STUDIES OF MYCOPLASMA - LIKE ORGANISMS IN PLANTS

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ABSTRACT

The disease clover phyllody (CP), caused by a mycoplasma - like organism (MLO), was studied from two wild clover populations. Disease organisms were transferred to Vinca rosea using the leafhopper Euscelis plebejus. V. rosea was a useful test plant and such infected plants were used for further MLO transmission experiments as well as for testing possible chemotherapeutants. Attempts to culture clover phyllody on artificial media were unsuccessful. Monstrose and cristate forms of some cacti were examined and found to contain MLO. Infected cacti were used for comparison with CP and for use in transmission and chemotherapy experiments. Other MLO diseases including those of Rosa sp., Fragaria vesca and Helenium hybridium were used in conjunction with CP, to find a specific light - microscope stain for MLO diseases. Dienes' stain was tested and successfully developed as a specific stain for MLOs, requiring no specialised equipment. A new fluorescent staining technique was also developed using europium chaelate. The MLO found in the phloem of a range of different MLO diseases were compared by electron microscopy. MLO from cacti, although larger than those from herbaceous plants, were otherwise very similar. In the course of the EM examination it was possible to observe replicative stages in the life cycle of the MLO and their movement through sieve pores which allows the MLO to spread through the host plant. Subcellular content and structural changes of the host plants were also noticed. The major change was the reversion of coloured plastids to green chloroplasts. The phenomenon was followed in a series of petals from Vinca rosea showing progressive greening.

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INTRODUCTION

A micro-organism causing bovine pleuropneumonia was first described by Nocard and Roux in 1898 (54). Similar organisms were discovered later from other animals and in the 1930s some were successfully cultured *in vitro*. This group of organisms was named pleuropneumonia-like organisms (PPLO). PPLO were later compared with wall-less or L-forms of bacteria. Such L-forms could be isolated from bacterial cultures and most, under suitable conditions, could revert back to the parental bacterial type (54). The wall-less PPLO were however found to be unrelated to any bacterial 'parent' and did not revert to a walled form. In recent years studies of DNA homology and comparison of nucleic acid base ratio determinations have emphasised that they are a distinct though heterogeneous group of organisms (198).

PPLO are distinct from bacteria in requiring sterols for growth in cell free media. They are identified by their extreme pleomorphism, limiting unit membrane, small ribosomes and central DNA strands (96). PPLO can pass through bacteria-retaining filters with a pore diameter of 0.45 μm although their linear dimensions are variable as a result of their plasticity. Electron microscopy has been used to show the size and shape of PPLO (111) and the size range has been accepted as 0.1 - 1.0 μm diameter (65). Size variations can occur between species and with stages of growth within a species.

In 1955 the International Committee on Nomenclature of Bacteria accepted the name *Mycoplasma* put foward by Edward and Freundt for the pleuropneumonia-like organisms (113, 54). These organisms were placed in a new class, *Mollicutes* which contains one order, *Mycoplasmatales*. The order is now recognised to contain four distinct genera: *Mycoplasma*, *Acholeplasma*, *Thermoplasma* and *Spiroplasma* (11). For

convenience the term mycoplasma will be used here in its original broader sense.

Recently mycoplasmas have been shown to be attacked by viruses (124) which are clearly distinguishable from bacteriophages and this is used as further evidence that mycoplasmas are distinct from bacteria.

The basic reproductive cycle of mycoplasmas starts with a small, densely staining spherical particle or elementary body. This body grows into a larger round or filamentous form (depending on species). It is generally accepted that new elementary bodies are budded off from these intermediate forms. Occasionally elementary bodies are found inside mature forms and it is assumed that these are released by the breakdown of the parent body. New elementary bodies can be formed in filamentous bodies by the constriction of a filament into a chain of small, dense, 'coccoid' forms. These are rounded off and released as elementary bodies.

Mycoplasmas are distinct in their reaction to antibiotics being sensitive to quite small amounts of tetracyclines but, unlike bacteria, resistant to penicillin (84). Penicillin is therefore often added to mycoplasma culture medium to prevent bacterial contamination.

Phytopathologists were interested in a large group of plant diseases characterised by stunting, rosetting, leaf yellowing, 'witches'-broom' symptoms and, in some cases, green flower symptoms. These were collectively entitled 'yellows' or 'European yellows' diseases. Because no known bacterial, fungal or protozoal agent could be found associated with these diseases they were assumed to be of viral aetiology. The 'yellows' symptoms were similar to those induced in plants by recognisable viruses although no virus could be purified from the plants showing 'yellows' symptoms.

The yellows group of diseases were also transmissable by grafting (214), by insects and, in some cases, by dodder (214). The yellows disease organisms were shown to infect their insect vectors (130, 131, 133, 188, 221) as well as their plant hosts (134, 176), infections being circulative in the insect vectors (188, 191, 220) and, in some cases, adversely affecting them (106, 222). Some cross-protection in vector insects (116, 135, 212, 213) and the ability of non-vector species of leafhoppers to act as reservoirs for the disease organism (132, 189) were additional phenomena known to occur with well-established plant pathogenic viruses.

However this group of so called 'virus' diseases was separated from other virus diseases by being transmitted by leafhoppers and rarely by other vectors (88, 180) and also by being sensitive to much lower temperatures than other virus diseases (105, 148, 151). Although the causal agent could be transmitted experimentally between insects by injection, they were not mechanically transmissable to plants. In 1967 Doi et al in Japan examined plants infected with Mulberry Dwarf, Potato Witches'-Broom, Aster Yellows and Paulownia Witches' Broom diseases using electron microscopy. They showed the presence of pleomorphic, wall-less micro-organisms in the sieve elements of the phloem of diseased plants but not in healthy controls (53). In the case of Mulberry Dwarf the treatment of diseased plants with tetracyclines brought about remission of symptoms presumably because the micro-organisms associated with the disease were sensitive to these antibiotics (103). It was therefore suggested that the disease inducing micro-organisms in these plants were mycoplasmas.

These findings aroused considerable interest and speculation as to the nature of the causal agent of other yellows diseases. Davis

and Whitcomb (47) discuss, in a comprehensive review, the possibility that mycoplasma, rickettsia or chlamydiae are the causal agent of such diseases. All three types of micro-organism are known to be pathogenic in animals. Except in a few cases where the disease organism has been shown to be a Rickettsia (60, 94, 158) or a virus (73, 187, 216, 218) it has been widely accepted that mycoplasmas, or mycoplasma-like organisms, are the pathogens causing many yellows diseases in plants.

In the years following Doi et al's discovery there has been a rapid growth of interest in yellows diseases as indicated by the number of reviews which have been written (65, 82, 99, 100, 139, 150, 197). By 1970 Maramorosch et al (139) were able to list 40 diseases which had been found to have mycoplasma-like organisms associated with them and this number is constantly increasing (5, 6, 7, 8, 9, 27, 28, 34, 43, 58, 85, 92, 97, 147, 159, 161, 163, 184, 196, 217, 224, 226). The papers quoted cover many different plant hosts and it is possible that many of the diseases are caused by strains of the same pathogen affecting different plant species.

In plants the pleomorphic organisms have been found to have the same size range, sensitivity to the same antibiotics, and show many of the varied growth forms associated with those mycoplasmas pathogenic to animals. They are also limited by a unit membrane, have similar sized ribosomes and central DNA strands. In the case of Aster Yellows a virus, similar to those parasitic on animal mycoplasmas, was found associated with the disease inducing organisms in the plant (3). Plant mycoplasma-like organisms are known to be able to complete their life-cycle in both insect and plant hosts (91).

Despite the considerable interest in these plant pathogens there is no firm evidence that these organisms belong to the same group as

animal mycoplasma. They have many similar properties but unequivocable evidence placing them in the order Mycoplasmatacae depends on their being cultured and it therefore seems preferable to call them mycoplasma-like organisms (MLO) (137).

Successful in vitro culture is also required to satisfy Koch's postulates and establish the aetiology of MLO-associated diseases. MLO are found, often in large numbers, in the phloem of yellows diseased plants and no other disease organisms have been found. They are not present in healthy control plants and remission of symptoms by tetracyclines is associated with a reduction in numbers of MLO in the plant. All the evidence indicates strongly that they are the causal agent, but until they can be isolated and shown to cause the same disease symptoms when re-introduced into healthy plants their aetiological role can only be assumed (137).

Some reports claim the successful culturing of plant MLO as with white leaf disease of sugarcane (122, 123) but this result has not been repeated in other laboratories (40). The major advance in this field came about when Saglio et al (175, 176, 177) were able to culture in vitro the causal agent of Citrus Stubborn (or Little Leaf) disease in both liquid and solid media. Citrus Stubborn showed symptoms characteristic of yellows diseases and EM studies showed mycoplasma-like bodies in the phloem of infected plants (14). Penicillin could be added to the growth medium to prevent bacterial contamination with no effect on the mycoplasmas. Re-introduction of the cultured organisms into healthy citrus plants caused symptoms indistinguishable from the original diseased plants thus fulfilling Koch's postulates.

The Citrus Stubborn organism was later identified as a mycoplasma by serology tests against known animal mycoplasmas (141). It had a distinct spiral morphology in culture however and was sufficiently well

characterised to be classified as a new genus Spiroplasma and named Spiroplasma citri.

Corn Stunt (CS) MLO have also been cultured successfully on artificial media (30, 223). Using phase contrast microscopy Davis (45) was able to show that the CS organism also has a spiral morphology. He therefore used a culture medium based on that of Saglio et al for his successful culturing experiments.

The spiral morphology of these organisms can now be readily demonstrated using thick EM sections or phase contrast microscopy (44) although earlier work gave no indication of this distinctive shape but merely showed pleomorphic structures (21, 78, 79, 80, 102, 215). It is possible therefore that other plant pathogenic MLO may be found to have a similar spiral morphology and belong to the same or closely related genera.

The CS organism has been classified as a Spiroplasma but has not been given a specific name. CS and S. Citri spiroplasmas can be distinguished from each other by electrophoresis of their proteins (156); this is an accepted method of showing differences between animal mycoplasma species (169). Cross-protection has also been shown between S. citri and CS (190) but as strains of S. citri differ serologically from each other (2, 211) this may not be sufficient to distinguish between species. Criteria for separating species must be set up before CS spiroplasma can be classified either as a new species or a strain of S. citri.

Now that spiroplasmas can be cultured routinely studies are being conducted on their metabolic activity and biochemistry (210) and on suitable media for their culture (108, 121). Such studies may help attempts to culture other MLO.

Many yellows diseases have been attributed to infection by MLO

but there is still some uncertainty in diseases such as Coconut Lethal Yellows (7) because the organism cannot be isolated, cultured or identified.

New MLO-type diseases are still being described so there is a requirment for a reliable means of detecting such diseases and of distinguishing them from diseases induced by other organisms. Possible methods of detection include the use of:-

a) Culture techniques so allowing the characterisation of the organism, fulfilling Koch's postulates and an understanding of the physiology of the organism.

b) Recognition of the organism in the electron microscope (EM) and recognition of possible specific MLO-induced changes in the host cell ultrastructure.

c) Specific light microscopy stain for MLO or their effects on host cells.

d) Use of specific chemotherapy not only to control the disease but also to provide a tool for distinguishing MLO from other plant pathogens.

e) Indicator plants or test plants which might provide a diagnostic test and a rapid and reliable method of distinguishing MLO from other pathogens.

