ETIOLOGY - PHYTOPLASMA

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Coconut root (wilt) disease has long been suspected to be induced by a sub-microsocopic agent (Nagaraj *et al.*, 1954). This hypothesis gained support with the experimental transmission of the disease to test seedlings employing the insect *Stephanitis typica* (Distant) under insect proof condition (Shanta *et al.*, 1960; Shanta *et al.* 1964). Comparative histological studies on palms with different intensities of root (wilt) disease revealed disorganisation and degeneration of vascular tissues. Phloem tissues showed increased chromophily and necrotic obliteration (Govindankutty and Vellaichamy, 1983), implying a vascular limited pathogen.

The presence of Phytoplasma, earlier referred as Mycoplasma Like Organisms (MLOs), was identified in sieve tubes of roots, tender stem, petiole and developing leaf bases of root (wilt) diseased palms (Solomon *et al.*, 1983). Phytoplasmas are plant pathogenic mycoplasmas that are non-helical non-culturable and trasmitted by arthropod insect vectors (Anon., 1984).

Constant association of Phytoplasma with the disease has since been established with the finding of the organism in tissues of all the 75 diseased palms as against their total absence in an equal number of healthy palms from disease free area studied (Table 8). The palms sampled cover the various intensities of disease and different locations. The mollicutes were found in increasing numbers in the sink regions. Mature tissues exhibited fewer numbers of degenerated forms (Solomon *et al.* 1987). This is in agreement with the findings in lethal yellowing disease of coconut in Florida (Parthasarathy, 1974). Conforming to the pleomorphic nature, forms varying from circular to oval and occasionally beaded or filamentous ones were observed in sieve tubes of diseased palms. The coccoid forms were in the range of 250-400 nm; bounded by a trilameller membrane and contained well defined internal structures such as DNA strands and ribosomes (Fig 7). The walls of invaded and closely neighbouring cells were thickened, the cytoplasm granulated and often contained vesicle like structures. Phytoplasmas were observed only in sieve tubes and often found in parietal position and more frequently close to the sieve area. Distribution of the organism within the vascular bundle was sparse and not all the sieve tubes in a phloem patch contained them. Similar trend of uneven distribution was also observed in the case of lethal decline of palms in Florida and Jamaica (Thomas, 1979; Parthasarathy, 1974), in Africa (Gianotti and Dollet, 1983) and coconut stem necrosis in North Sumatra and peninsular Malaysia (Turner *et al.*, 1978). Thomas (1979) after electron microscopic examination of over 36 declining palms belonging to 21 species observed that Phytoplasma concentration in

Table 8. EM examination of root (wilt) diseased samples *

Condition of palms	Location	No. of samples tested	No. of samples with Phytoplasma
Healthy	Disease free area	75	0
Disease early	Disease affected area	40	40
Diseased middle	Disease affected area	25	25
Disease advanced	Disease affected area	10	10

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

coconut was the lowest. Failure to find Phytoplasma in all the vascular bundles in root (wilt) affected palms could be attributed either to the low concentration *per se* or to an uneven distribution within the plant. None of the biological agents reported earlier to be associated with the disease (Radha, 1979; Lily, 1981) could be observed in the vascular tissues examined .

Govindankutty (1981) reported the occurrence of phloem anomalies in both roots and pinnae of palms affected with the disease. In subsequent studies abnormal bluish colouration in sieve tubes of diseased palms following Diene's staining and increased fluorescing sites in sieve area consequent to 4, 6 diamidino -2 phenylindole 2 HCl (DAPI) staining were observed (Solomon *et al.*, 1987). These histochemical staining reactions indicative of accumulation of DNA in extra nuclear sites showed the presence of Phytoplasma. Such characteristic reaction was not evident in tissues of healthy palms. Even in diseased palms positive reaction was not observed in all the sieve tubes of any phloem patch of root or every vascular bundle of rachillae. Such positive staining sites were more frequent in junctions of vascular bridges in rachillae. The occurrence of these reactions at scattered loci suggests the non uniform distribution of Phytoplasma in root (wilt) disease affected palms as corroborated in EM observation (Solomon *et al.*, 1987).

