ETIOLOGY

E. MYCOPLASMA-LIKE ORGANISMS

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Root (wilt) disease of coconut has long been suspected to be induced by a sub-microscopic agent (Nagaraj, Davis and Menon, 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, Menon and Pillai, 1960; Shanta, Joseph and Lal, 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).

The presence of mycoplasma - like organisms (MLOs) was identified in sieve tubes of roots, tender stem, petiole and developing leaf bases of root (wilt) diseased palms (Solomon, Govindankutty and Nienhaus, 1983). Constant association of MLOs with the disease has since been established with the finding of the organism in tissues of all the sixty five diseased palms as against their total absence in the thirty five healthy palms from disease free area studied. The palms sampled cover the various intensities of disease and from different locations. The mollicutes were found in increasing numbers in the 'sink' regions. Mature tissues exhibited fewer numbers of MLOs in degenerated forms (Solomon, Govindankutty and Mathen, 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 25-400 nm; bounded by a trilameller membrane and contained well defined internal structures such as DNA strands and ribosomes. The walls of invaded cells and of those close to them were thickened, the cytoplasm granulated and often contained vesicle like structures. MLOs 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, Jones and Kenten, 1978). Thomas (1979) after electron microscopic examination of over 36 declining palms belonging to 21 species observed that MLO concentration in

Veitchia merrilli (Becc.) H.E. Moore was the lowest. Failure to find MLOs in all the vascular bundles in root (wilt) affected palm 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 could be observed in the vascular tissues examined (Radha, 1977; Lily, 1981).

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 Dienes' staining and increased fluorescing sites in sieve area consequent to 4', 6-diamidino -2 phenylindole.2 HCl (DAPI) staining was observed (Solomon, Govindankutty and Mathen, 1987). These histochemical staining reactions indicative of accumulation of DNA in extra - nuclear sites showed the presence of MLOs (Fig. 1). 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 MLOs in root (wilt) disease affected palms as corroborated in EM observation (Solomon *et al.*, 1987).

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

The constant association of MLOs with the disease warranted search for insect vector(s). Earlier transmission experiments (Nagaraj and Menon, 1956; Shanta, Joseph and Lal, 1964) brought out the role of lace bug, *Stephanitis typica* Distant - Tingidae (Fig. 2) 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 MLOs with the disease implied a reinvestigation on the vectoral ability of the lace bug to imbibe and to transmit the phloem bound mollicute since MLO diseases are not known to be transmitted by true bugs (Heteropteran insects). Mycoplasma-like organisms 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