The research described in this thesis is based on studies of Clover Phyllody (CP), a disease characterised by the production of green leaf-like flowers (Fig I.1) and occasionally red-leaf symptoms (Fig I.1). CP was first described in Britain by Carr in 1960 (24) and more recently has been studied in Canada by Chikowski (31, 32, 33), and in France by Gianotti et al (67, 68, 69, 70, 71, 72) and by several other researchers (57, 128, 192, 193).

Soon after the first disovery of MLO by Doi et al MLO were found

to be associated with CP (69) and the disease was shown to be sensitive to treatment with tetracyclines (32, 33). As with most plant MLO the organism is circulative and persistent in the leafhopper vectors (128, 191, 193) and will multiply at all stages of infection in the insect (75). Faivre-Amiot et al (57) and Gianotti and Vago (72) claim to have successfully cultured the CP organism. Gianotti and Vago (72) also claim to have re-infected healthy plants from the cultured organism but the results have not been repeated.

In Britain CP occurs in wild clover plants which are commonly found on grassy verges. CP can also be found in isolated patches of clover in pasture particularly near the edges of fields where the plants are not so heavily cropped by browsing animals.

White clover is grown as a fodder crop and as a green manure to replace nitrogen in the soil. CP is therefore of economic importance where clover is grown commercially for seed (24). The disease can be spread by several species of leafhopper (31, 128, 191, 132) and its rapid spread through a crop can adversely affect seed production.

CP is a convenient MLO disease to study as it is relatively easy to find infected clover plants in the wild. As it induces symptoms similar to other MLO diseases it may be possible to relate any findings to these other diseases. If suitable vectors are present CP might well be transmitted from wild clover to nearby commercially important plants. This may have been the case where green primulas (202) and strawberry 'Green Petal' (6) diseases have been reported.

In the work for this thesis several different methods and approaches have been used to help characterise the CP disease inducing organisms. The experiments are described under five headings with the following objectives:-

I Transmission - These studies were designed with a view to investigating host range and for comparison of symptoms in different hosts.

II Culture - For the precise identification and characterisation of the causative agent of CP it is necessary to be able to culture the organism and to fulfill Koch's postulates. Attempts to isolate and culture the MLO from CP plants are described in this section.

III Chemotherapy - Experiments were undertaken to study the effects of various chemicals on MLO. Chemotherapy using antibiotics can be used not only to establish the mycoplasmal aetiology of the disease but also as a control measure.

IV Light Microscope Investigations - Attempts are described to find a rapid, inexpensive means of detecting MLO-induced diseases prior to the production of symptoms.

V Electron Microscopy - Electron micrographs of MLO from a variety of plants are compared and details of changes induced in host plants by the disease organism have been investigated.

Although CP has been the basis of most of the work described, in some experiments cacti have also been used to test their potential as experimental tools and also to examine monstrose and cristate forms for the presence of MLO or similar organisms. Other, possibly related, MLO diseases such as green flowered Helenium, roses and strawberries have been used for comparisons as indicated in the text.

I TRANSMISSION

Transmission experiments using MLO were designed to examine the possible effects of these organisms on a range of hosts and as a routine procedure for the maintenance of stocks of CP MLO. It was also hoped that some plants might prove to be useful indicator plants for MLO infections. After describing methods for the culture of plants and insects details of experiments studying the possible transmission of MLO by grafting, dodder and insects will be described.

A. Methods used in the culture of plants

a. Culture of herbaceous plants

Table I.1 shows the range of plants used in these experiments.

TABLE I.1

Plant species	Common name	Source
Fragaria versa var Alexandria	Strawberry	Thompson and Morgan
Callistephus chinensis var Sinensis	Chinese Aster	Local seed merchant
Iberis umbellata var Fairy	Candytuft	Local seed merchant
Primula auricola	Primula	Local seed merchant
Viola tricolor	Pansy	Local seed merchant
Mesembryanthemum crystallinum	Mesembryanthemum	Local seed merchant
Vinca rosea	Ornamental Periwinkle	Local seed merchant
Trifolium repens strain S124	Clover	Welsh Plant Breeding Station

Seeds were sown in trays of John Innes seed compost. The trays were covered with polythene or glass to increase the humidity until the seeds had germinated. Plants large enough to handle were potted

separately in 5" plastic pots in John Innes potting compost no. 2.

All plants were maintained in a glasshouse with a 16 hour photoperiod at approximately 20°C throughout the year. These conditions ensured satisfactory plant growth and flowering.

In the case of clover; after potting up plants were transferred to the cooler conditions of a cold frame. Plants were returned to the glasshouse for the duration of experiments or to force the plants to flower, but at the warmer glasshouse temperatures they were very susceptible to red spider mite so were not kept permanently in these conditions.

b. Culture of dodders (Cuscuta)

The Cuscuta species listed in table I.2 were each tested as experimental agents of transmission of MLO.

TABLE I.2

Species	Source
Cuscuta gronovii	London University Botanical Supply Unit
Cuscuta europea	Cambridge Botanic Gardens
Cuscuta epithymum	Seeds collected from wild plants

Seeds of C.gronovii were planted in pots containing healthy, well grown Kalanchoe diagrimontianum or V. rosea plants. On germination C. gronovii became parasitic on these host plants. Similarly C. europea was initially established on Flax (Linum sp) and C. epithymum on Gomphrena globosa.

The established Cuscuta plants were kept under similar conditions to the herbaceous plants.

c. Culture of cacti

Monstrose forms of the cactus Opuntia tuna, described as a witches'-broom infection, have been shown to contain MLO (36, 37, 197). Other cacti have monstrose forms and some, where the abnormal

stem growths are folded or convulated, are called cristate forms. No satisfactory explanation for the cause of such cristate forms has been given. In view of the MLO aetiology of O. tuna monstrosa it seems reasonable to suggest a possible MLO aetiology for other abnormal growth phenomena in other cacti. To test this possibility the following cacti were cultivated and examined for MLO.

TABLE I.3

Species	Source
Opuntia tuna monstrosa	Holly Gate Nurseries, W. Sussex
Opuntia lanceolata cristata	Holly Gate Nurseries, W. Sussex
Cereus variabilis monstrosus	Holly Gate Nurseries, W. Sussex
Cereus peruvianus	Holly Gate Nurseries, W. Sussex
Astrophytum myriostigma monstrosa	Holly Gate Nurseries, W. Sussex
Astrophytum myriostigma	Holly Gate Nurseries, W. Sussex

In experiments testing cacti as sources of MLO the plants were grown in small (2" - 3") pots containing sandy soil mixed with gravel. The temperature and light regime were as described for herbaceous plants. The cacti were watered as necessary to give satisfactory growth.

B. Sources of MLO

Table I.4 shows a complete list of the plants tested as possible sources of MLO.

TABLE I.4

Plant	Symptoms or Disease	Source	Fig no
Trifolium repens	Clover phyllody	Collected from wild	I.1
Primula auricola	Green flowers	Local nursery	I.1
Helenium autumnale L.	Green flowers	Royal Horticultural Society	I.2

Opuntia tuna monstrosa	monstrose growth form	Holly Gate Nurseries W. Sussex	III.1
O. lanceolata cristata	cristate growth form	Holly Gate Nurseries W. Sussex	-
Cereus variabilis monstrosus	monstrose growth form	Holly Gate Nurseries W. Sussex	III.1
Astrophytum myriostigma monstrosa	monstrose growth form	Holly Gate Nurseries W. Sussex	III.3
Vinca rosea	Clover phyllody	transmitted from clover	I.3
*V. rosea	Spiroplasma citri	John Innes Institute	-
*V. rosea	Corn Stunt spiroplasma	John Innes Institute	-
*Apple	Rubbery Wood	Long Ashton Research Station	-
*Pear	Rubbery Wood	Long Ashton Research Station	-
*Rubus	Rubus stunt	Long Ashton Research Station	-

*These plants were maintained with other diseased plants but only used for later experiments.

Clover Phyllody (CP) infected Trifolium repens plants were collected locally from two areas of grassland. One sample was from an area adjacent to the M3 motorway (map ref. 169 983657) and the other from a meadow at Sunningdale (map ref. 169 954683). As the two samples showed different symptoms they were designated the initials MW and SD respectively after their collection sites. MW plants had a compact green inflorescence (fig I.1a) whereas SD had a much more open, leaflike inflorescence (fig I.1b). These plants were the main source of CP for later experiments.

Turfs containing the infected clover plants were placed on John Innes potting compost no. 2 in seed trays. They were maintained in

FIGURE I.1

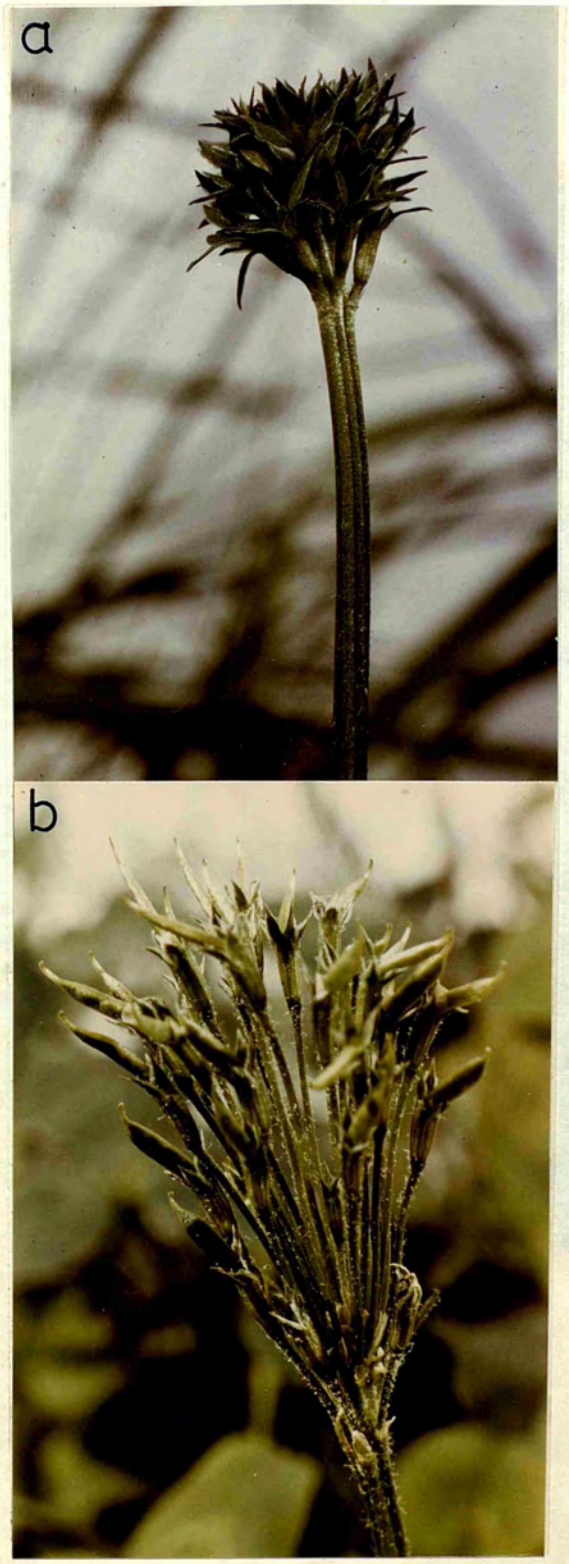
a. M.W. symptom of Clover Phyllody.

b. S.D. symptom of Clover Phyllody.

c. Red leaf symptom sometimes found associated with Clover Phyllody.

d. Green flowered Primula compared with a healthy plant.