The specificity of Diene's staining (Deeley *et al.*, 1979; Razin, 1983) and fluorescence staining of DAPI (Seemueller, 1976) to bind with nucleic acid component of the phytoplasma has been well documented and is advocated as a diagnostic tool for detecting phytoplasma infection in plants (Nienhaus *et al.*, 1982). These techniques are currently being used to detect Phytoplasmal infection in root (wilt) affected palms especially the symptomless palms in the diseased tract and the disease suspects.

The constant association of phytoplasma with the disease warranted search for insect vector(s). Earlier

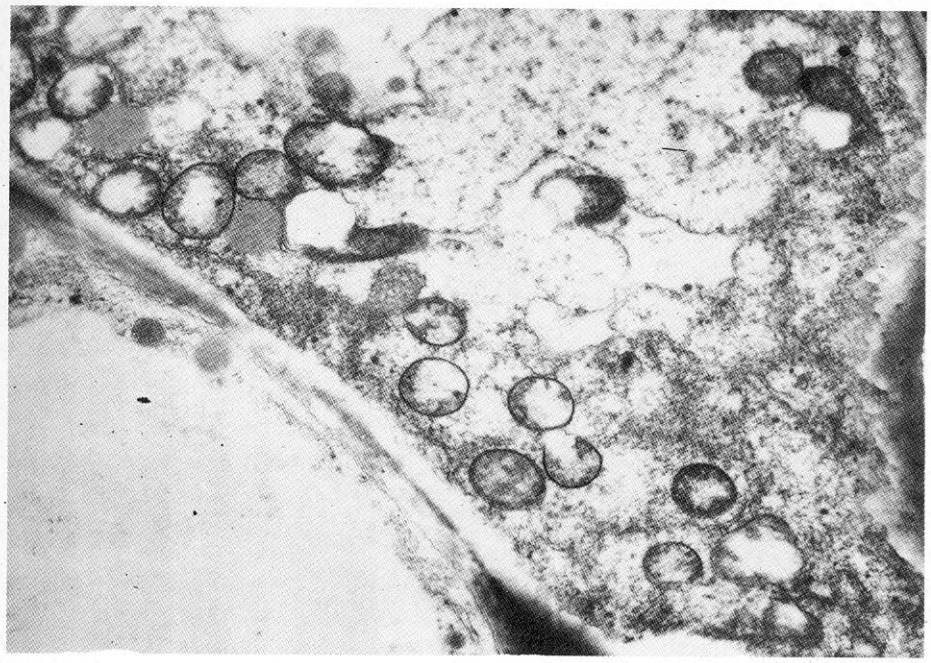

Fig. 7 Phytoplasma in sieve tubes of tender petiole from root (wilt) diseased coconut palm.

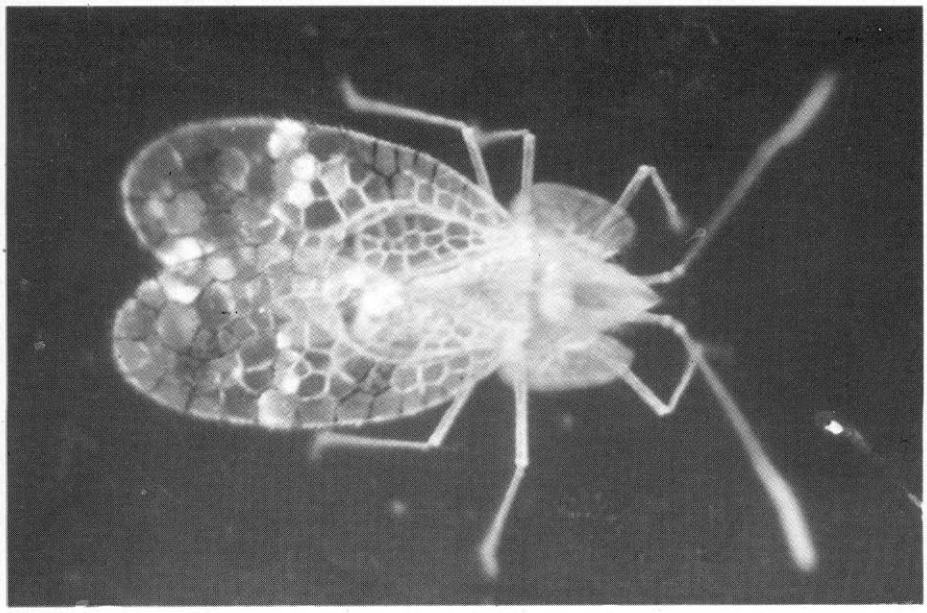

Fig. 8 Lace bug *Stephanitis typica* (Dist.)

