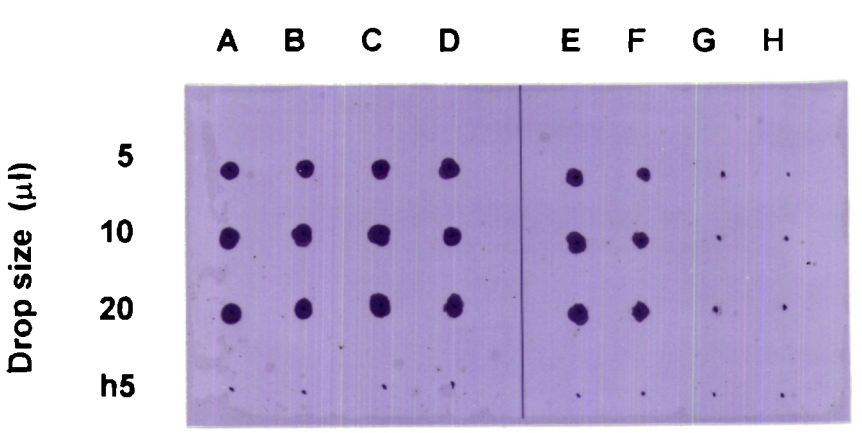


Fig. 5.7. Indirect dot immunobinding assay (DIBA) of purified sandal spike phytoplasma. The antigen (dilutions **A**- 1, **B**-1:2, **C**- 1:5, **D**-1:10, **E**- 1:15, **F**-1:25, **G**- 1:50, **H**- 1:100) was probed with Pab of dilution 1: 500, followed by anti-rabbit biotin (1:500 dilution) and streptavidin-HRP (1:500 dilution). (h=healthy sandal).

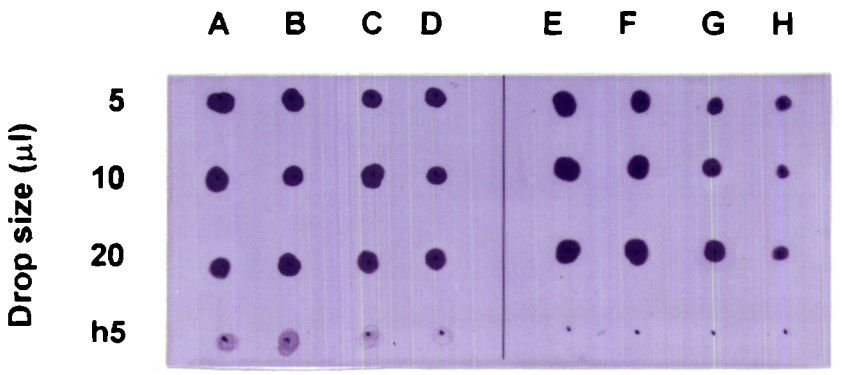
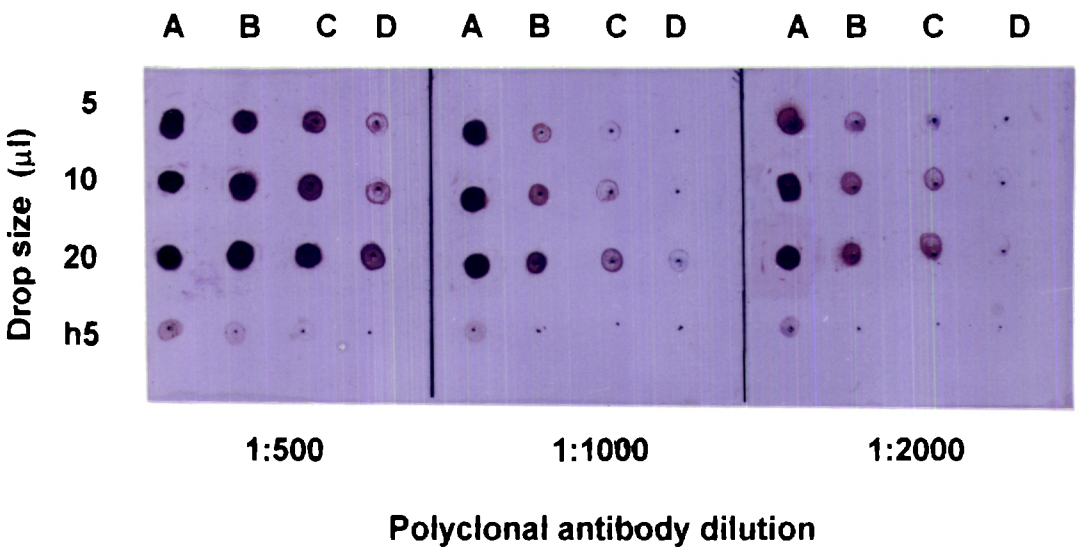


Fig. 5.8. Indirect dot immunobinding assay of crude extract of sandal spike phytoplasma. The antigen (dilution **A**- 1, **B**-1:5, **C**-1:10, **D**-1:25) was probed with Pab of dilution (Left 1:500; Middle: 1:1000; Right: 1:2000), followed by anti-rabbit biotin (1:500 dilution) and streptavidin-HRP (1:500 dilution). (h=healthy sandal).



5.3.4. Immunolight microscopy

The test could not provide direct evidence for the presence of the pathogen in phloem tissue. However, the phloem of diseased sandal tissues appeared reddish-brown in colour, whereas the phloem of healthy sandal appeared colourless (Fig. 5.9).

5.3.5. Immunofluorescence microscopy

The test could pinpoint the presence of the pathogen in phloem tissue. Fluorescent spots were easily distinguished in the phloem tissues of diseased sandal indicating the presence of the pathogen whereas no fluorescent spots were visualised in the phloem of healthy sandal tissues (Fig. 5.10). The test could not detect the presence of phytoplasma in the host plants of spike disease affected sandal, *Lantana* and witches' broom affected *Zizyphus* (Fig. 5.11).

5.3.6. Immunoelectron microscopy

The test was found to be highly sensitive because individual phytoplasma in the phloem tissues were seen densely coated with gold particles; whereas no pathogen was detected in the phloem tissues of healthy plants (Fig. 5.12). *Ex situ* detection of phytoplasma in extract of diseased sandal was found to be very rapid compared to *in situ* detection. Phytoplasma cells were labelled with protein-A gold, whereas gold particles were absent on grids coated with

healthy plant extract (Fig. 5.13). The phytoplasma specific antibody could also detect the pathogen in diseased sandal from Mysore confirming that phytoplasma affecting both Marayoor and Mysore (about 250 km apart) is of the same antigenicity (figure not shown).

5.3.7. Immunoblotting

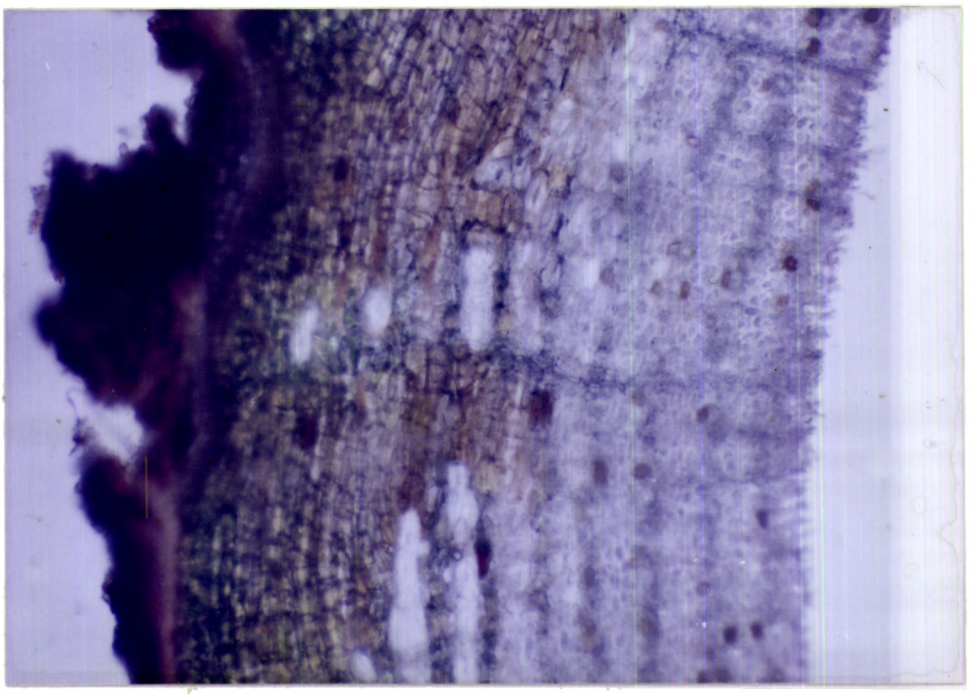
Silver staining of SDS-PAGE gels of total protein of purified or semi-purified phytoplasma could not reveal the specific antigenic protein associated with the microorganism. Only a few high molecular weight proteins were visible as bands while most of the low molecular weight proteins were not clearly visible, probably due to the low amount of the proteins (Fig. 5.14). Immunoblotting of the gel revealed the presence of a low molecular weight protein of 14,000 daltons in both semi-pure and purified phytoplasma (Fig. 5.15); the same was absent in healthy sandal.