Figure I.1



I.1(cont.)

cold frames and left to establish. Some plants were also planted in outdoor plots. Once plants were established they could be separated and potted up for transferring to the glasshouse where conditions induced flowering. As CP can only be reliably diagnosed by the green flower symptom it was necessary to induce plants to flower before using for experiments.

Once CP had been transmitted to Vinca rosea (see later experiments) it was necessary to find a rapid method of propagating the infected plants to increase the amount of diseased material available for experiments. It was possible to take cuttings by removing small side branches 5 - 10 cm in length from the infected plants. After dipping the end in rooting hormone (1 - napthylacetic acid) these cuttings were potted up directly.

Cuttings were however slow growing as the roots took several weeks to establish. Most of the resulting plants were less than 10 cm high after 12 months although a few grew rapidly once established. As the phloem of the cuttings was infected prior to rooting the resulting plants must have contained large numbers of MLO. This was demonstrated by the severe symptoms displayed by the plants and may have been the cause of the slow growth rate.

Green flowered Primula auricola plants (Fig I.1d) had been obtained from a local nursery for some earlier work in the department (202).

Green flower symptoms on Helenium autumnale L have been shown to be induced by MLO (8). This was confirmed later by EM. studies on some plants sent to us for investigation. The green plants were potted up in 5" plastic pots in John Innes potting compost no. 2 and maintained in the glasshouse. They were readily propagated by splitting off some plants which formed at the base of the stem and

all these plants produced green flowers.

The production of green flowers by roses is also known to result from infection with MLO (163). Such roses have been given the specific name Rosa chinensis var. viridiflora Dipp. Examples of this plant were obtained commercially and planted in 10" pots of potting compost in November 1977. Two were forced into flower by keeping them in the glasshouse. Four other plants were kept in cold frames and later planted in outdoor experimental plots.

The use and culture of cacti as possible sources of MLO have been described earlier (section A.c). The plants showing green flower symptoms were collected in order to examine for possible MLO and compare the symptoms with those caused by CP. These plants are listed in table I.4 above.

C. Methods used in Insect Transmission

a. General methods

Insect transmission studies were carried out with leafhoppers, aphids and whiteflies.

Insects were caged on plants in a variety of ways. Where small numbers of insects were used and required to be transferred later they were put in 3" x 1" plastic specimen-tube cages. These cages were made by cutting off the base of tubes and replacing them with muslin to provide ventilation and prevent water from condensing inside the tubes. The required number of insects, which had been anaesthetised with CO_2, were placed in the tube then the tube placed over a single leaf or inflorescence and the open end sealed with cotton wool. Whole potted plants were enclosed in clear plastic propagators. Circular areas of the propagators were cut out and replaced with muslin to increase ventilation and reduce condensation. Insects from a tube cage or from the tube of a poota could be shaken into the cage through

a 1" diameter port cut in the side of the propagator and closed with a cork.

All plants to be used for insect transmission experiments were transferred to an illuminated growth cabinet at least one week before the experiment started. Insecticides had to be used occasionally in the main glasshouse to control pests and isolation of test plants prevented the vectors being affected by insecticide residues. The double isolation of the growth cabinet and single-plant cages to retain experimental insects also prevented chance infection from outside.

b. Culture of Leafhoppers

A disease-free population of the leafhopper Euscellis plebejus Fallen, a known vector of CP (57, 67, 68) was obtained from John Innes Institute and maintained on healthy clover plants in an insect proof cage. The cage was maintained at 20°C with continuous illumination from fluorescent tubes to prevent diapause. As clover plants only survived for a few months under these conditions fresh plants were introduced into the cage occasionally. After allowing a few days for the insects to move onto the new plant any dead plants were removed.

Since clover plants proved difficult to keep under controlled conditions it was necessary to transfer CP MLO to plants more suited to the glasshouse conditions. To this end V. rosea, a widely used MLO host plant (1) was chosen. V. rosea could be readily propagated by cuttings and grafting. The transfer of CP MLO from clover to V. rosea was brought about by the following method.

Groups of ten leafhoppers were each allowed to feed on one MW and one SD-infected clover plant. The insects were caged in pairs in tubes fixed over single phyllodied inflorescences. After 7 days

all the MW-fed hoppers were released onto a caged V. rosea plant and the SD-fed hoppers were treated similarly. The insects were left on the plants for 30 days to ensure the completion of the incubation period and allow time for transmission feeds. Any surviving insects were then transferred to other healthy plants to see if they were still infective.

c. Transmission by aphids (Myzus persicae)

Twenty to thirty aphids were collected from Nicotiana tabacum plants in a disease-free glasshouse and caged on a CP-infected V. rosea plant. After 4 days they were transferred to a healthy V. rosea seedling using a paintbrush. They were caged on this plant for one week and then killed by spraying the plant with X-L contact insecticide. The time scale used allowed time for persistent transmission and, as the aphids were moved directly from an infected to a healthy plant, mechanical transmission might also have been possible.

d. Transmission by whiteflies

Whiteflies are important disease vectors mainly in tropical and sub-tropical areas (36, 37) where they transmit diseases of several important crop plants.

To study the possible involvement of whiteflies in the transmission of CP MLO large numbers of whiteflies on Streptocarpus sp. leaves supplied by London University Botanical Supply Unit were shaken into a cage containing a CP-infected V. rosea plant. After 24 hours several hundred whiteflies were collected using a poota and caged on a healthy V. rosea seedling. A week later they were killed with X-L insecticide spray.

D. Dodder Transmission

Cuscuta epithymum was established on healthy Gomphrena globosa

plants. Stems were allowed to grow out onto CP-infected V. rosea plants. After one week the C. epithymum was usually well established on the infected V. rosea so stems connecting the two plants were severed. Once the dodder was established on an infected plant the same method was used to establish a dodder connection between the infected V. rosea and a range of healthy plants. The connecting stems were left for three weeks to allow disease transmission to the healthy plants. All the dodder was then removed from the recipient plant and the plant was isolated and observed for the appearance of symptoms.

Cuscuta gronovii was maintained on Kalanchoe diagrimontianam and C. europea on Flax. Dodder stems from these hosts were allowed to grow out onto MLO infected plants i.e. green Helenium, green rose, green primula, O. tuna monstrosa, O. lanceolata cristata, Cereus variabilis monstrosus. The dodder would not establish easily on all the test plants however. When this happened a 10 cm length of dodder stem was broken off and wrapped round the stem of the infected plant. As the isolated stem had no other source of nutrient it usually became parasitic on the infected plant. Transmission of the diseases to V. rosea, strawberry, aster, candytuft, primula, pansy, mesembryanthemum and clover plants could then be attempted as before.

E. Transmission by Grafting

Transmitting CP to healthy V. rosea plants by grafting was a very useful method for increasing the amount of experimental material.

An infected shoot about 3 - 4 cm long was cut from a donor plant and the lower leaves removed. The bottom of the stem was cut into a wedge shape to expose a large amount of vascular tissue. The accompanying diagram shows the two methods by which this infected

shoot could be grafted into the stock plant. Grafts were held in place using PTFE plumbers tape which was self adhesive and reduced water loss from the cut surface. Grafted plants were kept under a propagator for at least one week after grafting as this increased the success rate possibly by increasing humidity and reducing water loss from the grafted scion.

Results

B. Sources of MLO

The symptom differences between SD and MW CP-infected clover plants are described above (p23). It was not possible to check if symptom differences between SD and MW clover remained constant in the field over several growing seasons because all the clover at the MW site died back during the drought in the summer of 1976. No clover grew again at this site during the period of this research. However, the SD clover did survive and the symptoms in this area remained constant for three years.

The green flower symptoms in Primula commence as virescence i.e. greening of the petals but as the plants age symptoms become more severe and the flower parts become leaf-like or phyllodied. Such symptoms have been shown to be associated with the presence of MLO in the plant (202) and this was confirmed by further EM work (see section V). As CP was present in clover adjacent to the nursery plots where the greening first occurred it was thought that CP MLO may have been transmitted to these plants and caused the flower greening. These plants were therefore used as a source of MLO for transmission experiments as it was hoped that the symptoms could be compared directly with CP symptoms in the same host plant to show if the diseases are of the same aetiology.

The green rose plants were small bushes with thin stems and

small leaves. This delicate growth form is similar in appearance to a floribunda bush rose but the flower heads are produced singly (fig I.2d). There was no apparent reason for this sudden change in growth form.

As can be seen in the relevant figures the green flowers on Primula, Helenium and rose plants are all distorted. The petals and sepals are very leaflike as in CP-infected clover and V. rosea plants. Experiments were designed to transmit MLO from the available diseased plants to a range of plants grown from seed (see table I.1) so that symptoms could be compared on plants of the same species. In this way it was hoped to show whether the CP MLO was the causal agent of any of the other diseases.

C. Insect Transmission

a. Leafhopper transmission

Leafhoppers which transmit MLO diseases are persistent vectors. Some vectors such as Macrosteles fascifrons can acquire disease organisms in as little as 30 minutes (31) but a high percentage of insect transmission only occurs after at least a 4 day feed on infected plants (31, 190, 220). An incubation period follows while the MLO multiply in the vector. During this time the insects, although containing MLO, are not infective to plants. Only when the MLO reach sufficient numbers in the salivary glands can they be injected into a plant during feeding. MLO have been found in vector leafhopper salivary glands within 5 days of an acquisition feed (90) but they usually do not occur in large amounts until 20 - 30 days after the start of acquisition feeding (90, 128, 190, 191, 192).

Leafhoppers were fed for 5 days on clover plants with either MW or SD symptoms. The vectors were seen to feed frequently on the infected plants and would have been expected to pick up MLO. They