transmission experiments (Nagaraj and Menon, 1956; Shanta, *et al.*, 1964) brought out the role of lace bug, *Stephanitis typica* (Distant) - Tingidae (Fig. 8) - the single major group of insects on coconut in the transmission of the disease. These observations were made when a viral etiology for the disease was suspected. But the report of Solomon *et al.* (1983) on the association of Phytoplasma with the disease implied a reinvestigation on the vectoral ability of the lace bug to imbibe and to transmit the phloem bound mollicute since phytoplasma diseases are not known to be transmitted by true bugs (Heteropteran insects). Phytoplasmas are mostly transmitted by leaf hoppers, plant hoppers (*Auchenorhyncha*) and rarely by psyllids. Record of insect fauna on coconut (Kurian *et al.*; 1979) however, did not include insects belonging to *Auchenorhyncha* from India. Therefore, a systematic inventory of insects in root (wilt) prevalent gardens using various traps and confirmation of their occurrence in coconut foliage by direct examination over a period of two years was carried out. As a result, besides lace bug, a leaf hopper, *Sophonia greeni* (Distant) and a plant hopper, *Proutista moesta* (Westwood) were recorded (Rajan and Mathen 1984; 1985). There was no disease occurrence independent of all the three insects. The potential of these insects to acquire the organism was verified electron microscopically. Phytoplasma was observed in brain and salivary glands of lace bug given an acquisition plus incubation period ranging from 18 to 23 days (Mathen *et al.*, 1987). Phytoplasma was not observed in lace bugs collected from disease free areas such as Kasaragod and Minicoy in Lakshadweep and also in bugs offered acquisition plus incubation periods less than 18 days (Mathen *et al.*, 1987). Phytoplasmas has also been observed in the salivary glands of *Proutista moesta* (Fig. 9) given an acquisition plus incubation period of more than 30 days on diseased palms (Anon., 1991).

In both the insects these polymorphic bodies are found in the acini of salivary glands and resembled in morphology and structure to those reported in vectors of other yellow diseases (Sinha and Paliwal, 1970; Nasu *et al.*, 1970). The morphology of Phytoplasma in root (wilt) disease affected field palms, insect salivary glands as well as brain tissues was comparable.

Although transmission of the disease from coconut to coconut through lace bug had been reported earlier (Shanta *et al.*, 1964), in the light of detection of Phytoplasma in the tissues of disease affected palms, transmission experiments were repeated under insect proof conditions (Fig. 10). Nine months after the first inoculation, coconut seedlings inoculated with lace bugs gave strong positive serological reaction in three out of four experimental seedlings and weak reaction in the fourth indicating contraction of root (wilt) disease. Light microscopy of root tissues subjected to Diene's staining, DAPI and Hoechst 33258 fluorochromes also indicated Phytoplasma infection in phloem. EM observation also confirmed the presence of Phytoplasma in all the four lace bug inoculated seedlings between 9 and 27 months after the first inoculation. By the 17th month, two of the seedlings developed flaccidity of leaflets, the diagnostic and