5.4. DISCUSSION

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 world-wide (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).

Fig. 5.9. Immunolight photomicrograph of the inner bark (CS) of (a) healthy and (b) diseased sandal. Note the red colour of phloem in diseased tissues. (X100).

a)



b)

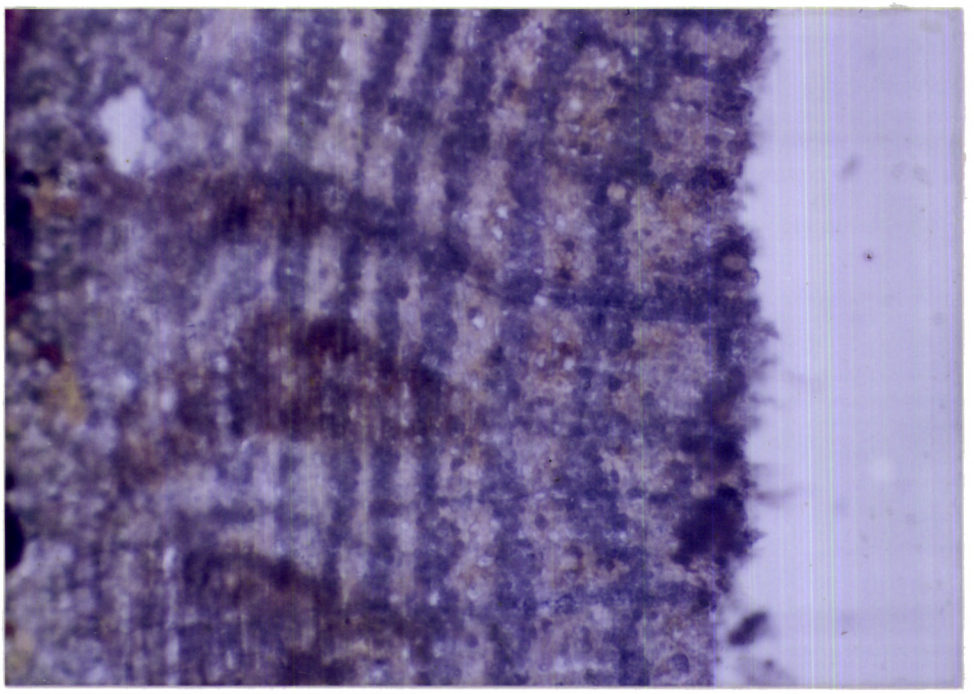
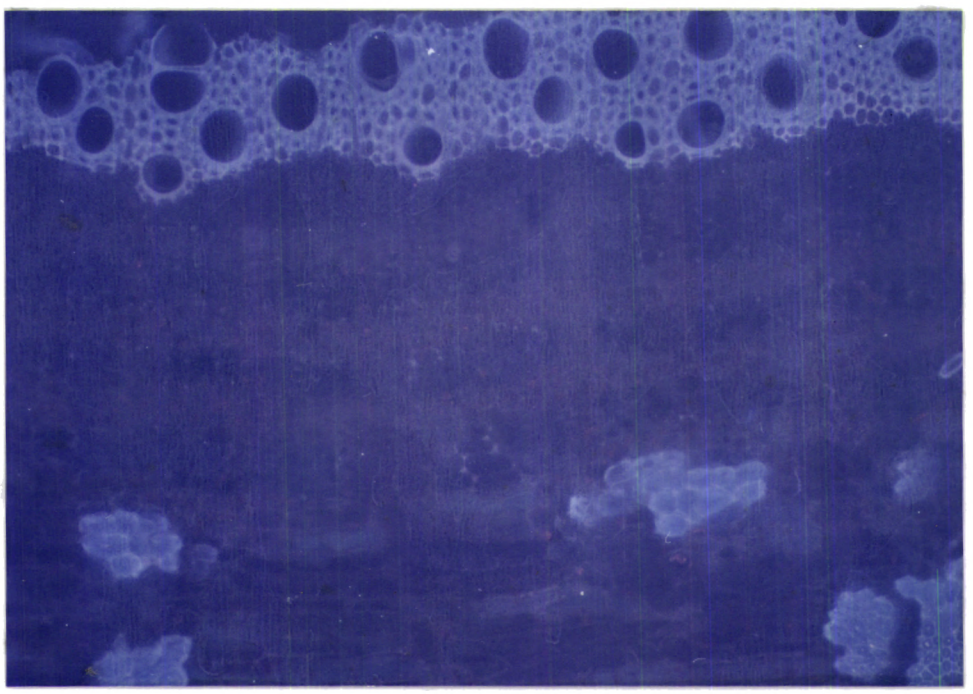


Fig. 5.10. Immunofluorescent photomicrograph of the inner bark (CS) of (a) healthy and (b) diseased sandal. (X100).

a)



b)

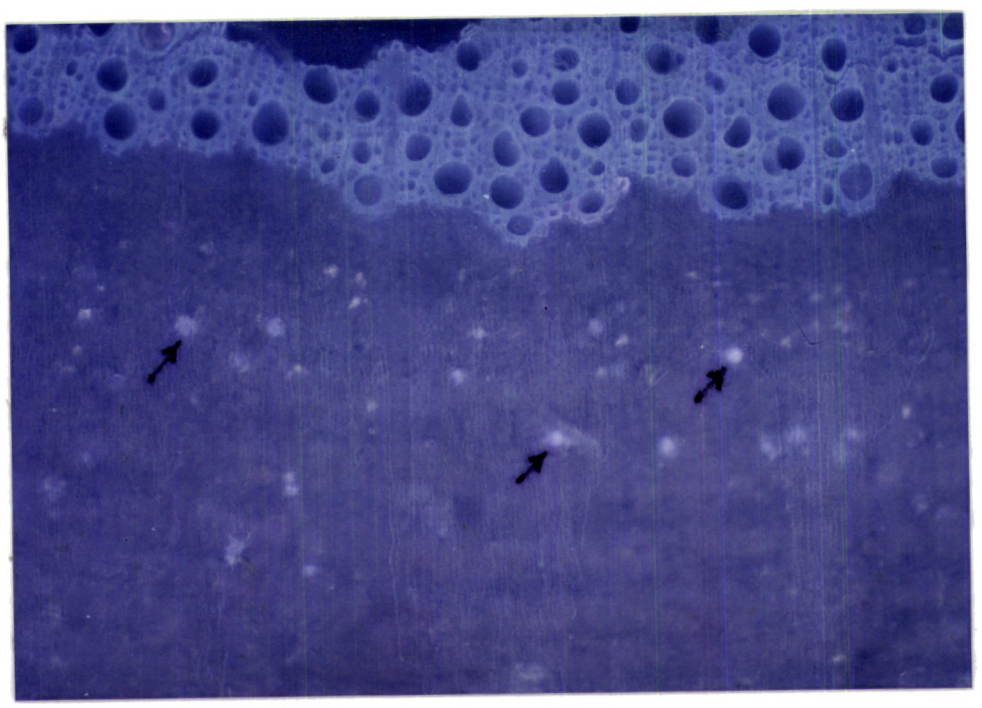
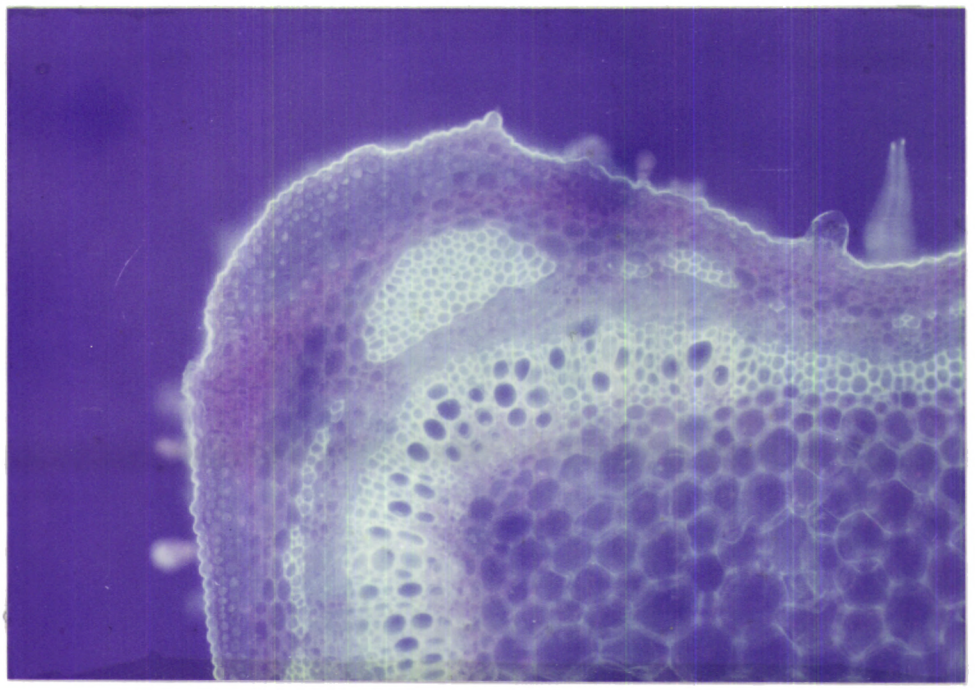