FIGURE. I.2

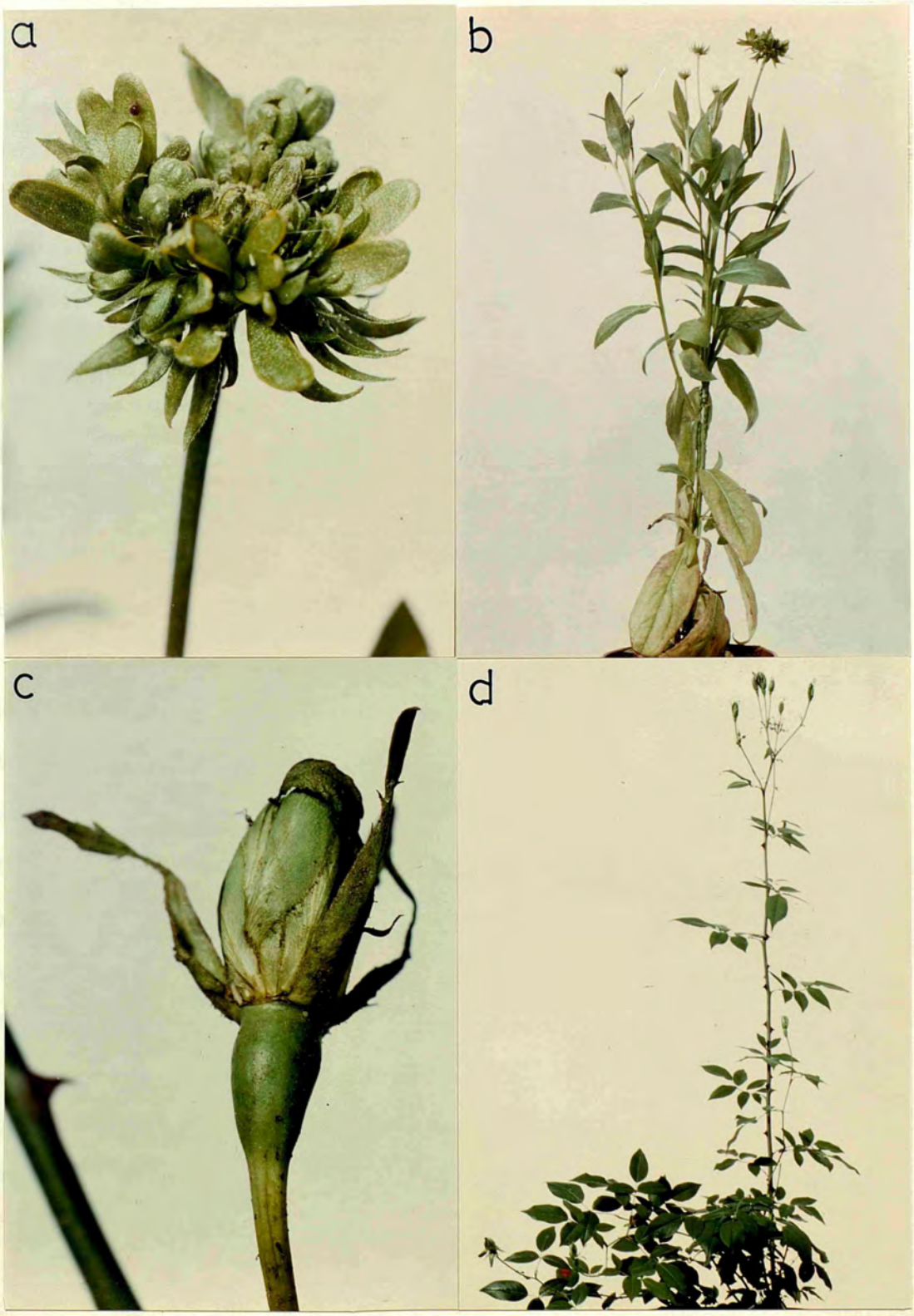
a. Close-up of a green *Helenium* flower.

b. Growth form of a green-flowered *Helenium* plant.

c. Close-up of a green rose.

d. Green-flowering rose bush showing one vigorously growing stem which also produced green flowers.

Figure I.2



were transferred to two V. rosea plants but only a few insects survived for more than a few days on these plants probably because they were adapted to feeding on clover.

Insects were observed closely during the first day after transferring to V. rosea and about 50% were feeding for at least short periods but only 20% of the insects survived the full 30 days on either plant. Because of the poor survival rate of the vectors the source of inoculum would have been small and this may explain the slow appearance of symptoms in the V. rosea plants.

Green flower symptoms began to appear in both plants 14 days after the removal of the insects (fig I.3). There were no apparent differences in either symptoms or the rate of symptom production between the two disease strains on this new host so the initial differences on clover might well have been a reaction to either environmental or host differences.

The leafhoppers still surviving at the end of this experiment were transferred to a healthy strawberry seedling to test whether CP in strawberries produced strawberry green petal disease. However the insects were not seen to feed on this plant and all died within 2 days. As these vectors were known to be infective and the strawberry plant remained healthy it is assumed that they did not feed at all or did not transfer sufficient MLO to produce symptoms. Alternatively the CP MLO may have been unable to multiply in strawberries.

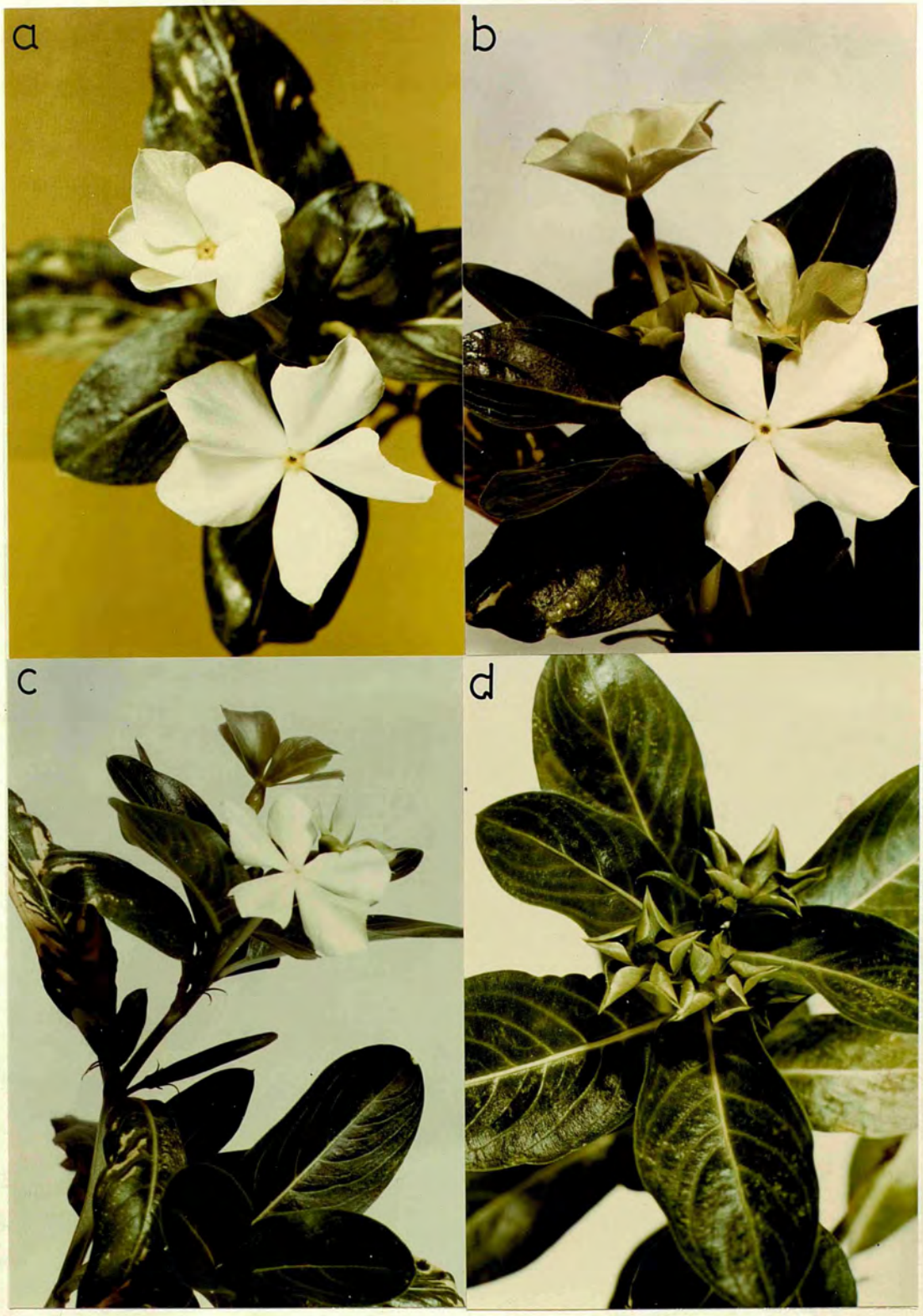
To test the chance of infection occurring in the wild 30 leafhoppers were collected from wild clover plants showing CP symptoms. These insects were caged directly onto two healthy V. rosea seedlings (15/plant) and left for 30 days as before.

Neither of the test plants showed CP symptoms even after several months so it would appear that very few of the potential vectors in the

FIGURE I.3

Progressive greening of Vinca rosea flowers infected with Clover Phyllody (see text).

Figure I.3



field are infectious.

The previous result also suggests that leafhoppers become adapted to feeding on one species of host plant and do not readily adapt to feeding on other species. Natural spread of CP from one plant species to another is therefore likely to be an infrequent event. It does however seem to have occurred in the case or primulas (202) where disturbance of hoppers from phyllodied clover plants resulted in transmission of MLO and subsequent production of green flowering symptoms.

Such a phenomenon may also have occurred with other green-flowered cultivated plants such as Helenium and Rosa so it is possible that other garden plants may be susceptible. As the leafhoppers were difficult to keep in culture it was not possible to test the susceptibility of herbaceous plant grown from seed using insect transmission. However this was tested later using dodder (section D).

c. and d. Aphid and Whitefly Transmission

During the winter several supposedly healthy V. rosea plants produced small, green tinged flowers similar to the early symptoms of CP. If the plants were infected the disease could only have been transmitted by aphids or whiteflies since both pests had been a problem in the glasshouse. Neither of these insects were known vectors of MLO diseases but experiments were set up to test their ability to transmit under glasshouse conditions.

Viruses which are transmitted by aphids can be stylet borne or circulative (206). Those carried on the stylets are, effectively, mechanically transmitted between plants. They are transmitted immediately after acquisiton but the vector loses infectivity soon after feeding on a healthy plant (160). Circulative aphid transmitted

viruses have a much shorter incubation period than leafhopper-transmitted diseases. A 2 day acquisition period followed by 5 days on a healthy plant is sufficient for transmission of all known aphid - transmitted plant diseases (173).

Vector whiteflies can acquire disease organisms in as little as 20 minutes (83) and a maximum transmission level is acquired within 24 hours (35, 83). They will then transmit almost immediately and will remain infective for several days without re-acquisition.

Despite repeated experiments giving aphids a 4 day acquisition feed and whiteflies a 24 hour acquisition feed no transmission was obtained. The plants which had shown mild greening symptoms flowered normally in the spring and summer showing that the symptoms were probably a reaction to the poor light and to lower temperatures unavoidable during the severe winter months. No chance infection occurred in the glasshouse even though there were occasional infestations by aphids and whiteflies. It therefore seems unlikely that either of these insects is a vector of CP.

D. Dodder Transmission

As the vector insect had proved unsuitable for transmission experiments between unrelated species of plants further experiments were carried out to test the effectiveness of dodder as a possible means of transmission.

Initial experiments using Cuscuta gronovii and C. europea showed that these species greatly weakened and could kill small herbaceous plants such as V. rosea seedlings. For this reason C. epithymum was the only dodder used for transmission between herbaceous plants. Even so C. epithymum had to be cut back to prevent excessive weakening of the host plant.

C. epithymum established well on CP-infected V. rosea and sent

out many vigorous branches but alhtough it would parasitise other V. rosea plants it would not establish on the other plants grown from seed (see table I.1). It was eventually established on aster, strawberry and primula plants by breaking off peices approximately 10 cm long and wrapping them round these plants. The initial piece of dodder transferred withered and became very much reduced in size but most formed haustoria close to the growing tip and became established. It was not possible to establish dodder on clover, pansy or mesembryanthemum plants. One C. epithymum plant was eventually established on a clover plant but only by germinating a dodder seed in the pot containing the clover plant. However the dodder did not grow well.

Inspite of the difficulty of establishing dodder on different hosts some C. epithymum was established on healthy primula, strawberry, aster and candytuft and subsequently allowed to grow out from each of these onto infected Primula sp., Helenium autumnale, Rosa chinensis and V. rosea. It has been shown that once a dodder bridge is established between two plants disease transmission can occur in either direction (107).