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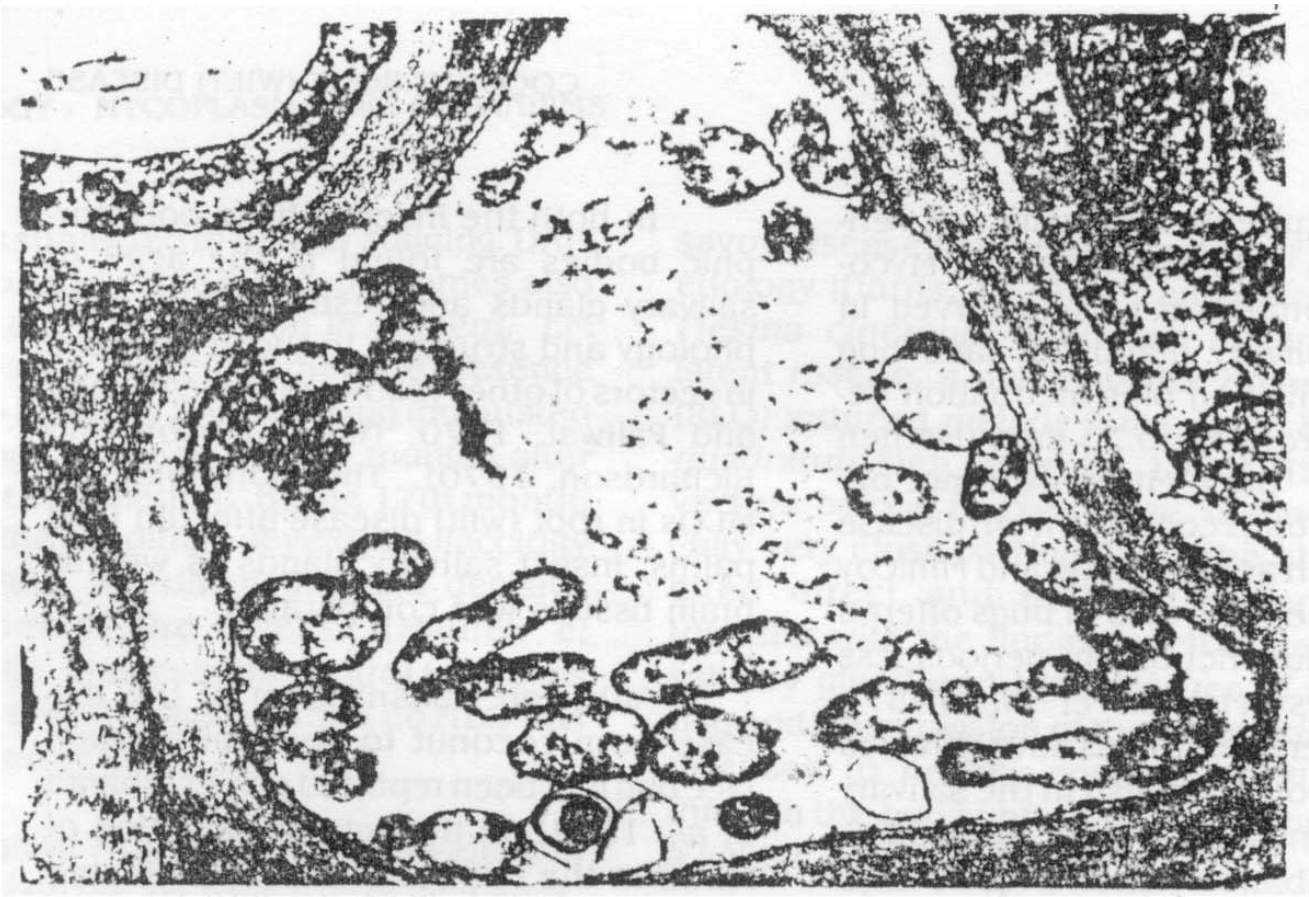
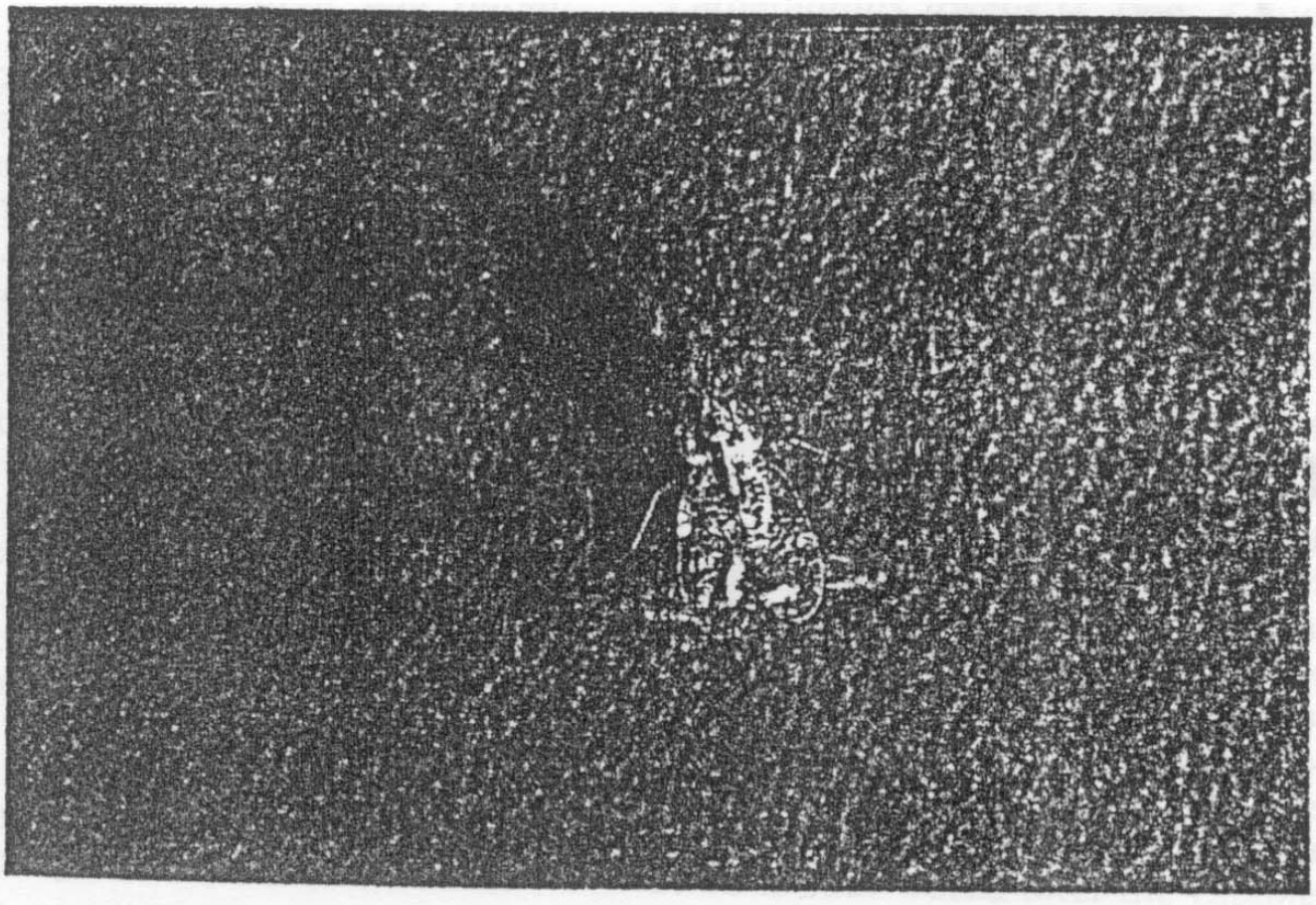