Fig. 5.11. Immunofluorescent photomicrograph of the inner bark (CS) of the host plants of spike disease affected sandal. (a) *Lantana* and (b) witches' broom affected *Zizyphus* (X100).

a)



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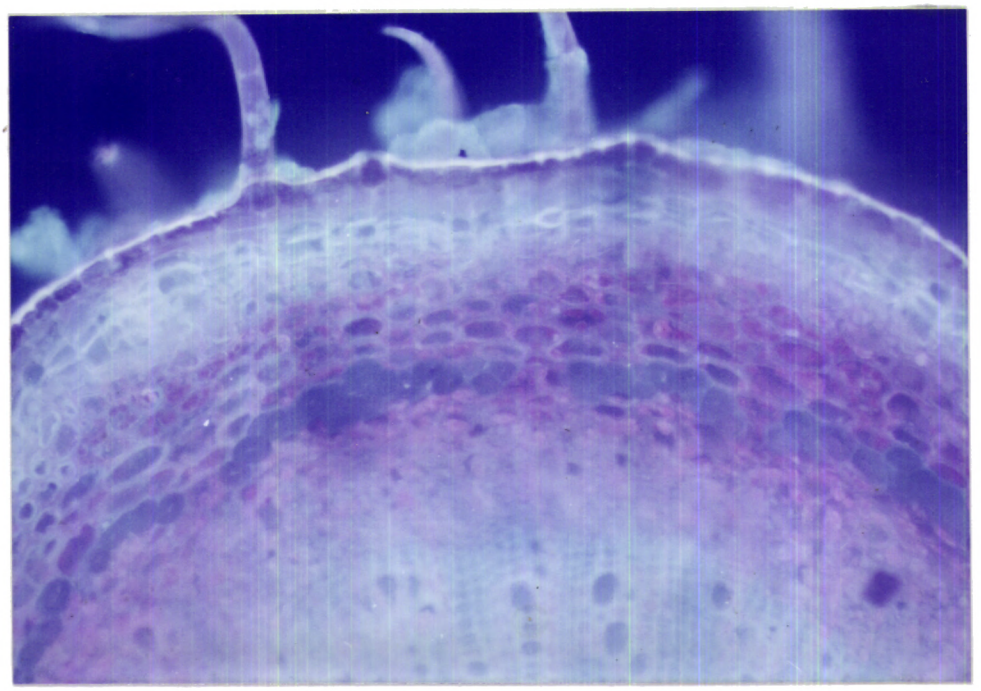
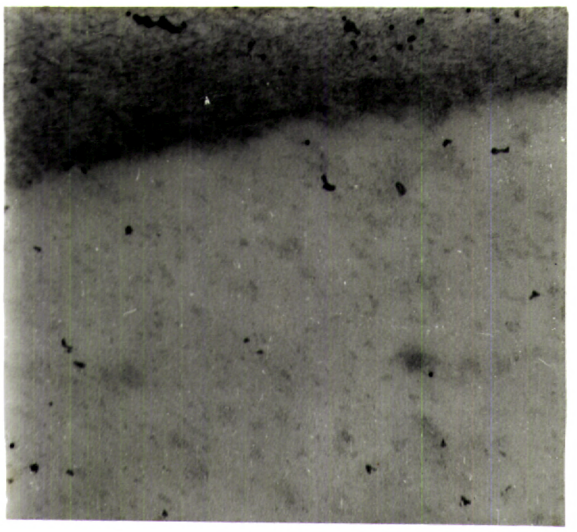


Fig. 5.12. Immuno electronmicrograph (*in situ*) of stem portion of a phloem tissue of (a) healthy and (b) diseased sandal. Note the dense coating of phytoplasma cells with gold particles (X50,000).

a)



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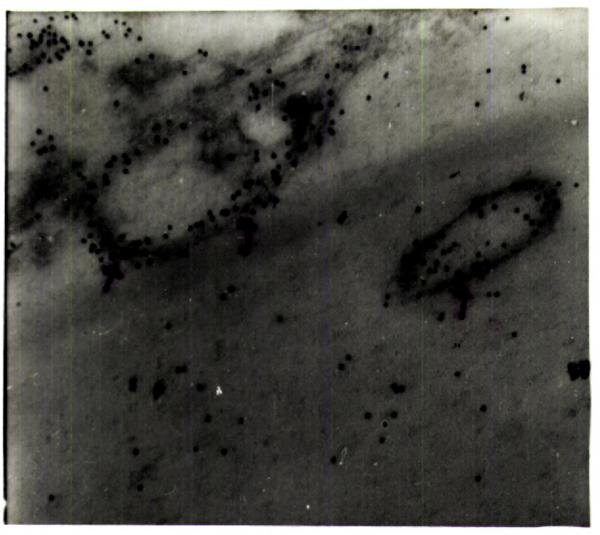
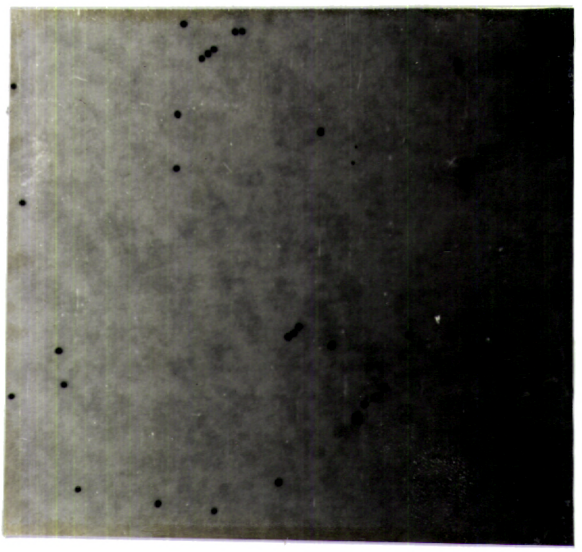


Fig. 5.13. Immuno electronmicrograph (*ex situ*) of extract of (a) healthy and (b) diseased sandal. Note the dense coating of phytoplasma cells with gold particles (X80,000).

a)



b)

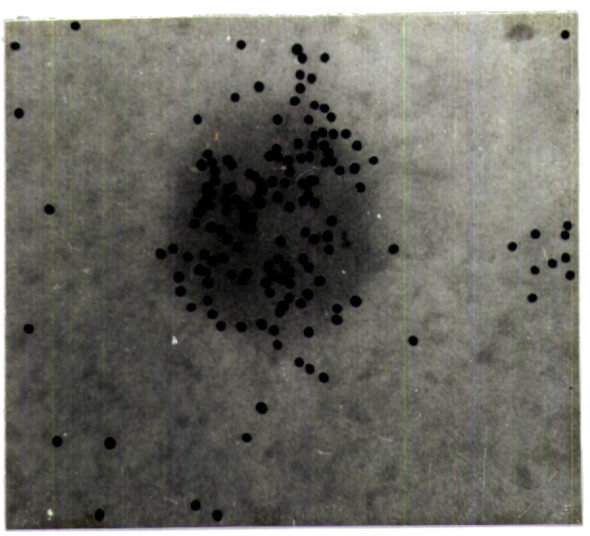


Fig. 5.14. SDS-PAGE profiles of proteins. Lane **1**: Marker, **2**: Purified healthy sandal, **3** and **4**: Partially purified diseased sandal, **5** and **6**: Purified phytoplasma.