Electron microscopy (see section V) showed that the C. epithymum parasitic on CP-infected V. rosea contained MLO in the phloem. No MLO could be detected in healthy V. rosea or in C. epithymum prior to parasitism. These MLO could only have been picked up from the infected host plant but no transmission was obtained even after plants were connected by infected dodder for a month or more. The pieces of dodder broken off to establish on other plants were not checked individually for the presence of MLO as a light microscope stain had not been developed and EM techniques would have taken too long to be useful. As no transmission was obtained with this method it

is supposed that there were either no MLO in the pieces of stem used or they did not survive the die-back of the stem before contact was established with the host plant. However this does not explain why transmission was not obtained between the healthy and infected plants linked by living stems of dodder.

Work by Carling (23) and Johnson (107) indicating that only a small percentage of transmission is obtained using dodder supports the results described here. Workers claiming dodder transmission to V. rosea from Ash trees (86, 87) and Erigeron canadensis (42) do not appear to have encountered the same difficulty although symptoms were slow to develop on the V. rosea infected with Ash witches'-broom (87).

In experiments designed to gain information regarding the possible MLO aetiology of different growth forms of cacti it was found that both C. europea and C. gronovii would establish on the cacti Opuntia tuna monstrosa, Opuntia lanceolata cristata and Cereus variabilis monstrose. Dodder had to be wrapped round the cacti and left undisturbed for several weeks before short, bright green pieces of the stem became parasitic between the spines of the cacti. Usually only pieces about 2 cm long became established although longer pieces were occasionally found at the growing points of O. tuna monstrosa.

The pieces of dodder parasitising the cactus had numerous haustoria but they did not grow rapidly and were never seen to produce tips more than a few millimetres long which were unattached to the host. MLO similar to those found in cacti were also present in the phloem of the parasitising dodder (see section V) so it is reasonable to assume that the haustoria must have been in intimate contact with the phloem of the cacti. The slow growth rate of the dodder may have been determined by the growth rate of the host plants but it is also likely to have been

caused by difficulty in establishing contact with the host vascular system since the vascular bundles in cactus stems are small and widely scattered.

The dodder stems did not grow to sufficient length to establish on other plants so the growing pieces were detached from the cacti and wrapped round stems of healthy V. rosea seedlings. Only C. gronovii could be treated in this way as the C. europea stems were too fragile.

After two months on the 3 cacti eight pieces of C. gronovii were transferred to V. rosea but only two taken from O. tuna monstrosa survived. They had become established after two weeks on the herbaceous host. One was put onto a V. rosea seedling at the two-leaf stage and within four weeks had killed the host plant. The other was established on a larger plant but no disease transmission occurred since the V. rosea grew and flowered normally.

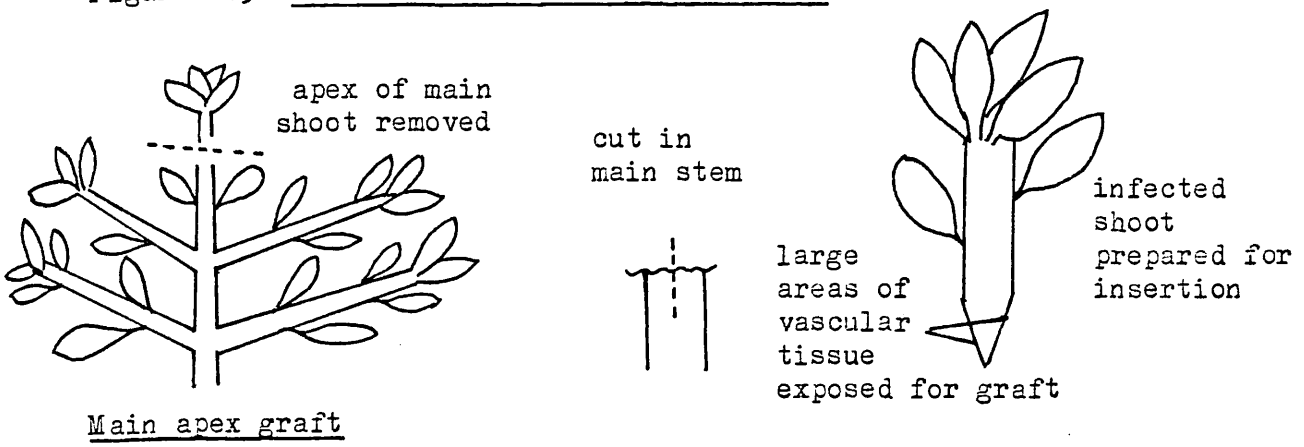
Attempts to use dodder transmission of pathogens from cacti have not been reported previously. Although no transmission was obtained in these experiments the dodder was able to acquire MLO from the cacti and be transferred to another plant. If the experiment could be expanded to allow for the low percentage transmission usually obtained it is possible that some transmission could be obtained.

The MLO in cacti are difficult to study because the host plants are difficult to work with. If the disease could be transmitted to a more convenient host research into them would be much easier.

E. Graft Transmission (see diagram overleaf)

V. rosea plants grafted while growing rapidly were found to be completely infected within 4 - 6 weeks. As quite large plants could be used this was a useful method for producing large amounts of diseased material. Plants grafted into the top of the main stem

Figure I.5 DISEASE TRANSMISSION BY GRAFTING

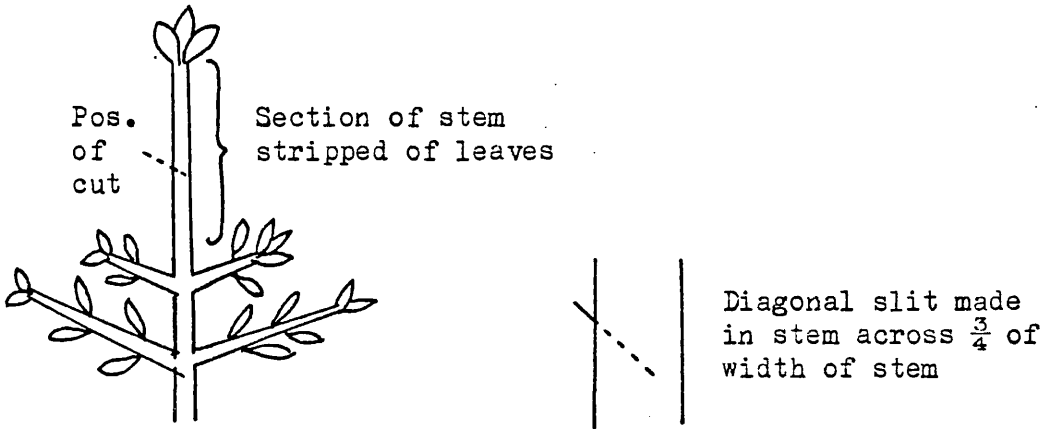


Main apex graft

Graft held in place with P.T.F.E. plumbers tape which also sealed cut against infection.

Side shoots grow out from main branch of stock plant and soon beccme infected.

Side graft into main stem



Infected shoot prepared as above and sealed in place.

Growth of main shoot continued. Less side growth than above as apical dominance not disturbed. No further growth of grafted scion. Less rapid infection of stock plant.

showed symptoms initially in the top branches close to the graft. Symptoms then spread rapidly to the rest of the plant. Grafts into the side of the main stem showed initial symptoms on the main stem and the spread of symptoms through the lower parts of the plant was slower than above. Occasionally one branch of a graft-infected plant would remain uninfected and flower normally.

It was found that a large infected V. rosea plant could be used to supply up to 40 scions for grafting. Large numbers of V. rosea seedlings could therefore be infected by grafting into the cut main stem which could subsequently be used in experiments such as chemotherapy and the use of diagnostic staining techniques. Seedlings at the 6 - leaf stage and about 10 cm high were used as smaller plants did not have large enough stems to hold the graft. Grafts were inserted into the cut main stem as above but symptoms were often slow to appear as the young plants were not flowering.

Initial symptoms of CP on V. rosea are recognised by green tinged or mixed coloured flowers which are smaller than normal (fig I.3a). Later flowers become progressively greener and smaller (fig I.3b and c) until the petals and sepals are quite leaflike (fig I.3d). Flowers which were formed before the MLO spread through the plant remained normal in appearance.

Figure I.4a shows ageing in a healthy V. rosea plant. Chlorosis of the lower leaves occurs followed by extensive abscission as the plant ages. After about 12 months in a 5" pot the plant looks very straggly with bunches of leaves only at the ends of the stems. At this stage the plant may flower occasionally.

CP - infected V. rosea becomes highly branched and stunted compared to a healthy plant. The branching is not caused by removal of the main stem for grafting as healthy plants which have had the

FIGURE I.4

a. Progressive ageing in healthy Vinca rosea plants showing leaf yellowing and, eventually, abcission.

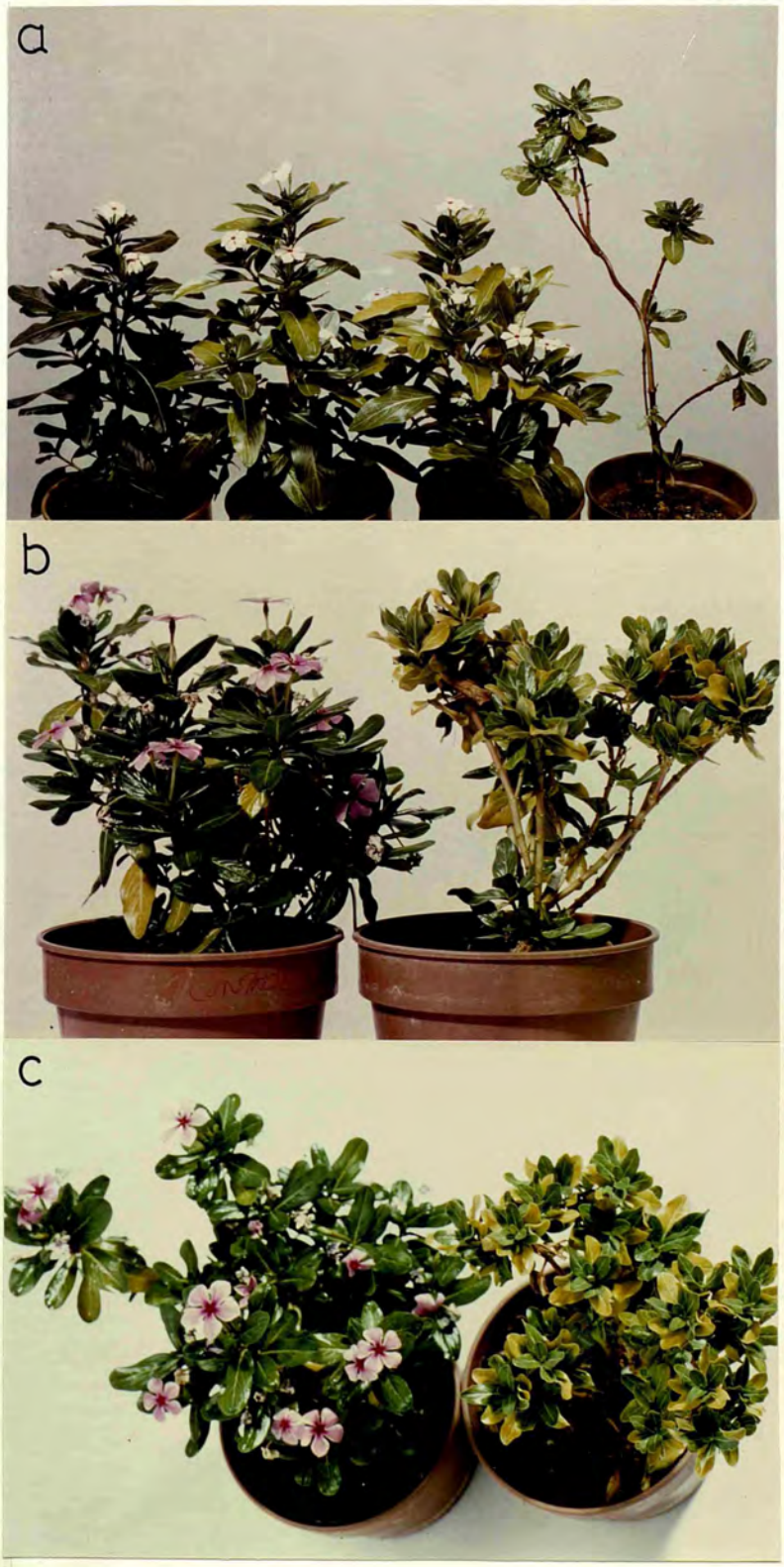
b. and c. One healthy Vinca rosea plant (left) compared with one of a similar age infected with Clover Phyllody (right). The diseased plant shows increased leaf yellowing and abcission, lack of coloured flowers and 'bunching' of the leaves at the ends of the stems.