Fig 1 Mycoplasma like organisms in sieve tubes of tender petiole from root (wilt) diseased coconut palm.



2 Lace bug

insects to acquire the organism was verified electron microscopically. Mycoplasma-like organism 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). Such MLOs were 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). However, recent EM studies have revealed the presence of MLOs also in the salivary glands of plant hopper with an acquisition plus incubation period over 40 days (Anon. 1989). The vectoral role of the plant hopper (Fig. 3) is to be assessed through a transmission experiment on coconut seedlings under insect proof condition.

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 yellows diseases (Sinha and Paliwal, 1970, Nasu, Jenson and Richardson, 1970). The morphology of MLOs 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 MLOs in the tissues of disease affected palms, transmission experiments were repeated under insect proof conditions (Fig. 4). Nine months after the first inoculation, coconut seedlings inoculated with lace bugs gave strong positive serological reaction in three and weak reaction in the fourth indicating root (wilt) contraction. Light microscopy of root

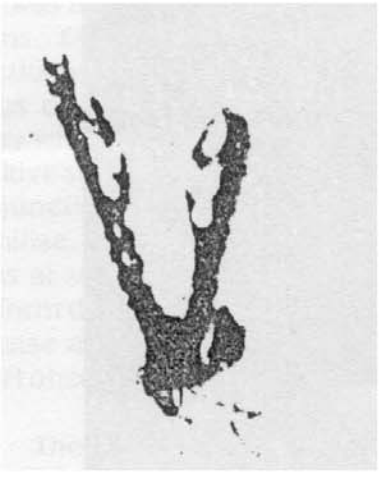


Fig. 3. Plant hopper

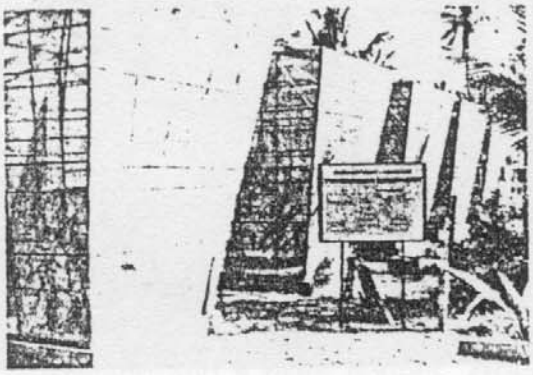


Fig. 4 Insect proof cages for transmission experiment

tissues subjected to Dienes' staining, DAPI and Hoechst 33258 fluorochromes also indicated MLO infection in phloem. EM observation also confirmed the presence of MLO in all the four lace bug inoculated seedlings between 9 to 27 months after the first inoculation. By the 17th month, two of the seedlings developed flaccidity of leaflets, the diagnostic and decisive symptom of the disease (Mathen *et al.*, 1990). However, in control seedlings there were no symptoms and no MLO 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, Mathew and Kurian, 1969). This pattern of distribution enhances the chances of the organism being acquired more efficiently by the bug since active forms of MLOs in higher concentration were found in tender tissues. It was also reported that the number of lace bugs in diseased palms was four times 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 MLO etiology (Harris, 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 MLOs 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.

Experimental transmission of MLOs 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. Dodder laurels established on periwinkle, maintained under insect-proof cage and bridged on to diseased coconut seedlings in the field, developed chlorotic spots in the interveinal areas and at vein endings of fully opened leaves, three to four weeks after the establishment of the haustorium. Passage of MLOs as confirmed by positive staining reactions and detection of the organisms through electron microscopy in the mid vein and petiolar tissues of the peri-

winkle, dodder strands and leaflets of diseased coconut seedlings, established the transmission of the disease from coconut to periwinkle. MLOs, were, however, not observed in dodder on disease free coconut and control periwinkle plants (Sasikala *et al.*, 1988). 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, Naidu and Gopalaraju, 1985) this is the first instance of an MLO 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 MLO *in vitro* is considered to be one of the pre-requisites to prove the pathogenicity of the organism. Mycoplasmas being restricted to the specialised vascular environment of phloem, a medium mimicking 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; McCoy, 1976, 1977) and *Phytomonas davidi* (McCoy, 1978).

Rajagopal *et al.* (1988) standardised a method for collection of vascular sap in ice packed vacuum flasks. The physico-chemical 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 MLO found associated with the disease. Sap from diseased palms had higher arginine level than in healthy palms. It is suspected that, the MLOs 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) mycoplasma in explants from diseased palms and infective insect tissues. MLOs could be maintained in rachillae explants from diseased juvenile coconut palms for more than 6-8 weeks in certain plant tissue culture media (Anon, 1989).

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. Resi-

due analysis of the antibiotic in root tips, un-opened leaves and nuts of the injected palms over a period of time 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 6 g ai) of Oxytetracycline hydrochloride (OTC - 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. 5.1 and 5.2) and Penicillin treatment deteriorated significantly over the pre-treatment condition (Pillai *et al.*, 1990). Thus, the remission of symptoms in OTC treated palms adds further evidence to the etiological role of MLOs in coconut root (wilt) disease.

Having proved the role of MLOs as the etiological agent of coconut root (wilt) disease, the emphasis is now on devising integrated management practices for root wilt disease affected palms. Field experiments are in progress to see whether the insecticidal control of aerial insects could regulate fresh incidence of disease in newly planted seedlings.

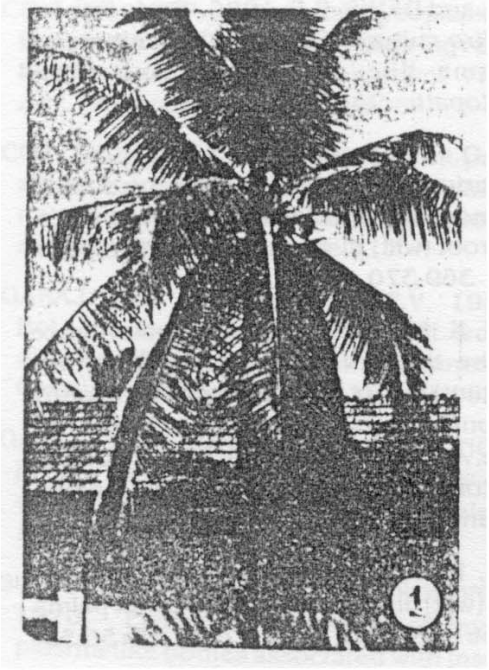


Fig. 5.1 Antibiotic therapy: OTC-treated palm

Fig. 5.2 Antibiotic therapy : Dist. water control

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