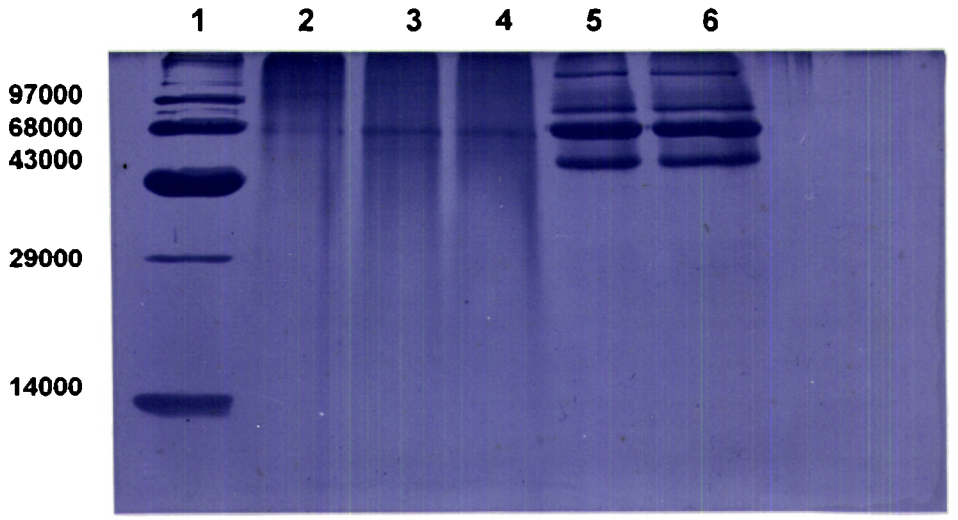
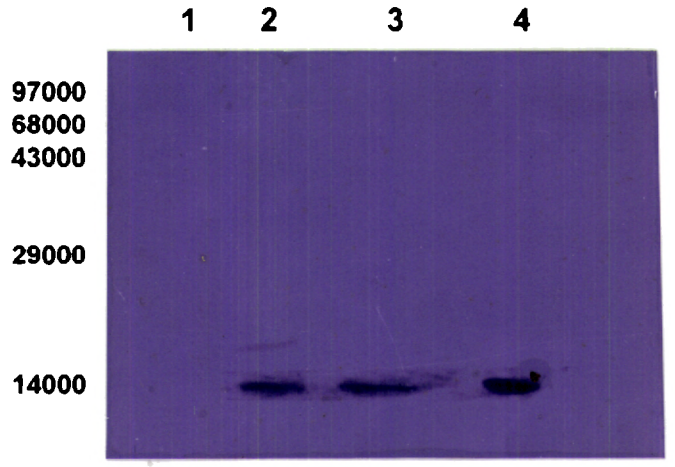


Fig. 5.15. Immunoblot of SDS-PAGE profiles of proteins of sandal spike phytoplasma. Lane **1**: Healthy sandal; **2** and **3**: Partially purified diseased sandal; **4**: Purified sandal spike phytoplasma. Note the single antigenic protein of 14,000 daltons.



Immunological assays are one of 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.

Sinha and Benhamou (1983) reported that phytoplasmas causing Canadian aster yellows and Canadian clover phyllody were serologically indistinguishable and neither was serologically related to Eastern X-disease. Whereas Clark *et al.* (1983) produced Pab against European clover phyllody phytoplasma and reported absence of cross reactivity between this antiserum and an isolate of European aster yellows.

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 & Scott 1985; Barnett *et al.*, 1987) and bacteria (Bouzar & Moore 1987; Azad & Schaad 1988). In the present study, eventhough 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 less compared to other techniques as it could only detect phytoplasma in concentrated and 1:2 dilution samples.

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 is 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 (Figs.5.3, 5.4). 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, nevertheless it could 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.5.5). 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. 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 5.6). 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.

Most of the workers employed indirect ELISA, but 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 up to a dilution of 1:400 only, whereas Clark *et al.* (1989) could detect tomato big bud phytoplasma at dilutions upto 1:600. Saeed *et al.* (1993) detected phytoplasma in faba bean phyllody up to a dilution of 1:300.

Eventhough 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 5.7). The results were identical when large number of samples were screened for the detection of sandal spike phytoplasma (Table 5.8). 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*.

Dot immunobinding assay (DIBA) differs from conventional ELISA in that the solid phase matrix used to adsorb the test material is a nitrocellulose membrane rather than a well in a polystyrene microtitre plate. It is widely used in the immunological characterization of phytopathogenic bacteria and fastidious prokaryotes from cultures or diseased tissues (Lazarovits, 1990). Using DIBA, a large number of samples can be processed within two to three hours rather than a day required for conventional ELISA procedures. Washing the membrane with deionized water rather than using PBS or TBS prior to application of sample was found to be better. Incubation of the membrane in substrate followed by gentle agitation was found to give good results than without agitation. Direct DIBA was found to be less sensitive compared to the indirect DIBA; therefore the latter technique was employed in the detection of phytoplasma in crude samples. There was no visible change in results when the drop size of purified phytoplasma (antigen) was increased in both direct and indirect DIBA, whereas the efficiency of the test was found to increase when the drop size was increased while using samples from crude extract (Fig.5.8). Tissue blotting or tissue printing

technique (Lin *et al.*, 1990), which utilises the sap squeezed out of the leaf to be blotted directly on to the membrane was not possible in sandal, since the plant, being a woody species, it was difficult to squeeze the sap from the leaf or stem tissues.

Immunolight microscopic techniques are widely used in medical microbiology (Bhan, 1995), but the technique was found to be less sensitive to detect phytoplasma in the sandal tissues. Hydrogen peroxide was added to the tissues after fixation to nullify the action of endogenous peroxidases. AEC was used as the dye since it is widely used in immunolight microscopy (Harlow and Lane, 1988; Hartmann, 1997). The test could not pinpoint the presence of phytoplasma; the whole phloem tissues of diseased plants appeared to be red in colour whereas the phloem of healthy sandal tissues did not show any colouration (Fig. 5.9).

Indirect immunofluorescence microscopy was found to be highly sensitive since the fluorescent spots indicating the presence of the pathogen is observed in the phloem tissues of diseased sandal against a dark background, whereas no fluorescent spot was observed in the phloem tissues of healthy sandal (Fig. 5.10). The technique was found to be very sensitive and the experiments could be completed within four hours. Immunofluorescence microscopy is highly popular in diagnosis (Keren and Warren, 1992). Da Rocha *et al.* (1986) and Hiruki (1988) used the technique

to detect phytoplasma in periwinkle, Cousin *et al.* (1989) employed the technique to detect phytoplasma in faba bean phyllody and stolbur disease of tomato; whereas Guo *et al.* (1996) used the technique to confirm the presence of phytoplasma in chokecherry. Shen and Lin (1993) used monoclonal antibody as the probe to detect the pathogenic phytoplasma causing sweet potato witches' broom using the technique.

A classical approach to identify disease caused by phytoplasmas in plants is electron microscopic detection of the phloem tissues, within which the phytoplasmas are easily distinguished. Immunoelectron microscopy is a highly sensitive technique to detect specific pathogens in diseased tissues (Milne *et al.*, 1995). For *in situ* detection, the diseased sandal tissues were embedded in L.R White due to its superior characters over epoxy resins (Hayat, 1989). Protein-A binds to Fc portion of immunoglobulins and the gold (diameter: 10nm) conjugated to protein-A is observed as black spots, distributed on the pathogen. Uranyl acetate treated specimens were found to be superior than those treated with lead citrate. Immunoelectron microscopy has been used to detect grapevine flavescence doree phytoplasma in the salivary glands of the insect *Euscelidius variegatus* (Lherminier *et al.*, 1990). The technique was used by Milne *et al.* (1995) to distinguish serologically differing phytoplasmas.

Ex situ detection of phytoplasma by immunoelectron microscopy was found to be a rapid technique and the experiment could be performed within six hours. Protein-A binds to the phytoplasma specific antibody after incubation and the conjugated gold particles are visualised as black spots distributed on the cell membrane of the pathogen. The phytoplasma found in the infected tissues from both Marayoor and Mysore were of the same antigenicity. No gold particle was visualised as clump in extract of healthy sandal. *Ex situ* detection of phytoplasma in extract of diseased periwinkle using protein-A gold has been reported by Vera and Milne (1994). The authors could detect phytoplasmas of different morphologies in the sample.