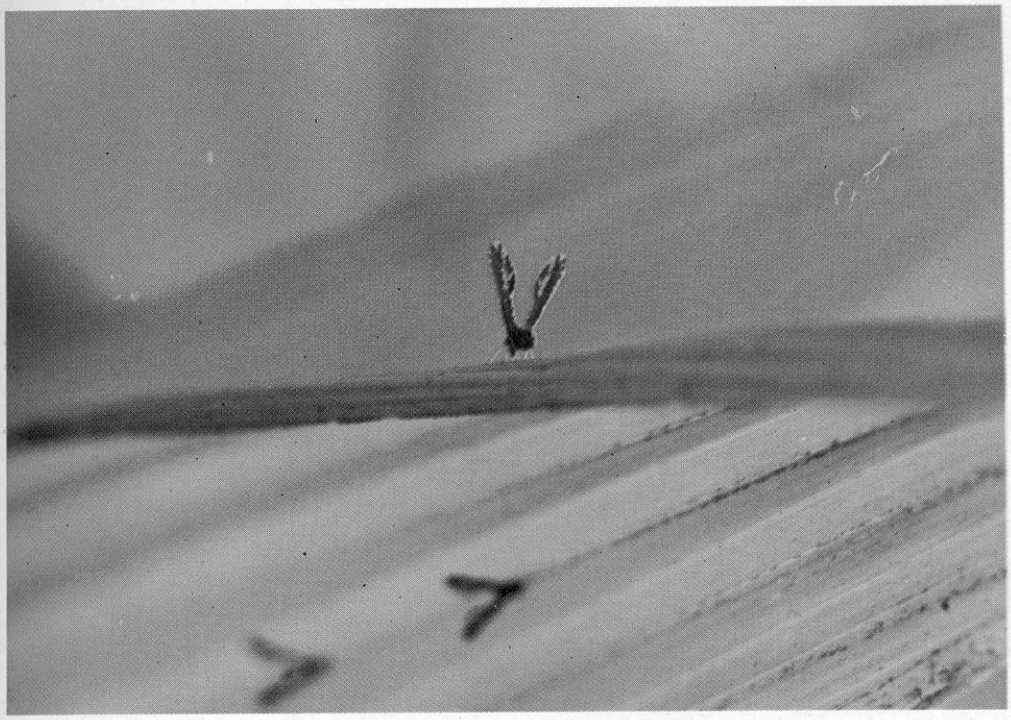

Fig. 9 Plant hopper *Proutista moesta* (Westwood)

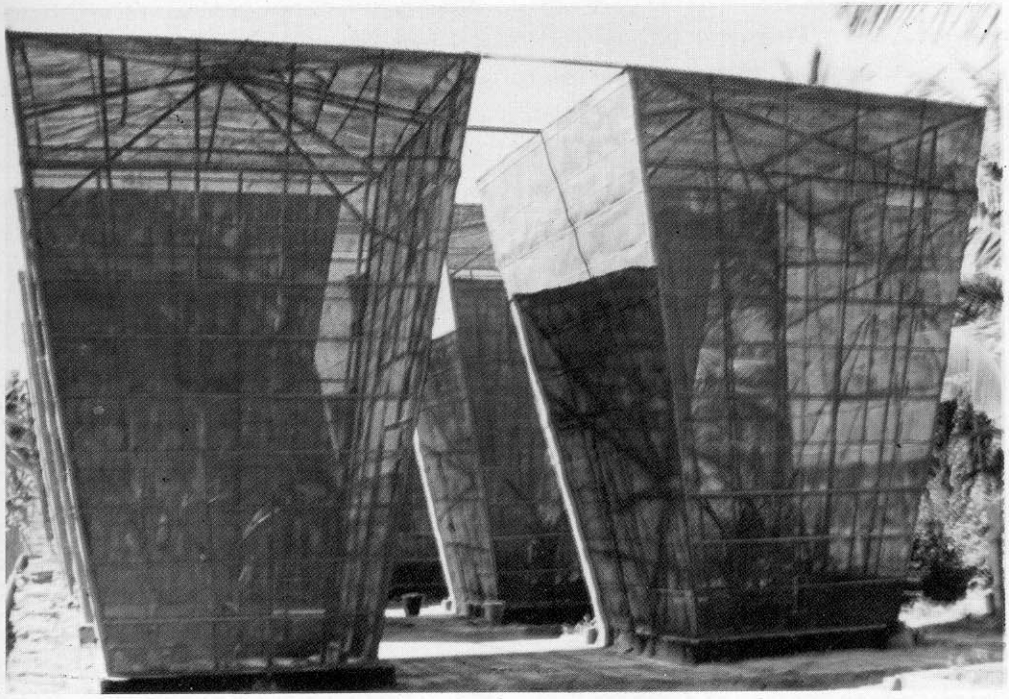

Fig. 10 Insect proof cages for transmission experiment

decisive symptom of the disease (Mathen *et al.*, 1990). However, in control seedlings there were no symptoms and no Phytoplasma was observed either.