FIGURE I.4

a. Progressive ageing in healthy Vinca rosea plants showing leaf yellowing and, eventually, abcission.

b. and c. One healthy Vinca rosea plant (left) compared with one of a similar age infected with Clover Phyllody (right). The diseased plant shows increased leaf yellowing and abcission, lack of coloured flowers and 'bunching' of the leaves at the ends of the stems.

Figure I.4



main stem removed are little different in appearance to normal plants. The bushy appearance of infected plants is enhanced by the large numbers of small leaves produced. Lower leaves become chlorotic at an earlier stage than in healthy plants but are retained longer giving a yellow appearance to the infected plant (fig I.4b and c). Flowers become very leaflike as symptoms progress and they do not terminate stem growth. A new pair of leaves will grow out through the centre of the "flower" and, as the internodes are very short, a rosetting effect occurs at the end of the stems. The production of flowers appears to stop altogether at this stage but this may be because the petals are indistinguishable from leaves. As with healthy plants leaves are shed rapidly from the base of the stems as the plant ages but the shorter stems, bushy arrangement of the leaves and chlorosis distinguish infected from healthy plants.

II CULTURE OF MLO ON ARTIFICIAL MEDIA

To be able to satisfy Koch's postulates and therefore positively identify the causal agent of a plant disease it is necessary to be able to culture the suspected pathogen *in vitro*. It is also useful to have the organism in culture in order to study it more thoroughly and to determine its reaction to environmental changes. The present lack of satisfactory identification and characterisation of plant MLO is a direct result of the failure to culture most of these organisms.

Before attempting to culture the causal agent of CP, cultures of *Acholeplasma laidlawii*, a known animal mycoplasma, and *Spiroplasma citri*, a plant mycoplasma, were set up to test the sterility of the media used and the suitability of techniques. The general isolation and culture techniques will be described first followed by more detailed methods and results of each type of culture used.

Animal parasitic mycoplasmas have complex growth requirements but have been cultured routinely for many years. Broth or agar cultures usually have a nutrient base of either heart or blood extract which can now be obtained commercially as PPLO medium. This medium has to be supplemented with yeast extract, blood serum, salts and sugars (84, 118). Because of the complex supplements such media cannot be defined. To reduce the risk of bacterial contamination penicillin and thallous acetate can also be added in small quantities.

Buffers, salts and proteins can be added to the basic medium as specific growth factors for the particular mycoplasma being cultured. The type of blood serum used is usually related to the mammalian host of the mycoplasma.

There are two main variations on the basic medium the first one involving the addition of sterols. The *Acholeplasmas* do not require sterols, supplied as blood serum, for their growth although they are

not adversely affected by its inclusion. The growth media for unknown mycoplasmas should therefore contain sterols.

Although thallous acetate is used to control bacterial growth in mycoplasma cultures the compound also inhibitis the growth of Thermoplasmas (82, 185). This fact may explain the failure of early experiments to isolate these organisms which were originally called T - strain mycoplasmas. Thermoplasma colonies are slow growing even under ideal conditions. As some organisms are inhibited by thallous acetate media were prepared with or without this chemical.

General methods

Because mycoplasmas grow down into the agar, unlike bacteria which form colonies on the surface, they cannot be readily transferred from agar using a loop. To sub-culture from agar a small block of agar containing colonies was either cut out and transferred to a tube of broth or inverted onto an agar plate and pushed over the surface. For sub-culturing from broth cultures a sterile loopful of inoculum was added to a tube of fresh broth medium or streaked on the surface of an agar plate.

The phenol red indicator added to most of the media is the most convenient method for detecting mycoplasma growth in liquid media. There is usually very little turbidity in the medium so the indicator shows up the pH drop in the medium caused by the acid end products of fermentation. The media were made slightly alkaline (pH 7.4 - 7.8) at which point the indicator is red. As the pH drops the indicator changes colour through orange to yellow. Mycoplasma colonies on agar are very small and transparent so adding indicator to the agar medium also aided the identification of colonies.

Method 1

A freeze dried culture of Acholeplasma laidlawii (NCTC 10116) was

FIGURE II.1

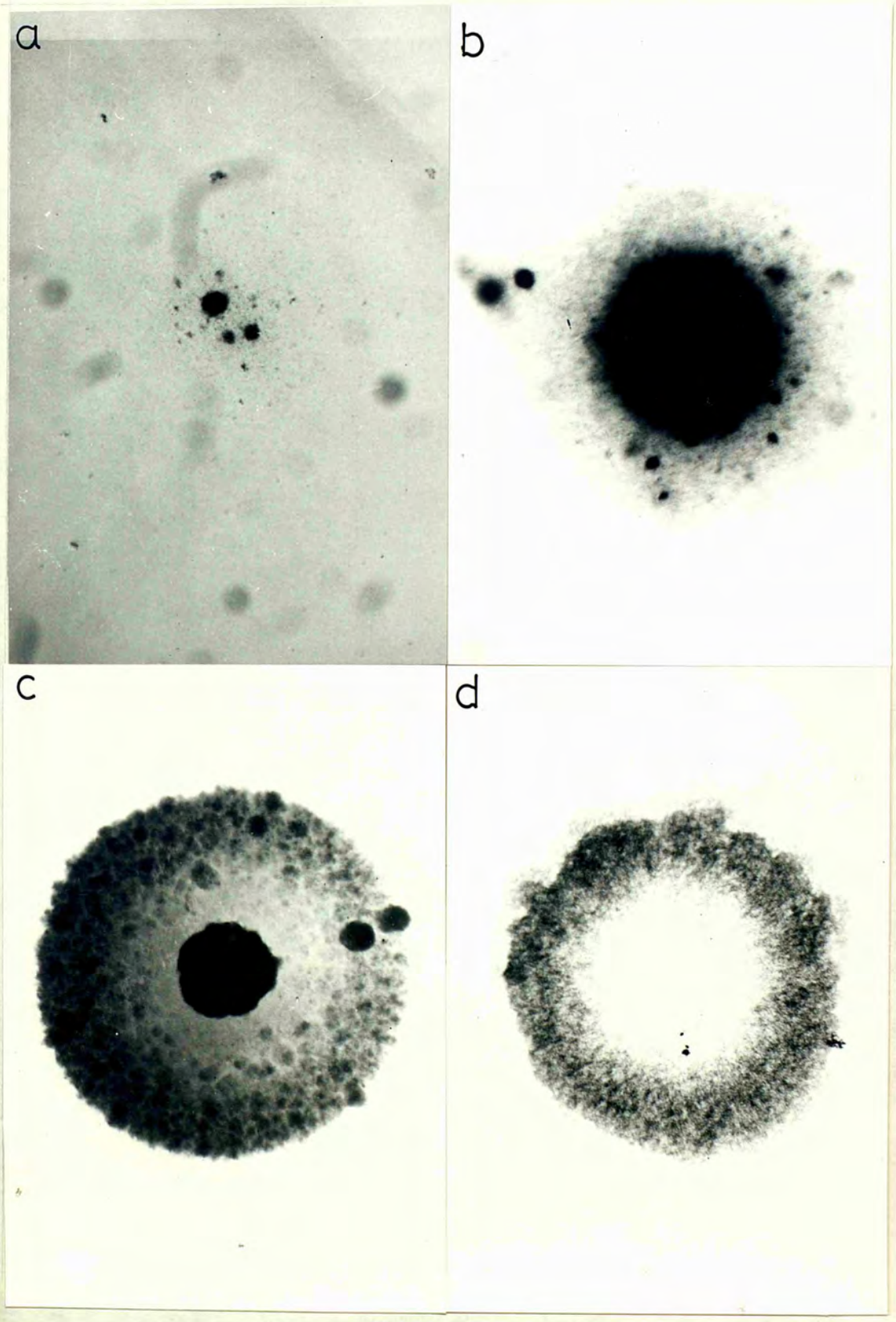
Micrographs of mycoplasma colonies on agar.

a. and b. Spiroplasma citri shown at low power in a. to show the granular 'halo'.

c. Acholeplasma laidlawii showing a typical 'fried egg' colony.

d. Clover Phyllody MLO.

Figure II.1



obtained from the National Collection of Type Cultures and inoculated onto a medium consisting of:-

2.1g Difco PPLO broth (or 3.4g PPLO agar)
0.1g fructose
0.1g glucose
0.1g tryptone
5.0g sorbitol
20ml horse serum (Wellcome horse serum 5 inactivated)
10ml 25% fresh yeast extract
16ml distilled water

This medium was based on that described by Fudl-Allah (90) and has been used as the base for more specialised media. For the broth medium the components were mixed together and sterilised by filtration through a series of Millipore filters ending in a filter with a 0.22 μ pore diameter. For the agar medium all components except the horse serum were autoclaved at 15 lb/in^2 for 20 minutes. The horse serum was added by filtration through a 0.22 μ filter.

At this stage final filtration was carried out using a 50 ml Millipore syringe and Swinnex filter holder so only small amounts of medium could be made up at one time. Broth and agar media were seeded from the freeze dried culture using a sterile loop. Cultures were incubated at 30°C.

Results 1

After a number of initial setbacks in developing a completely sterile culture medium Acholeplasma laidlawii was successfully cultured from the freeze - dried culture and could be sub-cultured repeatedly on agar and in liquid medium. On agar A. laidlawii produced typical 'fried egg' colonies (84), although these colonies are small and transparent (approx 0.35 mm diameter) and difficult to see. To aid observation and photography the plates were flooded with dilute Dienes stain (84) which produced pale blue colonies with a deep blue centre (fig II.1c). The centre of the colony is formed by the organisms growing down into the agar, while the edge growth is near

the surface of the agar resulting in a slightly frothy appearance.

Plant MLO

Media used in attempts to culture plant MLO have been based on media developed for animal mycoplasmas. Morphological similarities between plant MLO and animal mycoplasmas prompted attempts to grow plant MLO on known PPLO media rather than develop new media suitable for plant pathogens.

The idea proved successful to some extent when Saglio et al (175, 176, 177) were able to culture the Citrus Stubborn spiroplasma in a basic animal mycoplasma medium supplemented with horse serum, yeast extract, tryptone and sugars, with or without antibacterial agents such as penicillin and thallous acetate. A few years later the Corn Stunt spiroplasma was cultured using a similar medium (30, 61, 62, 223) and can now be cultured routinely on a much simpler medium containing only PPLO broth, horse serum and sucrose (121).

