STUDIES ON THE SPIKE DISEASE OF SANDAL

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CONTENTS

ABSTRACT

Sandal spike disease was first observed in the Reserve 51 of Marayoor forest range of Munnar forest division during June 1980. More than 50 per cent of the sandal trees in this reserve had been found to be affected with the disease. Rate of disease spread in two disease monitoring blocks, calculated in terms of apparent infection rate, was 0.080 and 0.066 per unit per month. *Zizyphus oenoplia, Stachytarpheta* sp. and *Jasminum rigidum* showing witches'-broom symptoms found in several places in the reserve were suspected to be the collateral hosts of the pathogen.

Histopathological studies, using aniline blue and Hoechst 33258 gave evidence on the occurrence of MLO in the diseased phloem tissues. The technique could be used as an easy method of disease detection. Electrophysiological studies using Shigometer indicated a positive correlation between severity of the disease and electrical resistance of inner bark in diseased trees. Though TEM studies showed large number of MLO in the phloem tissues, attempts to culture the organism *in vitro* were not successful. *Redarator bimaculatus* was identified as an insect vector of spike disease through disease transmission studies and by the presence of MLO in the tissues of intestine and salivary glands of the insects, fed on spiked sandal, and in the phloem tissues of plants infected through the vector.

Infusion of aqueous solution of 500 mg of tetracycline antibiotics, dissolved in 500 ml of water, in spiked trees gave remission of disease symptoms lasting for three to five months. Repeated infusion of tetracycline-HCl in alternate months for an year did not give complete recovery. Higher doses of tetracyclines i.e., 2 to 8 g/tree prolonged the remission period upto seven to eight months. Infusion of tetracycline at 12 g/tree was found phytotoxic. Combinations of different tetracyclines did not give any additional improvement of antibiotic action. Digitonin and 2-1, guanidinododecane acetate also gave temporary remission of disease symptoms. But cephalaxin did not give any disease remission.

INTRODUCTION

Sandal *(Santalum album L.)* is a semi parasitic, perennial tree belonging to the family Santalaceae. It possesses a highly valuable wood, known for its scented oil and carving. The oil found in the root and heartwood, is acknowledged as one of the most precious perfumery items from antiquity down to the modern times.

Sandal tree grows at altitudes from sea level to about 1200 m above mean sea level (msl). 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. The tree generally flowers twice a year, once in July-August and again in November-December. The tree, being a semi-root parasite, is known to have a wide range of host plants (Bhatnagar, 1965). Though it was controversial for some time, recently the necessity of a host for sustained healthy growth has been stressed by some authors (Ananthapadmanabha *et al.,* 1984; Sahai and Shivanna, 1984).

In India, Sandal is found mainly in the Deccan Plateau and its extension and in small numbers in almost all parts 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 (Fig. 1 & 2) (Varghese, 1971; Mathew, 1981). Limited distribution of sandal is also seen in the reserves of Arienkavu and Kasargode forest ranges (Chand Basha, 1977).

The sandal wood forests of Marayoor forest range can be classified under Champion's 5A/C-3 southern tropical dry mixed deciduous forests. These tracts, varying in elevation from 850—1050 m above msl, are situated on gently undulating hills and valleys. The soil is shallow and loamy with abundant rocky outcrops. The average annual rainfall in the area varies from 1000 to 1500 mm. The winter nights are cold (10°C) and summer days are hot (36°C). In Marayoor, sandal grows in forests which support dry deciduous vegetation as most of the species in the sandal reserves are xerophytic in nature. Among these, species of *Acacia, Terminalia, Ficus, Pterocarpus, Cassia* and *Emblica* are the predominant ones. *Dendrocalamus strictus* is also found in abundance. Most of the area of sandal reserves is filled by thickets of *Lantana* sp.
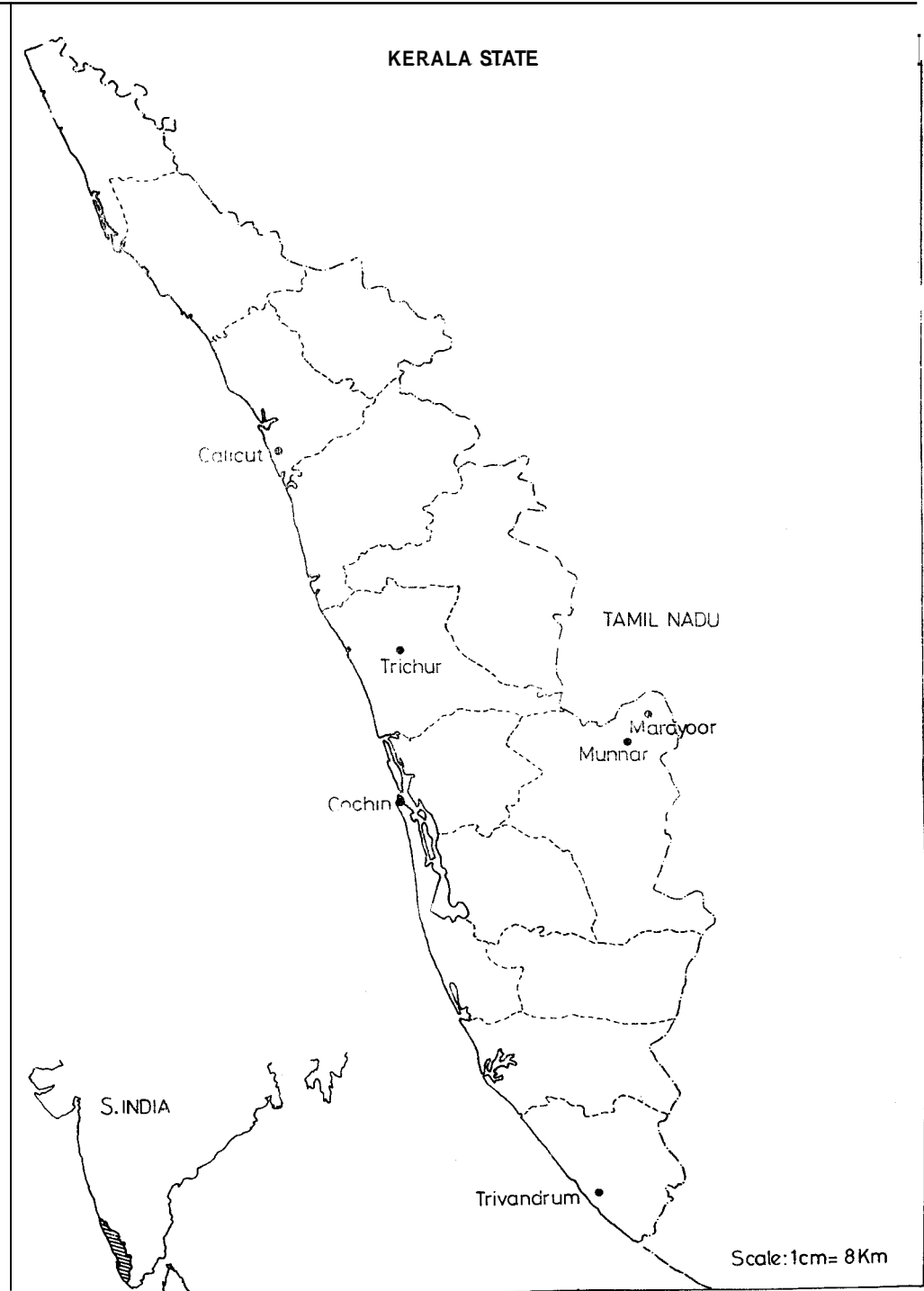
KERALA STATE

TAMIL NADU

Calicut

Trichur

Marayoor

Munnar

Cochin

S. INDIA

Trivandrum

Scale: 1cm = 8 Km

Fig. 1. Map of Kerala State showing location of Marayoor
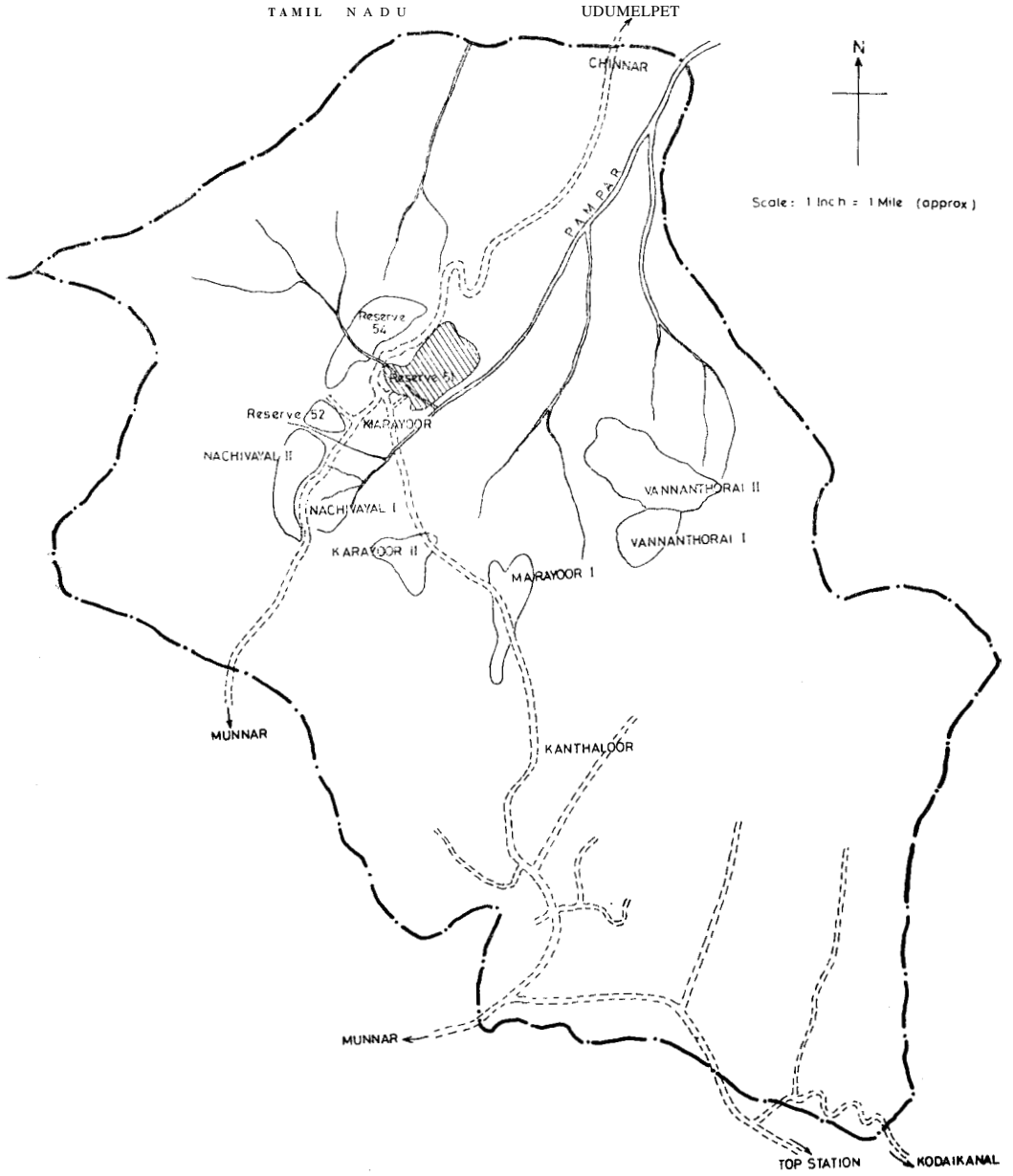
MARAYOOR RANGE

Fig. 2.   Sandal reserve forests of Marayoor range

Even though sandal is affected by only a few diseases, spike disease is the most important and destructive one and has attracted world-wide attention due to its unkown etiology. The disease is characterised by extreme reduction in size of leaves and internodes, accompanied by stiffening of leaves. In advanced stages, the whole shoot looks like a "spike" inflorescence. Spiked plants do not bear any flowers or fruits; occasionally only phylloid or abortive flowers are developed. Spiked trees usually die within 1 to 2 years (Fig. 3 & 4).

Spike disease of sandal is considered to be the most serious yellows-type disease of forest trees known in the world today (Seliskar and Wilson, 1981). The disease was earlier known to be graft-transmissible (Coleman, 1917), caused by an insect-borne virus (Anon., 1955). Recently. after the discovery of association of Mycoplasma-like organisms (MLO) with some yellows-type of diseases (Doi *et al.,* 1967; Ishiie *et al.,* 1967). mycoplasmal etiology of spike disease was confirmed by Dijkstra and Ie (1969), Hull *et al.* (1969) and Varma *et al.* (1969) through electron microscopic studies. Further confirmation of MLO as the pathogen was obtained from its positive response to tetracycline therapy (Raychaudhuri *et al.,* 1972; Rao *et al.* 1975).

Though the causative organism was discovered in 1969 and temporary disease remission obtained through tetracycline infusion in 1972, isolation, cultivation and characterisation of the pathogen and vector identification have not been achieved yet. Further, no report has come out on the field trial of the antibiotics for the control of the spike disease. The present status of the disease has been summarised recently by Parthasarathy and Venkatesan (1982).

Yellows-type of diseases are now well known group of plant diseases that infect over 300 genera of plants including trees and herbs. Most of the yellows diseases are associated with MLO or with spiroplasmas (Whitecomb and Black, 1982) and a few are associated with bacteria or rickettsia-like organisms (RLO) Except a few RLO, all the organisms are found only in the sieve cells (Markham, 1982).

Mycoplasma is the 'trivial' name given to all members of Class Mollicutes. It is distinguished from bacteria by the lack of peptidoglycan layer of bacterial cell wall and consequent resistance to penicillin and other antibiotics which inhibit bacterial cell wall synthesis. Because of absence of cell wall, the mycoplasmas are pleomorphic and can pass through pores as small as 220 nm even though the diameter of a viable organism is greater than 300 nm (Archer and Daniels, 1982).