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.* (1992b) 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. In immunoblotting studies, protein-A sepharose was used to adsorb any contaminating healthy sandal immunoglobulin which was earlier used during phytoplasma purification, and in the subsequent step the proteins were precipitated with TCA so that maximum resolution is obtained. 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 yellows phytoplasma and European aster yellows phytoplasma had a single major antigen of 22,400 daltons while aster yellows phytoplasma affecting lettuce had an antigenic protein of 18,500 daltons (Jiang *et al.*, 1988). Saeed *et al.* (1992) 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, as available methods of indexing diseased material are usually time consuming and often technically difficult (eg., grafting to indicator hosts, electron microscopy, etc.). Rapid immunological techniques like ELISA, immuno microscopy, etc., circumvent most of these problems. From the present studies it is inferred that polyclonal antibody raised against sandal spike phytoplasma was found to be efficient for all the immunological techniques. The major advantage of immunological techniques is that the same antibody can be used as the primary probe for all the tests. Secondary probes could be employed to enhance the sensitivity of the tests. Another positive factor of the technique is that the pathogen could be detected in the crude sample itself thus avoiding elaborate purification steps. Thus immuno diagnostics could be used as a tool for early detection of the pathogen in plant samples. Garnier (1997) and Chen and Chen (1998) proposed a unique approach to

develop phytoplasma resistant varieties; they were of the opinion that since the growth and metabolism of mollicutes are inhibited by antibodies or antibody fragments, insertion of its genes could be considered as an alternative approach for plant disease control. Indeed, it has been shown recently that transgenic plants expressing a functional single chain Fv antibody are specifically protected from virus attack (Tavladoraki *et al.*; 1993). Transgenic tobacco plants expressing an antibody against the stolbur phytoplasmas have also been developed (Garnier, 1997). These preliminary studies prove that immunological techniques have immense potential for future studies not only in diagnostics but also to develop transgenic plants, which resist pathogens.

6. DETECTION OF SANDAL SPIKE PHYTOPLASMA USING MOLECULAR TECHNIQUES

6. DETECTION OF SANDAL SPIKE PHYTOPLASMA USING MOLECULAR TECHNIQUES

Spike disease-affected-sandal and the host plants, *Lantana* and witches' broom affected *Zizyphus* were screened for the presence of sandal spike phytoplasma using polymerase chain reaction (PCR) technique. Oligonucleotide primers specific to the conserved region of 16S rRNA gene were used to amplify a 558 bp sequence of the phytoplasma. The technique could detect the presence of phytoplasma only in diseased sandal but not in the host plants or healthy sandal. When the PCR products after 20 cycles of amplification were subjected to restriction fragment length polymorphism analysis (RFLP) with *Alu* I restriction endonuclease, four DNA fragments were obtained. The technique confirmed that sandal spike phytoplasma belongs to group I (Aster yellows group).

6.1. INTRODUCTION

The inability to culture phytoplasmas *in vitro*, and their variable titre levels and uneven distribution in woody plants make their detection difficult. Recent advances in DNA technology have provided new and sensitive detection methods of the pathogen. DNA-DNA hybridization using phytoplasma specific probes increased the sensitivity of detection (Deng and Hiruki, 1990; Davis

et al., 1993); but this approach has now been surpassed by methods based on the polymerase chain reaction (PCR) (Andersen *et al.*, 1998). The development of PCR has enabled rapid and efficient analysis of specific DNA sequences present in low numbers in complex, heterogeneous mixtures of DNA (Mullis and Faloona, 1987; Coen, 1994; Hadidi *et al.*, 1995).

Deng and Hiruki (1991) first reported the use of PCR for detection of phytoplasma. The PCR technology has provided a sensitive means for detection of a broad array of phytoplasmas from infected plants using phytoplasma group-specific or universal primers derived from conserved 16S rRNA gene sequences (Ahrens *et al.*, 1993; Davies *et al.*, 1995). Phylogenetic analyses based on 16S rRNA and ribosomal protein gene sequences have revealed that the uncultured phytoplasmas formed a large discrete monophyletic clade within the class *Mollicutes* (Kuske and Kirkpatrick, 1992; Lim and Sears, 1992; Gundersen *et al.*, 1994; Sears and Kirkpatrick, 1994). The phylogenetic inter-relationships among representative phytoplasmas provided a basis for establishing a phylogenetically valid classification (Namba *et al.*, 1993; Gundersen *et al.*, 1994). By direct sequence analysis or RFLP analysis of PCR-amplified 16S rDNA, the phytoplasmas can be differentiated and classified (Schneider *et al.*, 1993). Seemuller *et al.* (1998) have classified phytoplasmas into twenty major taxonomic groups based on RFLP analyses of PCR-amplified 16S rDNA. This chapter reports the detection of phytoplasma from spike disease

affected sandal by polymerase chain reaction using primers specific to 16S rRNA gene, followed by identification of the pathogen by RFLP analyses.

6.2. MATERIALS AND METHODS

6.2.1. Extraction of nucleic acid from plants

Healthy and spike disease affected sandal and the host plants, *Lantana camara* and witches' broom affected *Zizyphus oenoplea* were collected from Marayoor, Munnar Forest Division, Kerala and Chamundi Hills, Mysore, Karnataka. Total DNA samples from healthy and diseased plants were extracted following the modified protocol of Doyle and Doyle (1990). One gram tissue of midrib and young stem was ground into fine powder using liquid nitrogen. Two ml of hot (65^0C) cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl) was added to the powder and samples incubated at 65^0C for one hour. The samples were extracted with chloroform/isoamyl alcohol (24:1) followed by low speed centrifugation (1600g) for 5 minutes and the aqueous phase eluted out. This step was repeated again; to the aqueous layer was added 1/10 volume 3 M sodium acetate (pH 5.2) followed by the addition of 2 volume cold (-20^0C) absolute alcohol. After incubation at -20^0C for 12 hours, the DNA precipitate was centrifuged for 10 minutes at low speed (1600g). Ethanol (95%) was added to the DNA pellet and recentrifuged at the same speed. The supernatant was discarded, air-dried and the pellet dissolved in

100 µl sterile distilled water. Total DNA was estimated at A_{260} (1 O.D =50 µg ml $^{-1}$) (Gallagher, 1996).

6.2.2. Polymerase chain reaction

The method followed by Ahrens and Seemuller (1992) that allowed the amplification of a 558 bp fragment of the 16S rRNA gene of phytoplasma was adopted for PCR with slight modification. The primers were designed from the conserved regions of the 16S rRNA gene of *Oenothera*-MLO (O-MLO) (Lim and Sears, 1989) located between 759 and 1,316 bp. The sequence of the forward primer was 5'-ACGAAAGCGTGGGGAGCAAA-3' and the reverse primer was 5'-GAAGTCGAGTTGCAGACTTC-3'. A total volume mixture of 50 µl contained 1 µl of test DNA (200ng), 1 µl of each primer (250ng) (Bangalore Genei, India), 2.5 mM each of four dNTPs, 1 µl (3 units) of *Taq* polymerase (B. Genei, India) or Dynazyme II (Finnzymes Oy, Finland) and 5 µl *Taq* buffer or Dynazyme buffer. The mixture was covered with two drops of mineral oil and subjected to 20 amplification cycles (PTC-150 Minicycler, MJ Research, USA), each of 30s denaturation (95^0 C), 30s annealing (55^0 C) and 30s extension (72^0 C). The final extension step was for 5 minutes.

6.2.3. Analysis of PCR amplification products

The PCR amplification products (10 µl) obtained from DNA of healthy and diseased sandal, the host plants - *Lantana* and *Zizyphus* and water control

after 20 cycles were electrophoresed in 1.5% horizontal agarose (Sigma, USA) gel in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide and bands compared with a 100 bp DNA ladder (B. Genei, India). The experiment was repeated six times. 15 µl of the reaction mixture obtained using *Taq* polymerase was digested with 1 µl of undiluted *Alu* I (B. Genei, India) following manufacturers instruction at 37^0 C for 12 hours. 10 µl of the digest was used to resolve the restriction fragments on a 3% horizontal agarose gel in TBE buffer (45 mM Tris-Borate, 1 mM EDTA, pH 8.0), and the bands were visualised after staining with ethidium bromide. The gels were documented using Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak, USA).

6.3. RESULTS

6.3.1. PCR amplification products

After 20 amplification cycles, DNA fragment of 558 bp was amplified from samples which contained DNA extracted from diseased sandal but not from healthy samples or water control. Bands were visualised for both *Taq* polymerase and Dynazyme II polymerase (Fig. 6.1). On comparing the DNA from spike diseased tissues from Kerala and Karnataka, the bands appeared to be identical (Fig. 6.2).

Restriction analysis of the amplified fragment with *Alu* I revealed the presence of a pattern characterised by restriction sites at position a, b, and c with fragments of 240, 191, 71 and 56 bp respectively (Fig. 6.3a,b - the 71 and 56 bp fragments are seen clumped together in the figure), as described by Ahrens and Seemuller (1992) for the first group of phytoplasmas. The size of bands were calculated with respect to the size of DNA marker. No DNA was amplified from tissues of the host plants of spike disease affected sandal, viz., *Lantana* and witches' broom affected *Zizyphus* indicating the absence of phytoplasma in these plants (Fig. 6.4).

6.4. DISCUSSION

Techniques based on symptomatology, host and vector range, and electron and fluorescence microscopy provide useful basic information on pathogens but offer little help in characterising organisms and are inadequate for diagnostic purposes (Firrao *et al.*, 1993). The PCR method of phytoplasma detection gained popularity after the classical work of Ahrens and Seemuller (1992). They transmitted phytoplasmas infecting several plants of different continents to *Catharanthus roseus* and maintained for several years. These phytoplasmas were detected and characterised using the primers designed from the conserved regions of the 16S rRNA gene of the *Oenothera* phytoplasma (O-MLO).