Apart from the direct evidences accrued on the vectoral role of lace bugs, a number of indirect evidences also lent support. Lace bugs were found colonising in increasing number towards the inner leaves (Mathen *et al.*, 1969). This pattern of distribution enhances the chances of the organism being acquired more efficiently by the bug since active forms of Phytoplasma in higher concentration were found in tender tissues. It was also reported that the number of lace bugs in diseased palms was four times more than that in symptomless palms (Mathen, 1982). Monitoring lace bug population for two years, Mathen (1985) reported a direct linear correlation between the number colonising the palms and percentage of fresh incidence of disease. Transections of coconut pinnae with lace bugs fixed in feeding position by a cold immobilisation technique revealed the termination of the stylet in phloem, thereby confirming the ability of the insect to pick up the phloem delimited organism (Mathen *et al.*, 1988).

Although, tingids as a group are not conventional mycoplasma transmitters, instances of tingids being vectors are encountered in literature. Sugarbeet savoy disease now recognised as of Phytoplasma etiology (Haris, 1979) is transmitted by *Piesma cinereum* (Say) and sugarbeet latent rosette, a rickettsia-like organism (RLO) induced malady transmitted by *P. quadratum* Fieb. (Proesler, 1980). These vectors belong to Piesmidae, taxonomically very close to Tingidae. The above cited direct and indirect evidences together with the finding of Phytoplasma in the salivary gland and brain tissues of lace bug and transmission of the disease from diseased to healthy coconut seedlings through the bug confirm the insect being a vector of the disease. The vector role of the plant hopper was also experimented in two year old WCT coconut seedlings, under insect proof condition. Phytoplasma was observed in six out of eight plant hopper inoculated seedlings 5-24 months after inoculation. Five of the seedlings exhibited the diagnostic symptom of the disease thus confirming the vector role of the plant hopper (Anon., 1997).

Results of the field experiments on the effect of vector control through foliar/ soil application of insecticides and plant products on the incidence of the disease clearly indicated the disease contraction even in plants sprayed at fortnightly intervals with ten times the normal recommended concentration of the insecticide (Anon., 1993; 1997). Similar results were also reported in lethal yellowing disease in Florida (Howard and McCoy, 1980; Howard and Barrant, 1989). This suggests that prevention of disease incidence through insect control is not a practical proposition.

Experimental transmission of Phytoplasma was attempted also employing certain phanerogamic parasites to periwinkle, *Catharanthus roseus* G. Don, a known mycoplasma indicator host. Dodder species, *Cuscuta campestris* Yunck., *C. chinensis* Lam.

and *C. subinclusa* Dur and Hilg. although established on coconut foliage, failed to put efficient haustorium to reach the coconut leaf vascular bundle. Tsai (1983) also failed in his attempts to transmit the lethal yellowing disease from coconut through *C. campestris*. A dodder laurel, *Cassytha filiformis*, however, established well on coconut putting forth haustorium to reach and form intimate contact with the vascular bundles. Periwinkle grown in sterilized soil in mud pots and protected inside insect-proof muslin cloth cages bridged through dodder laurel established on diseased coconut seedlings in the field, developed chlorotic spots in the interveinal areas at vein endings of fully opened leaves, three to four weeks after the establishment of the haustorium. Passage of Phytoplasma as confirmed by positive staining reactions and detection of the organisms through electron microscopy in the midvein and petiolar tissues of the periwinkle, dodder strands and leaflets of diseased coconut seedlings, established the transmission of the disease from coconut to periwinkle. Phytoplasma was however not observed in dodder on disease free coconut and control periwinkle plants (Sasikala *et al.*, 1980). Although *C. filiformis* had been employed to transmit citrus mosaic from sweet orange (*Citrus sinensis* (L) Osbeck.) to acid lime *C. aurantifolia* (Christm, Swingle) (Reddy *et al.*, 1985) this is the first instance of Phytoplasma being transmitted by an unconventional dodder species.

