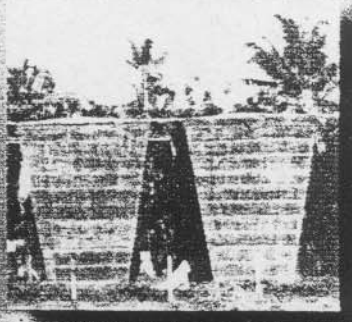
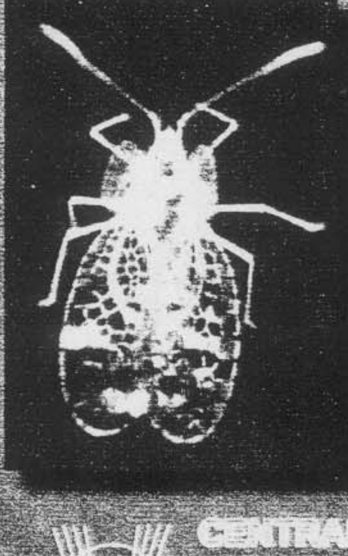
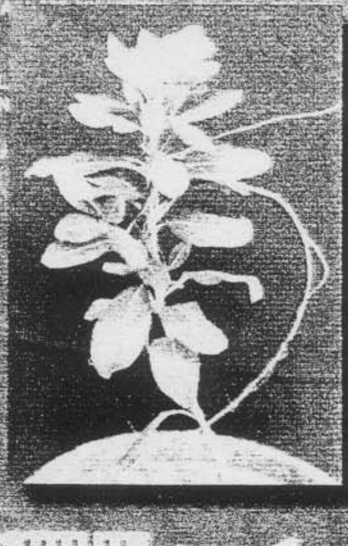
ROLE OF PHYTOPLASMA IN ROOT(WILT) DISEASE OF COCONUT







CENTRAL PLANTATION CROPS RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
REGIONAL STATION, KAYAMKULAM, KRISHNAPURAM
PIN - 690 533, KERALA, INDIA

ICAR

CPCRI

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ICAR

INDIAN COUNCIL OF AGRICULTURAL RESEARCH

Final Report

Project title

ROLE OF PHYTOPLASMA IN ROOT(WILT) DISEASE OF COCONUT

Date of start : 1983

Date of termination : 1997

CPCRI

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RPF III

FINAL REPORT

1. Institute Code No. : Path. 1.12(231)

2. ICAR Code No. : P1-83/1-ICI-H20/0311.

3. Name and address of Research Institute/Centre : Central Plantation Crops Research Institute, Kayangulam.

4. Project Title : **Role of phytoplasma in root(wilt) disease of coconut**

5. Name and Designation of Project Leader : Dr. J.J.Solomon, Principal Scientist

6. Name(s) and Designation(s) of Project Associates including Project Leader and Work Done.

S. No.	Name and Designation	Time spent (man months)	Work done
1.	Dr. J.J.Solomon, Principal Scientist	101	Electron microscopy of host and vector tissues
2.	Dr. M.P. Govindankutty, Sr. Scientist	75	Standardising and use of LM techniques for visualising phytoplasma in plant and vector tissues, culturing of phytoplasma in plant tissues.
3.	Dr. K. Mathen, Principal Scientist	74	Inventory of putative vector(s). Exptl. Transmission on coconut and test plants, field trial with insecticides and botanicals.
4.	Dr. C.P.R. Nair, Principal Scientist	86	
5.	Shri. P. Rajan, Sr. Scientist	142	Biology and bionomics of the insect vector.
6.	Dr. B. Sathiamma, Sr. Scientist	29	Inventory of mites and transmission studies.

7.	Smt M.Sasikala, Sr. Scientist	114	Dodder transmission to periwinkle and test plants, Serology.
8.	Dr. V. Rajagopal, Principal Scientist	11	Vascular sap collection and analysis.
9.	Dr. B. Chempakam, Sr. Scientist	26	" " "
10.	Dr. S.Robert Cecil, Sr. Scientist	8	" " "
11.	Smt. P.G.Kamalakshiamma, Scientist (Sr. Scale)	10	" " "
12.	Dr. N.Srinivasan, Sr. Scientist	81	Culturing
13.	Shri. M. Gunasekaran, Scientist (Sr. Scale)	69	Electron Microscopy, Culturing
14.	Dr. M. Kochubabu, Sr. Scientist	8	Culturing
15.	Dr.P.Chowdappa, Scientist(Sr.Scale)	36	Antibiotic therapy - uptake, translocation, distribution and residue analysis of OTC.
16.	Dr. A.K. Shukla, Scientist	29	Tissue culture
17.	Shri. N.G.Pillai, Sr. Scientist	84	Antibiotic therapy - field trial
18.	Shri. K. Madhavan, Sr. Scientist	6	Maintenance of EM
19.	Dr. Alka Gupta, Scientist	10	Electron microscopy

7.Location of Research Project with complete address : Central Plantation Crops Research Institute,

(Division/Section/Sub-Centre) Regional Station, Kayangulam.

8. Date of start : 1983

9. Date of termination : 1997

10. (a) **Objectives :**

Electron microscopical examination of different parts of coconut palms with characteristic symptoms of root (wilt) disease revealed the presence of phytoplasma in the sieve tubes of phloem tissues. Such intra-cellular organisms were not encountered in the tissues of healthy palms. Pathogenic nature of phytoplasmas has already been established for several plant diseases and reported for coconut diseases like lethal yellowing and related diseases in the Caribbean, Florida in the USA and West Africa , Coconut Stem necrosis reported from Malaysia and Sumatra as well as lethal declines in other palms.

The objective of the present study are :

i) Determine the role of phytoplasmas in coconut root(wilt) disease by establishing their constant association in different tissues and sap through electron microscopy,

ii) Develop rapid histochemical techniques for quick diagnosis of phytoplasma infection in plants,

iii) Study the structural alterations in the host at light microscopic and EM levels,

iv) Identify vector(s) and understand the mode of transmission ,

v) Analyse the vascular sap in order to develop suitable culture media that may support the growth of the organism in vitro,

vi) Culture the intraphloemic organisms to establish Koch's postulates,

vii) Undertake antibiotic therapy to establish the etiological role of phytoplasma.

(b) **Practical utility** (It should include background information and justification of the project in not more than 150 words):

Root(wilt) disease is the most serious, non-lethal but debilitating malady of coconut. The annual loss due to the disease is estimated to be about 968 million nuts. The total estimated monetary loss in terms of loss in husk, copra yield and number of leaves is of the order of about Rs. 5,000 million.

This century old disease was all along considered to be a disease of unknown aetiology. The results eminating from the project has conclusively established phytoplasma as the etiological agent, standardised light microscopic staining techniques for visualising phytoplasmal infection using optical microscope, identified the insect vectors of the disease and worked out their biology, bionomics and population dynamics, made attempts to culture the phytoplasma in cell-free media but attained success in maintaining it in plant tissues micropropagated *in vitro* and established the phytoplasmal etiology of the disease through differential chemotherapy and standardised serological techniques for disease diagnosis.

11. Technical Programme

I. ELECTRON MICROSCOPY

1. Thin Section Electron Microscopy :

   (a) Host tissues :

      i. Primary host - Healthy and root (wilt) disease affected coconut. Apical meristem,petiole, leaflet, root tips, inflorescence, embryo, endosperm, pollen etc.

      ii. Indicator plants - test plants employed in transmission trials.

   (b) Vector tissues : Salivary glands, brain and midgut samples of insects and mites, parasitic phanerogams.

2. Particulate preparations :

Semi-purified plant extract, vascular sap, vector haemolymph, culture samples and other negative stained preparations.

3. Serologically Specific Electron Microscopy :

4. Ultra-structural Alterations in Tissues :

II LIGHT MICROSCOPY

1. Histology and Histopathology

   a) Histology of the different parts of coconut and indicator hosts.

   b) Histopathology of protective, mechanical and vascular tissues esp. phloem in diseased coconut at different stages of disease, test hosts and parasitic plants.

   c) Microscopy of cells in tissue macerates, vascular sap and haemolymph of vectors.

   d) Diagnostic tests for phytoplasma infection, staining reactions and autoradiography.

2. Histochemistry

Localisation tests for nucleic acids, proteins, lipids, polysaccharides, phenols, enzymes-like hydrolases, oxidases and dehydrogenases in healthy and diseased tissues.

III TRANSMISSION

1. Inventory of Putative Vectors

   a) Traps : Sticky, rotary, suction, sweep net and light traps

   b) Direct examination

2. Vector tissues for Electron Microscopy : (As in I b.)

3. Experimental Transmission

   a) Coconut seedlings under insect proof cages

   b) Indicator plants

   c) Dodder

4. Biology and bionomics of suspected vectors

   a) Life history

   b) Feeding habits

   c) Population studies

5. Control of Vector

IV. VASCULAR SAP ANALYSIS

1. Standardization of methods for collection of vascular sap from inflorescence of apparently healthy and diseased palms.
2. Determination of rate of collection, pH and osmotic concentration of the sap.
3. Analysis of the sap for individual amino acids, nucleic acids, proteins, organic acids and sugars.
4. Estimation of lipids and sterols.
5. Determination of growth hormones by bioassay techniques.
6. Chemical analysis for major and micronutrients.

V. CULTURING

1. Standardizing methods of isolation of phytoplasmas from host and vector tissues.
2. Isolation and culturing of phytoplasmas in serum enriched mycoplasma media.
3. Assessment of coconut vascular sap as basal medium for survival and culturing of mycoplasmas.
4. Formulation of coconut vascular sap based media for isolation and culturing of phytoplasmas.
5. Propagation of phytoplasmas and candidate vectors in coconut tissue/organ culture.

VI. ANTIBIOTIC THERAPY

1. Standardization of injection techniques.
2. Bioassay to determine uptake, translocation and persistence of antibiotics.
3. Residual analysis of the chemicals in fruit components.
4. Field trials and assessment of remission.

12. Progress of work :

Coconut root (wilt) is a century old disease. The cause of the disease was unknown until the 1980's. A number of biological agents such as fungi, bacteria, viruses and nematodes were implicated as the etiological agent. However, none of these agents could be consistently found associated with the disease, nor their pathogenicity proved. Solomon *et al* (1983) reported the presence of Phytoplasma earlier referred as mycoplasma - like organisms in sieve tubes of meristem, petiole of juvenile leaves and root tips and indicated its presumable role as the etiological agent of the disease. This report and an earlier one by Nayar 1982 gained importance in the light of similar findings implicating Phytoplasma as the etiological agent of a number of coconut diseases . Lethal yellowing disease of coconut in Florida and Jamaica (Beakbane, Slater and Posnette 1972 ; Heinze, Petzold and Mawitz 1972; Parthasarathy 1972; Plavsic-Banjac, Hunt and Maramarosch 1972) and similar findings in Africa (Dollet and Giannotti, Renard and Ghosh 1977; Nienhaus and Steiner 1976; Giannotti, Arnaud, Dollet Delattre and Taffin 1975), lend further evidence. In this background this project was formulated with following lines of investigation.

- Electron microscopy of plant and insect tissues
- Light microscopy
- Transmission
- Vascular sap analysis
- Culturing &
- Chemotherapy.

I. ELECTRON MICROSCOPY

a) Plant tissues.

The objective of this study is to examine large number of samples of healthy and diseased palms of various age groups from different locations and of different intensities of disease to establish the constant association of Phytoplasma with the disease. The study also envisages EM examination of the putative insect vector tissues that have been offered acquisition and incubation period on diseased palms to ascertain the capability of the insect to acquire the Phytoplasma and act as a vector.

EM examination of coconut tissue:

Since the presence and distribution pattern of Phytoplasma in root (wilt) diseased coconut tissues is not known, to begin with, palms were subjected to destructive sampling and the following tissues were sampled. Sub-meristem, leaf bases of developing leaves (heart tissue), rachillae of tender inflorescence, spear leaf, mature leaf and freshly emerging roots - a cm away from the tip. The tissue samples were processed as per the following schedule (Thomas, 1979).