Mycoplasma-like organisms (MLO) refer to organisms seen in diseased tissues but not characterised through isolation and *in vitro* cultivation. Among the plant pathogenic mycoplasmas, only members of the genus *Spiroplasma* have been isolated
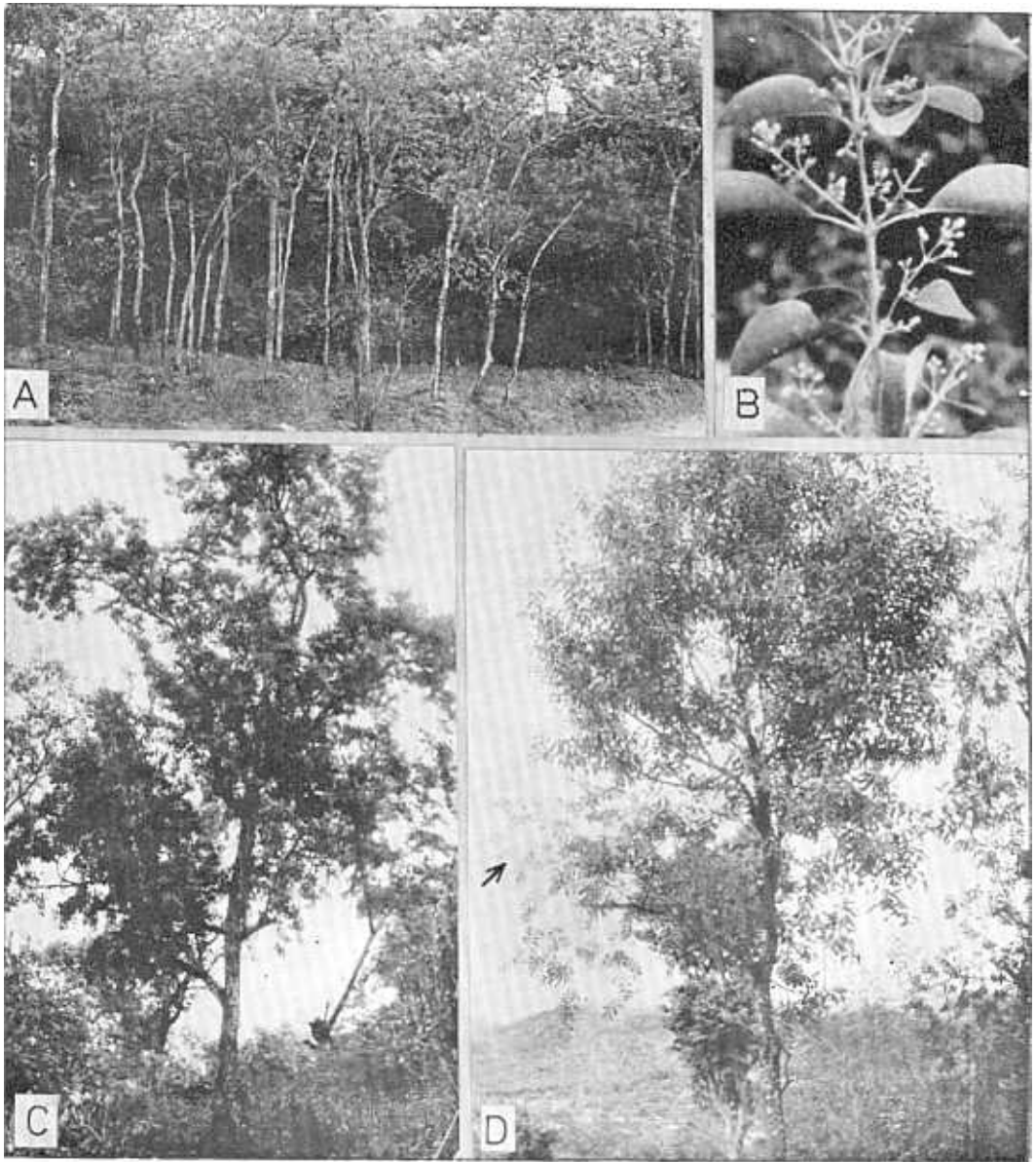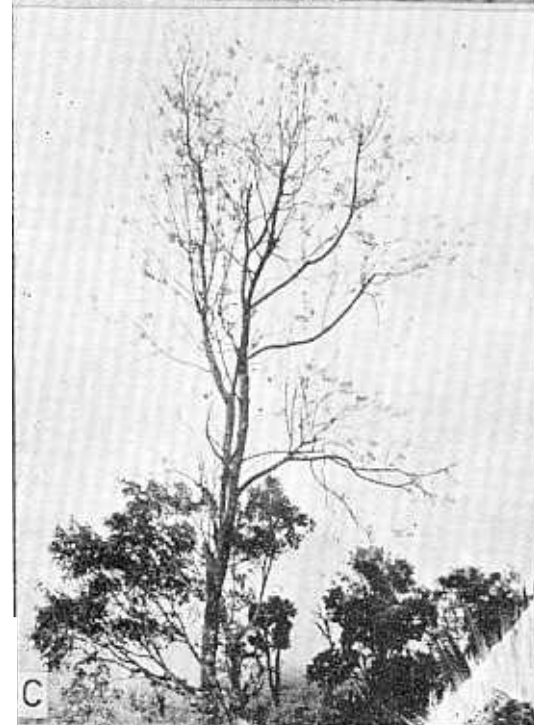
Fig   Healthy   spike diseased sandal in Sandal reserve fores   Maray   nse growth
         :es; B, portion of a healthy twig with flowers;   a health   D, a sandal
         infection (5-25%)

and characterised. Spiroplasmas are identified by their helical morphology. Usually MLO etiology of diseases is established by the presence of the organisms in phloem tissues through electron microscopy and by positive disease remission through tetracycline therapy (McCoy, 1979).

Sandal reserves in Marayoor forest range were considered to be free from spike disease (Chand Bacha, 1977; Surendran, 1977; Nayar, 1980) till Ghosh and Balasundaran (1981) reported the widespread occurrence of the disease in Sandal Reserve 51 In June 1980, on receiving information about some problem associated with sandal trees in the reserve from Mr. P. Radhakrishnan, the then forest range officer, the area was visited and the trees examined. It was found that a large number of trees were affected with typical spike disease.

Due to high incidence and varying intensity of disease and high density of trees (500 trees/ha), sandal reserves in Marayoor forest range were found to be ideal for field studies on spike disease. Further, the seed stand in Marayoor reserves is considered to be the best and it has been recommended for preservation (Venkatesan, 1981).

Detailed investigations were taken up with the view (i) to have a clear idea of the symptomatology of the disease not only through visual means but also through electrophysiological experiments using Shigometer and histopathological details mainly through fluorescent dyes and stains, specific for Mycoplasmas which may help in easy detection of disease, (ii) to monitor the spread of the disease in the field and to find out the possible vector of the disease, (iii) to isolate and characterise the causal organism, and (iv) to find out an appropriate chemical including antibiotics for the control of the disease through tree infusion technique.

SYMPTOMATOLOGY AND HISTOPATHOLOGY

INTRODUCTION

The occurrence of spike disease was first reported by McCarthy from Coorg in 1899 (Barber, 1903). The new leaves and shoots of a spiked tree become stiff and erect like bristles   As the disease advances, the internodes become shorter and leaves become smaller, narrower and more pointed until the new shoots give an appearance of fine spikes. Spiked branches bear no flowers or fruits. At the beginning of the symptoms manifestation, only a few branches may show spike symptom while others may be healthy with flowers and even fruits. The disease spreads to rest of the branches and ultimately the tree dies within one or two years.   The colour of spiked leaves becomes pale bluish-green which often changes to reddish-brown.   As the disease progresses, fine roots become dead and decayed.

Spike disease is generally diagnosed by the manifestation of the external symptoms.   Though attempts have been made to detect the diseased plants by deter- mining the length/breadth ratio of leaves (Iyengar, 196I) or with histochemical staining reaction (Ramaiah *et al.,* 1962; Parthasarathy *et al.,* 1966), none of these methods is practised in the field due to their unrelia bility. The importance of and the need for effective non-destructive means of disease diagnosis in the field were realised while planning chemotherapeutic measures in the field.   Classification of symptom manifestation into various infection intensity classes was found to be elaborate and laborious.   So, it was attempted to correlate the visual symptoms with electrical resist- ance of the inner bark measured with Shigometer. Recently this device is being used in experimental plant pathology (Tattar, 1976; Piirto and Wilcox, 1978; Davis *etal.,* 1979).

In addition to the use of Shigometer, fluorescent stains were also used for the differentiation of diseased tissues from healthy tissues.   Though electron microscopy is the only reliable and confirmatory method for demonstrating the presence of myco- plasma-like or rickettsia-like organisms associated with yellows diseases, this expensive and time consuming technique was not found to be convenient for routine disease diagnosis.   As   these organisms are essentiallyphloem pathogens histopathological aberrations of phloem tissues induced by their multiplications can be utilised for detecting the disease.   Certain characteristic host reactions which can be attributed to yellows diseases are phloem necrosis, excescive phloem formation, accumulation of starch in the plastids, deterioration of cell organalles, lysis of cytoplasmic inclusion bodies and so on (Ghosh, 1982).

MATERIALS AND METHODS

I. SYMPTOMATOLOGY

a) Symptom rating and correlation with Shigometer readings

Shigometer Model 7950 (North-East Electronics, New Hampshire, USA) was used to determine the electrical resistance. All measurements were taken in the trunk of the tree at breast height (1.2 m above ground) using needle type probes. Needles were pushed through the bark (till the meter reading was constant), at right angle to the trunk.

Based on external symptom manifestation (Fig. 3 & 4) disease intensity could be broadly classified into five groups.

Group	I	:	Healthy trees
	I1	:	Trees with partial infection
	III	:	Trees with moderate infection
	IV	:	Trees with heavy infection
	V	:	Tress with severe infection

Each group was assigned an infection intensity which indicates the degree of infection (Table 1).

Table 1. Rating of spike disease of sandal on the basis of external symptoms.

Infection Class	Intensity of infection (%)	Range of symptoms
I	0	No trace of spiked leaves; leaves oval to lanceolate, shining, olive green in colour; no defoliation or drying of branches; trees bear normal flowers and fruits.
II	5-25	Apparently healthy tree; only a few branches with spiked leaves; healthy branches bear flowers and fruits.
III	25-50	Most of the branches with spiked leaves; only a few branches with healthy leaves; In some twigs, only the lower, older leaves broader, healthy looking, and new leaves spiked; trees with no flowers or fruits.
IV	50-75	No trace of healthy leaves; trees full of spiked, weak drooping branches with stiff leaves projecting out; in some twigs lower leaves may be defoliated; foliage may be pale green to brown in colour.
V	75	Completely spiked trees; partially or fully defoliated; whole tree apparently dried and dead.

Trees with different infection levels were marked in the field and the electrical resistance of the inner bark was measured with Shigometer.

11. HISTOPATHOLOGY

Detection by fluorescence microscopy

(i) *Aniline blue (Dijkstra and Hiruki, 1974):* Free hand-cut sections from freshly cut twigs were heat-killed in boiling water for 8 to 10 minutes. The sections were stained with aniline blue (0.01%in 1/15 M K_2HPO_4, pH 8) for 10 to 15 minutes and viewed under Leitz Dialux fluorescence microscope.

(ii) *Hoechst 33258 (Seemuller, 1976):* For Hoechst 33258 staining, free hand-cut sections were fixed in cold 3% gluteraldehyde in 0.2 M sodium cacodylate buffer for I to 2 hours. They were then washed with 0.1 M phosphate buffer thoroughly and stained with 1 mg ml-1 solution of Hoechst 33258 in 0.1 M phosphate buffer for 15 to 20 minutes and viewed under Leitz Dialux fluorescence microscope.

RESULTS AND DISCUSSION

I. SYMPTOMATOLOGY

a) Symptom rating and correlation with Shigometer

Marked difference was observed between the electrical resistance of healthy and diseased trees. Based on electrical resistance, disease intensities could be classified broadly into four distinct groups as given below.

Intensity of infection	Range of electrical resistance
0% (Healthy)	3.0- 7.0 Kilo ohms
5-25%	7.1- 9.0 Kilo ohms
25-75%	9.1-13.1 Kilo ohms
>75%	>13.1 Kilo ohms