Though the disease could be experimentally transmitted through the lace bug to healthy coconut palms and through the dodder to periwinkle, culturing of Phytoplasma *in vitro* is considered to be one of the pre-requisites to prove the pathogenicity of the organism. Phytoplasmas being restricted to the specialised vascular environment of phloem, a medium simulating the physico-chemical environment of the phloem may be necessary for successful culturing of the organism in cell free medium. Phloem sap which is rich in nutrients has been found as an ideal medium either as such or with serum supplements for culturing *Acholeplasma laidlawii*, *Mycoplasma fermentans* and *Spiroplasma citri* (Eden-Green and Waters, 1982; Mc Coy, 1977) and *Phytomonas davidi* (Mc Coy, 1978).

Rajagopal *et al.* (1988) standardised a method for collection of vascular sap in ice packed vacuum flasks. The physicochemical condition of vascular sap from apparently healthy and diseased palms has since been analysed. The analysis of sap also gave an insight into the type of Phytoplasma found associated with the disease. Sap from diseased palms had higher arginine level than that in healthy palms. It is suspected that, the Phytoplasmas present in root (wilt) diseased palms may be of the non-fermentative type, which uses the arginine through dihydrolase pathway for its energy production (Chempakam and Rajagopal, 1989).

The vascular sap collected from apparently healthy palms was filter-sterilised and used as such or supplemented with growth factors for the preparation of culture media. In addition, about 40 different media with various combinations of growth factors,

nucleic acid precursors, co-factors, vitamins etc. were used for the culturing of the organism from tissues of diseased coconut, symptomatic periwinkle and infective lace bugs adopting a number of methods. Embryo-nated hen's eggs were also employed. However, the organism could not be cultured in any of the media (Anon., 1989). Currently, attempts are being made to maintain/propagate the root (wilt) phytoplasma in explants from diseased palms. Phytoplasma could be maintained in rachillae expants from diseased juvenile coconut palms for more than 6-8 weeks in certain plant tissue culture media (Anon., 1989). Co-culturing of dodder laurel with embryo cultured coconut plantlet for attempting *in vitro* transmission is also in progress.

Various methods of application such as ring barking, root feeding, gravity flow and stem injection with pneumatic pressure injection device were tried (Pillai and Raju, 1985). The pneumatic injector was found to be superior to all other devices/methods as the antibiotic injected with this could be detected in sufficiently high concentration in the foliage within 24 hr of application. Residue analysis of the antibiotic in root tips, un-opened leaves and nuts of the injected palms eventually revealed the retention of the chemical in the foliage for more than 12 weeks with the concentration petering out to minimum with the passage of time (Chowdappa *et al.*, 1989)

A field trial was initiated in 1984 with four concentrations (1,2,3 and 6g ai) of Oxytetracycline hydrochloride (OTC

Fig. 11 Antibiotic therapy : OTC-treated palm

Fig. 12 Antibiotic therapy : Dist. water control

Terramycin Tree Formulation of M/s Pfizer India Ltd.) a single concentration each of Neomycin, Penicillin and distilled water control. Fifteen palms each in the early stage of disease were given the different treatments at quarterly intervals. Fifty three palms treated with 3 and 6/g ai of OTC showed remission of symptoms. Contrastingly, palms in the distilled water (Figs. 11 and 12) and penicillin treatment deteriorated significantly over the pre-treatment condition (Pillai *et al.*, 1991). Thus, the remission of symptoms in OTC treated palms adds further evidence to the etiological role of Phytoplasma in coconut root (wilt) disease.

Non-cultivable nature of phytoplasma is a limiting factor in fufilling Koch's postulates. Nevertheless manifestation of disease symptoms through inoculation of insect vector(s) rendered infective and differential chemotherapeutic response of diseased palms to pencillin and tetracycline are considered adequate to offer the best circumstantial evidence to prove the phytoplasmal etiology.

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