Fig. 6.1. Agarose gel electrophoresis of polymerase chain reaction product of sandal spike phytoplasma after 20 cycles of amplification using both *Taq* polymerase (lane **2-4**) and Dynazyme II (lane **5-7**). Lane **1**: 100 bp ladder, **2** and **5** healthy sandal, **3**,**4**,**6** and **7** diseased sandal, **8**: water control.

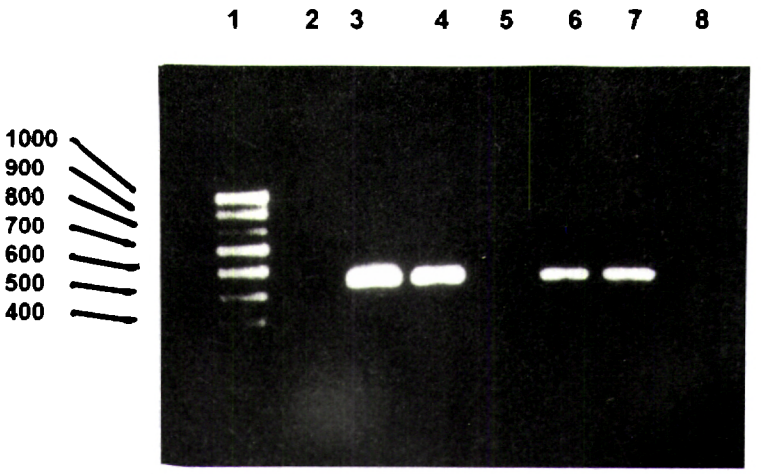


Figure 6.2. Agarose gel electrophoresis of polymerase chain reaction product of sandal spike phytoplasma after 20 cycles of amplification using spike infected sandal tissues of Kerala (lane **3-5**) and Karnataka (lane **6-8**). Lane **1**: 100 bp ladder, **2** healthy sandal.

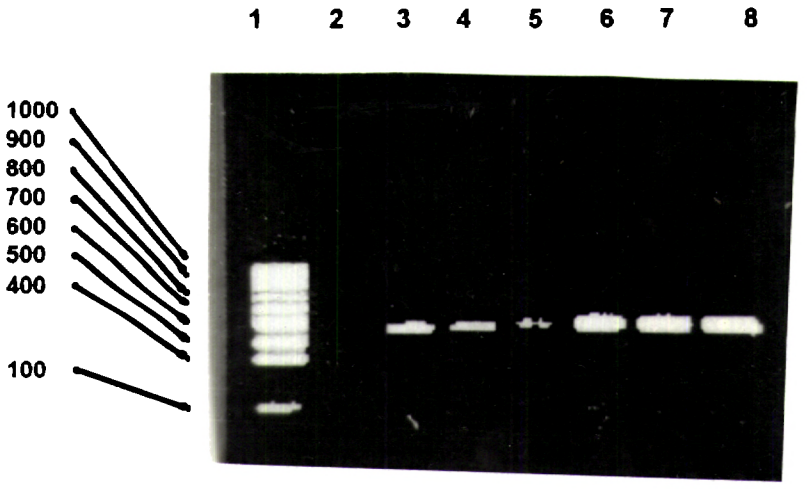


Figure 6.3.a. Agarose gel electrophoresis of *Alu* I digests of PCR products of sandal spike phytoplasma. Lane **1**: 100bp ladder, **2** and **3**: sandal spike phytoplasma of Kerala and **4** and **5**: sandal spike phytoplasma of Karnataka.

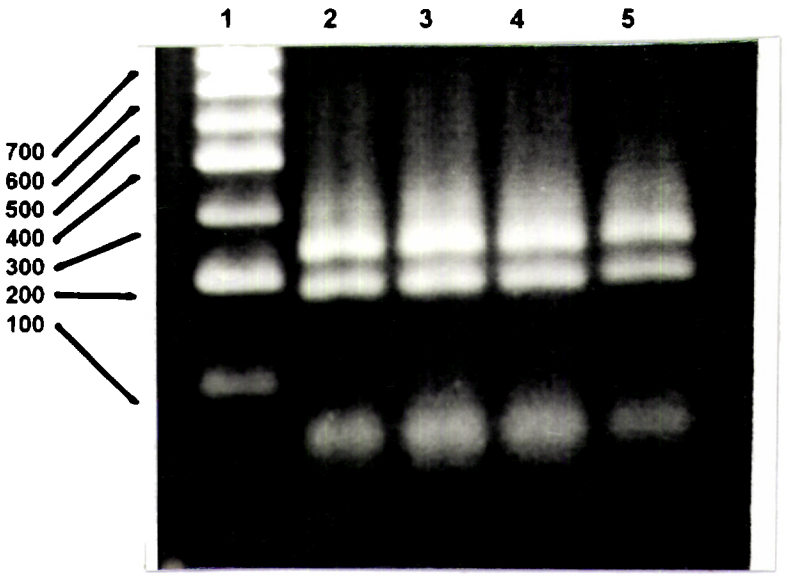
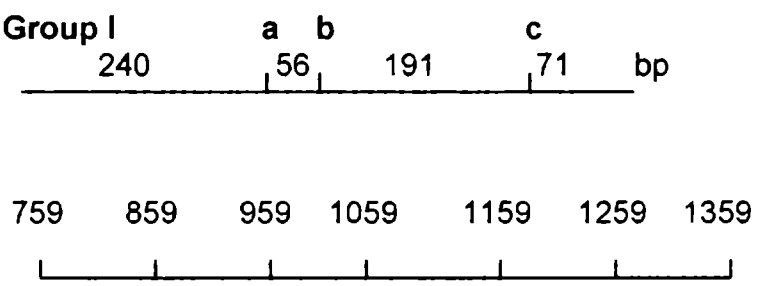
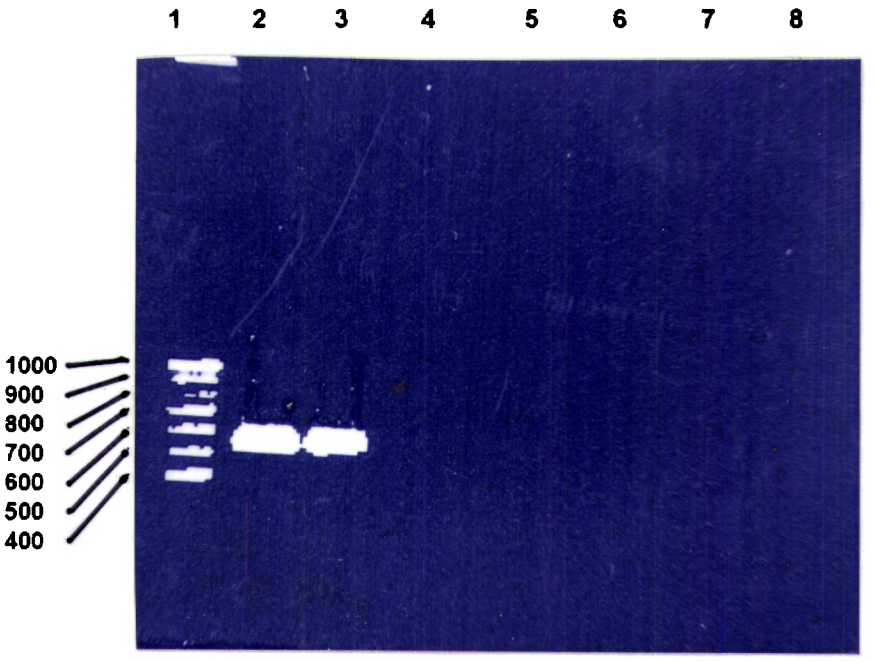


Figure 6.3.b. *Alu* I restriction maps of the 558 bp 16S rDNA fragments of group I phytoplasma (Ahrens and Seemuller, 1992).



Positions on the 16S rDNA of the O-MLO

Fig.6.4. Agarose gel electrophoresis of polymerase chain reaction product of host plants of spike disease affected sandal after 20 cycles of amplification using *Taq* polymerase (Lane **1**: 100 bp ladder, **2** and **3**: diseased sandal, **4** and **5**: *Lantana*, **6** and **7**: witches' broom affected *Zizyphus*, **8**: water control).



Eventhough there are reports claiming detection of sandal spike phytoplasma, maintained in *Catharanthus roseus* for several years, using PCR and RFLP (Ahrens and Seemuller, 1992; Schneider *et al.*, 1993) none of the workers has attempted detecting the pathogen from spike diseased sandal tissues. According to Woese *et al.* (1980) the mollicutes have a high mutation rate resulting in many totally unique oligonucleotides. Hence, the present study, conducted to detect the presence of the pathogen in fresh tissues of spike diseased sandal has more relevance. From the study, it was inferred that the pathogens did not undergo any mutation in spite of repeated maintenance in periwinkle as reported by Ahrens and Seemuller (1992).