The spurr embedded blocks were sectioned in LKB ultramicrotome IV and the sections picked on uncoated 200 mesh copper grids. The grids were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined under Carl Zeiss EM 109 Turbo transmission electron microscope operating at an accelerating voltage of 50 kv.

EM examination of phloem tissues of 80 diseased and 75 healthy palms revealed the consistent presence of Phytoplasma in tissues of all the diseased palms and their total absence in healthy palms from disease free area (Table 1).

Table. I : EM examination of root(wilt) diseased coconut samples

Condition of palms	Location	No. of samples tested	No. of samples with phytoplasma
Healthy	Disease free area	75	0
Apparently Healthy	Diseased area	20	3
Disease early	Diseased area	40	40
Disease middle	Diseased area	25	25
Disease advanced	Diseased area	10	10

Tissues sampled : Root, Rachilla, leaves in case of non - destructive sampling. Heart tissue, in case of destructive sampling.

The palms examined are of various age groups, intensities of disease and from diffrent location. Irrespective of the age, location and intensities of disease, Phytoplasma was observed in tissues of all diseased palms. The concentration and distribution of Phytoplasma in the different plant parts of the palm was studied. The organism was found to be unevenly distributed between the vascular tissues. They were found in increasing numbers in the 'Sink' region particularly in the heart tissue (immature leaf bases and sub-metristematic tissue). This was followed by root tips and rachilla of unopened inflorescence. Degenerated or moribund forms are often observed in mature tissues (Solomon *et al* 1987). Neither every sieve tube nor all the vascular bundles contained Phytoplasma. Some of the individual vascular elements were totally free of the organism or contained fewer bodies in parietal position. Such uneven distribution has been reported in other Phytoplasmal diseases also, lethal yellowing disease of palms (Parthasarathy 1974 ; Thomas 1979), X-disease of Prunus (Garnet and Gilmer 1971) and certain other witches broom diseases (Hiruki and Shukla 1973 ; Seliskar *et al* 1973). In yellow leaf diseased areca palms, Nayar and Seliskar (1978) observed unexpanded tender rachilla to have more Phytoplasma than fully expanded leaves. Empty structures lacking internal contents only were evident in older leaves. The distribution of Phytoplasma in feeder roots, area close to tip region and mature portion of the root was studied. Phytoplasma in increased number and frequency was observed in the root tips.

Phytoplasmas are bound by a triple layered unit membrane and contain DNA strands and peripherally dispersed ribosomes (Solomon 1991). Pleomorphic forms ranging from circular to oval and occasionally beaded or filamentous ones are also observed (Fig 1). The coccoid forms are in the size range of 250-400 nm. Phloem paranchyma and companion cells were devoid of the organism. The cell walls of the Phytoplasma harbouring cells and the adjoining ones were often thickened, the cytoplasm granulated and contained vesicle like structures (Solomon *et al.* 1983).

Possible dual infection with flagellates was also studied in six palms each in the early, middle and advanced stages of the disease. The sieve tubes of rachilla of tender inflorescence of all the six diseased palms invariably contained Phytoplasma and were free of flagellates, viruses or any other micro-organisms. This results again suggest in unequivocal manner that the infection is due to a single pathogen namely the Phytoplasma and no other pathogen is involved.

Ultra-structural changes consequent to disease contraction was also studied. It was observed that sieve tubes that contained Phytoplasma and the bordering cells often had fibrillar tubules. The tubules occasionally had electron dense granules. They were identified as P-proteins (Solomon and Geetha 1993). Such fibrils have been recorded in Kaincope disease affected coconut palms (Dollet *et. al.* 1976). The P-proteins which are similar to microfilaments are supposed to provide the motive force to assimilate movement in sieve elements. (Hepler and Palevitz 1974). Although P-proteins are normal host components, their massive accumulation in sieve cells of diseased palms may be part of the host-parasite interaction.

The protophloem elements in roots and rachilla of diseased palms are compressed and had electron dense contents indicating necrotic obliteration. Deposition of Callose, a response to injury is often observed in sieve pores of Phytoplasma infected cells. Sieve plate pores of infected sieve elements were lined with callose.

Another prominent ultrastructural change is the elaboration of the membrane system. Plasmalemma which lines the cell wall makes deep intrusion into cell lumen forming invaginations. The invaginations often enclosed vesicular or membraneous structures lacking contents and are described as paramural bodies. The sieve tube plastids contained crystalline inclusions

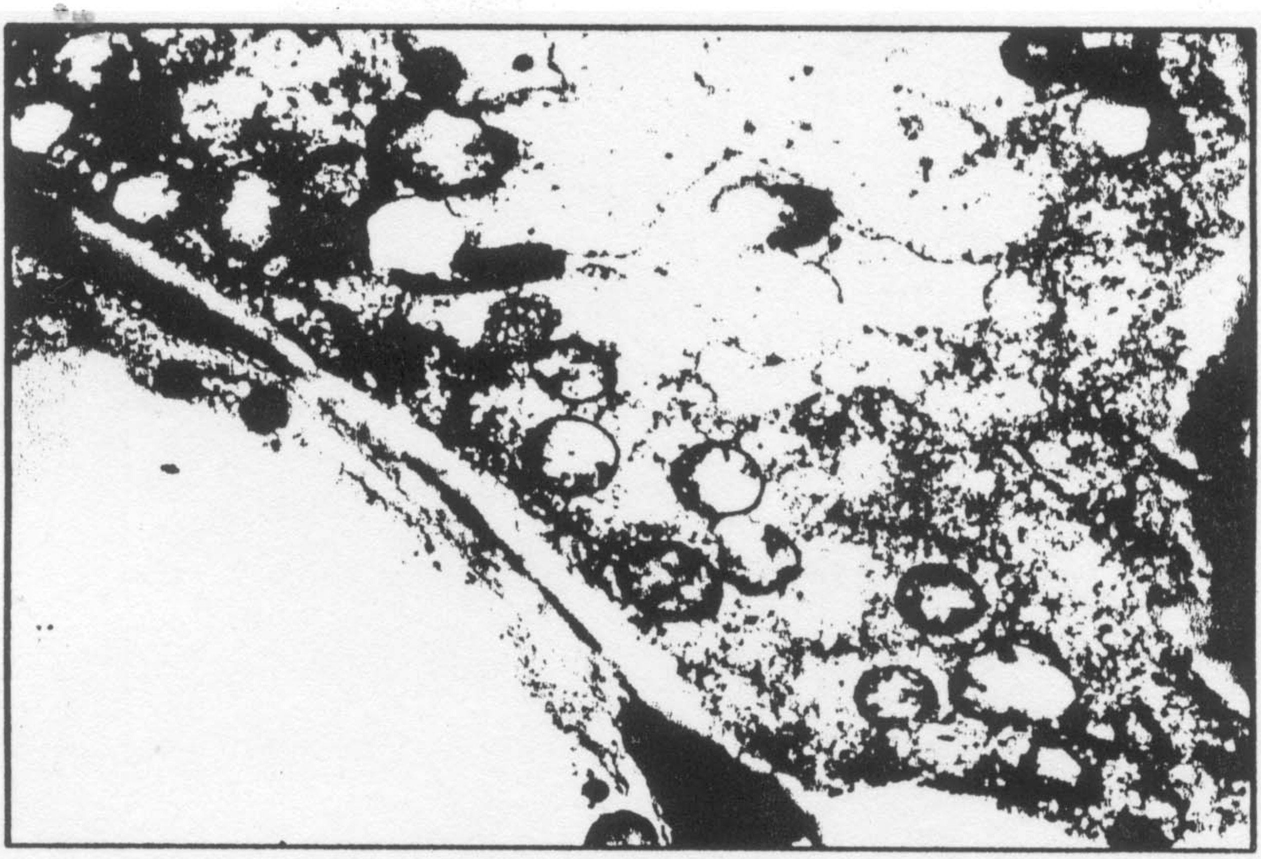

Fig. I. Phytoplasma in sieve tube of rachilla of root (wilt) diseased palm.

with well-defined crystalline lattice identified as 'Cuneate crystalline inclusions'. Clusters of electron dense osmiophilic membrane bound crystals rhombic or hexagonal are observed in rachilla of diseased palms. Wildman and Hunt (1976) interpreted these crystals as phytoferrittin, a normal host component that often accumulates in plants infected with biological agents. Most of these alterations denote degenerative changes as a result of pathogenesis.

Transmission of the disease through pollen and embryo was also investigated. Anthers of 10 diseased palms containing pollen grains were fixed, sectioned and the ultrathin sections examined under EM. Phytoplasma was not observed in the pollen grains. Similarly, embryos of nuts from diseased palms and tender roots of four sprouts of nuts of diseased palms were examined. Phytoplasma could not be observed in the embryos and sprouts. Recently, Phytoplasmal DNA has been detected in embryo of nuts collected from a few lethal yellowing diseased palms in Mexico (Oropeza *et. al.*, 1991). Similarly, Cadang-Cadang viroid is also detected in embryos and in pollens and reported to be seed transmitted at a low rate of about one in three hundred (Hanold and Randles 1991). As these sub-microscopic agents present in very low concentration can escape dectection through conventional microscopic techniques, more sensitive diagnostic techniques such as PCR and DNA hybridisation assay needs to be employed to determine transmission of Phytoplasma through seed.

Rachilla and Root tissues of five root (wilt) diseased palms in 2 locations were examined prior to the application of antibiotics and subsequently after second and third year. Phytoplasma in fewer numbers were observed in OTC treated palms. Rachilla of juvenile inflorescence of six antibiotic treated palms, three treated with Terramycin and three with Terramycin + Bavistin (T + B) were examined prior to the application of the antibiotic and subsequently, after first and second year of initiation of treatment. Phytoplasma was observed in all pre-treatment samples. After treatment sparse distribution of Phytoplasma was observed in T + B treated palms compared to pre-treatment. Two palms were found to be free of Phytoplasma.

b) Insect tissues :

The objective of this study is to examine selected organs such as gut, salivary gland and brain tissues of putative insect vectors to ascertain whether they could acquire Phytoplasma while feeding on diseased palms and can sustain multiplication.

Phytoplasma, in general are transmitted by insects that have mouth parts adapted for feeding on phloem tissues. Plant hoppers and leaf hoppers (Homoptera) are the phloem feeders that are known vectors of Phytoplasma. Lace bug being a Heteropteran insect its potential to feed on phloem had to be determined. Lace bugs fixed on feeding position on coconut pinnae by a cold immobilization technique was sectioned serially to trace the course of the stylet. The sections revealed the termination of stylet in phloem. However, lace bug is not an exclusive phloem feeder. It feeds mainly on palisade cells of the mesophyll tissue and also on phloem.

Having observed the phloem feeding nature of lace bugs, the next logical step was to determine whether the insect has the potential to acquire the phloem bound pathogen and sustains its multiplication to function as a vector.

Field collected adult lace bugs were confined to leaflets of diseased coconut palms in the field by covering with muslin sleeve (Fig. II) to have access to feed on and acquire the phloem bound pathogen. After acquisition feeding the insects were recaptured and allowed to feed on detached leaflets of healthy palms for completing the incubation period. Twenty-four samples involving 107 bugs representing 122 combinations (with 3 to 5 insects per combination) were studied.

Phytoplasma was observed in brain tissues and salivary glands of lace bugs offered 5 days acquisition feeding and 13-18 days incubation period on diseased palms (Fig. III). It was absent in bugs collected from disease-free area-Kasaragod and Minicoy in Lakshadweep Islands. The organism was also absent in lace bugs offered acquisition and incubation period of less than 18 days. Presence of Phytoplasma in insects that had access to diseased palms indicates that the insect can acquire the pathogen during feeding and can sustain its multiplication.