There have been some claims of successful culture of other plant MLO (57, 71, 168) on similar animal mycoplasma media but until the organisms have been identified by comparison with known mycoplasma their aetiological role cannot be substantiated. The culture of plant MLO other than Spiroplasmas has proved rather elusive and it is not clear from the published results if successful isolations can be repeated. Some of the results have also been contradicted by the failure of other workers to duplicate them (40).

Method 2

Having established an aseptic routine for A. laidlawii attempts were made to culture a known plant mycoplasma. A culture of Spiroplasma citri was supplied by the John Innes Institute and successfully cultured on the following medium:-

J. I. medium		
	21g	Difco PPLO broth (or 34g PPLO agar)
	70g	Sorbitol
	1g	fructose
	1g	sucrose
	20mg	phenol red
	700ml	distilled water
	100ml	fresh yeast extract (5g/litre)
	200ml	horse serum (Wellcome, 5 inactivated)

For both broth and agar media all components except the horse serum were autoclaved at 15 lb/in^2 for 20 minutes. The horse serum was then added through a 0.22 µ Millipore filter using a pressurised vessel and via a pre - filter stack to enable large amounts to be dispensed. Penicillin G at 1,000 µ/ml and 10 ml of 2.5% thallous acetate solution were added to each 1 litre of medium to prevent bacterial growth.

The complete medium was dispensed in 10 ml aliquots into sterilised universal bottles or 9 cm petri dishes under sterile conditions. Bottles and petri dishes of media were stored at 4°C or used immediately.

Results 2

Spiroplasma citri was successfully cultured and sub-cultured many times at 32°C using J.I. medium. The culture was maintained in the laboratory for several weeks without contamination. The colonies are rather smaller (approx 0.2 mm diameter) than those of A. laidlawii and have a granular halo (fig II.1a and b). They were also stained with Dienes stain to aid identification.

Method 3

Having established a reliable routine for culturing both an animal and a plant mycoplasma, experiments were commenced in an attempt to culture the MLO causing CP symptoms.

Extracts of CP - infected V. rosea plants were made by grinding 1 g of surface sterilised plant material with 5 ml sterile CP medium

(see below) using a pestle and mortar. The crude extract was centrifuge at 200 G for 5 minutes to remove the larger pieces of debris. Using aseptic techniques the supernatant was placed in a Millipore syringe and forced through a sterile filter with a pore diameter of 0.45 u directly into broth medium or onto agar plates. This filter pore size is too small for the passage of bacteria but mycoplasmas were assumed to pass through because of their size range and pleomorphism.

The culture medium used in this experiment was designated CP medium. It was made up to the recipe for J.I. medium but omitting the antibacterial agents as it was possible that these might affect the growth of the micro-organism. Cultures were incubated at 15°C, 20°C, 25°C and 30°C to determine the optimum temperature for growth.

Result 3

The first attempt at isolating the CP organism from V. rosea on agar resulted in small colonies (approx 0.3 mm diameter), at the higher temperatures, which stained with Dienes' stain. No colonies developed at 15°C. The agar cultures held at 30°C had colonies 5 days after inoculation, and some colonies were also found at 25°C. After 7 days some growth was also found at 20°C. The colonies were slightly larger than those of S. citri but smaller than A. laidlawii. They did not have a deep centre but the edges were frothy and, in some cases, very irregular (fig II.1d). The slow growth rate of these colonies and their method of staining with Dienes' stain suggested that they were mycoplasmas.

The organisms were sub-cultured on agar but at a second attempt the sub-culture became contaminated with bacteria. The older cultures which had been stored at 4°C were no longer viable so they could not be sub-cultured again and the culture was lost.

There was no apparent growth in the broth medium so small flasks

containing 10 ml of broth medium were inoculated from the agar cultures and incubated at 25°C on a shaker to allow free gaseous exchange. The flasks were sealed with sterile cotton wool bungs but again there was no growth. Obviously oxygen deficiency did not prevent growth in broth but the reason for growth on agar only is not known.

Method 4

For further attempts at isolating plant MLO 5 media were used. Four were made up as for J.I. medium but the antibacterial component was varied. The media were identified by designating them initials as follows:-

1. S.C. Complete S. citri medium containing thallous acetate and penicillin G.
2. T.A. As above containing thallous acetate only.
3. P. As above containing penicillin G only.
4. C.P. As above with no antibacterial agents (used successfully in method 3).

The media were made up, dispensed and stored as for J.I. medium. The fifth medium was a commercial mycoplasma medium supplied by Flow Laboratories as a kit designed for detecting mycoplasma contaminants in tissue culture.

The Flow medium contained:-

20g	PPLO broth base
100ml	pretested horse serum
50ml	yeast extract (25% w/v)
200mg	calf thymus DNA
1g	arginine
1g	glucose
20mg	phenol red
850ml	water
(8g	Difco bactoagar - plates only)

All 5 types of media were seeded with extracts from CP - infected clover and V. rosea plants and green flowering Helenium autumnale, Primula sp and Rosa chinensis var virediflora. The plant extracts were

made as for V. rosea in method 3 above.

Results 4

All further attempts at isolation of plant MLO were unsuccessful. The initial successful isolation was made from flowering shoots of rapidly growing plants so it is possible that this material contained unusually large numbers of MLO.

However, the erratic results seem to correspond with other workers' attempts to culture. Occasionally culture of plant MLO is successful but the cultures are difficult to keep and the original isolation cannot be repeated reliably.

Electrophoresis

Whilst attempts were being made to culture the CP MLO on artifical media experiments were also undertaken to see if the MLO could be detected using electrophoretic techniques. It has been shown that mycoplasmas produce distinct species - specific electrophoretic patterns on polyacrylamide gels (169) so it was decided to see whether electrophoresis might be used as a technique to distinguish between healthy and MLO - infected V. rosea plants. Furthermore it was hoped that with the possible culture of MLO on artificial media it might prove possible to compare the protein profiles of cultured MLO with profiles from infected plants and in this way be able to develop a technique for characterising the protein changes associated with MLO infection.

One gramme of plant material was ground up in 5 ml of phospate buffer pH 7.4. The extract was centrifuged for 5 minutes at 200 G to remove large pieces of debris. The supernatant was withdrawn and half the volume of each sample was filtered through a 0.45 µm Millipore filter to remove any micro-organisms, larger than MLO, which might be present and alter the protein content.

Equal volumes of each of the four samples of the supernatant were applied to the cathode side of a polyacrylamide gel with a pH range of 3.5 to 9.5 (PAG plates from LKB). The gel was run for 1.5 hours on an LKB 2117 Multiphor electrofocusing unit.

After fixing for 1 hour in a mixture of sulfosalicylic and trichloracetic acids in 33% methanol the gel was stained for 10 minutes at 60°C in 100 ml of a 0.115% solution of Coomassie Brilliant Blue in 25% ethanol and 8% acetic acid. Several changes of a similar solution without the Coomassie Blue was used to destain the gel. If required the gel could be preserved in 10% glycerol in destaining solution, allowed to air - dry until sticky to the touch then stored between two sheets of clear plastic. The gel was photographed while in preserving solution.

Results

Electrofocusing of plant samples showed a series of many protein bands concentrated towards the anodle side of the gel. The infected plants contained more protein than the healthy as shown by the darker staining. Filtered samples also contained less protein than the unfiltered ones. The infected samples showed two very close, heavily staining bands which were not present in the uninfected samples (fig II.2). The bands occurred in both the filtered and unfiltered infected samples so the extra protein could not have resulted from bacteria.

Since the plants were identical in all other respects it seems likely that the different proteins have resulted from the MLO or changes initiated in the plants due to the presence of the MLO pathogens. Unfortunately due to lack of time and failure to culture CP MLO '*in vitro*' it was not possible to determine the nature of the extra bands. The successful distinction of healthy from MLO - infected

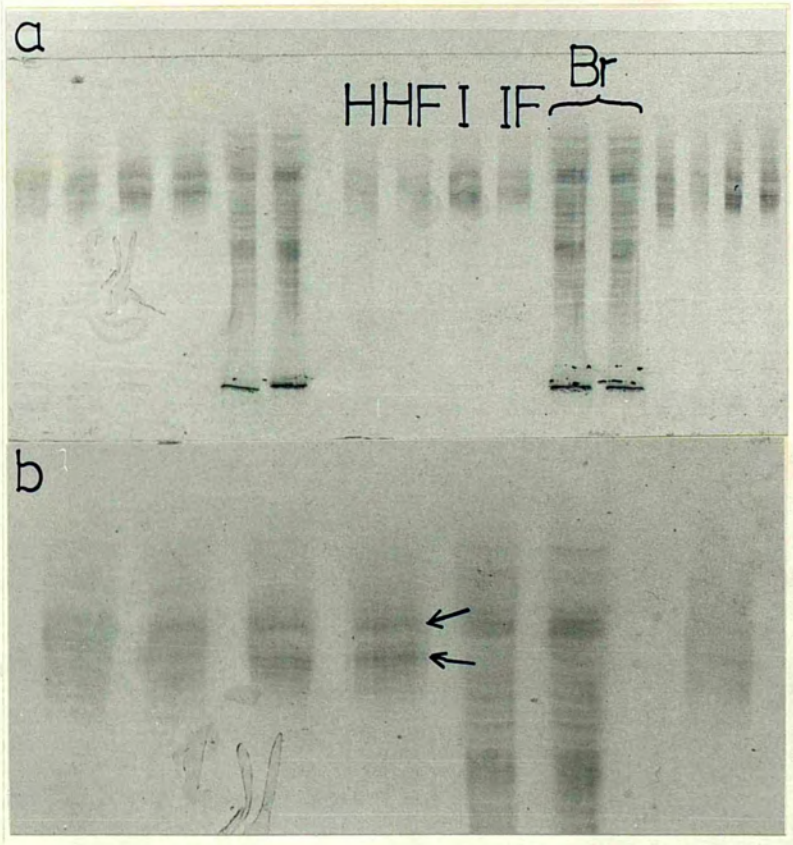
FIGURE II. 2

Iseelectrefocusing gel of proteins from healthy and Clover phyllody-infected Vinca rosea plants.

a. Complete gel showing H. healthy Vinca rosea; HF. healthy Vinca rosea extract filtered through a 0.45µm Millipore filter; I. Clover Phyllody infected Vinca rosea; IF. Clover Phyllody infected Vinca rosea filtered as above; Br. broth used for culture of MLO.

b. An enlargement of one area of the same gel showing more clearly the stronger protein bands in the infected material (arrows). These bands are not affected by filtering.

Figure II.2



plants using electrophoretic technique is important however since it might well, if developed, provide a method of reliably diagnosing MLO - infection in plants.

III CONTROL BY CHEMOTHERAPY

The observation that many yellows diseases could be successfully controlled by heat treatment, together with the knowledge that viruses were inactivated by heat led to all yellows diseases being attributed a viral aetiology. Later it was shown that diseases with confirmed viral aetiology required heat therapy for prolonged periods of days or weeks for complete cure (151) whereas other diseases, now known to be caused by non-viral pathogens, responded by symptom remission after only short term treatment at temperatures between 40° and 50°C (13, 105, 115, 151). Non-viral yellows diseases include those induced by MLO and recovery from the disease is complete and permanent if heat treatment is carried out correctly (20, 115).

Heat treatment can be used commercially to control heat - labile disease organisms and usually involves a hot water soak of cuttings prior to planting out. This method is used routinely for example to control White Leaf disease of sugarcane (125). Such procedures are however expensive, time consuming and generally restricted to small plants or portions of plants used for propagation purposes. They could not be used in a mechanised planting system or on large plants such as trees.