Young stem and leaf midrib tissues from diseased and healthy sandal and its hosts were used for isolating DNA, since the greatest yields of DNA was obtained using the youngest, freshest tissue (Reichardt and Rogers, 1996). For extraction of nucleic acid from plant tissues, liquid nitrogen was used because this method was found to produce DNA yields of higher average molecular weight when compared to direct grinding of fresh tissue in buffer (Doyle and Doyle, 1990). The liquid nitrogen powdered tissues were incubated at 65^0 C for one hour rather than 30 minutes for larger DNA yields (Moore, 1996). 3 M sodium acetate was used to induce DNA precipitation (Reichardt and Rogers, 1996). The yield of DNA pellet improved after incubating the tissues overnight at -20^0 C. The DNA pellet was dissolved in sterile distilled water as suggested by Ruiz *et al.* (1997) and not in Tris-EDTA buffer since the same can interfere in PCR amplification, as EDTA was found to effectively reduce the magnesium concentration.

Both the polymerases, *Taq* polymerase and a DNA polymerase from *Thermus brockianus* (Dynazyme II) amplified the phytoplasma DNA (Fig. 6.1). In the subsequent studies, only *Taq* polymerase was used. A 558 bp fragment of DNA was amplified from both the Kerala and Karnataka sandal spike population (Fig. 6.2). The last extension step was prolonged to 5 minutes as suggested by Kramer and Coen (1996) to ensure that all the PCR products are of full length.

The restriction enzyme *Alu* I has the recognition sequence 5'-AG/ CT-3'. The incubation of restriction enzyme with the PCR product overnight at 37^0 C gave better results rather than incubating for 2 hours. As the PCR product in the study was 558 bp, TAE buffer and 1.5% agarose gel were used since the gel had an effective resolution in the range 3-0.2 kb (Voytas, 1996). For visualising the RFLP fragments, TBE buffer and 3% agarose gel were employed to visualise smaller DNA fragments less than 0.2 kb, while TBE buffer provided good resolution than TAE buffer (Martin, 1996).

The RFLP analysis with *Alu* I restriction endonuclease revealed the presence of four DNA fragments of 240, 191, 71 and 56 bp, and the results confirmed that sandal spike phytoplasma could be attributed to the group I (Aster yellows group), sub group B (Ahrens and Seemuller, 1992; Schneider *et al.*, 1993; Seemuller *et al.*, 1998). Group I differs from group II only by a shorter fragment at 3' end (Fig. 6.3b). Group III is missing the site "a", while group IV is missing restriction site "b", which results in two fragments of about the same size (240 bp) (Ahrens and Seemuller, 1992). Different workers have identified phytoplasmas of the first group associated with declining apricot (Marcone *et al.*, 1995a), phytoplasma diseases in lettuce (Marcone *et al.*, 1995b), pear decline (Pollini *et al.*, 1994), dieback in papaya (Gibb *et al.*, 1996), periwinkle (Marcone and Ragozzino, 1995c) and corn poppy (Marcone *et al.*, 1996e). The Aster yellows group (Group I) is the largest group among the phytoplasma groups (Seemuller *et al.*, 1998).

Biological properties such as symptomatology, range of susceptible plant hosts and relationships to insect vectors had been the major criteria for diagnosing the phytoplasmal diseases and the associated phytoplasmal strains before molecular based methods became available (McCoy *et al.*, 1989; Lee *et al.*, 1998a). In practice, they remained important tools for preliminary identification of putative phytoplasmal diseases. The identities of the putative causal agents can now be accurately identified and defined on the basis of phylogenetic criteria using PCR and RFLP techniques. Lee *et al.* (1998a) was of the opinion that a given type of phytoplasma can potentially inflict various diseases in different plant hosts. Griffiths *et al.* (1999) using the non-ribosomal primer pair FD9f/r followed by RFLP analyses found that the elm yellows phytoplasmas inhabiting elm trees in North America are distinct from those inhabiting elm trees in Italy, whereas Nakashima *et al.* (1999b) could detect phylogenetically closely related phytoplasma (group II) affecting phyllody disease in sesame (sesamum) and *Richardia* growing in the same field. The present study using molecular techniques could identify phytoplasma of the same group affecting both Marayoor and Mysore sandal populations, but could not identify the pathogens in the host plants of spike disease affected sandal confirming that sandal spike phytoplasma is confined only to sandal.

7. COMMERCIAL EXPLOITATION OF TECHNIQUES DEVELOPED TO DETECT SANDAL SPIKE PHYTOPLASMA

7. COMMERCIAL EXPLOITATION OF TECHNIQUES DEVELOPED TO DETECT SANDAL SPIKE PHYTOPLASMA

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

7.1. COMMERCIAL EXPLOITATION OF PLANT DIAGNOSTICS

Both wild and cultivated plant species are subjected to diseases to such an extent that about 80,000 diseases of plants have been recorded throughout the world. Microorganisms causing plant diseases can be classified into six major groups viruses, viroids, fastidious prokaryotes (which include phytoplasmas and spiroplasmas), bacteria, fungi and protozoa (Agrios, 1997). The control of plant diseases depends on accurate and rapid detection and identification of the pathogens. In this context, detection is the process of testing for the presence of pathogens, while identification is defined as specific grouping of the causal agent (Chu *et al.*, 1989).

Traditionally, diagnosis of plant diseases has been based on recognising characteristic symptoms expressed by diseased plants and

looking for the presence of pathogens on the surface of diseased material (McIntyr and Sands, 1977). This together with other observations and evaluation of the environmental conditions, generally allow the causative agent to be identified. While considering pathogen detection, problems can arise due to the multiplication of pathogens in internal tissues, ability of the pathogens to survive as saprophytes, obscure and seasonal expression of disease symptoms etc.

In such cases, special methods are required to isolate or detect pathogens amidst the cell components and other saprophytes. This usually involves performing a series of diagnostic tests. 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). Diagnostics is considered as one of the three major applications in agricultural biotechnology, the other two being biopesticides and transgenic plants (Mannion, 1998).

7.1.1. Diagnostics

Diagnostics can be viewed as a discipline in its own right, combining a wide range of techniques in developing simple, fast and reproducible procedures that measure a feature of the biological material in hand in a way that is easily interpretable. Improved diagnostics are useful in epidemiological

studies to determine the distribution and abundance of pests and pathogens (Skerritt and Appels, 1995).

Biotechnology offers forestry the possibilities of an array of new procedures for overcoming major constraints in woody plant improvement, protection and utilisation (Krugman, 1990). It also provides an array of new methods for the early detection and identification of pathogens of woody plants. 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).

Nucleic acid probes have been developed for detection of many plant pathogens. The availability of nucleotide sequences has made possible the development of PCR assays for the detection and diagnosis of several viroids, viruses and other pathogens. Because of its great sensitivity, the PCR provides a good alternative to other diagnostic methods and can speed up diagnosis, reduce the sample size required, and often eliminate the need for radioactive probes (Hadidi *et al.*, 1995).

7.1.2. Comparison of nucleic acid probes and antibody assays

In some cases, a decision will have to be made as to whether it is more appropriate to pursue an antibody or nucleic acid probe in the development

of a diagnostic test. Clearly, small molecules such as lipids, mycotoxins or agrochemicals are amenable only to antibody based detection, but for macromolecules and plant breeding applications, the choice is not so clear cut. Nucleic acid probes are generally better for genotype characterization, while antibodies could provide a better test for phenotype - especially if the target is a product of a gene whose expression is influenced by environment (Skerritt and Appels, 1995).

7.2. 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, direct and indirect ELISA, direct and indirect DIBA and various immuno microscopic techniques 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. Eventhough, the number of steps were higher in indirect ELISA, the sensitivity of the test was high when biotin-streptavidin system was used.

The indirect DIBA could be completed within three hours. The *ex situ* detection of phytoplasma by immunoelectron microscopy was very rapid since the whole test could be completed within four hours.

For molecular studies the DNA could be isolated using a modified CTAB method which used minimum amount of chemicals and was found to be very rapid. The specific primers could easily detect the presence of phytoplasma using polymerase chain reaction within four hours. The identity of the organism could be proved by restriction fragment length polymorphism analysis. The reagents used in these techniques could be marketed as kits as shown in figure 7 1.

Thus three major kits could be developed based on the present studies viz., sandal spike phytoplasma purification kit, immuno detection kit and molecular detection kit. The reagents that can be supplied are listed in table 7 1.