To find out whether the nymphs of lace bugs can acquire the Phytoplasma and sustain its multiplication or only adults can do it, third instar nymphs of lace bugs allowed to feed on diseased palms for 5 days were recaptured and maintained on leaflets of healthy palms. The emerging adults fixed after 13-18 days were sectioned and examined under EM. Phytoplasma was not observed in the salivary glands and other tissues. Since the adults were not allowed to feed on diseased palms, the above results indicate that nymphs probably do not acquire Phytoplasma or even if they do, the organisms are not transtadially carried over to imago. Eggs and follicle of adult lace bugs offered 18 days A+IP on diseased palms were studied. Phytoplasma was not observed in the limited number of samples examined. Attempt was also made to collect and examine haemolymph of lace bug. But this was not successful.

The potential of the plant hopper to aoquire Phytoplasma from leaflets of diseased coconut palm was studied. Tissues of different organs - salivary glands, accessory glands, foregut, midgut, ovary, testes and malphigian tubules of laboratory reared plant hoppers offered A + IP of 15, 20, 21, 25, 28 and 30 days and salivary glands of plant hoppers offered 30-59 days A+ IP period were examined. Phytoplasma was observed in gut and salivary gland tissues of plant hoppers offered more than 30 days A + IP on diseased palms. Phytoplasma was not observed in hoppers offered less than 30 days A + IP and also in freshly emerging adults maintained on

Fig. II. Lacebug confined to coconut leaflets by muslin sleeve.

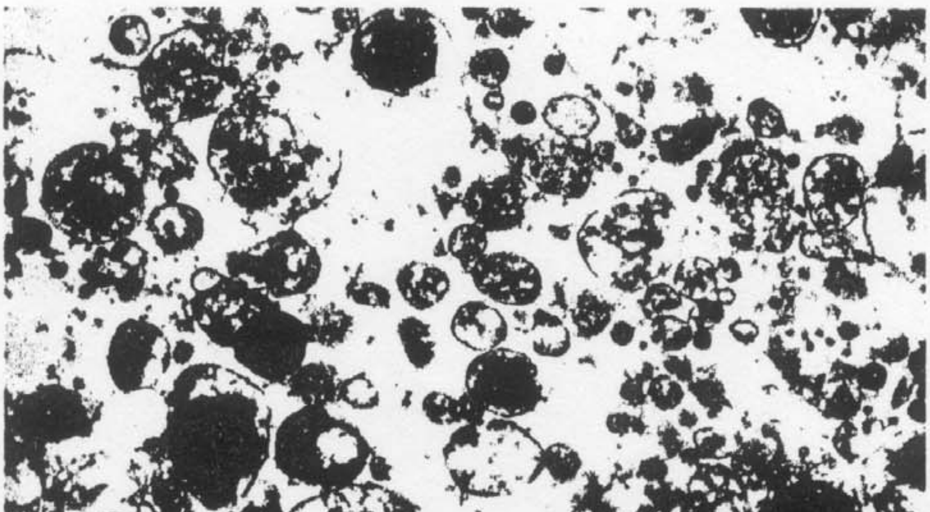

Fig. III. Phytoplasma in acini of salivary glands of lace bug.

healthy palms. Attemps to collect haemolymph from the plant hopper was not successful. Honeydew secreted by plant hopper was smeared on carbon coated copper grids, stained with KPTA and examined under EM. Nothing resembling Phytoplasma could be identified.

In an identical study, the vector role of the leaf hopper, *Sophonia greeni* to acquire Phytoplasma was studied. Salivary glands and gut tissues of leaf hopper offered 4 to 37 days were examined. Phytoplasma was not observed in the samples.

A limited number of tetranychid white mite (*Oligonychus* sp.) liberated on diseased palms known to harbour Phytoplasma and collected back after different A + IP were sectioned and examined under EM. It was found difficult to identify the different organs. A gross EM examination of ultrathin sections of the mites did not reveal the presence of Phytoplasma.

Mealy bug is one of the insects consistently observed in unopened spear leaves and tender rachilla of palms with middle whorl yellowing symptoms and in some Root (wilt) disease affected palms. Turner *et al* 1978, had reported a constant association between Coconut Stem Necrosis diseased palms and the presence of mealy bugs. They are phloem feeders on unopened spears and are absent in apparently healthy palms. It is thus implicated as a possible vector of the Coconut Stem Necrosis Disease. Hence a limited number of mealy bugs (6 lots) collected from diseased palms were sectioned and examined under EM. Phytoplasma was not observed in the samples.

Coconut seedlings inoculated with lace bug and plant hopper were periodically sampled to ascetain the presence of Phytoplasma to confirm transmission. Similarly, periwinkle bridged to diseased coconut palms with dodder laurel and connecting dodder strands were examined. Sieve tubes of mid rib and petiolar tissues showed the presence of Phytoplasma.

II. LIGHT MICROSCOPY.

Electron microscopy although is an ideal tool for detection of Phytoplasma in plant and insect tissues, the technique is laborious, time consuming and only limited area of a tissue could be studied at a time. Certain histochemical techniques, Dienes' staining and staining with fluorochromes such as DAPI (4' - 6' diamidino - 2 phenyl indole 2 HCl) Hoechst 33258 have been used for detection of Phytoplasmal infection in plants. DAPI binds with DNA to form fluorescent complexes under ultraviolet light enabling the detection of Phytoplasma. Similarly, Bisbenzimide (Hoechst 33258) a DNA binding fluorochrome binds preferentially to the adenine-thymine rich DNA characteristic of mycoplasma.

Free-hand sections of tender rachilla and roots of diseased and healthy palms when stained with Dienes' stain exhibited bluish colouration of sieve tube contents. Such staining reaction was not observed in tissues of healthy palms. Similarly, fixed tissues, sectioned and stained with DAPI or Hoechst 33258 showed fluorescing areas in extra-nuclear sites of diseased palms. Positive staining reactions were more frequently observed in junctions of vascular bridges and also close to the periphery of the sieve tubes. Similar observations were gathered from electron microscopy (vide supra). Nucleic acid specific stains like Schiff's reagent, Azure B, Toluidine blue O and Giemsa were also found useful in locating DNA rich material in phloem sieve tubes of diseased palms.

A method combining DAPI and Aniline blue was also followed. This aids in identifying phloem tissue with ease and looking for positive DNA accumulation in extra-nuclear positions indicative of the presence of mycoplasmal infection.

Callose deposits in sieve tubes, especially in sieve area, a response to injury is noticed. The excessive callose deposition indicated by phosphorescent aniline blue reaction is a characteristic feature of some sieve tubes in disease affected palms.

During the course of investigations a total of 202 coconut samples were studied. The samples include healthy palms from disease-free area, elite palms from hot-spot area, palms in the early, middle and advanced stages of disease. Invariably, all diseased palms had positive staining reaction and no reaction was evident in samples of healthy palms. Of the 69 elite palms from heavily diseased gardens tested, six showed distinct staining reaction. The reaction was always found to be weak in palms in the advanced stage of disease (Table. II).

Table. II : Detection of phytoplasma in RWD coconut tissues by LM

Condition of palm	Location	No. of samples tested	No. of samples positive
Healthy	Disease free area (Kasaragod)	11	0
Elite palms	Hot spot area	69	6
Disease early	Diseased area	68	68
Disease middle	,,	26	26
Disease advanced	,,	28	28

Tissues sampled : Tender root and rachilla
Histological dyes used : Dienes', Aniline Blue
Fluorochrome : DAPI, Hoechst 33258

Experimental coconut seedlings inoculated with lacebug and plant hopper were perodically sampled and studied. This helped in monitoring transmission of disease to healthy seedlings by the putative insect vectors. Stem and petiolar tissues of periwinkle inoculated with lace bug and plant hopper were also stained to ascertain positive transmission of the disease. Petiolar tissue of periwinkle bridged to diseased coconut seedlings with dodder laurel and the source coconut palms were studied. Positive staining of source palm, connecting dodder laurel and recipient periwinkle confirmed transmission of Phytoplasma from coconut to periwinkle.

Tender rachilla of six palms with middle whorl yellowing symptoms were subjected to Dienes' and Giemsa staining. Positive staining reaction was observed in all the samples. Palms treated with oxytetracycline hydrochloride were studied prior to the start of application and subsequently.

To study the transmission of Phytoplasma through seed, 29 embryos of nuts from 8 diseased palms and the roots emerging from nuts of these palms were sectioned, stained and examined for Phytoplasma. The sections did not show positive Dienes' staining and fluorochrome reaction.

The fluorescence staining reaction to detect Phytoplasmal infection in plant tissues was also extended to the putative insects used for transmission of the disease. Dissected salivary glands of 27 infective and five non-infective lace bugs and 25 infective and five non-infective plant hoppers were macerated and subjected to Hoechst fluorochrome reaction. Extra-nuclear fluorescing areas could be made out only in salivary glands of infective lace bug and plant hoppers. The technique thus could be used for determining the vector role of the putative insects.

The histochemical staining reaction in detecting Phytoplasmal infection in coconut was also compared with other known Phytoplasmal diseases of economically important crop plants - sesamum phyllody, little leaf of brinjal, sandal spike and grassy shoot disease in sugarcane. The reaction observed in coconut was similar to the other plants tested except from the sparse staining reaction that indicated low concentration of the organism. Such sparse distribution of Phytoplasma in root (wilt) diseased coconut has been confirmed in EM studies also.

Centrifugates of unfermented vascular sap collected from diseased palms were also subjected to phase contrast and fluorescence microscopic studies.

Attempts were also made for histochemical localisation of acid and alkaline phosphatases in tender rachilla and root tissues. Cryocut sections were used for this study. Wide variation of the acid phosphatase enzyme was noticed irrespective of the healthy and diseased nature of palms. Cortical cells, sieve tubes and phloem parenchyma cells contained copious amount of the enzyme acid phosphatase.

III. TRANSMISSION

The objective of the study is to identify the insects of transmission importance and establish their vector role. Phytoplasmas are mostly transmitted by leaf hoppers and plant hoppers (Auchenorrhyncha) and in a few cases by psyllids.

Record of insect fauna on coconut in India at the time of the start of the project did not include insects belonging to the Homopteran group. Therefore, a systematic inventory of insects of transmission significance in root (wilt) prevalent gardens was made using various traps. A suction trap similar to that used in United Kingdom and Europe for forecasting aphid population and the rotary trap modified from the one employed in Florida, were fabricated and used for collection. Three types of sticky traps - wide polythene sheet wrapped around trunk upto two meters, portable sheets held at crown height and an eight faceted multi - directional traps were used. For sticky traps, petroleum jelly, chasis grease and castor oil were tried. Castor oil application ensured maximum trapping. Insects were also trapped by light trap erected in coconut gardens. The trapping was also supplemented by sweep net collection made from diseased coconut gardens.

As the insects collected in traps need not necessarily be the ones feeding on coconut, direct examination of about 500 seedlings which had their fronds at eye level was made for two years. The study had brought out a total of 34 species of insects under 23 families belonging to eight orders besides 11 species of mites. Lace bug earlier recorded was the major insect species on coconut. A leaf hopper and plant hopper identified as *Sophonia greeni* (Distant) Cicadellidae and *Proutista moesta* (Westwood) - Derbidae - Fulgoridea were new records of transmission significance. It was observed that the leaf hopper preferred to feed on tender fronds and plant hopper on older leaves. Lace bug was found in increasing numbers from the outer to inner whorls of leaves. Besides, mango leaf hopper, paddy leaf hopper and sugarcane plant hopper were also noticed occassionally on coconut foliage, probably as migrants. Collections were also made in the night to assess whether the nocturnal collections differed from the diurnal fauna. The insect fauna did not differ significantly. In subsequent studies an aphid and two species each of leaf and plant hopper were noticed in addition to the earlier record.

The biology and bionomics of the putative vectors were also studied. The leaf hopper Sophonia greeni feeds from the abaxial side but feeding marks are not evident. It breeds on coconut leaflets.

The natural breeding site of the plant hopper could not be located in spite of vigorous systematic search. But it is said to lay eggs and breed on decaying organic matter. Eggs have been located externally on older leaves and also on moist soil.

In a parallel study conducted at CPCRI, Palode, the plant hopper was observed to breed on oil palm bunch wastes from the palm oil factory. Eggs and nymphs of all stages and adults could be collected from this substrate. The biology of the plant hopper was studied in the laboratory using male inflorescence of oil palm. The egg to adult period of the insect was completed within 38-40 days.