The range of electrical resistance measured gave an indication that as severity of spike disease increased electrical resistance also increased. Wargo and Skutt (1975) found that resistance to pulsed electric current was higher in trees defoliated by gypsy moth than in undefoliated trees. Also vigorous and dominat trees had lower resistance than intermediate and over topped trees. This study also showed that healthy trees had lower resistance to pulsed electric current than stressed trees. Strong inverse correlation between phloem thickness and electrical resistance in red maple has been recorded by Carter and Blanchard (1978) using Shigometer. Dixon *et al.* (1978) found a linear correlation between electrical impedence and water potential at a given temperature in the inner bark of avocado and spruce. In sandal affected by spike disease also, there is a quantitative increase in the phloem tissue (Dijkstra and Van der Want, 1970) and the water potential of the plant system is
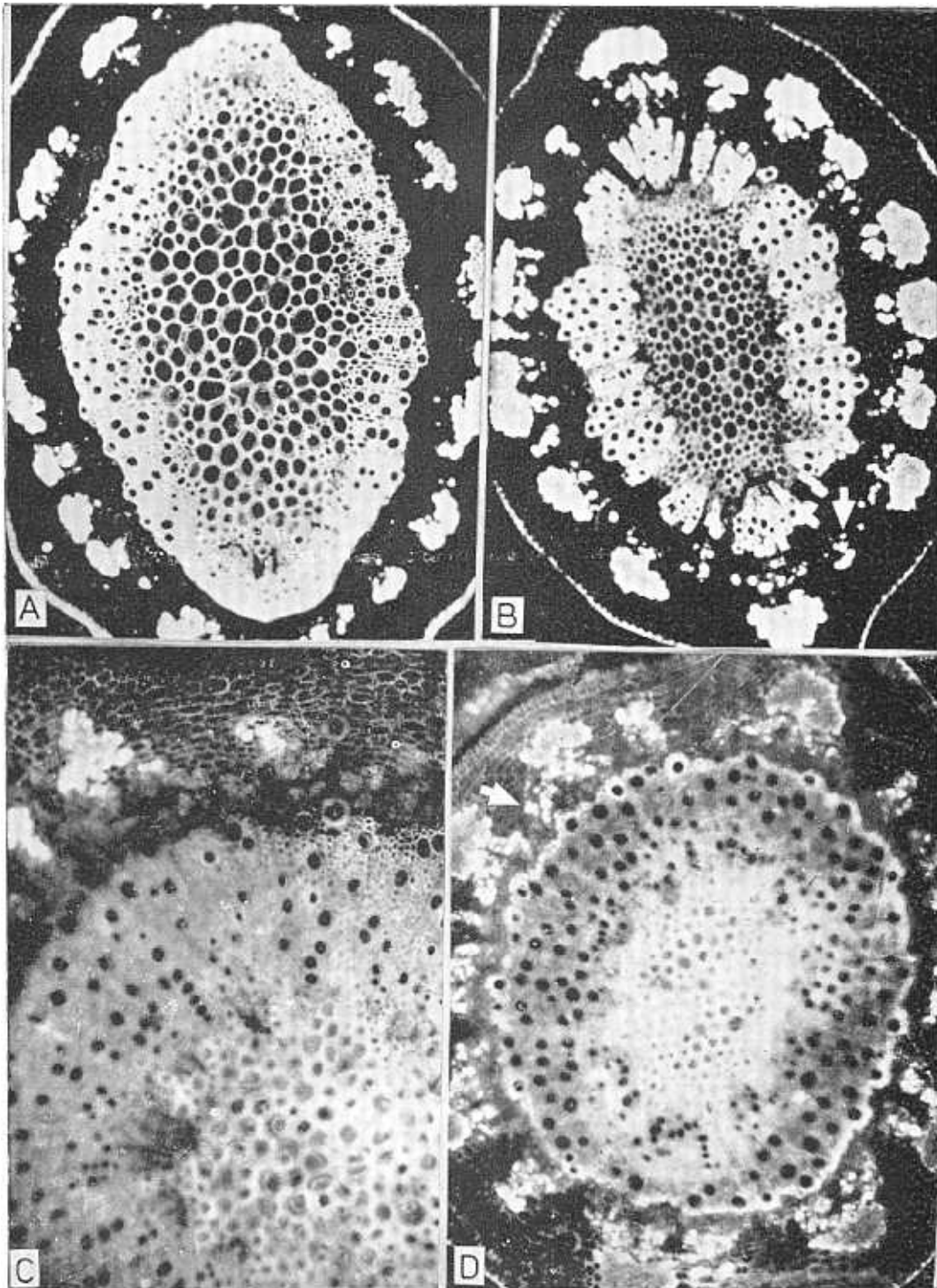
Fig. 5. Fluorescent micrographs of diseased and healthy tissues. A, C. S. of stem from healthy plant, stained with aniline blue (x100); B, C. S. of stem from diseased plant stained with aniline blue (x100); note the large number of fluorescent spots in the phloem region. C, C. S. of stem from healthy plant stained with Hoechst 33258 (x250); D, C. S. of a diseased stem stained with Hoechst 33258 (x175); note the large number of fluorescent spots seen in the phloem tissue.

altered.   Alteration in the mineral uptake and change in Ca/Fe ratio (Iyengar, 1972) in roots also may contribute towards variation in the pattern of electrical resistance between healthy and diseased sandal.   The electro-physiological techniques emerged as a simple and non-destructive method of monitoring changes in the water relations of plant systems.

Our results indicate the usefulness of Shigometer in differentiating spike diseased sandal in the field and in giving a quantitative measure of the intensity of the disease.   The technique served as a quick and non-destructive method of detecting spike disease for taking curative measures.

11. HISTOPATHOLOGY

Detection by fluorescene microscopy

(i) *Aniline blue:*   Transverse sections of young twigs and leaves from severely diseased trees, when treated with aniline blue showed a bright yellow fluorescene throughout the phloem tissue (Fig. 5 A & B).   Transverse sections of a comparable twig from a healthy tree showed only a few fluorescent spots in the outer zone of phloem.   Cell walls of xylem and their sclerenchymatous tissue from diseased as well as healthy tissue showed dull greyish yellow fluorescence.

(ii) *Hoechst 33258:*   Sections treated with Hoechst 33258 showed numerous small bright yellow green fluorescent spots in phloem of diseased twigs whereas no spots could be detected in healthy sections (Fig. *5* C & D).

Detection of abnormal level of wound callose produced in response to injury to phloem cells had been suggested as an indirect method for diagnosing the MLO infected plants (Waters, 1982). Dijkstra and Hiruki (1974) used fluorescence microscopy and reported that there is a marked difference in fluorescence in the phloem area of healthy and spike diseased sandal tree and discussed its diagnostic value.   Ghosh *et al.* (1974) observed the best differentiation of diseased and healthy sections when phloem tissues of healthy and witches'-broom affected *Mirabilis jalapa* were stained with aniline blue.

Russell *et al.* (1975) successfully used DAPI (4'-6-diamidino'-2-phenylindole), a DNA binding fluorochrome, for detecting mycoplasmal contamination of tissue culture   By using DAPI or Hoechst 33258, a benzimidole derivative, Seemuller (1976) obtained positive DNA/DAPI fluorescence with pear decline disease, a disease associated with MLO. Recently Hiruki (1952) used aniline blue and DAPI as fluorochromes for detecting MLO in mulberry dwarf and paulownia witches'-broom and obtainzd positive fluorescence with DAPI and not with aniline blue in the case of mulberry dwarf and vice versa in the case of paulownia witches'-broom.

There was sufficient evidence that aniline blue staining could be used for distinguishing MLO infected phloem tissue from that of healthy tissue. But this technique cannot be used in isolation as it does not detect MLO as such. However, though electron microscopy is the most reliable and confirmatory technique for establishing association of MLO with yellows diseases, positive fluorescence by aniline blue and Hoechst 33258 can also be used to detect MLO infection associated with yellows diseases.

EPIDEMIOLOGY

(1) Disease Progress

INTRODUCTION

Though extensive literature has accumulated on the occurrence of virus and mycoplasmal diseases of various crop plants, only very few publications deal with the epidemiology of these diseases, especially those of trees.   Only recently some attention has been paid on the epidemiology of viral diseases and a few publications have come out (Scott and Bainbridge, 1978; Plumb and Thresh, 1983; Thresh, 1983).

The dearth in the literature on epidemiology of mycoplasmal disease could be partly attributed to comparatively recent discovery of the true etiology of such diseases.   Whatever data that are available have not been analysed in sufficient detail to obtain information on the various factors influencing the pattern and rate of spread.   This proves a serious limitation.   Disease progress curve represents an inte-gration of the complex interaction between MLO and their host plants in a given environment that frequently involves insect vectors.   Disease progress curves are essential in deciding the necessity and adopting appropriate method for control measures (Thresh, 1983.)

In the past, studies on the spike disease were mainly confined to understanding the nature of the pathogen and the vector involved in the disease transmission.   Very little attention was given to monitor the progress of disease in a particular area. Raychaudhuri and Varma (1980) attributed the lack of any organised effort to record the progressive spread of sandal spike disease, to the difficult terrain, the small population of sandal per unit area, and the absence of quick method of disease detection.   Muniyappa *et al.* (1980) studied seasonal spread of sandal spike disease in experimental plots of 100 x 100 m size in the sandal reserve forests of Karnataka for two years.   The average initial incidence and final incidence of the disease were recorded.   However, the progress of the disease was not evaluated critically.   This chapter presents results of spread of spike disease, as monitored in Marayoor.

MATERIALS AND METHODS

Spread of spike disease: After recording spike disease in sandal Reserve 51 in June 1980 (Ghosh and Balasundaran, 1981) all the other eight sandal,reserves at Marayoor (Table 2) (Varghese, 1976) and the Chinnar Reserve were visited to ascertain whether
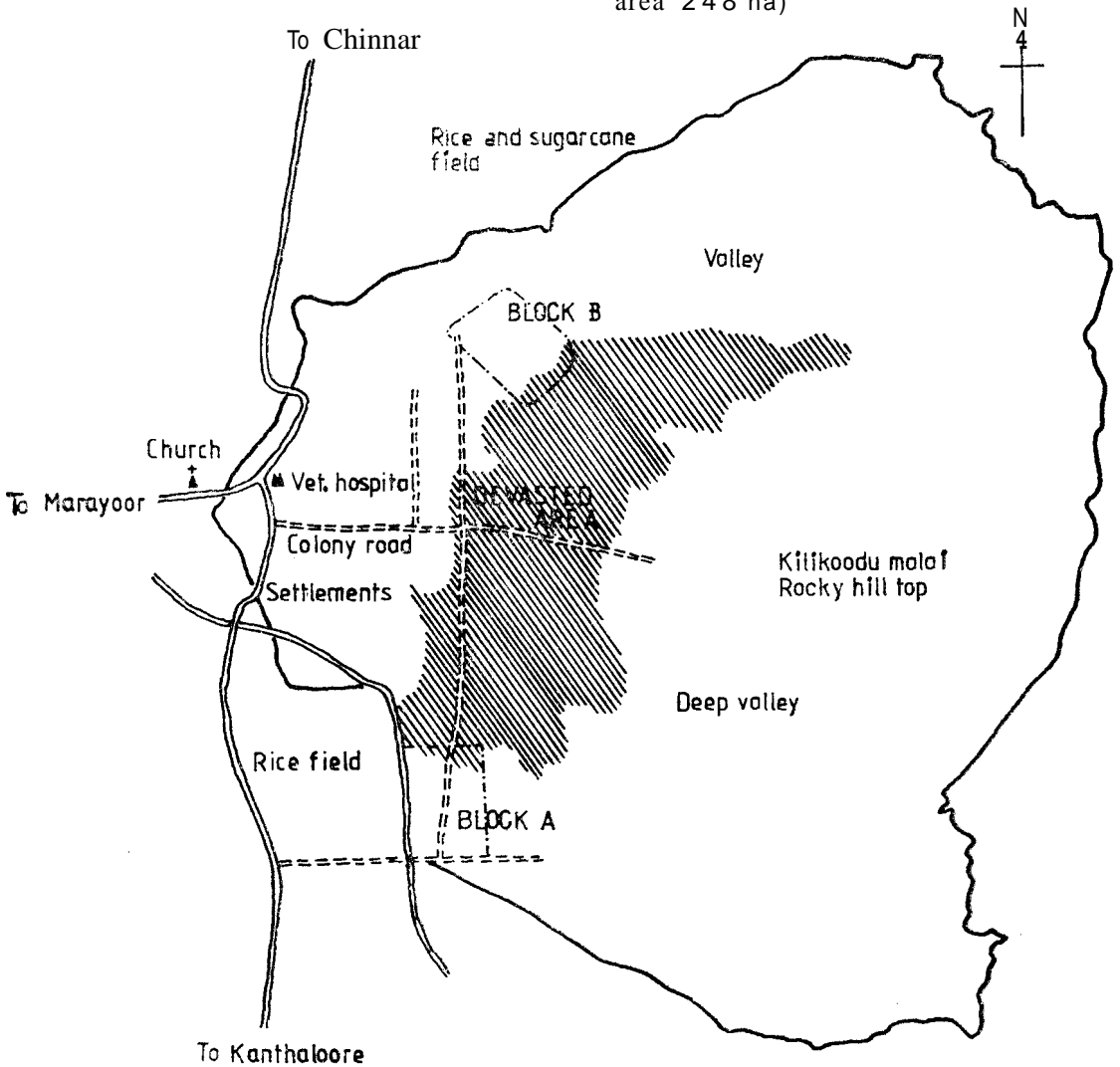
the disease had spread to those areas (Fig. 2). Later all these reserves were visited regularly every year to find out whether the disease had spread to new areas. SandaI trees growing in the reserves and revenue lands adjacent to the Reserve 51 were observed at three-months intervals.

Table 2.   Sandal Reserve Forests of Marayoor Range

Felling series		Reserve	Area ha	Number of trees	Number of trees /ha
I	i	Sandal Reserve 51	382	23,227	61
	ii	Sandal Reserve 52	53	5,338	101
	iii	Sandal Reserve 54	18	1,268	60
II	i	Karayoor Sandal Reserve			
		Block I	120	27,839	232
	ii	Block II	97	5,141	53
	iii	Nachivayal Sandal Reserve			
		Block I	146	62,543	428
	iv	Block II	101	19,865	197
III	1	Vannamthorai Sandal Reserve			
		Block I	115	9,344	81
	ii	Block II	5 10	32,029	63
		Total	1,542	186,594	121

Disease monitoring plots: In order to find out the pattern of disease-spread, if any, in Reserve 51, two experimental plots. one (Block A of 200 trees) with an area of about 4.5 ha and the other (Block B of 175 trees) having an area of about 4 ha were selected.   These experimental plots were demarcated in such a way that one side of the blocks was in close proximity to the advancing perimeter of the heavily diseased area (Fig. 6 & 7).   Some diseased sandal trees were also included in the experimental plots.   The western border of Block A was revenue land with a large number of sandal trees.   A third plot (Block C) measuring about 2 ha with 350 sandal trees was selected in Nachivayal Reserve   about 2 km away from Reserve 51 (Fig. 2).   The area between these reserves is under paddy and sugarcane cultivation.   All the sandal in the three plots were marked and their relative positions plotted on a graph paper. The observation began in February 1982 and continued till February 1984. Incidence of spike disease in all the trees of the three plots was recorded once in three months, with respect to the appearance of the initial symptom and the rating of the disease severity of trees already diseased.

SANDAL RESERVE 51-SPIKE DISEASE AFFECTED AREA
area 248 ha)

N
4

To Chinnar

Rice and sugarcone field

Valley

BLOCK B

Church

Vet. hospital

DEVASTED AREA

To Marayoor

Colony road

Kilikoodu mala!
Rocky hill top

Settlements

Deep valley

Rice field

BLOCK A

To Kanthaloore

Map not to scale

Fig. 6. Nature of disease spread; note the location of disease monitoring blocks A and B.