For purification of sandal spike phytoplasma the major instruments needed includes filtration apparatus and ultracentrifuge. An ELISA reader is the only instrument needed for detecting the pathogen when the ELISA technique is employed, whereas, a thermal cycler and horizontal electrophoresis system are the instruments needed for molecular detection of the pathogen. Fluorescence microscope and electron microscope are essential for visual confirmation of the presence of the specific pathogen when the immunomicroscopy technique is employed.

Table 7.1. 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** **3. DIBA kit** 4. **Immuno microscopic kit**	Phytoplasma specific antibody, Phytoplasma specific antibody-HRP, Anti-rabbit antibody-HRP, Anti-rabbit antibody biotin, Streptavidin-HRP, Anti-rabbit FITC, Protein-A gold, Agarose, Phosphate buffered saline, Positive control (phytoplasma).
MOLECULAR DETECTION KIT	16S rDNA primer, Alu I, Taq polymerase, Taq buffer, dNTP solution, Positive control DNA.

Eventhough, India has a monopoly in the production and export of sandal and its products, the marketing of the species is primarily 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 can be used in tree improvement projects of sandal, particularly for screening disease resistant trees. Many states are planning to remove restrictions on possession and trade of sandal. In such a scenario, most of the farmers will be very enthusiastic to cultivate the species due to the high price fetched by sandalwood. In that situation, the kits can be used for ensuring disease-free planting stock for introduction to non-sandal area. Any farmer can easily detect the pathogen using double diffusion test or dot immunobinding assay; so the reagents for these techniques can become popular among the farming community. All the other techniques require sophisticated instruments and technicians and may not be popular since only a few laboratories have the necessary infrastructure. Nevertheless, the reagents will be of much use to quarantine laboratories for screening sandal, especially seedlings, for the presence of pathogen using ELISA and molecular techniques.

Most of the companies supply antibodies either as freeze dried powder or with preservatives which has a shelf life of about 12 months at 4^0C 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$600. These kits contain reagents for ELISA only. For molecular detection, the company charges around US$ 120 per sample (sample size: 30 stems/100g grain). The reagents of sandal spike phytoplasma detection kit (both immunological and molecular kits), when produced in large scale, could be supplied at a lower price due to lower labour and other input costs in India.

Fig.7.1. Kits developed for purifying and detecting sandal spike phytoplasma.

8. SUMMARY

8. SUMMARY

Sandal (*Santalum album* L.), the root hemi-parasitic tree is famous for its highly valuable heartwood and oil. India has a monopoly in the production and export of sandalwood and its products. Spike disease is the most serious disease of the species. Production of sandalwood has plummeted from around 3000 tonnes per annum during 1985 to around 1000 tonnes in 1997; similarly oil production also declined from 140 tonnes in 1985 to 40 tonnes in 1997. The disease is characterised by extreme reduction in size of leaves and internode and in advanced stage the whole shoot looks like a spike inflorescence. Spiked trees die within one to two years after the appearance of disease symptoms. Sandal spike disease though noticed for the first time in 1899, it was in 1969 that the causative organism of the disease was detected by transmission electron microscopy (TEM). The disease is caused by a phytoplasma, seen exclusively in the phloem tissues. Attempts to isolate and culture the pathogen *in vitro* has been futile, thereby limiting the study of the pathogen. Except TEM the techniques adopted so far to detect the pathogen in plants are indirect and insensitive methods which could not distinguish the specific pathogen. The present study was undertaken to develop immunological and molecular techniques for the detection of sandal spike phytoplasma.

8.1. *IN SITU* DETECTION OF SANDAL SPIKE PHYTOPLASMA

Initially, the healthy and diseased sandal tissues were screened for the presence of phytoplasma *in situ* using fluorescence microscopy employing the fluorochrome, 4',6-diamidino-2-phenyl indole (DAPI) which binds specifically to the A-T rich sequence of the DNA of phytoplasma. The fluorescent spots were detected exclusively in the phloem of diseased tissues and not in healthy sandal tissues. The efficiency of the stain was compared with that of Dienes' stain; DAPI staining was found to be superior since the fluorescent spots were visualised against a dark background. No fluorescent spots were visualised 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.

8.2. PURIFICATION OF SANDAL SPIKE PHYTOPLASMA

Phytoplasma from diseased sandal tissues was purified using a differential filtration technique. The technique took advantage of the property of phytoplasma to pass through 0.45 μm pore size membrane filters. The purity of phytoplasma pellet obtained after ultracentrifugation at 65,000g was confirmed using transmission and scanning electron microscopy.

8.3. AMINO ACID AND TOTAL PROTEIN ESTIMATION

The total amino acid estimation was performed using HPLC. The microorganism had lower amount of aromatic and sulphur containing amino acids compared to the other classes of amino acids. The total protein content was calculated to be 256.54 μg ml^{-1} using HPLC. Protein estimation based on conventional methods like Lowry method or spectrophotometric assays at A_{205} and A_{280} was comparatively insensitive for total protein quantitation of the pathogen; however the silver binding method of protein estimation was highly sensitive probably due to the property of silver to bind to sulphydryl and carboxyl moieties of proteins.

8.4. IMMUNOLOGICAL TECHNIQUES TO DETECT THE PATHOGEN

The purified phytoplasma (antigen) was injected to rabbits to raise polyclonal antibody. Precipitin bands were visualised against the extract of diseased sandal only and not against healthy sandal when the antisera containing polyclonal antibody was subjected to Ouchterlony double diffusion test. No precipitin band was seen against the extract of host plants of spike disease affected sandal indicating absence of sandal spike phytoplasma in host plant tissues.

Phytoplasma was detected in diseased sandal using direct and indirect enzyme linked immunosorbent assay (ELISA) techniques. Indirect ELISAs utilised both HRP conjugated anti-rabbit antibodies as well as avidin and streptavidin amplification systems. Indirect ELISA was found to be more sensitive for detecting the pathogen compared to direct ELISA. For indirect ELISA, one hour each was found to be optimum for both coating antigen and incubation of antibody. Antibodies at a dilution of 1:2000 could be used as probe in indirect ELISA and could detect a minimum of 25ng of purified phytoplasma protein. The test could detect phytoplasma from extract of diseased sandal upto a dilution of 1:10,000. The techniques could not detect phytoplasma in the host plants of spike disease affected sandal.

Dot immunobinding assay (DIBA), a rapid technique employing nitrocellulose membrane rather than a polystyrene strip as in ELISA for detecting fastidious prokaryotes was standardised for detection of the pathogen. Using this technique it was possible to detect the pathogen within three hours after coating the diseased plant extract.

The polyclonal antibody was used to detect phytoplasma *in situ* using the immuno microscopic techniques - immunolight microscopy, immunofluorescence microscopy and immunoelectron microscopy. Fluorescent spots were visualised

in the phloem of diseased sandal but not in healthy sandal when the tissues were subjected to indirect immunofluorescence microscopy. Immunoelectron microscopy employing protein-A gold was the most sensitive of the test since individual phytoplasma cells were found to be densely covered with gold particles. An *ex situ* detection method of immunoelectron microscopy was also standardised and was found to be very rapid compared to the *in situ* detection method.

To determine the antigenic protein size of sandal spike phytoplasma using immunoblotting (Western blotting), the protein profiles of healthy sandal and spike disease affected sandal after SDS-polyacrylamide gel electrophoresis were blotted onto nitrocellulose membrane and probed using the phytoplasma specific antibody. The sandal spike phytoplasma had an antigenic protein of 14,000 daltons.

8.5. MOLECULAR TECHNIQUES TO DETECT THE PATHOGEN

The DNA of healthy and spike disease affected sandal was purified using a modified CTAB method. Oligonucleotide primers specific to the conserved region of 16S rRNA gene was used to amplify a 558 bp sequence of the phytoplasma. The amplified products obtained after 20 cycles of polymerase

chain reaction (PCR) were visualised only in the tissues of spike disease affected sandal but not in healthy sandal or the host plants of spike disease affected sandal after electrophoresis. The amplified DNA product when subjected to restriction enzyme digestion using *Alu* I restriction endonuclease revealed four fragments; restriction fragment length polymorphism (RFLP) banding pattern was similar to group I (Aster yellows group). Sandal spike phytoplasma present in both the Marayoor and Mysore sandal forests were found to be of the same group.

The techniques confirmed that sandal spike phytoplasma is specific to sandal and is not transmitted through the host plants.

8.6. COMMERCIAL EXPLOITATION OF TECHNIQUES DEVELOPED TO DETECT SANDAL SPIKE PHYTOPLASMA

The reagents developed for detecting sandal spike phytoplasma could be commercially marketed as kits, which may be beneficial to the sandalwood industry. The kits, viz., sandal spike phytoplasma purification kit, immuno detection kit and molecular detection kit could be used for purifying sandal spike phytoplasma and detection of the pathogen.

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