Lace bug was found to breed on coconut. It lays eggs singly and inserts it into leaf tissue. The position of the egg is made out externally by white specks and by ridge corresponding to its contour inside. Egg is ellipsoidal; incubation period is on an average 12 days (a fortnight) and the nymphal period 13 days. Thus the total life cycle is completed in 25 days (the first, second and third instar nymphal stages occupy 8-10, 7-8 and 10 days). Annual count of lace bug on coconut seedlings confirmed direct linear correlation between its abundance and fresh incidence of disease.

Studies on feeding habit of lace bug showed that it prefers to feed on chlorophyllous leaves and insert its stylet invariably through stomata. Damage to cells lining the sub-stomatal chamber in the form of cell wall lysis and evagination of cell protoplast was noticed in free-hand sections of feeding spots. Stylet penetration was often found to be deep enough to reach any tissue within the leaflet. The maximum thickness of coconut leaflet is around 400μ m whereas lace bug has a stylet of about 600μ m.

To determine whether lace bug feeds on phloem to be a vector of the disease, field collected adult lace bugs were starved for 16 h and then released on detached coconut leaflets kept in glass tubes with the cut ends dipping in water. After the insects settled on the abaxial side of the leaflets and started feeding the tubes kept in a canister were lowered into a vessel containing liquid nitrogen. About 30% insects died in feeding position with their stylets inserted into the lamina. Serial sectioning of lace bug fixed in feeding position with the stylet in situ in the leaflet traced its course terminating in phloem. This indicated that lace bug can feed on phloem tissue. But it is not an exclusive feeder on the phloem tissue. It inserts its stylet through stomata, ruptures the cell walls of mesophyll tissue and drain the contents of pallisade cells resulting in cavities immediately inner to the lower epidermis.

The constitution of leaf vasculature as such makes it easy for the bug to feed on phloem tissues. Each leaflet has several main and diminutive vascular bundles running parallel with occassional transverse commissures. The smaller bundles are oriented towards the abaxial region and the phloem tissues are also in this direction. Hence access to vascular bundles is not at all difficult.

Population of lace bug and plant hopper was monitored in 47 cultivars in CPCRI Kayangulam Kayal Farm. and 10 cultivars at the institute farm. These cultivars have been planted in the diseased gardens to assess their resistance/tolerance to root (wilt) disease. The 10 cultivars in the institute farm were observed four times a year. All the cultivars were found to lodge both lace bug and the plant hopper.

The population build up and seasonal abundance of the putative insect vectors, lace bug, plant hopper and leaf hopper were studied since 1992 on 100 seedlings for 3 years in a cultivator's garden. Lace bug though found to be present throughout the year, had two peak period of abundance - March-April and September-October. Plant hopper was not present during April-May. Population build up was noticed with the onset of summer showers in late May-June and highest population was recorded during October-November. Leaf hopper population reached maximum during July-August.

Leaf hopper, *Sophonia greeni* was found to breed on brinjal and *Areca triandra* in natural condition. Plant hopper was occassionally observed on banana and Licula sp.

A rapid survey of the contiguous disease tract and freshly reported pockets of disease incidence indicated the presence of all the three putative insect vectors. In other words there was no disease occurrence independent of all the three insects.

Mites : Mites as possible vector of coconut root (wilt) disease was also investigated. An inventory of mites on coconut was made. Red palm mite (*Raoiella indica*) and tetranychid white mite (*Oligonychus sp.*) occur on coconut foliage. Examination of 48 leaves of ten selected palms confirmed the record of tetranychid and eriophyid as phytophagous mites of coconut. Seven other species were also studied. Laboratory cultures of the mites were maintained by excised leaf bit dip method. The mites newly recorded were *Oligonynchus iseilemae and Notostrix attenuata.*

Tetranychid mites recaptured from RWD palms after acquisition and incubation period of 6, 14, 15 and 16 days were examined under EM. Phytoplasma could not be identified in the mite tissues.

Biology and bionomics of the mite, *O.iseilemae* was studied. It completed its life history in seven to ten days. Experimental transmission using the white mite *O.iseilemae* on periwinkle, an accepted mycoplasmal indicator host was also tried. Periwinkle inoculated with *O.iseilemae* that received 15 to 20 days A+IP on diseased palms although showed general yellowing but failed to produce symptoms typical of mycoplasmal infection.

Exptl. transmission from diseased coconut to healthy coconut seedlings using lace bug

Lace bug, being the single major group of insects occurring in higher numericals and throughout the year on coconut posed as the putative vector of the disease. Experimental transmission of the disease on adult palms in the field and under insect proof condition done in the past lend further credence to its claim as vector. However, tingid being a heteropteran insect, its vector role needed to be reinvestigated in the light of the implication of a phytoplasmal etiology for the disease. Phloem feeding nature (vide supra) of the insect as determined by serial sectioning of the lace bug fixed in feeding position and the location of phytoplasma in salivary glands and brain tissues thus needed further confirmation through transmission studies.

Two year old West Coast Tall coconut seedlings obtained from disease-free area and planted in methyl bromide fumigated loamy sand from paddy field in underground field tanks of 1.8x1.8x1.2 m and protected by netted field cages of 3.7 m height were used in this study. Four seedlings were inoculated with lace bug and four were maintained as uninoculated control.

Initially, adult lace bugs collected from coconut palms and renderd infective by allowing them to feed on diseased coconut palms were recaptured and liberated on leaflets of four coconut seedlings in IP cages. This inoculation was supplemented by inoculating with lace bugs offered 5 days acquisition feeding on diseased palms and offered incubation period of 13-18 days on coconut leaflets to ensure infectivity. Details of lace bug inoculation are furnished in Table III.

Table. III : Number of lace bugs inoculated on experimental coconut seedlings in field cages numbered 3,4,9 and 10.

	5 days' acquisition					5 days' acquisition plus 13 days' incubation				
Date	3	4	9	10	Total	3	4	9	10	Total
31 Dec 1985	184	180	180	174	718					
31 Mar 1986	200	196	202	180	778					
30Jun 1986	631	637	639	664	2571					
Total	1015	1013	1021	1018	4067					
30 Sep 1986						626	621	616	595	2458
31 Dec 1986						427	431	434	458	1750
31 Mar 1987						90	76	80	76	322
Total						1143	1128	1130	1129	4530
30 Jun 1987	100	101	106	97	404					
30 Sep 1987	278	285	257	287	1107					
30 Nov 1987	-	705	-	706	1411					
Total	378	1091	363	1090	2922					
Grand total	1393	2104	1384	2108	6989					

The experimental seedlings, both lace bug inoculated and uninoculated were sampled at bimonthly intervals from nine months after the initial inoculation for serology, light and electron microscopy. Samples of three lace bug inoculated seedlings sampled nine months after the start of inoculation showed strong serological reaction by producing a crisp precipitin line midway between the antigen and antiserum depot. Feeble reaction was observed in sample from the fourth seedling. However, no precipitin line was formed against sample from the uninoculated control.

Light microscopy of sections of root apex of uninoculated seedlings subjected to Dienes' stain developed abnormal bluish colouration in sieve tubes. Similarly, sections stained with DAPI and Hoechst 33258 showed abnormal fluorescence of the sieve tube contents. This indicated phytoplasmal infection of phloem and thus confirmed the transmission of the organism.

Electron microscopic examination of the ultra-thin sections of roots revealed the presence of phytoplasma in one of the lace bug inoculated seedlings on the nineth month, in two more seedlings by twelfth and in the remaining seedling by the 27th month after the start of inoculation. By seventeenth month, two of the inoculated seedlings have shown 'flaccidity' of leaflets the decisive and diagnostic symptom of the disease. Phytoplasma was not observed in the uninoculated control plants.

Experimental transmission from diseased coconut to healthy coconut seedlings using plant hopper, *Proutista moesta*

Electron microscopic examination of ultra-thin sections of the plant hopper offered acquisition access feeding and incubation period on diseased palms for more than 30 days revealed the presence of phytoplasma in the salivary gland and gut tissues of the insect.

The vectorial ability of plant hopper to transmit the disease was studied in a transmission expt. Two year old West Coast Tall seedlings brought from disease-free area were planted in 16 underground field tanks of 1.8x1.8x1.2 m. filled with methylbromide fumigated paddy field soil. The seedlings were protected by field cages of 5 m height netted with galvanized iron gauze of 40 mesh/linear inch^{-1}. Eight seedlings were inoculated with infective plant hoppers offered 30 days A+IP on diseased palm and eight were maintained as uninoculated control.

From five months after the start of inoculation the seedlings were periodically sampled for electron microscopy and serology. The plants were also monitored for visual symptoms. Newly emerging roots were sampled for electron microscopy and spear leaves were used for serological testing. Phytoplasma was observed in one of the seedlings sampled five months after the start of inoculation, in three more after eighth month and one each after 16 and 24 month of inoculation. By the fifth month each seedling had received inoculation with 537 infective hoppers, by eighth month with 610 and by 16th month with 1400 plant hoppers. Phytoplasma has thus been detected on six out of the eight inoculated seedlings and five of them had shown flaccidity the diagnostic symptom of the disease between 8 to 24 months. All the seedlings also showed positive serological reaction. None of the uninoculated eight control plants either had phytoplasma or shown symptoms of the disease.

Exptl. transmission from diseaased coconut to test plants through putative insect vectors

Lace bug:

Periwinkle plants were inoculated with field collected lace bugs offered 18 days acquisition and incubation period on diseased coconut palm. Similarly another set of plants were inoculated with a cumulative total of 200 bugs of natural population at the rate of 10-20 insects per day. Another set of periwinkles were inoculated with lace bugs collected from root (wilt) diseased palm. None of the inoculated seedlings showed any symptom of the disease. Dienes' staining of the periwinkle stem segments also did not show any mycoplasma positive staining reaction. EM examination of ultrathin sections of petiole and stem segments also did not reveal phytoplasma in sieve tubes.

In addition to Periwinkle the following plants *Sesamum indicum, Zizyphus, Stachytarpheta and Solanum melongena* were also individually inoculated with infective lace bugs to assess whether any of them could be used as a test plant. None of the lace bug inoculated plant species showed any symptoms. LM and EM observation of sections of inoculated plants also did not indicate the presence of phytoplasma.

Leaf hopper

Periwinkle plants were inoculated each with 50 field collected leaf hoppers. The inoculated plants showed mosaic symptoms in 5 to 6 days after the liberation of the hoppers, developing into coalesced patches in 10 to 12 days. The plants also showed growth abnormalities. However, EM examination of the plant tissue did not reveal phytoplasma. The changes observed in the inoculated plants could be due to toxaemia. Leaf hoppers were found to breed on brinjal and Areca triandra in natural condition.

Plant hopper

To assess whether plant hopper can feed on periwinkle, the feeding probe of P. moesta was studied using Mc Bride's stain. Feeding marks of the plant hopper could be located on periwinkle plants.

Based on this information a transmission expt. using plant hopper was done on periwinkle. Two periwinkle plants were inoculated each with 25 infective plant hoppers offered A+I of 36 days. None of the plants showed any symptoms.

With a view to identify an alternate host preferably a short statured palm species the feeding acceptability of the following were studied: *Licula grandis, Pinanga sp., Areca triandra, Elaeis guineensis, Trinex sp. and Cyrtostachys renda.* The insect could survive on *Elaols* for 55 days, 27 days on Licula and also on *Cyrtostachys.*

Acceptability studies with plant hopper on known hosts of mycoplasmal diseases was also tried. Zizyphus, Dodonaea, Sesamum, Stachytarpheta, banana, sugarcane, maize, brinjal, cowpea, periwinkle, Pandanus and citrus are the hosts. Sixty percent of the plant hoppers survived after 48h of release on maize and sugarcane.

Dodder transmission

Apart from insect transmission which is the natural mode of transmission for phytoplasmas they also could be transmitted experimentally through grafting and using phanerogamic parasites. Coconut being a monocot lacking cambium, grafting is not feasible, and hence transmission using species of Cuscuta was tried.