PROGRESS OF SPIKE DISEASE IN BLOCK A

(AREA 4.5 ha approx   NUMBER OF TREES 200)

RESEARCH PLOT
SILVICULTURAL RESEARCH WING
KERALA FOREST DEPARTMENT

N

HEAVILY SPIKED AREA

→VALLEY

RICE FIELD

CANAL

FOREST ROAD

◉ DISEASED TREES RECORDED IN FEB 1982

DURING MAR TO
AUG 82

DURING SEP 82
TO FEB 83

DURING MAR TO
AUG 83

DURING SEP 83
TO FEB 84
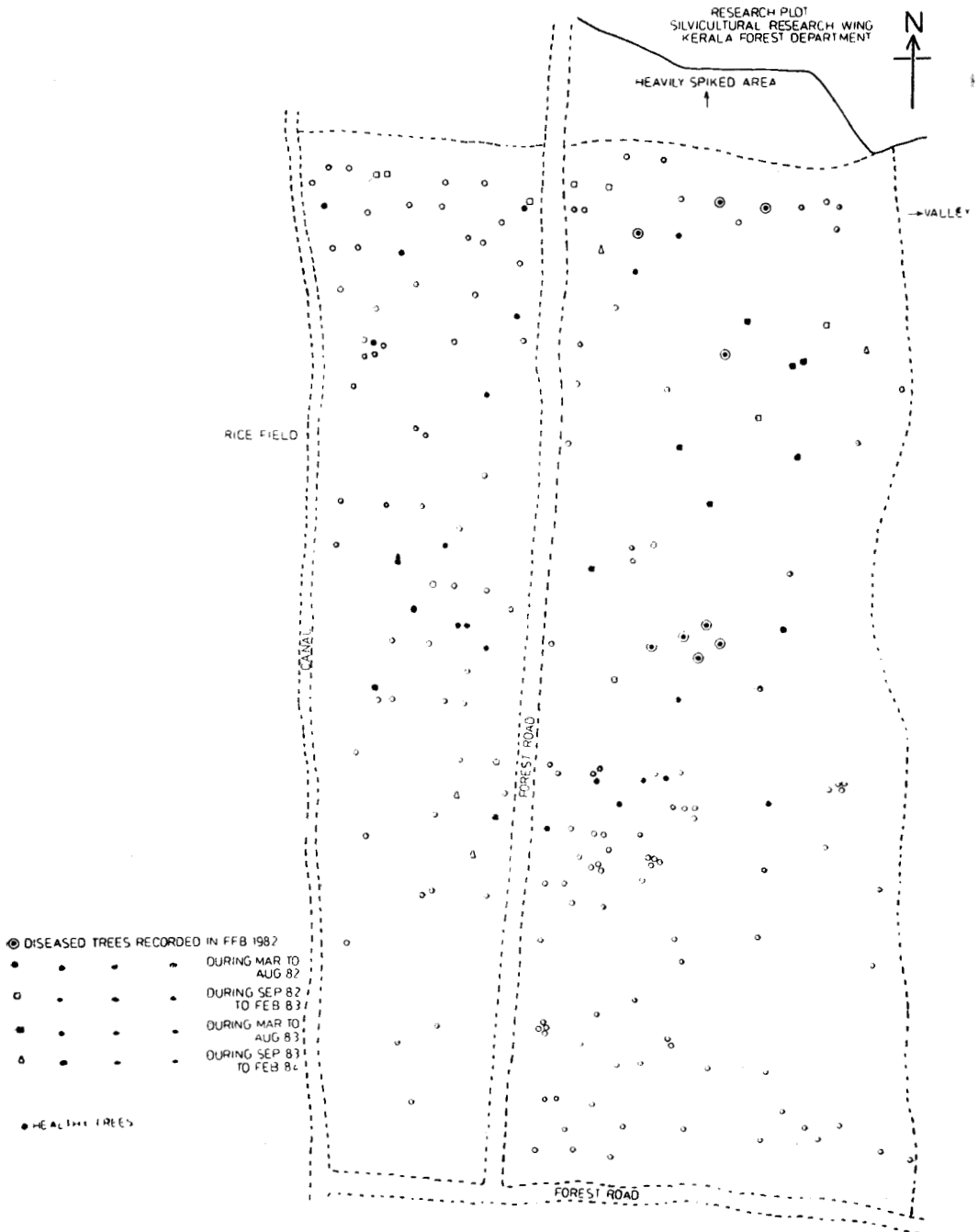
• HEALTHY TREES

FOREST ROAD

Fig. 7.   Map of Block A showing the disease progress.

The apparent infection rate (r) was calculated based on the differential equation of Van der Plank (1963) for compound interest diseases:

$$\frac{dx}{dt} = rx(1-x)$$ where the change in proportion of disease 'x' with time 't' is equal to a rate value 'r' times the proportion of the disease present at any time (**x**) multiplied by the factor (1-x). The apparent infection rate 'r' is the slope of the curve obtained by plotting, log, (x/l-x) against time (Thresh, 1983), since spike disease could be considered as polycyclic disease (see discussion). The estimated time for 95% incidence of the disease in each plot was calculated by substituting 0.95 for x in the regression equation

$$\log_e \left(\frac{x}{1-x} \right) = \hat{a} + \hat{r}\, t$$ where $\hat{a}$ and $\hat{r}$ are estimated constants.

The average time required for progress of the severity from infection class II to V and until the death of the tree were calculated.

RESULTS AND DISCUSSION

Spread of spike disease in the Reserve 51 and adjoining areas

The disease is now confined to Reserve 51 and is slowly spreading to adjacent revenue lands and the Chinner Reserve (Fig. 2). Initially in June 1980, a large number of dried and very heavily spiked trees (>75%) were found at the north west slop of the Kilikkoodumalai of Reserve 51 (Fig. 6). It appears that the disease might have established here several years before 1980 and later spread in all the directions. The progressive expansion of the disease is by 'radial' spread from the limit of the diseased area. Year after year, the boundary of the diseased area, where fresh infections are found, advanced towards the limit of the reserve. By February 1984, the disease had advanced upto a radius of about 1 km from the focus of the initial infection and about 700 m from the limit when disease was detected in 1980. Now the disease has advanced upto the church compound and the border of the Chinnar Reserve and the boundary of Block A (Fig. 2). When the disease approached the limit of Reserve 51 and the adjacent revenue lands, the spread was not strictly radial because of the human interference and the farming activities in the adjoining field. The disease could not spread towards the eastern side where there were only very few trees on the rocky Kilikoodumalai and the deep valley east of it. However, the spread was typical towards south where one of the disease monitoring plots (Block A) was located.

The source of initial inoculum by which the spike disease would have originated and spread in Marayoor could not be ascertained. In India, spike disease was

confined to an area bounded by Chickballarpur in the north, Javadi Hills in the east, Nilgiris in the south and Coorg in the west (Venkatesan, 1978). The only possibility of its introduction appears to be from this area, more than 200 km away from Marayoor.

Disease spread in the monitoring plots

Details of the spike disease spread in disease monitoring plots are given in the Table 3 and Fig. 7 & 8.

Table 3. Spike disease spread in the monitoring plots.

| Block	Apparent infection rate r (per unit per month)	Coefficient of determination R^2	Estimated time for 95% incidence (months)	Estimated time for disease progress from infection Class II to V (months)	Estimated time for disease progress from infection Class II to death (months)
A	0.0803±0.012*	0.87	70	13.39±0.72	14.67±0.68
B	0.0660±0.005	0.96	87	11.83±0.86	14.67±1.27
C	—	—	—	—	

* Standard error

The disease advanced considerably in Blocks A and B and not at all in Block C. The data given in Table 3 clearly indicated that the rate of spread of spike disease in all the plots was not similar. There is significant difference between Block A and B in respect of apparent infection rate. The difference between Blocks A and B might be largely due to the difference in micro-climatic conditions of the plots. The topography of Block A was almost level compared to the sloping terrain of Block B. A large number of miscellaneous trees in Block B provided more shade to the sandal in Block B; higher soil moisture of Block B due to its proximity to a stream compared to dry condition in Block A could also be a factor contributing to varying infection rates. This was not in agreement with the observations of McCoy (1976) on coconut lethal yellowing in Florida, where the highest r values (apparent infection rate) occurred under high cultural maintenance and the lowest were adjacent to salt water. Observations on all the experimental blocks are being continued so that a clear picture of the spread of spike disease will emerge.

Undergrowth and collateral hosts

Besides the above mentioned factors, presence of certain weeds such as *Lantana camara, Zizyphus oenoplia, Jasminum rigidum, Dodonea viscosa* and *Srachytarpheta* sp. might also contribute to the spread of the disease and influence the infection rate (Fig. 9 A & B). *L. camara* was suspected to be a symptomless

DISEASE PROGRESS CURVE OF SANDAL SPIKE IN BLOCK B OF
SANDAL RESERVE 51 DURING FEB 82 TO FEB 84
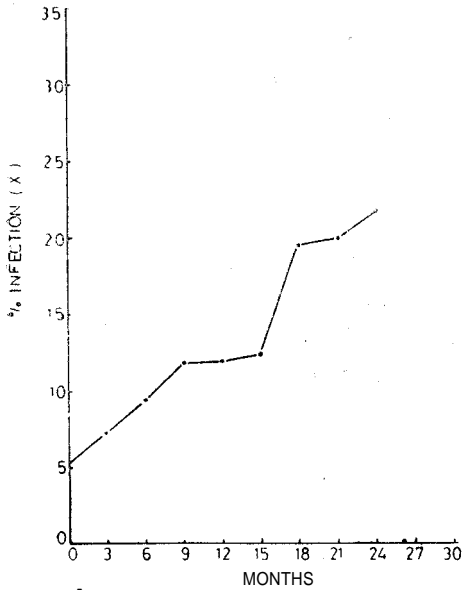
A
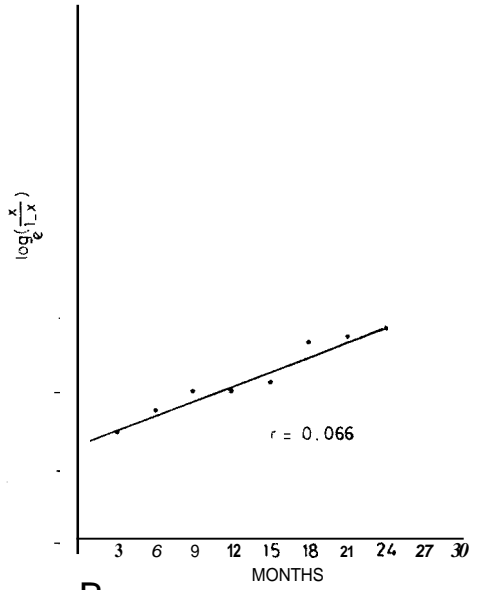
B

DISEASE PROGRESS CURVE OF SANDAL SPIKE IN BLOCK A OF
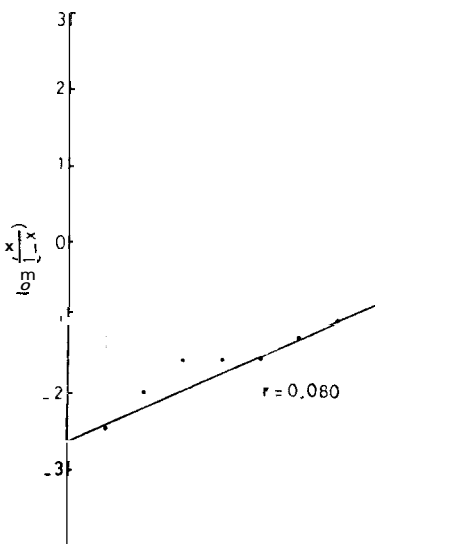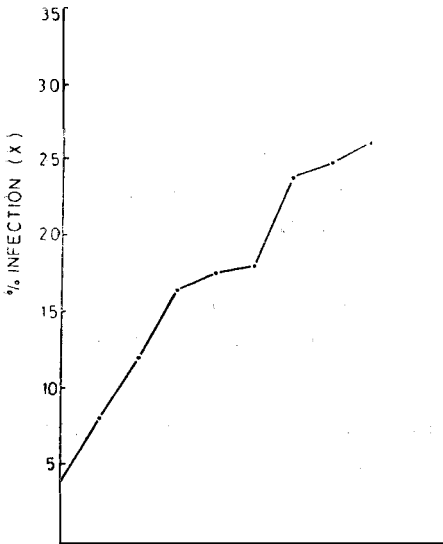SANDAL RESERVE 51, DURING FEE 1982 TO FEB 1984

Fig. 8. Disease progress curves. A, Disease progress in block B; B, Regression line and apparent infection rate of disease in block B; C, Disease progress in Block A; D, Regression line and apparent infection rate of disease in Block A.

alternate host for the sandal spike MLO by Nayar and Srimathi (1968). However, there was no proof for this assumption. In all the three blocks, witches'-broom affected *Z.oenoplia* was found. *Z. oenoplia* was suspected to be a collateral host of sandal spike pathogen (Hull *et al.,* 1970). However it might be noted that though witches'-broom affected *Z.oenoplia* were found in several places in Nachivayal Sandal Reserve I and 11, spike disease was not found on sandal there. Witches'-broom affected *J. rigidum* and *Stachytarpheta* sp. were also common. However, *D. viscosa* was not found attacked by witches'-broom disease. Sandal being a semi-root parasite, spike disease might be transmitted by haustorial contact also. Coleman (1923) showed haustorial connection between diseased and healthy trees and suggested that spike disease transmission occurs through haustoria also. However the chief way of disease transmission is through vectors. This subject is dealt in the next part in detail.

Polycyclic disease

According to Thresh (1983), compound interest diseases (Van der Plank, 1963) or polycyclic diseases are those where sources of inoculum increase as progressively increasing numbers of plants become infectious. This definition holds good for sandal spike disease also. It was observed that fresh infection of spike disease was prevalent in the experimental area throughout the year and also that at any given time the diseased trees were in various levels of infection. Presence of collateral hosts and the vector throughout the year in the same area further confirmed the polycyclic nature of the disease.

(ii) Vector

INTRODUCTION

Prokaryotic plant pathogens associated with yellows-type of diseases are generally found in the vascular tissue of infected Plants. This confers a need for specialised methods of transmission such as grafting, dodder and insects (Markham, 1982). Most of the insect vectors of plant diseases are included in two taxonomic groups, the Sternorrhyncha (aphids and psyllids) and the Auchenorrhyncha (leaf hoppers, plant hoppers and frog hoppers). Tsai (1979) has listed vectors of about 50 diseases caused by MLO, Spiroplasmas and RLO.

Intensive research on spike disease and its vector was initiated in 1927 as a joint effort of the then Madras, Mysore and Coorg Governments assisted by the Indian Institute of Science, Bangalore and the Forest Research Institute, Dehra Dun (Anon., 1955). Mr. S. Rangaswami Iyengar, the then research range officer at Denkanikota, working during 1934 to 1939, suggested that *Coelida indica,* a small sap sucking insect, is the vector of the disease (Rangaswami and Griffith, 1941). Dr. N. C. Chatterjee, the then entomologist, Forest Research Institute, Dehra Dun,

working on this problem between 1927 and 1934 found that two Orthopterus insects, *Derodes sparsus* and *Lytana inflata* caused symptoms resembling severe etiolation in sandal as a result of their feeding on the leaves. Such weakened plants, when exposed to *C. indica* readily showed symptoms of spike (Anon., 1955).