Attempts were made to establish *Cuscuta campestris,* C. *chinensis and* C. *subinclusa (Convolvulaceae) on coconut foliage. Although the three* species of *Cuscuta* developed infection pads on coconut leaflets, they failed to establish haustorial connection with vasculature.

Reddy *et. al.*, (1985) reported trasmission of citrus mosaic through dodder laurel *Cassytha filiformis* (Lauraceae) from Sathugudi orange *(Citrus sinensis)* to acid lime *(Citrus aurantifolia).* Hence attempts were made to transmit phytoplasma from diseased coconut to periwinkle using *Cassytha filiforms.* Initially the dodder laurel was established on *Aralia* sp., *Bougainville* sp., *Coleus* sp., *Croton* sp., *Helianthus annuus, Solanum melongena, Sesamum* and periwinkle to be used as feeder plants to establish on diseased coconut. A 'dodder garden' was established with twenty young coconut palms transplanted from the cultivators' fields and planted in cement pots/field soil and parasitized with dodder, in order to attempt transmission to a number of coconuts.

Dodder laurel established on coleus and periwinkle maintained in IP cages was bridged to juvenile diseased coconut palms. The laurel established haustoria on coconut leaflets within 4-8 days during wet season (June to December) and 22-25 days during dry season. Within 14-21 days of establishment of haustorium the recipient periwinkle plants showed diffuse chlorotic flecks in the interveinal areas and at vein endings of fully opened leaves. The spots later enlarged and coalesced to form yellow patches eventually covering the entire leaf.

EM examination of leaves of the donor diseased coconut palm, recipient periwinkle and the connecting dodder laurel showed presence of phytoplasma confirming transmission.

The phytoplasma also could be serially transmitted from symptomatic periwinkle to healthy periwinkle through dodder and by grafting.

Effect of insect control on incidence of disease

Seven field experiments were conducted to ascertain whether the control of insects through insecticides and botanicals could prevent fresh incidence of disease. Details of the expt. are furnished in Table IV.

Table. IV : Effect of insect control on incidence of disease
Insecticide/concentration : Monocrotophos alternate with endosulfan 0.01% Conc.

Expt. No. & Details	Treatment / Frequency	No. of seedlings	No. of. Seedlings diseased
01 Clearance Planting Dt. of Start : June 1985 Dt. of termination : June 1993	T_1 Monthly spraying T_2 Six - monthly spaying T_3 Unsprayed control Guard rows/Reserve unsprayed	24 24 24 29	19 15 12 8
02 Under Planting Dt. of Start : Oct. 1987 Dt. of termination : June 1993	T_1 Monthly spraying T_2 Six - monthly spraying T_3 Unsprayed control Guard rows	32 32 35 22	9 2 7 3
03 Under Planting Dt. of Start : July 1988 Dt. of termination : June 1993	T_1 Source palm Sprayed (Thrice a year) T_2 Source Palm Un sprayed	45 45	5 7
04 Under Planting Dt. of Start : July 1988 Dt. of termination : june 1993	T_1 Source Palm+Seedings sprayed (thrice a year) T_2 Source Palm+Seedings Unsprayed	50 50	6 10
05 Under planting Midly affected area Adichanalloor Dt. of Start : July 1988 Dt. of Termination : June 1993	T_1 Quarterly spraying T_2 Six - monthly spraying T_3 Unsprayed control	33 33 30	3 2 3

Insecticide/concentration : Monocrotophos alternate with endosulfan 0.1% Conc.

Expt. No. & Details	Treatment / Frequency	No. of seedlings	No. of. Seedlings diseased
06 Under Planting	T_1 Fortnightly spraying	40	6
Dt. of Start : July 1990			
Dt. of termination :	T_2 Unsprayed control	44	17
June 1995	Guard row	8	2

Expt. No. & Details	Treatment / Frequency	No. of seedlings	No. of. Seedlings diseased
07 Under planting	T_1 Phorate 10G	40	4
Dt. of Start : Oct. 1993			
Dt. of termination :	T_2 Carbofuran 3G*	40	4
June 1997	T3 Neem cake*.*	40	5
	T4 Control	40	8
	*I yr 10g ai. II yr 20g ai,		
	III yr 30g ai / seedling		
	**I yr 1.5Kg. II yr 3.0Kg,		
	III yr 4.5 Kg / seedling		

Frequency of application : Three times a year (ie.) January, May and September. In each application 1/3 of the total dosage for the year was given.

The first expt. was done in an area where the garden was cleared of all palms and replanted with healthy WCT seedlings brought from disease-free area. There were three treatments, T1 Monthly spraying, T2 six monthly spraying to coincide with peak period of abundance of the putative insects and T_3 unsprayed control. Monocrotophos and endosulfan, a systemic and contact insecticide at 0.01% concentration were sprayed alternately on the treatment seedlings. Between the treated palms guard rows of untreated seedlings were maintained to prevent vitiation of the exptl. results. Irrespective of the treatments seedlings in both insecticide sprayed and unsprayed control plants had contracted the disease.

As underplanted seedlings contract the disease much earlier than in clearance planting a second expt. was done with same treatments in underplanted situation. Here again the treatment effect was not significant. Both sprayed and unsprayed plants had contracted the disease.

The third expt. was done on seedlings underplanted in an existing diseased garden. The treatments are (i) source palm sprayed thrice a year and (ii) source palms unsprayed. The insecticide concentration was same as the previous experiment. Significant difference was not noticed between treatments. Seedlings in both treatments had contracted the disease.

In the fourth expt. both the source palm and the underplanted seedlings were sprayed thrice a year and the control had both source palm and underplanted seedlings unsprayed. Both treated as well as control seedlings had contracted the disease.

The fifth experiment was similar to the second experiment with three treatments. This was conducted in a mildly disease affected area at Adichanalloor. In one treatment the seedlings were sprayed at quarterly intervals, another treatment with six monthly spraying and compared with untreated control. Seedlings in all the three cases have contracted the disease and there was no significant difference between treatments.

Since spraying of insecticides at 0.01% concentration at monthly intervals also did not have any effect on incidence in the aforementioned field experiments another experiment was conducted with higher concentration of insecticides (at 0.1%) applied at fortnightly intervals. Even fortnightly application with ten times higher concentration of insecticides could not prevent fresh incidence of disease.

Soil application of insecticides and botanicals was tried in another experiment. Here again significant difference between treatments could not be observed.

Similar results were recorded in lethal yellowing disease in Florida. Biweekly spraying of insecticides resulted only in slight reduction in vector population and in spread of LY (Howard and Mc Coy 1980; Howard and Barrant 1989). Howard (1987) is of the view that the insecticides currently available are not persistent and thus the treated palms are quickly reinfested resulting in disease contraction.

Bioassay with insecticides

For trying newer insecticides against the putative insect vectors, bioassay studies were conducted in comparison with insecticides in vogue. Fenvalerate (Sumicin), Cypermethrin (Cymbush), Malathion (Malathion), Methyl parathion (Metacid), Dimethoate (Rogor), Monocrotophos (Nuvacron), Feripraparin (Danitol), Fenthion (Lebaycid) were the eight insecticides tested against lace bug. The methodology for conducting bioassay studies with insecticides against plant hopper was standardised. Six insecticides namely, Endosulfan, Monocrotophos, Methyl parathion, Malathion, Dimethoate and Fenvalerate were screened against plant hopper.

Repellant action of botanicals and insecticides against putative insect vectors

Repellant action of four concentrations of garlic, naphthalene and neem seed extracts were tested against the plant hoppers. The concentration tried were 0.1, 0.2, 0.5 and 1%. None of these products showed any repellant action. Oils of Neem, Punna and Marotti had significantly higher repellant action at 5% concentration than oil cakes.

Studies on soil insect fauna

Soil insect fauna was examined by Berlese funnel method, drawing soil samples (200-250 g) from root zones of palms. Fifty samples of soil collected from diseased gardens in Alappuzha and Kollam districts were examined. Consistent presence of collembolans and soil mites was noticed in samples of all the locations without any significant difference among treatments.

Studies on movement/flight range of lace bug and plant hopper

For studying the flight range the insect candidates were labelled with markers such as microscopic stains, paints and uranyl acetate. The markers were found to be lethal to the insects. However, Printer's gilt was found to be nonlethal. Labelling with radioactive material is another option that could be pursued. The vertical movement of the insect was studied by erecting 10 sticky traps.

Critical studies with lace bug using coconut sprouts

To determine the optimum number of lace bugs required for effecing transmission of root (wilt) disease coconut sprouts were inoculated with known number of infective lacebugs (lace bugs offered 18 days A+I on diseased palms) 1, 5, 10, 20 and 40. Roots from the sprouts were sampled 8 and 14 months after the commencement of the inoculation for EM examination.

IV. VASCULAR SAP ANALYSIS

Since phytoplasmas are phloem limited, and are non-culturable, the objective of this study is to analyse the physical and biochemical constituents and environment of the phloem so that a medium mimicking phloem constituents could be synthesised and the conditions suitably made.

In this study, three healthy and three diseased palms were processed for tapping by the traditional method of beating and slicing the inflorescences. A method of collection of unfermented vascular sap in frozen condition was standardised. In general the healthy palms yielded sap for longer duration (70 to 90 days), than the diseased (22 to 56 days). Apparently healthy palms had a rate of flow of 5 ml/hr until 25 days, but shot up at 32 days upto a rate of flow of 22 to 25 ml/hr. This trend continued for 70 days followed by a rapid decline. With the increase in intensity of disease, the rate of flow decreased. Diurnal differences in the rate of sap collection during developmental stages of palm and from different inflorescences of the same palm was studied. Irrespective of the condition of the palm, the rate of flow was generally more during day than during night. The pH of healthy sap ranged between 6.1 and 6.4 while that of diseased palm was between 6.5 to 7.5. The osmotic concentration was higher in healthy (730 to 750 m. mol Kg -1) than that in diseased (590 - 700 m. mol Kg -1).

Vascular samples were fractionated into acidic, basic and neutral fractions. Employing two dimensional paper chromatography aminoacids, organic acids and sugars in the sap from healthy and diseased palms were identified. Thin layer chromatography was also employed to quantitatively estimate aminoacids. The sap from apparently healthy palms had higher contents of reducing sugars, total amino acids, proteins and lipids than that from diseased palms. Total sugars, however did not show much difference (Table V). For further analysis individual fractions of amino acids, organic acids and sugars, in the samples were fractionated through Dowex columns and lyophilised. Ten amino acids were detected through TLC in the sap from, both healthy and disease affected. Amino acids, organic acids and sugars were further quantitatively estimated by gas liquid chromotography . Arginine and aspartic acid among amino acids, malonic acid and Lactic acid among organic acids and glucose and galactose among sugars registered higher values in the sap collected from the inflorescences of diseased palms than that from the apparently healthy ones (Table VI). Arginine levels were found to be about 30% more in disease affected palms. It is suspected that the phytoplasma present in RWD palms may be of the nonfermentative type which uses the arginine for its energy production and growth thus accounting for the higher concentration (Table VII).

Similarly, sap from four healthy palms and three diseased palms was centrifuged and fractionated using organic solvents for analysing major elements. Elemental analysis indicated differences only in potassium content between healthy and diseased sap. Among the micronutrients zinc and copper had shown marked differences.

Bioassay of giberellin-like substances in vascular sap using rice second sheath method and close response curve with GA3 have been performed. Giberellin activity tended to be high in the sap from disease affected palms. Bioassay of auxins and giberellins in vascular sap was performed for three apparently healthy and two diseased palms. Both these growth substances were found to be in comparatively higher concentration in sap from diseased category (Table VIII).

Vascular sap from diseased palms was also collected on carbon coated copper grids and stained with phosphotungstic acid and examined under EM. Spherical to ovoid bodies and occasionally beaded ones were observed. The structures are to be conclusively identified.

An attempt was also made to collect sap from trunk of a juvenile palm as per the method followed by Eden Green. No sap however could be collected. The unfermented sap was used for culturing of the organism.

Table. V : Biochemical costituents of vascular sap. Values expressed as mg g $^{-1}$ sap solids (Chempakam *et al.*, 1991).

Constituents	Apparently healthy	Diseased
Total sugars	299.50	286.20
Reducing sugars	98.10	65.90
Proteins	4.90	2.50
Free amino acids	45.90	18.30
Arginine*	0.41	0.68
Phenols	0.60	0.20
Lipids	0.83	0.57
Sterols*	0.40	0.22

* Values expressed as mg ml $^{-1}$ sap.

Table. VI : Composition of amino acids, organic acids and sugars identified in the phloem sap from the inflorescences of apparently healthy and root (wilt) diseased coconut palms. Values are expressed as mg g $^{-1}$ sap soilds. (Chempakam *at al.*, 1991)

Compounds identified	App. healthy	Diseased
A) Amino acids (average of 5 palms) :		
Cystine + Cysteine	0.25	0.20
Arginine	0.19	0.28
Serine	0.13	0.09
Glycine	0.11	0.09
Aspartic acid	0.19	0.25
Glutamic acid	0.12	0.06
Leucine	0.14	0.12
Methionine	0.17	0.13
Phenylalanine	0.27	0.24
Tyrosine	0.06	0.23

Compounds identified	App. healthy	Diseased
B) Organic acids (Avarege of 5 palms) :		
Oxalic acid	1.54	1.03
Malic acid	1.29	1.02
Fumaric + Succinic acids	0.41	0.29
Maleic acid	0.90	0.64
Malonic acid	75.07	82.05
Citric acid	11. 73	12.93
Lactic acid	13.67	23.70
C) Sugar (Average of 4 palms) :		
Sucrose	135.50	128.30
Glucose	25.50	39.40
Galactose	18.10	28.90
Mannose	11.50	9.50
Lactose	16.30	14.70
Raffinose	20.40	17.50

Table. VII : GA_3 - like substances in the sap from healthy and diseased palms.

Palm No.	Condition	GA3 equivalent (µg/ml sap)
37	AH	4.68
69	,,	3.56
54	,,	4.08
		Mean 4.08
38	Diseased	8.81
72	,,	4.28
		Mean 6.55

Table. VIII : Free arginine levels in the inflorescence sap and spear leaves of apparently healthy and root(wilt) diseased palms.

Palm condition	Vasucular sap (mg/ 100ml sap)	Spear leaf (mg/ g dry wt.)
Apperently healthy	0.4138	0.194
Diseased	0.6846	0.318
Healthy (Kasaragod)	-	0.256

V. CULTURING

As isolation of the disease agent from diseased palm and reproducing the disease symptom and assessing its pathogenicity are pre-requisites to establish Koch's postulates, attempts were made for in vitro culturing of phytoplasma.

(a) In cell-free media:

Efforts were made to culture the organism from rachilla of young unopened inflorescence, meristem, root tips, mid vein of spear leaves, phloem sap from root (wilt) affected palms, petiole, dodder laurel and mid vein of symptomatic periwinkle. Samples from the different plant parts were partially clarified and inoculated to broth media under serial 8 fold dilutions.

Media enriched with horse serum, phloem sap of healthy palms independently and in combination with horse serum, DNA, yeast extract and serum based media were tried. Further considering the fastidious nature of phytoplasma associated with CRWD, a nutritionally enriched semisynthetic medium was prepared. The medium consisted of mycoplasma broth, normal horse serum, glucose, D-ribose, sucrose, DNA, NAD, and supplemented with purines, pyrimidines, nucleosides, nucleotides, ATP, fresh yeast extract, yeastolate, vitamins etc. The pH and molarity of the medium was adjusted to simulate conditions in coconut vascular sap. Phloem sap as such or supplemented with nutrients and horse serum was also tried in the media.

Culture media incorporating foetal calf serum, tissue culture medium 199 (10x); serum substituted with ascitic fluid, linoleic acid, palmitic acid, cholesterol, hemic chloride, tween etc. and enrichments like mucin, casitone, casaminoacids, free nucleotides, vitamin B12, NADN etc. Chick embryo extract, brain heart infusion, minerals, organic acids, aminoacids, nucleic acid precursors, cofactors and chemicals such as sodium thioglycollate which reduce oxidation reduction potential were prepared in liquid, semisolid and solid states, and used for culturing of the organism.

Media on liquid, solid and biphasic (having activated charcoal in agar layer) supplemented with contaminant inhibitors, penicillin and thallium acetate were also tried.

Inoculation to the media was done by different techniques:

i) Aspetic tituration of sample followed by serial dilution in liquid media

ii) Direct inoculation of pieces of plant parts into liquid media followed by reinoculation of the pieces to fresh media

iii) Direct inoculation of sample pieces on solid media

iv) Direct inoculation of phloem sap to various media

v) Inoculation of tissue extract into biphasic media by serial dilution

All the cultures were incubated at $30^{\circ} \pm 1^{\circ}C$. Reinoculation was also done from liquid to liquid, liquid to solid and solid to liquid. Microbial growth in the culture media was periodically monitored. Except for occasional decline in pH and turbidity no growth of phytoplasma was evident.

Response of phloem sap from healthy and root (wilt) affected coconut palms to growth of two cultivable conventional mycoplasma was assessed in comparison with the root (wilt) phytoplasma. The sterol dependent Mycoplasma sp. and sterol independent Acholeplasma sp. were the conventional mycoplasmas tested. Both the mycoplasma species could survive for prolonged periods in vascular sap from healthy palms but in the sap from disease affected palms, the cells lost their viability thus indicating the inhibitory effect of the latter on the organism. The vascular sap as such or supplemented with nutrients however could not support growth of the RWD Phytoplasma.

Coconut milk from nuts in different stages of maturity was asceptically collected and used for the isolation of the organism. Culture media formulated with coconut milk, vascular sap and horse serum enriched with nutritional factors were used for the isolation of the intraphloemic organism. Tender rachilla, root meristem and spear leaf of infected palm and petiole and midvein of symptomatic periwinkle were used. The liquid cultures after appropriate incubation were centrifuged and the sediments transferred to carbon coated copper grids, negatively stained and examined under EM. Phytoplasma could note be observed in any of the cultures. In the course of investigation more than 40 different media under different cultural conditions were used for the isolation of phytoplasma. Another approach made was the maceration of coconut, periwinkle and dodder plant parts (root, rachilla, petiole and laurels) with enzymes and separation of phloem strands and inoculate them to appropriate culture media. Here again the organism could not be cultured.

Attempts were also made to isolate the phytoplasma from macerates of lace bug offered 18 days acquisition and incubation period on diseased coconut palm. The organism was however not culturable.

(b) In chick embryos:

Since the root (wilt) Phytoplasma was not amenable to cultivation in cell-free media, efforts were made for the propagation/ cultivation in an in vitro modal system. "Embryonated hen's

[redacted]

eggs". Tissue extracts from coconut, periwinkle and lace bug were independently inoculated on to embryonated eggs through different routes viz. yolk sac, allantoic and amniotic cavities Egg contents (yolk, yolk sac allantoic and amniotic fluids) recovered from the inoculated eggs after specified incubation period were inoculated onto serum based media. No sign of growth was observed in the inoculated embryonated egg and also in the reinoculated media. Egg contents were transferred to coated grids and examined under EM. However, no organism resembling Phytoplasma could be identified. This again confirmed the fastidious nature of RWD phytoplasma.

The same technique was also extended to isolate mycoplasma from Sesamum phyllody infected sesamum plants and periwinkle which had received the pathogen through dodder. EM examination of allantoic fluid recovered from the embryonated hen's eggs inoculated with sesamum phyllody material revealed the presence of mycoplasma with distinct helical morphology. The isolated organism also could be serially sub-cultured in vitro. The mycoplasma associated with sesamum phyllody also could be cultured in serum enriched media. The organism is identified as a Spiroplasma.

(b) Insect tissues:

Having failed to culture the RWD Phytoplasma in cell-free media, the possibilities of culturing/maintaining the organism in specific organs of infective insects was explored. Excised salivary glands and midguts were inoculated to MM and L-15 (Leibritz) medium supplemented with 20% normal horse serum.

Salivary glands of the plant hopper proliferated within 4 days and formed monolayers covering the culture vial surface in 6-7 days. Light microscopic observation of of the cells fixed and stained with Giemsa revealed the cells to be epithelial type with mono or bi or multinucleate condition. Efforts were also made to initiate primary cultures from salivary glands and midgut of lace bug and plant hopper aseptically reared in the laboratory.

Salivary glands from about 400 wild lace bugs were inoculated to MM growth medium to initiate primary cultures. Although no cell proliferation was found, the explants could be maintained upto one week in viable form.

To prevent microbial contamination in the inoculated insect organs used for inoculation, efforts were made to rear the insects - lace bug, plant hopper and leaf hopper - aspetically on alternate hosts and artificial diets. However, the alternate host of lace bug *Alpinia purpurata* could not be propagated aspetically in sterilised soil, MS and White's media.

It was tried to rear the insects in a meridic and holidic diets in 5 and 10% sucrose solutions. Adults of lace bug and leaf hopper could be maintained upto one week on holidic diet and 5% sucrose solution but the survival rate was found to be very low in meridic diet, 100% mortality was observed in 24 h. Parafilm was found to be ideal for membrane feeding of artificial diets to insects.

An all glass apparatus for rearing of insects under aseptic condition has been got fabricated. This item of work had to be terminated due to technical reasons.

In coconut tissues

As phytoplasmas are not amenable to cultivation in vitro in cell-free media, attempts were made to maintain the organisms in plant tissues micropropogated in vitro. Cousin *et al.* (1990) could maintain Poplar witches' broom Phytoplasma on *Populus alba* propogated in tissue culture for more than 3 years. Bertaccini *et al.* (1992) could maintain a collection of Phytoplasmas in plant tissues micropropagated in vitro. Davies and Clark (1994) could maintain Phytoplasma associated with Pear in tissues propagated in Murashige and Skoog (MS) medium supplemented with Gibberellic acid (GA), Indole butyric acid (IBA) and Benzyl aminopurine (BAP) for more than three years. Similarly Jarausch *et al.* (1996) could maintain Phytoplasma associated with apple proliferation in micropropagated tissues for more than 8 years. With this background, an attempt was made to maintain Phytoplasma in coconut, periwinkle and dodder tissues micropropagated in vitro.

A number of tissue culture media incorporating various levels of growth substances were tried to determine the morphogenetic potential of root (wilt) diseased palms. Tender leaf, petiole, rachilla and root tissues of coconut seedlings were tried as explants. During the course of the study explants from 5 healthy palms and 64 diseased palms were used for culturing.

Among the explants tried, tender unexpanded leaves from 2-3 year old coconut seedlings responded better. Inoculated root tips invariably shrivelled up. Unexpanded tender leaves were inoculated to half strength MS medium supplemented with (mg/L 50 NAA, 300 Casein hydrolysate and thiamine hydrochloride having 3% sucrose, 0.5% agar and charcoal 1%. Explants showing response were sub-cultured to MS medium with low auxin content 10-14 days after inoculation. Some of the explants showed swelling along cut ends and subsequently developed root initials in 4 weeks of inoculation. Only 10% of the cultures showed root initiation. The cultures were transferred to liquid MS medium with BAP and NAA after 8 weeks of inoiculation. Addition of GA induced better root growth. Leaf cultures with root growth were periodically subcultured. Adventitious roots developing from these cultures were cut into small pieces and sub-cultured in the same medium. Explants with root tips grew actively. Survival and propagation of phytoplasma in leaf explants and the roots emerging from the leaf explants were periodically monitored by examining ultrathin sections of the samples under EM. Phytoplasma was observed in sieve tubes of leaf explants, swollen cut ends and in the emerging roots upto 9 months of inoculation. Thereafter moribund forms accompanied with necrotic changes only were observed. Root cultures could be maintained in the medium upto 1 1/2 years.