However, later work carried out during 1943 to 1948 to repeat Mr. Rangaswamy Iyengar's experiments failed to give a conclusive proof and the results obtained were not statistically significant (Anon., 1955). Similarly attempts to repeat Dr. Chatterjee's experiments from 1949 to 1953, also did not lead to any positive results. In the mean time, *Moonia albimaculata* was also recognised as a vector of sandal spike disease (Dover and Appanna, 1933). But, these reports were, however, considered doubtful (Lasrado, 1955). After more than three decades of lull on the studies of vector transmission, Shivaramakrishnan and Sen-Sarma (1978) suggested *Nephotettix virescens* as a vector of this disease. However, Raychaudhuri and Varma (1980) questioned the procedure adopted by them. Muniyappa *et al.* (1980) also could not confirm their finding. Besides, Muniyappa *et al.* (1980) found that *C. indica* also failed to transmit the disease Sen-Sarma (1982; 1984) has reviewed the present status of knowledge in respect of insect vectors of sandal spike disease.

Since the earlier studies did not give any conclusive evidence of vector trans-mission of spike disease, an investigation for finding out the insect vector of the spike disease was conducted.

MATERIALS AND METHODS

Insect Collection

Insects, especially the phloem feeders, were collected from Reserve 51, using sweep nets during day time and light traps of different colours during night time in different seasons. Collections were also made using aspirator closely observing diseased and healthy trees. The insects were fixed in formaldehyde, brought to the Entomology Division of the Institute and identified upto the generic level. The feeding behaviour of the insects was determined by examining the mouthparts and only phloem feeders were selected for further study.

Qisease transmission experiments

The transmission of the disease was attempted on 1-to 2-year-old seedlings raised in insect proof cages and 4-year-old plants maintained in the nursery at Adult insects were collected from diseased and healthy sandal trees from spiked area of Reserve 51 and released either on excised diseased shoots kept in water or on spiked branches for 24 to 168 hours for acquisition feeding and brought to the insectary. Two to eighteen of these insects irrespective of sex, were released on the healthy

seedlings in an insect proof cage for 7 to 50 days. Periodical observations were recorded for the symptom expression.

Light and transmission electron microscopic studies of diseased plants

(i) *Fluorescent microscopy:* Thin transverse sections of leaf and stem tissue from plants infected through vector: transmission and also from those of healthy plants were stained with 0.01% aniline blue and examined under fluorescent microscope as described earlier under Symptomatology and Histopathology.

(ii) *Transmission electron microscopy (TEM):* Petiole and stem tissues from plants infected through insect transmission as well as from field infected plants were fixed in 2.5% gluteraldehyde in 0.2M sodium cacodylate buffer (7.2 pH) for 24 hours. They were then post-fixed in 1% osmium tetroxide in the same buffer for 2 hours, de-hydrated in ethyl alcohol series and embedded in Epon-araldite mixture. Sections were cut in an LKB IV microtome using diamond knife, stained with uranyl acetate and lead citrate and viewed under JEOL-100 transmission electron microscope.

(iii) *TEM of insects fed on spike diseased sandal:* Twenty seven adult insects collected from spiked sandal from reserve 51 were again confined to severely spiked sandal tree branches under insect proof nets. After allowing them to feed on the diseased branches for 10 days, 24 live insects were transferred to insect proof boxes, containing shoots from healthy plants and transported to the laboratory. Immediately these insects were released on selected branches of 4-year-old healthy sandal trees under insect proof nets. After 10 days the live insects were dissected and their intestine and salivary glands removed. Insects collected from healthy trees and maintained on healthy plants in the nursery served as control. Insects immobilised through chilling for three minutes were dissected under gluteraldehyde solution and the excised intestine and salivary glands fixed in 3% gluteraldehyde in 0.2 M sodium cacodylate buffer for 6 hours. Pieces of the tissues were washed in the buffer three times and post-fixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer overnight. The tissues were washed in buffer and dehydrated for 15 minutes in graded solutions of ethanol and water and finally in propylene oxide for 30 minutes. Small bits of tissues were embedded in Epon-araldite mixture (Hall, 1978). Sections were cut on LKB IV microtome using glass knife, stained in uranyl acetate and lead citrate and examined under Philips EM 400 transmission electron microscope.

RESULTS AND DISCUSSION

The sweep net did not yield any suspected phloem feedicg insects. Collections in the night traps mostly consisted of moths and winged termites, except a few

N . virescens. However, *N .virescens* was not used for transmission studies because of the negative result reported earlier by Muniyappa *et al.* (1980). A pentatomid bug, identified as *Halys dentatus* Fb., collected by using aspirator. was seen feeding and thriving well on healthy and spiked sandal trees However, transmission experiments with this insect did not give any positive result. Another insect, which was quite common on the diseased and healthy sandal tree was an Issid, identified as *Redarator bimaculatus* Dist. by Commonwealth Institute of Entomology, London (Fig. 9 C & D)

The insect, *R. birnaculatus* at various stages of growth was observed on diseased and healthy sandal trees of the reserve 51 and adjacent revenue lands throughout the year. However they were found to be more prevalent during October to March and the insect catches were more during these months. Generally, in sandal forests, insect populations were found to be more during August to December (Muniyappa *et al ,* 1980). During day time, *R. bimaculatus* was found to camouflage between either the crack and crevices of the bark of large trees or just underside of small branches or leaves. They were usually observed on the young branches during night time and early morning. *R. bimaculatus* had been earlier reported on spiked sandal from Karnataka (Chatterjee and Bose, 1933). Presence of this insect in large numbers in Reserve 51 and its near absence in other sandal reserves, made to suspect *R. bimaculatus* to be a vector of spike disease in Marayoor.

Disease transmission through *R. bimaculatus*

After 3 months, typical spike symptoms were noticed on a few 1· to 2-year-old seedlings and one 4-year-old plant. Details of the results are outlined in Table 4. The leaves of the affected plants showed browning which was gradually followed by defoliation and dedth of the seedlings after a few months Appearance of spike symptoms in the 4-year-old plant was slower than that in seedlings. Initially the disease appeared only on the vector inoculated branches, but later it appeared on a few others with characteristic symptoms of spike disease. A longer duration taken in the expression of the disease, its slow progress and lower severity on the plants at Peechi, compared to the rapid increase of the disease on plants in the field at Marayoor may possibly be attributed to the different climatic conditions at Peechi and Marayoor.

Heat-killed hand sections (C. S.) of tissues from seedlings showing spike symptoms were stained with 0.01% aniline blue and examined under fluorescence microscope. Presence of bright fluorescent spots in the phloem region showed callose formation, which indirectly indicates the presence of MLO in the phloem tissue (Ghosh *et al.,* 1984). No such fluorescence was observed in tissues obtained from healthy seedlings.

Table 4. Disease transmission through *R. bimaculatus*

Plant material	Acqui-sition feeding time (hr)	No. of insects fed	No. of days insects fed	Disease symptoms if any
1 to 2-year-old Seedling	24	3	7	Doubtful symptoms
,,	24	3	14	"
,,	24	5	14	Nil
,,	72	3	14	Spike disease symptoms
,,	48	5	14	Nil (dried)
,,	96	7	12	Spike disease symptoms
,,	96	2	7	Nil
,,	96	7	12	Nil
,,	120	6	50	Nil
4-year-old plant	120	4	45	Spike disease symptoms
"	168	16	40	Nil
"	168	18	40	Nil

Further confirmation of association of MLO with the vector transmitted spike symptom was obtained in transmission electron microscopy. The phloem tissue of diseased plant parts contained numerous pleomorphic MLO inside sieve elements (Fig. 10). The morphology of the MLO was comparable to that found in the phloem tissues of naturally infected sandal (Fig. 11). Complete absence of MLO in the phloem of healthy plants further confirmed that the disease was caused by the MLO, transmitted by the insect.

Electron microscopy of the insect vector

Intestinal and salivary gland tissues of *R. bimaculatus* also showed the presence of pleomorphic bodies varying from 200 to 500 nm in diam (Fig. 12). The aggregation of MLO was more in intestine than in salivary glands. The bodies were bounded by unit membrane and they were usually found near the basement membrane of the intestine and the salivary glands. From various cell organelles, viz., mitochondria, golgi bodies, etc., vesicles and some large bodies with several concentric layers of membrane enclosing one or a few small bodies of the same shape, MLO could be distinguished by the size, pleomorphic nature, membrane structure, and the network of chromatin within.

A

B

C

D
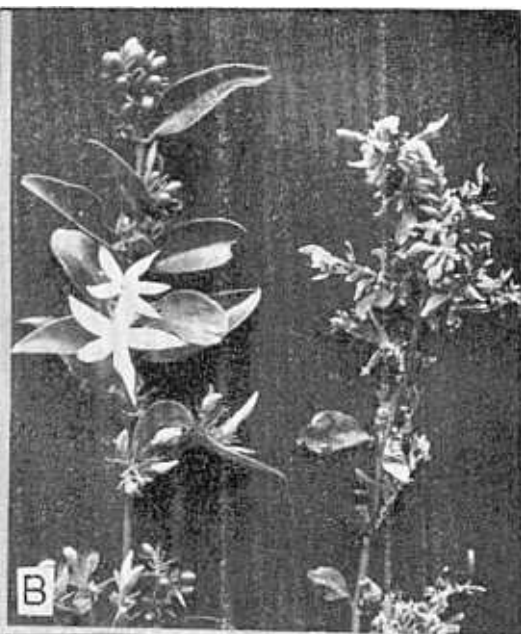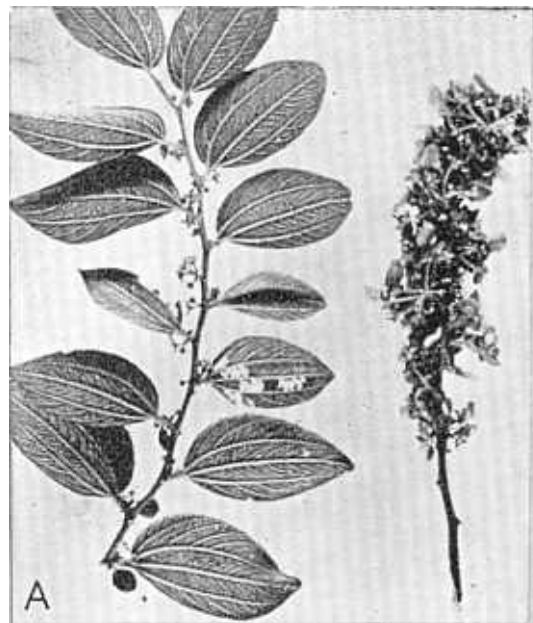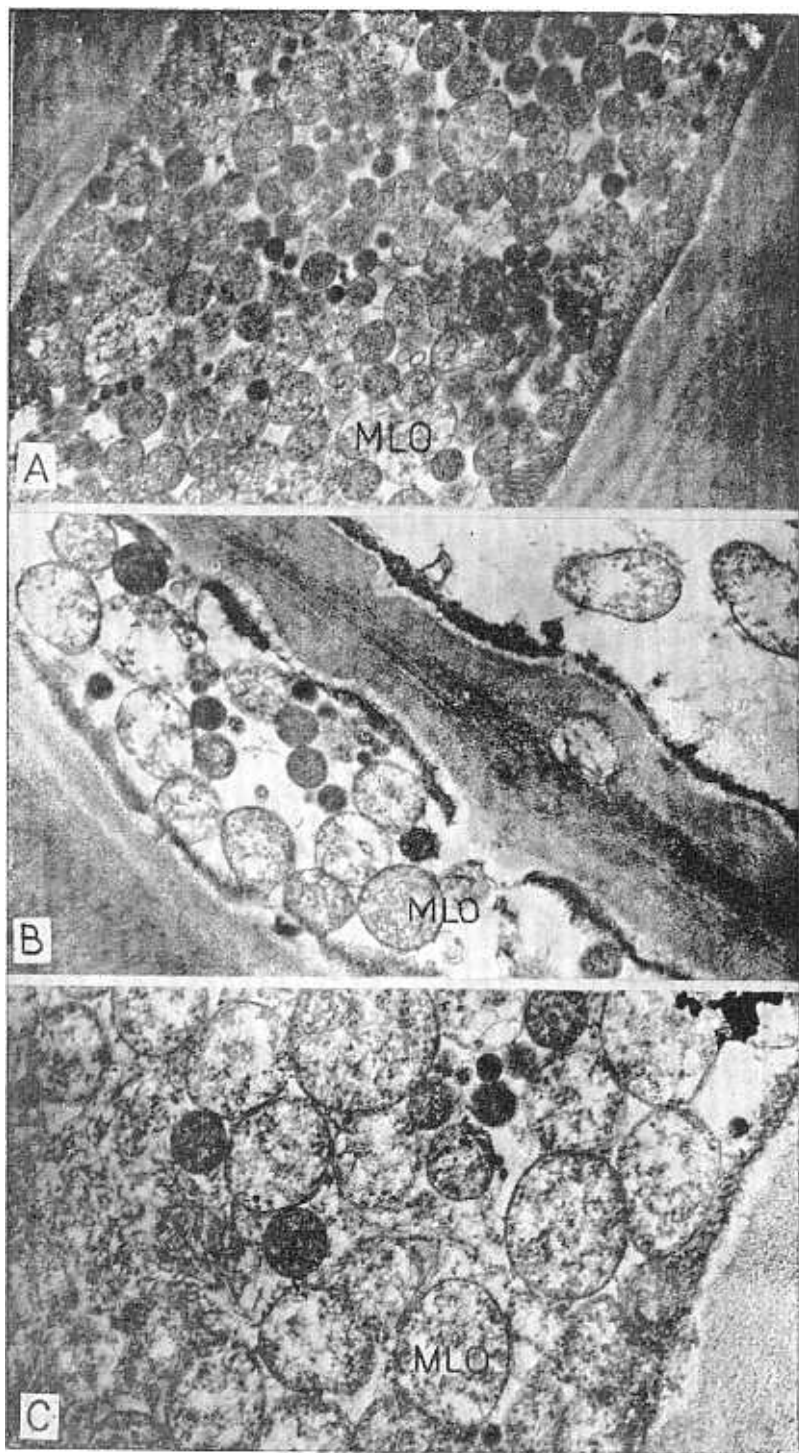
Fig. 10. Transmission Electron Micrographs (TEM) of tissues from sandal infected by insect vector. A, TEM of L. S. of sieve cell profusely filled with MLO (x16,000); B, C portions from different sieve cells magnified (B=x28,000; C=x40,000).
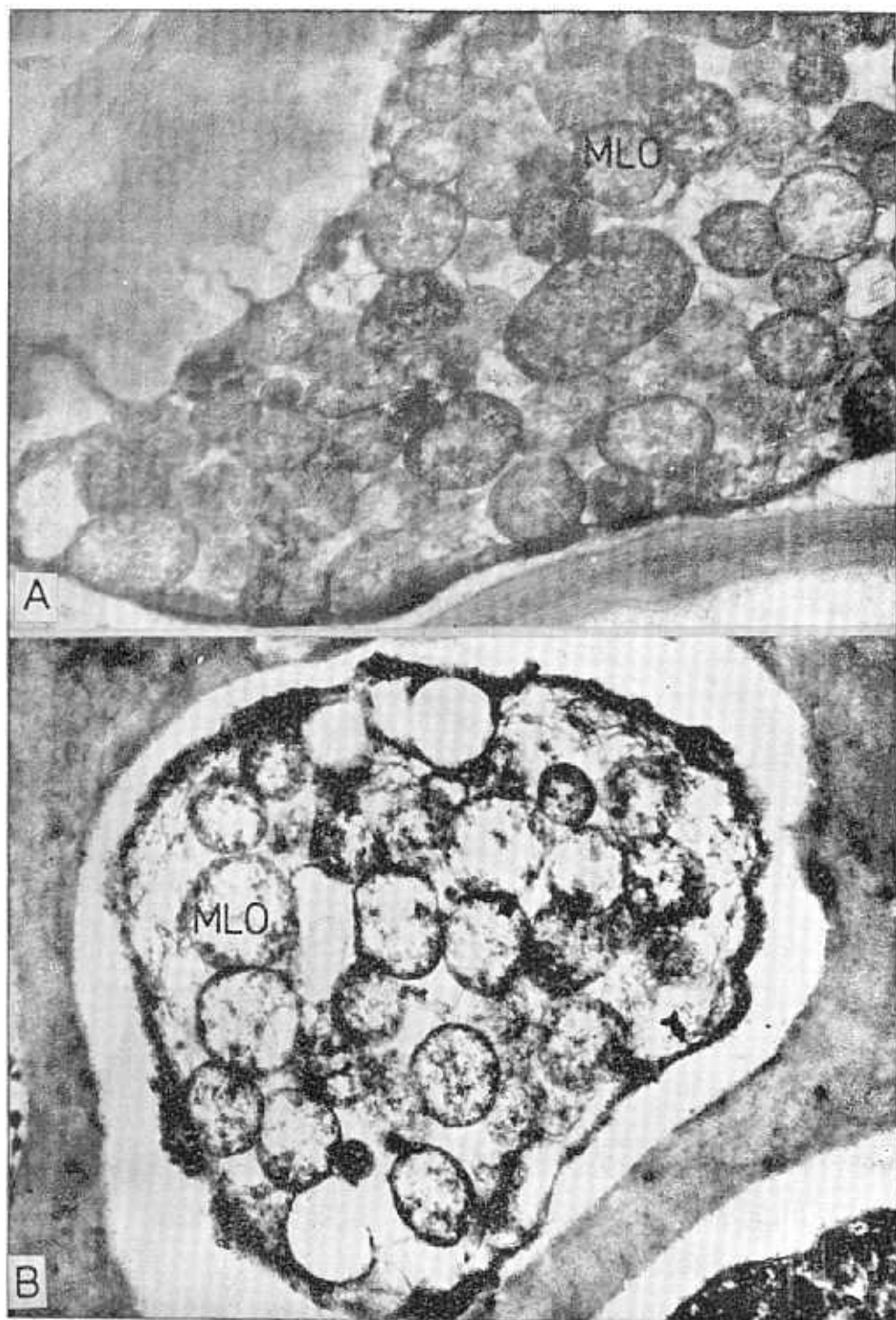
Fig. 11. TEM of tissues from field infected sandal. A. TEM of L. S. of sieve cell filled with MLO (x30.000); B. TEM of C. S. of sieve cell filled with MLO (x25,000).