In periwinkle tissues

Tender axillary buds of symptomatic and asymptomatic periwinkle surface sterilised with 50% chlorine water for 20 min. followed by 70% ethanol for 10 min. were inoculated to half strength Y-3 medium. It was found that medium supplemented with Streptomycin (200 ppm) and Nystatin (120 ppm) yielded 50% cultures without contamination.

Axillary buds and stem segments from healthy periwinkles showed active growth and produced broad green leaves. In contrast, cultures of symptomatic periwinkles showed proliferation of axillary shoots, resulting in a cluster of pale green narrow leaves that withered soon. Samples from the cultures have been fixed from EM examination. Two of the tissue culture propogated healthy periwinkles have been transferrred to soil plus soilrite and are being maintained.

Dodder laurels for conducting in vitro transmission

Efforts were made to culture dodder explants in tissue culture media with a view to attempt in vitro transmision of phytoplasma from diseased coconut tissues in culture to coconut embyro cultured plantlets.

Tender tips of dodder laurel was used as explants and surface sterilised with 70% ethanol yielded 50% contamination-free cultures. Dodder laurels cultured in Rangaswamy's medium with BAP and IBA actively grew. Dodder embryos excised from seeds germinated in this medium. However further grouth was poor. Similarly, tips of dodder laurel initially maintained in Hoagland's solution for 2 weeks and later transferred to solid media are growing . About 50% of the cultures showed shoot development.

Embryos of nuts from healthy and root (wilt) diseased palms were inoculated to a modified Y-3 medium. The embryos germinated in two weeks time.

Attempt was made to co-culture dodder along with embryo cultured coconut plantlet. Both coconut and dodder showed active growth. But the dodder failed to establish haustorial connection on the coconut leaf.

VI. ANTIBIOTIC THERAPY

Since phytoplasmas are not amenable to cultivation in vitro, antibiotic treatment of plant diseases associated with the mollicutes is recognised and practised as a diagnostic aid. The known susceptibility of mycoplasmas as a group to tetracycline antibiotics and their insensitivity to Penicillin is considered as supporting evidence for proving the mycoplasmal etiology of a disease. Response of plants to differential chemotherapy is considered equally as important as the visualisation of the organism in providing evidence for the presumptive mycoplasmal etiology of a disease.

The most important prerequisite for the antibiotic experiment is that there should be an efficient tool and method to administer the antibiotic. The chemical should reach the target site in unaltered state within a reasonable period of time. As foliar application is reported to be less effective other means of application were tried.

The efficacy of different methods of application, root feeding, stem injection by gravity flow and stem injection and petiole injection were compared. A pressure injector made of PVC having 10 kg/cm2 pressure holding capacity specially got fabricated for this study was used The quantity absorbed was measured and the uptake and translocation studied by use of a tracking dye Rhodamine B and bioassay.

Table: Efficacy of different methods of application

S.No.	Method of application	Replication	Average quantity absorbed out of 500 ml administered in 6 h.	Bioassay
1.	Root feeding	4	108 ml	—
2.	Stem injection			
	a. Gravity flow	5	268 ml	+
	b. Pressure	5	407 ml	+
3.	Petiole injection	5	350 ml	+

Maximum quantity of antibiotic was found to be absorbed by pressure injection. Using this method of application the ideal site of applicatioin was assessed. The antibiotic OTC 3g ai was administered by pressure injection at the bole region just at the soil surface, 50 cm and 100 cm above ground level. The uptake and translocation was assessed by bioassay. The results showed that the bole region at the surface of soil was found to be the ideal site for injecting the antibiotic. The efficacy of two pressure injectors, a metallic screw type and a PVC air pressure injector was compared.

The presence of OTC in different parts of crown of treated palms viz. petiole, leaflets of outer, middle and inner whorl was assessed using a tracking dye, Rhodamine B and by microbiological bioassay using *Bacillus cereus* sub. sp *mycoides* as the test organism. The dye could be detected in the stem and petiole of the outer, middle, inner whorls of leaves when injected with the pressure injector.

The microbiological bioassay with *Bacillus cereus* was done by paper disc method and by tissue (leaf/stem) disc method. Concentric zones of inhibition were noticed in all the parts of treated palms injected with the air-pressure injector. Such zones of inhibition was not observed in tissues of untreated control and also in palms injected with the metallic screw injector. The results of the study thus clearly indicated.that PVC air pressure injector is the most efficient method to administer the antibiotic into the vascular system.

In a preliminary trial OTC 3g and 6 g ai dissolved in 500 ml distilled water was injected into 11 diseased palms of different age and intensity. Seedlings and young palms upto an age of 14 years developed chlorosis followed by drying of leaflets within 3-4 weeks. In adult palms phytotoxicity was not evident.

Four other antibiotics - tetracycline hydrochloride (M/s Hindustan Antibiotics, Pimpri) oxystecline (M/s Sarabhai Chemicals, Baroda), Nebacin (tobramycin sulphate of Dista Products Co., Indianopolis, USA) and Ancef (Cephazolin of Smith Kline Co. Philadelphia) were tried on two each of disease affected palms. An equal number of palms injected with distilled water maintained as control. These trials however could not be continued for lack of supply of the antibiotics.

Method for quantitative estimation of.Terramycin residues in treated palms and optimum test of bacterial population required for bioassay were standardised. Uptake, translocation and presence of the antibiotic in tissues leaflet and petiole of different whorls and parts of nuts of different maturity including husk, embryo, endosperm and nut water of two palms treated with 6 and 9 g a.i. were studied. The antibiotic was observed in all leaves of the treated palms except the spear leaf 24 hr after injection. Similarly, except husk of mature nuts, embryo and coconut water, all leaf samples representing different whorls and endosperm revealed the presence of the antibiotic. Maximum concentration was evident in the middle whorl of leaves especially in the basal portion of the pinnae. Out of 45 nuts collected from 14 palms that received 1, 2, 3, 6 and 9 g a.i. Terramycin residues were detected only in seven nuts that received 6 and 9 g a.i. OTC. In meat from palms that received higher concentration OTC , no residual effect was detected beyond 37 days.

Reverse Phase High Performance Liquid Chromotographic method was attempted to analyse the residues of OTC in coconut. Various parameters required for OTC detection were determined and instruments standardised. Standard sample clean up procedure was attempted and the percentage of recoveries were calculated to determine the efficiency of the method. A perfect HPLC method was standardised for the analysis of OTC in coconut water.

In another trial OTC residues in the foliage and roots of 3 root (wilt) diseased palms injected with 3 g a.i. was monitored. The findings are 1. The antibiotic could be detected in the foliage within 24 h after application indicating the efficiency of administration of the drug to reach the target site. 2. Maximum level of OTC was translocated to leaves in the third whorl 3. Leaves in the 4th, 5th and 6th whorls accummulated less but more or less at equal levels 4. Lower concentration has been detected in the foliage of outer two and inner whorls 5. Very little OTC activity was noticed in non-transpiring tissues such as inflorescence and roots 6. Detectable level of OTC was observed for more than 12 weeks and another important observation was that 7. With the onset of senescence of mature leaves, OTC gets redistributed to the foliage of third whorl.

A large scale field trial was conducted at two sites with the following treatments.

Table. IX : Details of palms treated with antibiotics.

No.	Treatment	Location / No. of palms			Response
		Mavelikkara	Vytilla	Total	
1	Ti Terramycin 1g ai.	15	-	15	+
2	T2 Terramycin 2g ai.	15	-	15	+
3	T3 Terramycin 3g ai.	7	8	15	+
4	T4 Terramycin 6g ai.	7	8	15	+
5	T5 Penicillin 30,00,000 units	7	8	15	–
6	T6 Distilled water	7	8	15	–
7	Neomycin 3g ai.	7	8	15	Phytotoxic

The antibiotics were administered to the trunk by the pressure injector at quarterly intervals. Although palms treated with Neomycin showed improvement over the pretreatment condition, the application had to be discontinued after one year due to severe phytotoxic symptoms. Treated palms showed severe bleeding patches and breaking of the petiole resulting in the ultimate death of a few palms. The experiment was continued for 3 years so that the entire canopy was replaced with a fresh set of leaves. The palms were indexed prior to first application in June 1984 and subsequently at half yearly intervals. Disease intensity score was computed for each of the palms based on method of George and Radha (1973). Final observations were recorded at the end of 3 years after the tender most leaf tagged at the commencement of the experiment had shed. Comparison of the final index with the pretreatment index revealed remission of symptoms in 53.3% of the palms treated with Terramycin 3g a.i. (T3) and 53.9% of palms treated with 6g a.i. (T4). The disease indices increased over pretreatment condition in both Distilled water and Penicillin treated palms (Table. X). Results of the experiment has thus conclusively established that the disease is induced by a wall less organism presumably phytoplasma.

Table. X : Response of Diseased Palms to Antibiotics

Treatment	No.of palms treated	No. of palms with remission	Percentage showing remission
T_1 - 1g ai. OTC	15	5	33.3
T_2 - 2g ai. OTC	14	3	21.3
T_3 - 3g ai. OTC	15	8	53.3
T_4 - 6g ai. OTC	13	7	53.9
T_5 - Penicillin 3 lakh units	15	Nil	-
T_6 - Distilled water	15	Nil	-

OTC : Oxytetracycline

Although Neomycin treated palms in the trial showed improvement in the foliar condition it had to be discontinued due to phytotoxicity. Hence a lower concentration of the antibiotic 1g a.i. administered at monthly intervals was tried. These palms also showed severe phytotoxic symptoms in the form of bleeding patches wilting and ultimate death of the palms.

A reverse formulation of Steclin 9 parts of tetracycline and one part of streptomycin was tried on a limited number of palms at monthly and quarterly intervals. Although the stecline treated palms showed remarkable improvement in the foliar condition, the experiment had to be discontinued after a year of start due to non supply of this research formulation by M/s. Hindustan Antibiotics, Pimpri.

Tylosin tartarate, which has been shown to block aster yellows infection in vector insects and to reduce the ability of the insects to transmit the disease agent was also tried on a limited number of palms at a dosage of 3g a.i. at quarterly intervals. Tylosin tartarate treated palms showed intense yellowing of all leaves and the treatment had to be discontinued for lack of any improvement.

Based on the results of the large scale field trial conducted during 1984-1987 and the decision of the Phytoplasma group meeting held during march 1989, a new field trial was conducted as detailed below :

No.	Treatment	Number of palms	Periodicity of application	Response
1.	Terramycin 3g ai.	5	Monthly	Phytotoxic
2.	Terramycin + Bavistin (3:1)	5	"	2/5 Remission
3.	Bavistin 1g ai.	5	"	No improvement
4.	Polyalthia leaf extract	5	"	Deterioration
5.	Gentamycin 3g ai.	15	Quarterly	Phytotoxic
6.	Penicillin 30 lakh	5	Monthly	Deterioration
7.	Distilled water	5	"	Deterioration

Monthly application of OTC did not have any added advantage over quarterly application in inducing state of remission. Two out of 5 palms treated with Terramycin + Bavistin showed remission of symnptoms. Tissues from, selected palms were also examined prior to the application of antibiotics and subsequently. Phytoplasmas in fewer numbers and degenerated forms were observed in palms treated with Terramycin + Bavistin. Palms treated with Bavistin, Distilled water and Polyalthia leaf extract however deteriorated over pretreatment condition. Gentamicin was found to be highly phytotoxic. Seven out of 15 treated palms died and the remaining eight showed gradual decline in yield and did not exhibit any improvement in foliar condition.

Heat therapy

It was suggested in the phytoplasma group meeting that a few diseased seedlings may be subjected to heat therapy. This was done on 3-4 year old diseased seedlings by covering them with polyethylene covers of 3x3x3 M. Two seedlings each were given the treatment for 24, 48 and 72 h. Uncovered seedlings were maintained as control. Covering the seedlings with polyethylene sheets increased the temperature by 14-20° C. Seedlings given the treatment for 48 and 72 h showed wilting of foliage and drying of 50% leaflets.