Fig. 12. TEM of tissues of insect vector.   A, TEM of tissue from intestine;   note the aggregate of MLO near the basement membrane (x27,000); B, a portion of A magnified (x60,000); C, TEM of tissue from salivary glands; note the aggregate of MLO near the basement membrane (x45,000).

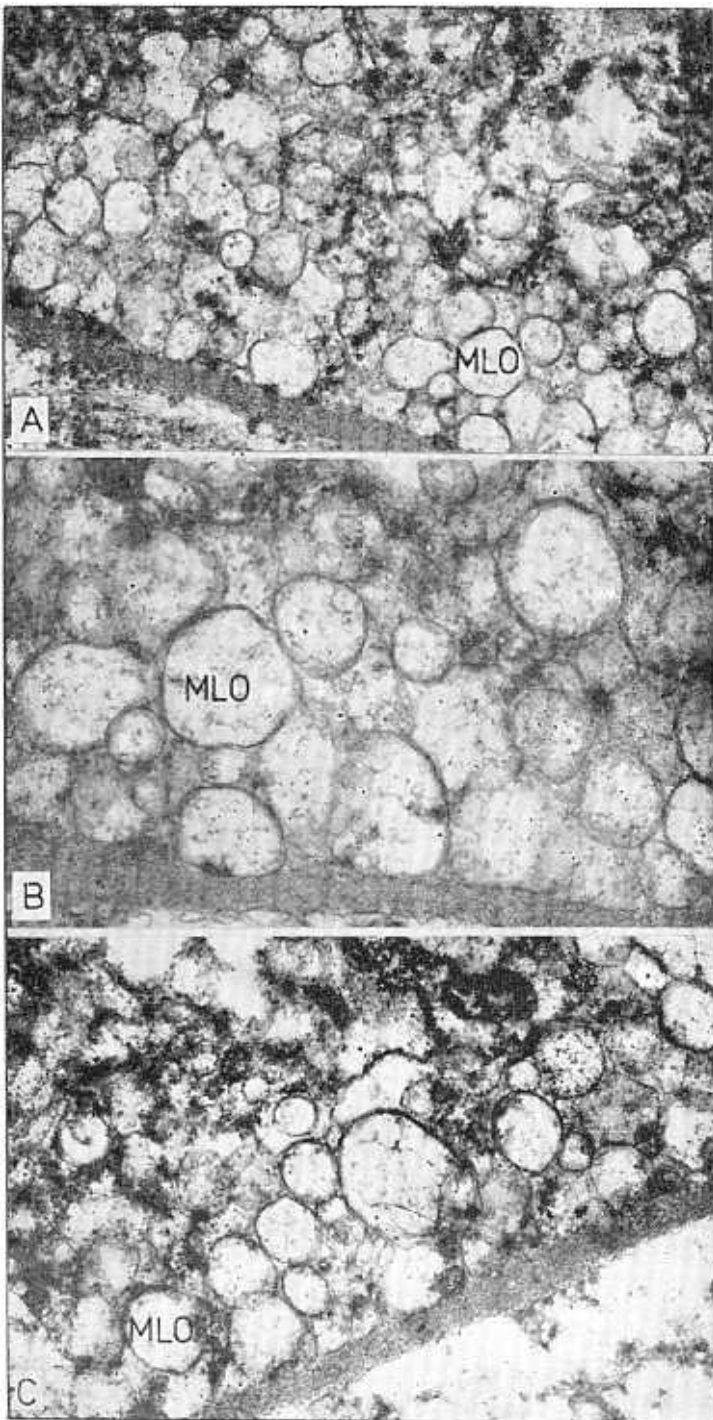   Large aggregations of MLO near the basement membrane of the organs had been reported from other insect vectors also (Nasu, *et al.,* 1970). Usually MLO enters the tissues of the intestine through the lumen of the alimentary canal and the salivary glands through the hemolymph (Tsai, 1979). Fewer number of MLO in the salivary gland as compared to that in intestine could be due to the early dissection of insects before considerable multiplication of the MLO could occur in the salivary glands. However this explanation is not conclusive as the acquisition period of the vector and the minimum incubation period required by the pathogen inside the vector are not known. Also since the insects were collected from the field it was not possible to exclude the possibilities of their acquiring sandal spike pathogen through feeding on diseased sandal or even the alternate hosts in the field prior to their deliberate confinement to the spiked plants as done in the present study. However, positive results achieved in the transmission of the disease through the vector, the presence of MLO in the intestine and salivary glands of the vector and in the phloem tissues of sandal, diseased through insect transmission, confirm that *R. bimaculatus* is capable of transmitting spike disease of sandal. In spite of the presence of insect growth inhibitor in sandal (Sankaranarayanan *et al.,* 1979), *R. bimaculatus* feeds and breeds well on sandal. The biology and behaviour of the insect, virulence, minimum duration for successful acquisition feeding, incubation period, etc. need to be studied in detail so as to understand the full potentiality of *R. bimaculatus* as a vector of sandal spike disease.

ISOLATION AND CULTURING OF SANDAL SPIKE MYCOPLASMA

INTRODUCTION

The causative organisms of yellows-type diseases have been recognised as structures resembling mycoplasmas. However, spiroplasmas are the only plant pathogenic mycoplasmas that have been cultured in *vitro* (Daniels. 1983). Cultivation of Citrus stubborn spiroplasma was achieved in 1971 (Fudl-Allah *et al.,* 1971; Saglio *et al.,* 1971) and corn stunt spiroplasma in 1974 (Chen and Liao, 1975; Williamson and Whitecomb, 1975). Nayar and Ananthapadmanabha (1970) claimed *in vitro* culturing of sandal spike pathogen in a semisynthetic medium Recently Nayar (1984) claimed proof of Koch's postulates and mentioned that the liquid culture containing the sandal spike mycoplasma was deposted in 1980 at the FAO/WHO Reference Centre in Denmark. But on enquiry, it is understood that such a culture is not available with this Centre. Hence reports on *in vitro* cultivation of sandal spike mycoplasma is yet to be confirmed. Some preliminary attempts, were made in this laboratory to cultivate the organism *in vitro.*

MATERIALS AND METHODS

For *in vitro* culture of MLO associated with spike disease of sandal the following culture media were tried.

1. Plant mycoplasma medium No. ATCC 675 ⁃ **Kondo** *et al* **,** 1977)

Difco PPLQ broth	21g
D-Fructose	I g
Glucose	1 g
Sucrose	I g
Sorbitol	50 g
Peptone	I g
Bactotryptone	l g
Glass distilled water	700 ml
Horse serum	200 ml
pH 7.2-7.4	

2. C-3G Medium (Liso and Chen, 1975; 1977)

PPLO broth base	15
Sucrose	120 g

Horse serum	200 ml
Double distilled water	720 ml
Phenol red (1% solution)	2 ml
pH 7.2-7.4	

3. **SMC Medium (Saglio** *et al.,* 1971; 1973)

PPLO broth base	34 g
Tryptone	10 g
Glucose	1 g
Fructose	1 g
Sucrose	10 g
Sorbitol	70 g
Fresh yeast extract 25%	100 ml
Horse serum	200 ml
Phenol red (1% solution)	2 ml
Double distilled water	574 ml
7.2-7.4	

Preparation of media

All the ingredients except horse serum and fresh yeast extract were dissolved in distilled water and autoclaved at 15 psi for 15 minutes.   Horse serum and yeast extract were separately filter-sterilised   passing through Seitz filter, and phenol red solution separately autoclaved and later aseptically added to the broth base.   Five millilitre of the medium was transferred aseptically to screw cap bottles.

Isolation method: Stem bits 1.0 to 1.5 cm in length, were cut from the young diseased stem and immediately surface sterilised in 2.5% sodium hypochlorite solution for 2 to 5 minutes.   After rinsing twice or thrice in sterile water, the stem pieces were dipped in 75% ethyl alcohol for 15 seconds and flamed quickly.   The surface sterilised bits were then placed in sterile petridishes containing 2 ml of culture medium and cut into small pieces.   The pieces were macerated and squeezed using forceps so that the inoculum was released into the medium.   This source inoculum was then added to 20 ml of fresh medium and thoroughly mixed and filtered through millipore filter (450nm) (Millipore Corporation, Massachussetts, USA) to remove possible contami-nation of bacteria.   Half a millilitre of the final inoculum was then added to 5 ml of fresh medium and inoculated into screw cap bottles.   In order to prevent the anti-spiroplasma action of plant tissue extracts, the primary culture was serially tenfold-diluted in fresh medium at two days' intervals.   The bottles were incubated at 28°-30°C and observed for growth indicated by colour change of the medium from red yellow.

RESULTS AND DISCUSSION

No colour change was observed in the bottles indicating absence of MLO multiplication. Samples from the inoculated tubes were also regularly examined in dark field microscope for any microbial growth. However, no such growth was observed.

Culturing of sandal spike pathogen has been attempted earlier also by Muniyappa *et al.,* (1980) using the medium of Nayar and Ananthapadmanabha (1970) in addition to plant Mycoplasma medium No. 675 and BS-4 medium. But these media also did not support the growth of MLO of sandal spike disease. Muniyappa *et al.,* (1980) were unable to confirm the results reported earlier by Nayar and Ananthapadmanabha (1970)

Mycoplasmas are very fastidious organisms. The number of spiroplasmas that have been grown in artificial media represent only a very minor proportion of the total number of MLO that have been demonstrated by electron microscopy in different plants and insects (Freundt, 1981). Most of the noncultivable MLO do not appear to possess the helical morphology of the cultivable spiroplasmas. The non-helical MLO have resisted till now any attempt of their cultivation in artificial media. Although several claims of cultivation of MLO have been reported, none of them have been confirmed.