Solar pasteurisation

Solar heating of soil by mulching with transparent polyethylene during hot season was done. Soil around the base of two diseased seedlings, 2 meter diameter was ploughed, watered and covered with polyethylene sheets. This increased the soil temperature from 32.5°c to

44.8°c at a depth of 15 cm. However, at 30 cm depth the mean increase in temperature was only marginal (about 3.7°c). The mulching was continued for 45 days. Solar heating of the soil around the basin of diseased seedlings by mulching did not have any impact on the disease.

VII. SERO-DIAGNOSIS

Preparation of root(wilt) antigen.

Root(wilt) disease specific antigen was isolated from spear leaves of root (wilt) diseased palms in the early, middle and advanced stages of disease by Polyethylene Glycol (PEG) precipitation and differential centrifugation method. The Ultraviolet absorption spectrum of the purified preparation was recorded in ultraviolet-visible spectrophotometer. The preparation had 260/280 ratio of more than 1. Although no clear peak was evident the maximum abosorption at 260 nm and minimum at 280 nm indicated the preparation to be a nucleoprotein.

Polyacrylamide gel electrophoretic analysis (PAGE) of nucleoprotein isolated from palms with varying disease intensity revealed a similar pattern. A higher protein content was observed with increase in disease severity. Total protein and nucleoprotein isolated from spear leaves of healthy, apparently healthy and palms with different disease intensities were compared by PAGE. Two additional bands could be detected in samples from diseased palms. Antigenecity of the separated fraction was also assessed.

Preparation of root (wilt) disease specific antiserum

Root(wilt) disease specific antiserum was prepared by immunising more than 50 rabbits during the course of the project period. The animals were bled ten days after the fourth weekly intramuscular injection. The immunised animals were bled at weekly intervals and the titre assessed. The highest titre that was recorded was 1/4096. Maximum antigen titre was observed in spear leaf followed by the next outher leaf. A booster injection was administered when a drop in the titre was noticed.

The specificity of the root (wilt) antiserum was assessed by Ouchterlony's double diffusion test. Spear leaf extracts from healthy and diseased palms were tested in 1% agar gel against root (wilt) antiserum. Precipitin lines were observed midway between the antigen and antiserum wells. A single precipitin line was observed against the healthy leaf extracts and two precipitin lines against diseased samples. The single line merged with a similar precipitin line formed against diseased samples indicating the common antibodies in the antiserum in addition to disease specific antibodies.

Cross absorption

A number of methods were tried to eliminate common antibodes from the antiserum. Total protein isolated from leaves of healthy coconut palms were tried. Similarly, leaf extracts from healthy coconut palms and also from four other palm species, *Caryota urens, Borassus flebellifer, Elaeis guineensis, Elaeis oleifera* and *Cyrtostachys renda* were tried in intragel cross absorption to remove cross reacting antibodies.

An antihost serum was prepared against antigen isolated from leaves of healthy palms for purifying the root (wilt) antigen. Similarly, an antihost serum prepared against protein isolated from another palm species (*Borassus flabellifer*) was also tried. None of the methods tried could offer complete removal of common antibodies.

Attempts were also made to enhance the specificity of root (wilt) antiserum by cross absorbing the antiserum with host proteins or by passing through immuno absorbent column. However, total elimination of common antibodies could not be achieved.

Serological testing.

Tender leaf samples from a total of 2154 coconut palms as detailed in Table XI tested with the antiserum.

Table. XI : Results of Coconut Leaf Samples Tested

Sl.No	Details of palms sampled	No.of samples	No. of samples with positive reaction
1.	Elite palm (Hot spots)	1905	804
2.	F1 hybrid seedlings of elite palm	31	16
3.	Diseased	27	25
4.	Palms with middle whorl yellowing symtoms	17	14
5.	Experimental Coconut Seedlings	24	10
6.	Disease suspects	40	29
7.	Nematode pathogenicity experiment	7	4
8.	Apparently healthy coconut	98	53
9.	Healthy coconut	5	0
	Total	2154	955

Of the 2154 samples tested, positive reaction was recorded in 955 samples indicating their disease nature. Majority of the samples tested numbering 1905 are from apparently healthy elite palms identified in the four southern Districts of Kerala, namely, Kottayam, Kollam, Pathanamthitta and Alappuzha districts. The coconut palms tested are West Coast Tall, Chowghat Green Dwarf and Chowghat Orange Dwarf varieties. The palms with negative reaction were used as parents in evolving hybrids that are resistant to the disease.

The specificity of the antiserum to root (wilt) disease was also assessed by testing leaf samples of 13 yellow leaf diseased arecanut and thirty two spear rot affected oil palm. None of the samples gave positive reaction, thus confirming the specificity of the antiserum to only root (wilt) disease. Leaf samples from 40 disease suspects from Tamil Nadu and Kerala were tested. Twenty nine samples reacted positively indicating their disease status. The results thus confirmed the presence of Root (wilt) disease in Cumbum valley, Kasaragod and Calicut districts.

Sixty eight experimental palms under antibiotic treatment were tested prior to the start of antibiotic application. All the palms gave intense positive reaction for the disease. Forthy one of the the treated palms were retested after two years of commencement of the experiment. Faint reaction was observed in all the samples tested. This confirmed that antibiotics are not mycoplasmacidal but have mycoplasmstatic effect only on treated diseased palms.

Leaf samples collected from 8 coconut, I arecanut and 2 oil palm seedlings used for cross inculation studies, inoculated with either the plant hopper, *Proutista moesta* or bridged with dodder laurel were serologically tested: The source palm used were root (wilt) diseased coconut, Yellow leaf diseased arecanut and spear rot diseased oil palm. Positive reaction was observed in 6 coconut seedlings - one each from root(wilt) and yellow leaf diseased and two each from spear rot inoculated with *P. moesta* as well as bridged by dodder. Plant hopper inoculated arecanut and oil palm samples did not yield any positive reaction.

Preparation of phytoplasma specific antiserum:

To develop a phytoplasma specific antiserum, two antiserum were prepared. One was against the membrane fraction of phytoplasma prepared from symptomatic periwinkle and the other against its leaf grindates. The former antiserum had a higher titer than the latter.

An antiserum was also prepared against the antigen isolated from infective lace bugs. The cross reacting factors from the antiserum were removed. The antiserum was evaluated against wild lace bugs and plant hopper. It was observed that a minimum of 20 lace bugs and 25 plant hoppers were required to give visible precipitin reaction in agar gel double diffusion test.

Enzyme linked Immunosorbent Assay (ELISA)

For performing ELISA, α–globulin was isolated from the whole antiserum by sodium sulphate precipitation followed by fragmentation of $F(ab^1)_2$ by pepsin of digestion. Indirect DAS ELISA and protein - A indirect ELISA were done with crude antigen of healthy and diseased palms. Additives in various concentrations were incorporated in extration medium to eliminate tannins and other inhibitory substances. However, only marginal differences was perceptible in the absorbance values between healthy and diseased samples. Of the various types of ELISA tried, indirect ELISA and among the buffers, PBS-Tween 7.4 were found to give maximum difference in the absorbance values between healthy and diseased samples. Optimum dilution of primary antibody was determined as 1:400 and maximum antigen titre was observed in spear leaf followed by the next outer leaf.

Further ELISA was conducted with crude antigen of root (wilt) diseased and healthy samples. Different types of ELISA, $F(ab^1)_2$ ELISA, Indirect ELISA using Alkaline phosphatase (ALP) and Horse Radish peroxidase (HRP) as congugates were performed. Direct Antigen coated (DAC), Indirect ELISA using carbonate bicarbonate buffer of pH 9.6 as extraction and first antibody diluting buffer with HRP as enzyme conjugate yielded significant difference in the absorbance values between healthy and diseased samples.

Immunoglobulin fractions were isolated and purified from the whole antiserum by different salt precipitation techniques. High concentration α– globulin was obtained by ammonium sulphate precipitation method.

ELISA has been ultimately standardised for detection of root (wilt) disease. Antigen coated indirect ELISA using horse radish peroxidase as enzyme conjugate gives clear cut difference in the absorbance values between healthy and diseased samples. Addition of gelatin and ovalbumin in the extraction buffer significantly reduced the non-specific reaction and thereby enhanced the specificity of the test. The difference is highly significant when the ELISA plates were incubated overnight at 4° C after the addition of substrate.

Comparative efficiency of crude leaf extract over total protein isolated from healthy and diseased coconut palm was evaluated. Crude atigen invariably recorded higher absorbance value than total protein both in healthy and diseased palms while the crude antigen of diseased palm recorded 4 times higher values, total protein samples recorded thrice the values than healthy samples only

13. Approximate expenditure incurred in the project (Give reason for variation if any, from original estimated cost) : Rs. 95 lakhs.

14. Publications and material (one copy each to be supplied with this proforma) :

a) Research Papers : 37

b) Popular articles : 2

c) Reports : 15

d) Seminars and workshops (Relevant to the Project) in which the scientists have participated :

i. Sixth Workshop on All India Co-ordinated Coconut and Arecanut Improvement Project held at University of Agricultural Sciences, Bangalore 20-28 October, 1983.

ii. Seventh All India Workshop on Plantation Crops held at Trivandrum Nov. 6-8, 1985.

iii. Indo-US Workshop on Mycoplasma Diseases of Crops - Basic and Applied aspects held at Dehra Dun, Dec. 3-6, 1985.

iv. Third Regional Workshop on Plant Mycoplasma held at Delhi during February 24-28, 1987.

v. International Symposium on Coconut Research and Development (ISOCRAD-II) held at CPCRI Kasaragod during 26-29 Nov. 1991.

vi. FAO/ IBPGR meeting in Indonesia during 4-6 October 1991.

vii. Tenth Plantation Crops Symposium (PLACROSYM-X) during 2-4 December, 1992.

viii. International Workshop on Coconut Lethal Yellowing Like Diseases, Elmina, Ghana, during 5-10 November, 1995.

ix. National Consultations for improving productivity and utilisation of Coconut, Coconut Development Board, Kochi, during 12-13 Aug. 1998.

x. Indo-US Workshop on viriods and Diseases of Uncertain etiology held at Advanced Centre for Plant Virology, Division of Mycology and Plant Pathology, IARI, New Delhi, during 15-18 Nov. 1989.

e) Material developed such as new varieties of crops or breeds of farm animals, implements, products etc.

Disigned and fabricated a pneumatic pressure injector for atministering chemicals to palms.

15. Details (Nos. etc.) of Field /Laboratory note books and final material and their location :

Field /Laboratory note books are kept in the section.

16. Comments/Suggestions of Project Leader regarding possible future line of work that may be taken up arising of this project :

a. Molecular techniques for detection of phytoplasma in plant and insect vectors may be developed.

b. Phytoplasma specific antiserum may be prepared ; ELISA standardised for disease detection.

c. Plant products and chemicals with curative potential may be tried.

17. Signature with Name of Project Leader and Associates :

J.J.Solomon	-	Project Leader	JJSolomon
M.P.Govindankutty	-	Associate	
K.Mathen	-	"	
C.P.R.Nair	-	"	
P.Rajan	-	"	
B.Sathiamma	-	"	
M.Sasikala	-	"	Sasikala
V.Rajagopal	-	"	
B.Chempakam	-	"	
S.Robert Cecil	-	"	
P.G.Kamalakshiamma	-	"	PGK
N.Srinivasan	-	"	
M.Gunasekaran	-	"	
M.Kochubabu	-	"	
P.Chowdappa	-	"	
A.K.Shukla	-	"	
N.G.Pillai	-	"	
K.Madhavan	-	"	
Alka Gupta	-	"	

18. Signature (with comments if any) of Head of Division /Section/Station :

The work has been completed in a satisfactory manner. All items of the technical programme have been addressed in detail.

16.04.2001.

अध्यक्ष
फसल रोग प्रभाग
केन्द्रीय रोपण फसल अनुसंधान संस्थान
कासरगोड़, केरल 671 124

19. Signature (with comments, if any) of Director :

17.04.2001.
(Acting Director).

Director
CENTRAL PLANTATION CROPS RESEARCH INSTITUTE
P. O. KUDLU, KASARAGOD 670124

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