CHEMICAL CONTROL

INTRODUCTION

Though research on spike disease and its management started several decades ago, no satisfactory progress had been made towards its effective control or prevention of fresh outbreak. Attempts in the past for control of the spike disease through eradication of all diseased sandal in an area, providing a buffer zone of cleared area where all the healthy and diseased trees were extracted in between healthy and diseased zones, weeding and clear-felling of all miscellaneous trees other than sandal in a sandal reserve to suppress alternate hosts, etc. were not successful. In fact clear-felling of trees other than sandal aggravated the disease situation by rapid destruction of spiked trees (Anon, 1955).

Antibiotic treatment of plant diseases associated with MLO was attempted mainly after the discovery of suppressive effect of tetracycline group of antibiotics on symptom development of mulberry dwarf disease (Ishiie *et al.,* 1967) and of tetracycline and chloramphenicol against aster yellows diseases (Davis *et al.,* 1968). Recently Sinha (1979) and McCoy and Williams (1982) have reviewed the work on the control of Plant mycoplasma diseases using chemicals including antibiotics.

Various methods such as foliar sprays, direct injection and infusion have been used for treating plants with antibiotics. Though remission of disease symptom has been induced by foliar sprays, its utility appears to be limited because of larger quantity of antibiotics needed for therapeutic response and the widespread environmental contamination (McCoy and Williams, 1982). Infusion or injection of antibiotics into the diseased trees has been approved for commercial use on several diseases in USA. Injection of oxytetracycline-HC1 as a prophylactic treatment to protect healthy palms in areas of high lethal yellowing disease in Florida has been found to be quite successful (McCoy, 1982). The various tree injection methods adopted earlier for the control of lethal yellowing of coconut involved gravity flow, air pressure injection and the Mauget injector (Hunt *et al.* 1974; McCoy, 1974). Lacy (1982) reported that when oxytetracycline-HCl was infused into X-diseased scaffolds of peach and nectarine. using gravity flow technique, the damage to the treatment site was less when compard to the pressure injection method and Mauget infusion method. The advantage of injection and infusion methods is such that a smaller amount of antibiotic only is necessary as compared to that in spraying and also the environmental pollution hazards are minimised because the chemical is confined internally to the treated plants.

The degree of response of tetracycline against MLO diseases varied with plant age, severity of symptoms and method of treatment. Although the response is temporary, in a few cases like coconut lethal yellowing (McCoy, 1974; 1982), pear decline (Nyland and Moller, 1973) and paulownia witches' - broom (Yi *et al.,* 1981) this temporary remission period is of sufficient duration and antibiotic application has become a practical field control measure.

Raychaudhuri *et al.,* (1972) and Nayar *et al.,* (1973) reported temporary remission of spike disease symptoms for 75 to 90 days after infusion of tetracycline group of antibiotics. Rao *et al.,* (1975) found that a combination of benlate and terramycin or terramycin alone showed recovery in disease symptoms 25 to 30 days after treatment; but the period of remission lasted from 30 to 130 days only. There are a number of reports of application of mycoplasma-static substances by girdling method (Raychaudhuri *et al.,* 1972; Raychaudhuri, 1977; Rao *et al.,* 1975) foliar spray, paste method after debarking and by introducing through cut end of twigs (Nayar *et al.,* 1973). Raychaudhuri, *et al.,* (1972) found that application of anti-biotics by 'girdling' method was better than spraying, soil drenching and cut and dip method. But McCoy (1976) reported that solid implants of oxytetracycline- HCI had little effect, possibly due to the phytotoxicity of high concentrations of the antibiotic during a period of slow solubilisation. Spraying method sometimes produced no appreciable intake and this method has the drawback of affecting non-target microorganisms.

A tree infusion technique developed earlier as a method for controlling teak mistletoe (Ghosh and Balasundaran, 1980) was found to be suitable for applying antibiotics into spike diseased sandal trees. In this chapter the results of infusion of various antibiotics and some mycoplasma-static substances, singly, repeatedly and in different doses are described.

MATERIALS AND METHODS

Experimeotal area

The experiments were conducted in Sandal Reserve 51 of Marayoor forest range of Munnar forest division.

Standardisation of Tree infusion technique

The tree infusion method developed in this laboratory is a gravity flow technique (Ghosh and Balasundaran, 1980). It consists of locally fabricated metallic nozzles (N) which are tightly screwed into holes, hand-drilled in the main trunk of the trees at a height of 1 m above the ground. Nozzles are connected to a distributor (D) through polyethylene pressure tubes which in turn are connected to a

disposable glucose saline set used as reservoir (R) with dripping device (C) and stop cock (S). The reservoir is filled with an aqueous solution of tetracycline and the flow of solution can be monitored through the dripping device. After the completion of infusion, the holes are sealed with bee wax to avoid colonisation by pathogenic micro-organisms and insects (Fig. 13).

The study to find out seasonwise uptake of antibiotic was conducted during 1980 to 1983. The infusion experiment was started in July 1980 immediately after the detection of spike disease in Marayoor.   During 1982-83 the infusions were done once in alternate months beginning from July 1982.   The diseased trees infused with various tetracyclines and other compounds were included in the three infection intensity classes viz., mild (Class 11-5 to 25% infection), moderate (Class 111-25 to 50% infection) and heavy (Class IV-50 to 75% infection).

Screening of tetracyclines for disease recovery

Five tetracycline antibiotics, viz., tetracycline hydrochloride (Tetracycline-HC1-Indian Drugs and Pharmaceuticals Ltd.), Oxytetracycline-HC1 (Terramycin-Pfizer India Ltd.), Demethyl chlor-tetracycline-HC1 (Ledermycin-Lederle), Chlortetracycline-HCl (Aureomycin-Cyanamid) and Doxycycline (Mortidox, Mortel Hammer) were used in this study. Aqueous solution (500 ml) of each of these antibiotics was infused into diseased trees.   Treated trees were observed 30 days after infusion and based on remission of disease symptoms, they were grouped into three arbitrary percentage classes as follows:

% recovery	Symptoms
1-25	New sprouts of the leaves large and normal in size; the lower leaves still smaller showing spiked symptom.
25-75	New shoots with normal broad and healthier leaves.
75-I00	Completely recovered from the disease with flowers and fruits.

Iofusion of antibiotics repeatedly and in combination

Repeated infusions using tetracycline-HCl, oxytetracycline-HC1 and demethyl-chlor tetracycline-HC1 were carried out to ascertain whether there was any permanent control or long lasting disease remission of the spiked trees. In this experiment spiked trees were infused with each of the tetracycline compound separately at 500mg/tree in 500 ml of water once in 60 days starting from July 1982. The infusions with each antibiotic were given in such a way that there were 6 group of trees which received 1, 2, 3,4,5 or 6 infusions in a year.   Water was infused as control.

In another experiment tetracycline-HC1 oxytetracycline-HC1 and demethyl chlortetracycline-HC1 were mixed in equal ratio and infused into trees at 500 mg in 500 ml of water during June 1983.

Effect of higher does of tetracyclines

In order to find out the optimum dosage of tetracyclines and whether high dosage gives long lasting remission, tetracycline-HCI was infused into spiked trees at higher concentrations, viz., 2, 4, 8 and 12 g per tree. The solutions for first two dosages were prepared by dissolving the chemicals in 500 ml of water and for third and fourth concentrations, respectively 1 and 1.5 l of water was used.

Screening of oxytetracycline-HCI Tree Injection Formula and other chemicals

The Tree Injection Formula (Oxytetracycline-HCI 20% soluble powder, Pfizer India Ltd., Bombay), digitonin, 2-1, guanidinododecane acetate and cephalaxin were also screened. In the case of tree formula, dosage of oxytetracycline-HCI was calculated in terms of active ingredient. All chemicals were dissolved in 500 ml of water and infused into the trees around 8 AM.

RESULTS AND DISCUSSION

Standardisation of infusion technique

Tree infusion method for administering aqueous solution of antibiotics was successfully used in the field. Trees with girth range of 20 to 30 cm needed 2 nozzles, whereas a girth of 31 to 50 cm 3 nozzles and 51 cm and above, 3 to 4 nozzles. No infection or damage to tissues surrounding the holes drilled for fixing the nozzles could be observed. However the bee wax used to plug the holes got removed after one year.

The results of the study made during 1982 to 1983 indicated that the uptake of antibiotics depended on a number of factors such as season, girth of the tree and also the number of nozzles used. During bright sunny days preceded by a few showers, the uptake of antibiotic was rapid. The uptake (500 mg to 4 g/500 ml) was maximum during June to November (Table 5). On a bright sunny day, 500 of aqueous solution of antibiotics could be infused into medium sized spiked trees within 3 to 6 hours during these months, whereas it took 24 to 48 hours during December-February and more than 48 hours during March-May. However, water alone was taken up faster than the antibiotic solutions in the respective seasons. It is evident from Table 6 that June to November is the main rainy season with warm climate and this season coincides with period of maximum uptake. Probably during this season there will be increased physiological activity leading to faster growth of

plants. The rain may increase the root pressure and the sunshine the transpiration rate and hence the rapid uptake. The uptake of antibiotics was relatively slow in heavily diseased trees whereas it was quite rapid in trees in the initial stages of infection with full foliage. The gravity flow technique was earlier adopted for infusing water soluble chemicals into teak (Ghosh and Balasundaran, 1980) and it was found that the maximum uptake was during the rainy seasons.

Table 5. Uptake of tetracyclines, oxytetracycline tree injection formula and other chemicals in spiked sandal tree of GBH>51 cm in different seasons during 1981‑1983.

Season	Chemical	Dosage in 500 ml of water	Time taken (hr)
June-November	Tetracycline Compounds	500 mg to 4 g	3—6
	Tree injection formula	3 g	3—6
	Other chemicals"	Upto 500 mg	3—6
	Water		2—5
December-February	Tetracycline Compounds	500 mg to 4 g	24—48
	Tree injection formula	3 g	24—48
	Water		24—30
March-May	Tetracycline Compounds	500 mg	>48
	Water		24—48

* Digitonin, Cephalaxin, 2- I, Guanidinododecane acetate.

Table 6. *Rainfall and temperature data for Marayoor*

Month	Rainfall (mm)			Temperature (c)			
	1980	1981	1982	1981		1982	
				Maximum	Minimum	Maximum	Minimum
January	---	17.0	—	30	10	30	I1
February	—	—	—	32	10	33	12
March	88 5	93.1	59.2	34	12	35	14
April	87.5	98.8	32.0	35	16	35	14
May	98.6	144.4	94.0	36	18	35	18
June	111.6	169.2	39.4	32	18	34	20
July	119.4	113.2	84.6	33	16	34	19
August	57.0	149.2	63.0	31	16	32	18
September	156.4	208.0	110.0	31	15	33	16
October	185.3	238.2	185.0	30	15	32	17
November	214.2	85.4	167.0	29	12	30	16
December	113.4	123.0	34.0	29	11	27	11

Compared to pressure injection method and Mauget injection method, the gravity flow method used in the present study is less expensive. The components of the infusion device are locally made and they are reusable for a number of times. This technique can be used for treating the diseased trees in sandal reserves on a large scale (Fig. 13).

Screening of various tetracycline compounds for disease recovery

All tetracycline compounds gave temporary disease remission falling in different percentage classes (Table 7). The remission was usually observed 25 to 45 days after infusion. In all the cases percentage recovery was between 25 to 100%, Sandal trees with moderate infections flowered but there was no fruit setting. The period of remission lasted for 3 to 5 months irrespective of the chemical after which the disease reappeared. The trees with mild infection responded better than the ones in advanced stage of infection.

Temporary remission of the symptoms of several mycoplasmal diseases has been demonstrated by the treatment with tetracyclines. Oxytetracycline-HC1 is the most common in use. The length of remission period varied greatly with type of antibiotic, their concentration, method of administration, host, and severity of the disease at the time of treatment (Davis and Whitecomb, 1970.) The rate of development of lethal yellowing disease in coconut palms was slowed after the injection with tetracycline (Hunt *et al.,* 1974). Rosenberger and Jones (1977) reported that a single injection of 1.25 to 3.75 g of oxytetracycline-HC1 per peach tree induced remission of peach X-disease symptoms for one year. But Ying (1979) reported that infusion of 2 1 of 500 ppm achromycin (tetracycline-HCI) per plant once in March and the other in September, completely cured 2-year-old witches'-broom diseased trees; 3-to 4-year old trees showed only partial recovery. McCoy (1975) found that doses as small as 50 to 100 mg/tree induced symptomatic remission in early stages of coconut yellowing. Furthermore, though MLO got degenerated during the disease remission period, they reappear in new growth at the end of such remission period (McCoy, 1982).

Repeated infusions of tetracyclines

Repeated infusion of the three tetracycline antibiotics (Table 8) (tetracycline-HCI, oxytetracycline-HCI and demethyl chlortetracycline-HC1) and their combinations as mixtures (Table 9) failed to give complete or prolonged remission and it lasted only for four months after the first infusion as in the case of single infusion. The failure to give sustained recovery after repeated infusions may possibly be due to the loss in activity of the antibiotics given in subsequent administrations.

This was partly confirmed from a bioassay study carried on the tetracycline solution in the reservoir using *Escherichia coli* as the test organism. The results
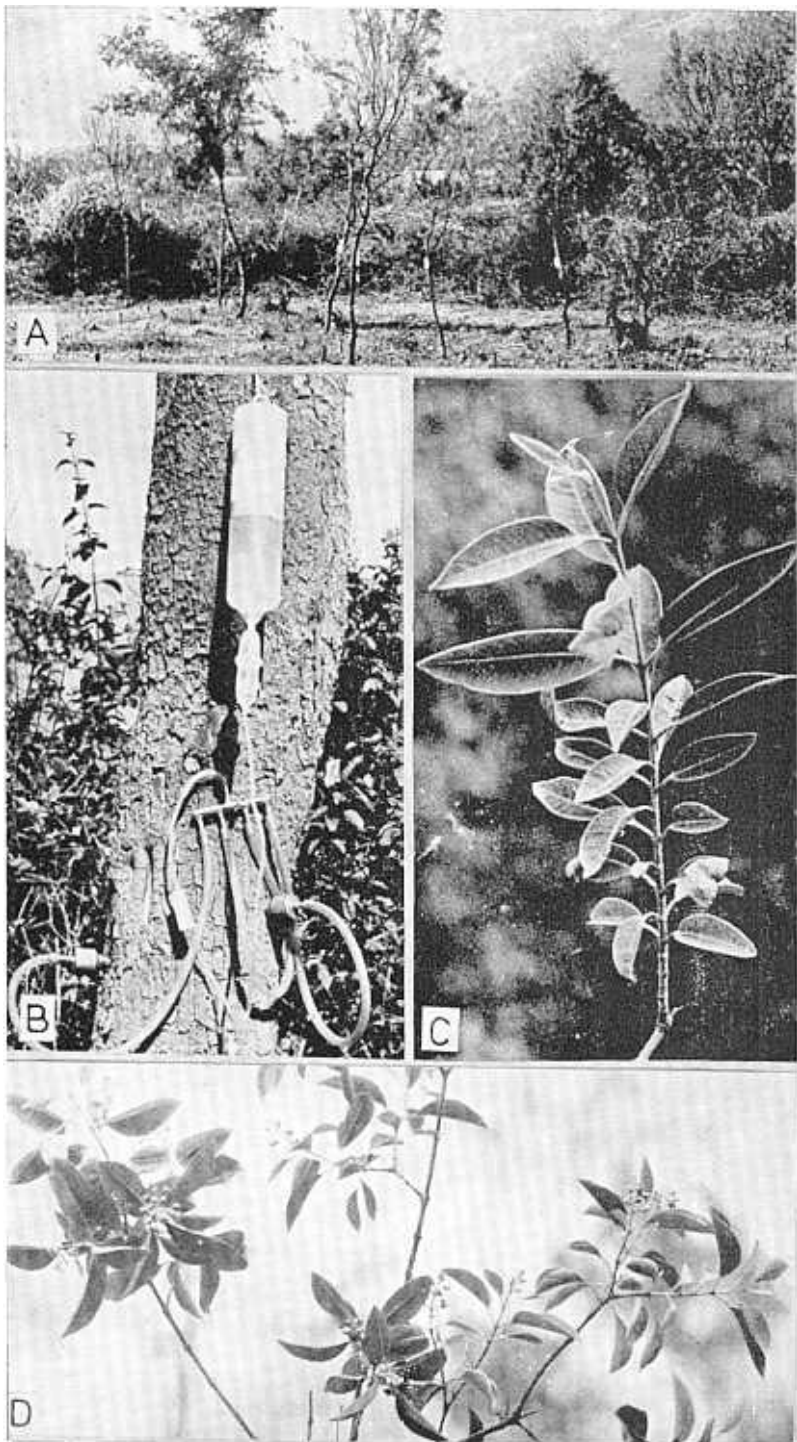
Fig. 13.   Tetracycline therapy of spike diseased sandal.   A, Infusion of antibiotic solution into diseased trees in the field; B, close-up of infusion device; C, disease remission; note the narrow leaves at the lower part of the twig, and the broad leaves formed after the infusion of the antibiotic solution; D, portion of a tree showing normal leaves; note the flowers on some branches.

indicated that degradation of tetracycline was 14.67, 19.90, 20.36 and 25.48% within 24, 48, 72 and 96 hrs respectively over control. Evidently, the loss in activity of tetracycline caused by the long duration taken for infusion during November-May possibly rendered the antibiotics ineffective to give sustained remission. However, this aspect needs further investigation.

Table 7. Number of spiked trees showing disease remission after infusion of various tetracyclines.

Treatment	Dosage per 500 ml of water	Girth range (cm)	Symptoms range"		
			Mild	Moderate	Heavy
1. Tetracycline-HC1	500mg	30-69	3/3	1/1	2/2
2. Oxytetracycline-HC1	„	27-55	1/1	....	3/3
3. Demethylchlor tetra- cycline-HCI	"	20-62	1/1	2/2	1/1
4. Chlortetracycline-HC1	"	19-35	1/1	2/2	0/1
5. Doxycycline	"	18-64	....	3/3	0/1

*   Numerator denotes the number of trees showing recovery symptoms and the denominator total number of trees treated.

Table 8. Effect of repeated injection of tetracycline on spike disease

Sl.No.	Chemical	Dosage in 500ml of water	Girth range in cm	No. of infusions (once in 60 days)*					
				1	2	3	4	5	6
1.	Tetracycline-HCI	500 mg	25-69	4/4	0/4	0/4	0/4	0/4	0/4
2.	Oxytetracycline-HCI	500 mg	21-65	4/4	0/4	0/4	0/4	0/4	0/4
3.	Demethylchlor tetracycline-HCI	500mg	18-62	4/4	0/4	014	0/4	0/4	0/4
4.	Control (Water)		22-71	0/4	0/4	014	0/4	0/4	0/4

*   Numerator denotes healthy growth and denominator, number of trees treated.

Table 9. Effect of combinations of various tetracyclines on remission of spike disease

Treatment	Dose	Symptoms range*		
		Mild	Moderate	Heavy
1. Tetracycline-HCI + demethyl-chlortetracycline-HCI	500+ 500mg/500ml	0/1	0/2	...
2. Tetracycline-HCI + Oxy-tetracycline-HCI	500 +500mg/500ml	2/2	1/1	...
3. Oxytetracycline-HCI+ demethylchlortetra-cycline-HCI	500+ 500mg/500ml	2/3	...	...
4. Tetracycline-HCI + Oxytetracycline-HCI + demethylchlortetra-cycline–HCI	500+ 500+500mg/500ml	...	3/3	...

* Numerator denotes number of trees showing recovery symptoms and denominator number of trees treated.

Remission of symptoms of lethal yellowing in coconut palms lasts from 4 to 7 months and retreatment of such palms at 4-month intervals brings about sustained disease remission.   Whereas in pear decline, it lasts for 2 to 3 years and repetitive applications are given at a 2-year interval to achieve complete remission (McCoy, 1982).

High dose of tetracyclines

The remission could be prolonged upto 7 to 8 months by infusing higher dose of tetracycline-HC1 (Table 10). There was no visible phytotoxic symptoms on trees upto a dose of 8 g. However, a dose of 12 g showed phytotoxicity in the form of browning and yellowing of leaves and the trees dried eventually.

High dose of tetracyclines has also been used to prolong the remission in other diseases. McCoy (1975) showed that lower doses responded well but higher dose of 20 g/tree gave longer lasting remission of lethal yellowing of coconut. Ying (1979) found that injection of 2 litres of 1 to 2 g of achromycin (tetracycline-HC1) in summer and spring could control paulownia witches'-broom in 2 to 3-years-old trees whereas 2 to 3 litres of 2 to 4 g of achromycin twice a year was necessary for 4 to 5-year-old trees.

In sandal spike disease the minimum dosage of tetracycline to bring about noticeable remission was earlier reported to be 300 to 500 mg/tree (Raychaudhuri, 1977; Nayar *et al.,* 1973). Nayar *et al.* (1973) found that antibiotics at 1.5 g/tree caused phytotoxicity in the form of foliage scorching, dropping of leaves and even drying up in a few trees. However, our studies indicated that dosage as high as 8 g/tree in the girth range of 31 to 37 cm did not cause any visible phytotoxic simptom and it was only 12 g/tree which gave phytotoxic symptoms.

Table 10. Effect of higher doses of tetracycline-HCI on remission of spike disease

Treatment	Dose	Girth range (cm)	Symptoms range *			Remission Period
			Mild	Moderate	Heavy	
1. Tetracycline-HC1	2g/500 ml	34-52	2/2	2/2	....	7 months
2. "	4g/500 ml	52-57	1/1	3/3	....	7 "
3. "	8g/500ml	31-37	2/2	1/1	....	8 "
4. "	12g/1500ml	41-71	1/1	1/2	....	Phytotoxic

* Numerator denotes number of trees showing recovery symptoms and denominator number of trees treated.

Screening of other chemicals including oxytetracycline hydrochloride Tree Formulation

Oxytetracycline-HC1 tree formulation, digitonin and 2-1, guanidinododecane acetate gave temporary remission for 3 to 5 months after 25 to 45 days since infusion, as in the case of other tetracycline compounds (Table 1I).

The oxytetracycline tree injection formula did not help in prolonging the remission period further from four months. This antibiotic had responded well in suppressing lethal yellowing of coconut palm when infused in doses ranging from 0.5 to 20 g/tree (McCoy, 1975).

Oxytetracycline-HCl tree injection formula (Terramycin-pfizer Inc), which has been registered for use in the control of lethal yellowing of coconut palm in Florida, USA, is stabilised by the addition of citric acid and so were the tetracycline compounds tested in treatment of eastern-X-disease of peach (Sands, 1974; Sands and Walton, 1975). Acidic tetracycline formulations are more effective with injection/infusion treat ment methods because acidic tetracyclines are more readily transported by xylem (Dimond, 1965). Basic substances tend to be absorbed by the negative charges on xylem walls (Marsh, 1977).

Table 11. Effect of oxytetracycline-HC1 tree injection formula and other chemicals
on the disease remission in spiked trees

Treatment	Dosage per 500 ml of water	Girth range (cm)	Symptoms range *		
			Mild	Moderate	Heavy
1. Tree Injection Formula	3 g	31-39	1/1	2/2	1/I
2. "	6 g	25-38	1/1	3/3	...
3. Digitonin	25mg	20-28	1/1	...	1/1
4. "	50mg	23-47	—	1/2	...
5. "	100 mg	22-25	1/1	...	1/1
6. 2-1, Guanidinododecane acetate	500mg	46-48	1/1	1/1	...
7. Cephalaxin	500 mg	46-71	0/2	...	...

* Numerator denotes the number of trees showing recovery symptoms and deno-
minator number of trees treated.

Application of digitonin did not cause any phytotoxicity. Digitonin
sensitivity is used in the laboratory to differentiate acholeplasmas from the other
mycoplasmas. Mycoplasmas which are sterol dependent get inhibited by digitonin
whereas acholeplasmas do not get inhibited as they are sterol nondependent (Archer
and Daniels, 1982).. 2-1, Guanidinododecane acetate may be acting as a structural
analogue of guanidine and the mycoplasma-static effect might be due to the incorpor-
ation of the chemical into MLO DNA and the consequent suppression of MLO DNA
replication.

Cephalaxin as expected, did not give any remission because it acts on
cell wall synthesis and mycoplasmas do not have any cell wall.

SUMMARY AND CONCLUSIONS

In Kerala, Sandal Reserved forests (area 15.45 km^2) and the adjoining revenue lands (area 47.26 km^2) of Marayoor forest range of Munnar forest division constitute the main sandal tract. The number of sandal trees per unit area in Marayoor is the highest among the sandal reserves of India; it is also considered to be the best seed stand. Spike disease of sandal, causing heavy damage to sandal trees in Karnataka and Tamil Nadu, is reported from Marayoor sandal reserves also. The pathogen of the disease is identified as mycoplasma-like organisms (MLO)

Though the spike disease was first observed in Marayoor forest range during 1980 only, a survey conducted subsequently indicated that the disease might have appeared in the Sandal Reserve 51 (382 ha) a few years earlier Till now, more than 50% of the sandal trees in this Reserve have been found to be affected with the spike disease. In the diseased area, most of the trees were either already dead or in various stages of infection. The disease is spreading towards the boundary of the reserve, the adjacent Chinnar Reserve and the revenue lands. Rate of disease spread in two disease monitoring blocks, calculated in terms of apparent infection rate, was 0.080 and 0.066 per unit per month respectively. *Zizyphus oenoplia*, *Stachytarpheta* sp. and *Jasminum rigidum* showing witches'-broom disease symptoms found at several places in the reserve, are suspected to be the alternate hosts of the spike pathogen.

Histopathological studies, using aniline blue and Hoechst 33258 gave evidence on the occurrence of MLO in the diseased phloem tissues. The technique can be used as an easy method of disease detection. Electrophysiological studies using Shigometer indicated a positive correlation between severity of the disease and electrical resistance of inner bark in diseased trees.

Though the presence of a large number of MLO in the phloem tissue was confirmed in transmission electron microscopy of diseased tissues, attempts to culture the organisms *in vitro* were not successful.

Through insect transmission experiments on healthy seedlings, *Redarator bimaculatus* was identified as the possible vector. The role of *R. bimaculatus* as an insect vector of spike disease was confirmed through electron microscopy by the presence of MLO in the intestine and salivary glands of the insects, fed on spiked sandal plants and in the phloem tissues of plants infected through the vector.

Chemotherapy of sandal spike disease was attempted, employing a cheap and efficient method of infusing various tetracycline compounds into diseased trees. Infusion of aqueous solution of 500 mg of tetracyclines dissolved in 500 ml of water in spiked trees gave remission of the disease symptoms lasting for 3 to 5 months. Repeated infusion of tetracycline-HCl in alternate months for an year, did not give complete recovery. Higher doses of tetracyclines prolonged the period of remission upto seven to eight months. Infusion of tetracycline at 12 g per tree proved to be phytotoxic. Compounds like digitonin and 2-1, guanidinododecane acetate also gave temporary remission of disease symptoms.

Although, in general, complete recovery of MLO infected plants through antibiotic treatments were not achieved, remission of disease symptoms upto two years had been achieved in some tree species. Hence more studies need to be conducted to prolong the period of disease remission in spiked sandal so as to make the technique economically feasible for use in the forest on a large scale.

Infusion of tetracyclines in apparently healthy trees in spiked area as a prophylactic treatment can be tried which may be helpful in keeping the disease under check. Such a practice is successfully adopted in Florida, USA to save coconut trees from lethal yellowing, another serious MLO disease. Since a vector is identified now, studies on the management of spike disease through vector control using chemical or biological agents should also get priority.

For a long term solution of the spike. disease, production of disease resistant sandal trees through breeding could be the best method. In a heavily diseased area, the apparently healthy sandal trees will be promising in this respect. Such trees might have escaped the disease either through genetic resistance or evaded the disease through some other mechanism. Such trees should be preserved and utilised for production of seedlings through suckers and grafts for planting afresh in the diseased area. However, before any large-scale breeding work is initiated, such plants should be subjected to rigorous tests for establishing their genuine genetic resistance.

The inability to prove the Koch's postulates and the lack of knowledge on the biology of the vector could be the two impediments in resistance breeding and also in the biological control of the insects. Hence, serious attempts should be initiated for *in vitro* cultivation of the pathogen and for detecting the most efficient natural enemies of the vectors. Systematic studies on the life cycle of the vector, vector-pathogen relationship including minimum acquisition period, incubation period of the pathogen in the vector, feeding behaviour, percentage of viruliferous insects in the vector population etc. also should be carried out.

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