There is an obvious need for an effective chemical treatment to control MLO diseases since this would probably be cheaper and might be effective on plants of all sizes and ages. It may be possible to utilise the knowledge of the chemical control of animal mycoplasmas to control plant MLO.

Plant MLO, like animal mycoplasmas, are resistant to penicillin but sensitive to quite small amounts of tetracyclines (84). Penicillin acts on bacteria by affecting wall production so has no effect on mycoplasmas which are wall - less. Thus resistance to penicillin can

be used to distinguish mycoplasmas from bacteria.

Many different antibiotics have been tested against animal mycoplasmas (104) and, although several have been shown to adversely affect mycoplasma growth *in vitro* they are only active at high concentrations which would be toxic to the host. Tetracyclines and the antibiotic Erythromycin are active against MLO at concentrations suitable for clinical use.

The report of Doi et al (53) showing pleomorphic bodies in plants infected with Mulberry Dwarf, Potato Witches' Broom, Aster Yellows and Paulownia Witches' Broom was closely followed by a report by Ishie et al (103) showing that tetracyclines could suppress symptom development in Mulberry Dwarf Disease. These observations, together with the known effects of tetracyclines on animal mycoplasmas, were considered to support the theory of mycoplasmal aetiology of at least some yellows diseases of plants.

Since Ishie's observations many papers reporting MLO in infected plants have also shown symptom suppression by tetracyclines (15, 17, 19, 32, 33, 39, 41, 46, 48, 55, 63, 86, 98, 101, 123, 144, 142, 166, 168, 178, 179, 184, 195, 200, 201, 205, 219). Some of these reports merely show recovery of the plant host while others also show reduced transmission by vector insects. The suppression of disease symptoms by tetracyclines has been used to support the mycoplasmal ætiology of some plant diseases. However in the case of Pierce's disease of grapevine (93, 94) and Peach Phony disease (60) some doubt still exists as to the identity of the causal agent. It is now suggested that they are Rickettsia. This indicates that caution must be exercised in attributing a mycoplasmal ætiology to tetracycline - sensitive plant diseases. Nevertheless antibiotic sensitivity is useful as an indicator of the type of micro-organisms involved.

A range of tetracycline antibiotics have been shown to be useful in suppressing yellows disease symptoms. The most efficient chemical and the method of treatment vary between diseases and can depend on the preference of the researcher (32, 33, 46, 48, 63, 98). The most popular methods of application are by leaf sprays or root soak. Unfortunately tetracyclines are slightly phytotoxic so the level of application must be below the tolerance level of the plants (32).

Remission of symptoms following tetracycline treatment has been correlated with a reduction in numbers of the MLO found in plants (46) indicating that the antibiotics affect the micro-organisms and do not simply mask the symptoms. However complete remission of symptoms only occurs with continuous treatment and both symptoms and MLO will re-appear if treatment is stopped (46). This shows that tetracyclines do not completely eliminate the MLO from plant tissues. Some plants can be protected from acquiring MLO - induced diseases by treatment with tetracyclines immediately prior to exposure to infective vectors (48). Such treatment is therefore not suitable in the field as natural insect infestation can occur at any time. Continuous application of antibiotics which would be needed to prevent acquisition from insect vectors would be as expensive as it is undesirable.

Experiments are described below which were designed to test the effect of some potentially useful chemicals on MLO diseases and also to test the effectiveness of different methods of treatment. Oxytetracycline hydrochloride (OT HCl) was used to test methods of application as this chemical is known to be effective against MLO diseases (98, 112, 144). Its effectiveness also gives some support to the idea that the aetiological agents involved are MLO.

Four chemicals were used in the experiments:- OT HCl, Carbendazim (Car) and two experimental chemicals designated 115799 and 130261. The last two chemicals were supplied by I.C.I. and had been shown to have some activity against bacteria. It was felt that it would be interesting to check for any possible activity against plant MLO. Because of their experimental nature they could only be identified by their numbers.

Experiments were carried out testing some or all of the four chemicals on CP - infected *V. rosea* and cacti with growth abnormalities thought to be caused by MLO. Details of the methods, chemicals and plants used are shown in table III.1 below. Experimental results are given later.

TABLE III.1

	STEM SOAK	ROOT SOAK	INJECTION
CP - infected *Vinca rosea*		OT HCl Car 115799 130261	
Opuntia tuna monstrosa	OT HCl	OT HCl	
Opuntia lanceolata cristata			OT HCl
Cereus variabilis monstrosus		OT HCl	OT HCl
Cereus peruvianus		OT HCl	OT HCl
Astrophytum myriostygma			OT HCl
A. myriostygma monstrosa			OT HCl

EXPERIMENTS WITH CACTI

The abnormal growth form of O. tuna known as O. tuna monstrosa has been shown to result from infection by MLO (25, 26, 119, 140). Remission of the monstrose or 'witches' - broom' symptoms was obtained after treatment with tetracycline hydrochloride (25, 140). Viruses have also been shown associated with this disease but they do not cause the symptoms (26). Opuntia subulata monstrosa has also recently been shown to contain MLO (49).

Other cacti with monstrose or cristate forms may also contain MLO. This possibility can be tested by observation of their reaction to treatment with tetracycline and also by E.M. examination. For this reason O. lanceolata cristata, Cereus variabilis monstrosus and Astrophytum myriostygma monstrosa, cacti showing abnormal growth forms and likely to contain MLO were used in the chemotherapy experiments.

Maramorosch et al (197) showed that tetracycline treatment caused a reduction in the number of MLO in the plant. Repeated treatment of cuttings (25) resulted in a reversion to the normal growth form. Although it was anticipated that the slow growth rate of cacti might make them poor test plants for chemical treatment it was hoped that release from possible MLO effects would result in detectable morphological or growth changes making them suitable for long term experiments. Initial experiments were performed using O. tuna monstrosa not only to verify the results of earlier workers but to substantiate the techniques to be applied to other untested cacti.

Treatments were carried out using three different methods of applying the chemicals:- stem soak, root soak and injection.

Stem soak

Branches of O. tuna monstrosa about 3 cm long were broken off from a large plant. Ten were soaked for two hours in a 100 ppm

solution of oxytetracycline hydrochloride with the broken ends of the stems kept immersed. Ten control pieces from the same plant were soaked in distilled water for the same length of time. All these pieces were then potted up to root in small individual pots of sandy loam and maintained in the glasshouse.

Results

80% of the antibiotic treated and 70% of the control cacti rooted successfully and survived for the duration of the experiment. There was no toxic effect from the antibiotic since treated plants showed no obvious differences from the controls. For three and a half months after treatment there was no difference in growth rate. By March, six months after treatment, the water treated controls were beginning to branch showing the typical monstrose growth form. At this time the tetracycline treated cuttings were growing less vigorously than the water treated plants and showed no branching. However ten months after treatment the tetracycline treated cacti also started branching and by twelve months no difference could be detected between the two treatments.

The antibiotic treatment, it would appear, had a very temporary effect on symptoms and there was no obvious reversion to the healthy growth form as reported by Casper (25). However he gave repeated treatments and found that symptoms re-appeared several weeks after treatment was stopped.

Root soak

Root soak in a chemical solution was used as an alternative method of chemical treatment. Plant roots take up nutrients from soil water so it is likely that some chemicals can be taken up in the same way.

Figure III.1 shows the cacti used for treatment by root dip.

FIGURE III.1

Cacti used for treatment with oxytetracycline hydrochloride by root soak (see text).

Figure III.1

Plants A and B (*O. tuna monstrosa*), E (*Cereus peruvianus*) and G (*Cereus variabilis monstrosus*) were taken out of their pots and the roots washed clean of soil. The roots were then immersed in a 100 ppm solution of oxytetracycline hydrochloride and left to soak for two hours. The treatment was repeated once a week for three weeks. Plant C was treated similarly in distilled water and D, F and H were untreated controls.

Results

The root soak treatment was stopped after three weeks because of root damage. The damage was caused by the constant uprooting and washing. Figure III.2 shows the plants two months after the start of treatment. Plant A and B show longer, unbranched stems compared with their growth form before treatment but when compared to plants C and D the differences are not convincing. Plant E, the healthy control, remained rather thin and pale in colour possibly because of toxic effects of oxytetracycline hydrochloride on the plant. Plant G did not appear to be affected by the treatment.

Injection

As MLO are only found in the phloem the chemicals must get into the phloem to be effective as chemotherapeutic agents. It is not certain if the oxytetracycline was absorbed into the phloem by root soak so the chemical was injected directly into the stem in the anticipation that it would come into direct contact with the phloem tissue.

One each of the following cacti was treated by injecting 1 ml of a 100 ppm solution of oxytetetracycline hydrochloride into the base of the main stem:- *Opuntia lanceolata cristata*, *Astrophytum myriostygma*, *A. myriostygma monstrosa*, *Cereus peruvianus* and *Cereus*

FIGURE III.2

Cacti two months after treatment with oxytetracycline hydrochloride by root soak (see text).

Figure III.2

III.2(cont.)

variabilis monstrosus. One plant of each variety was kept as an untreated control.

Injections were carried out using a small plastic syringe fitted with a 0.6 mm (23g) disposable needle. The needle was inserted just under the epidermis of the plant and the antibiotic solution forced in until either 1 ml had been injected or it was impossible to force in any more fluid. In the latter case a second injection was made to complete the application of 1 ml. On injection the liquid could be seen soaking into the adjacent tissue. The treatment was carried out once a week for six weeks.

As vascular tissue is scattered in cacti it could not be guaranteed that the injection went into the phloem tissue. However, as the vascular tissue is so widely distributed and can occur fairly near the epidermis, it was assumed that some of the antibiotic would penetrate to the phloem.

Results

After six weeks of treatment by injection the damage caused by the insertion of the needle was considerable so treatment was stopped. Three weeks after the end of the treatment most of the cacti had necrotic areas around the injection sites surrounded by much larger areas of wet rotting tissue. The rotted areas may have been caused by water soaked tissue being invaded by soft rotting organisms or damaged cells breaking down.

In the experiment using an Astrophytum myriostygma monstrosa plant the cactus was only mildly affected by the injection and morphological changes suggested that it was recovering.

The monstrose form of this cactus is difficult to root and it is grafted onto an Epiphyllum for propagative purposes. The cactus

is elongated and has many small buds growing near the top. Figure III.3a shows the treated and untreated plants and the enlargement of the buds on the tetracycline treated plant is clearly visible. The treated buds resembled minature forms of the healthy plant. Healthy plants can be rooted without difficulty. Buds from the treated infected plant, if recovered from the disease, should also root easily.

To test this hypothesis some buds were removed from both infected plants (fig III.3b) and placed on sandy soil in pots. The untreated buds soon withered and died when left to root. The treated buds survived for several months and produced some roots.

The buds on the treated plant continued to enlarge for two years after treatment and there was no sign of any new, smaller buds, typical of the infected plant, being produced. It seems therefore that tetracyclines do have an effect on this cactus.

Except for this one encouraging result it does not seem that cacti can be used as suitable indicator plants for testing chemical treatments. Their growth is too slow and it is difficult to be sure of getting antibiotics into the phloem.

Experiments with Vinca rosea

All further experiments were carried out using CP - infected V. rosea plants. Previous experiments suggest that root soaking should be the best method for maximum uptake of chemical by the plants. Injection could not be used on these small herbaceous plants as the stems are too fragile to withstand damage caused by the needle.

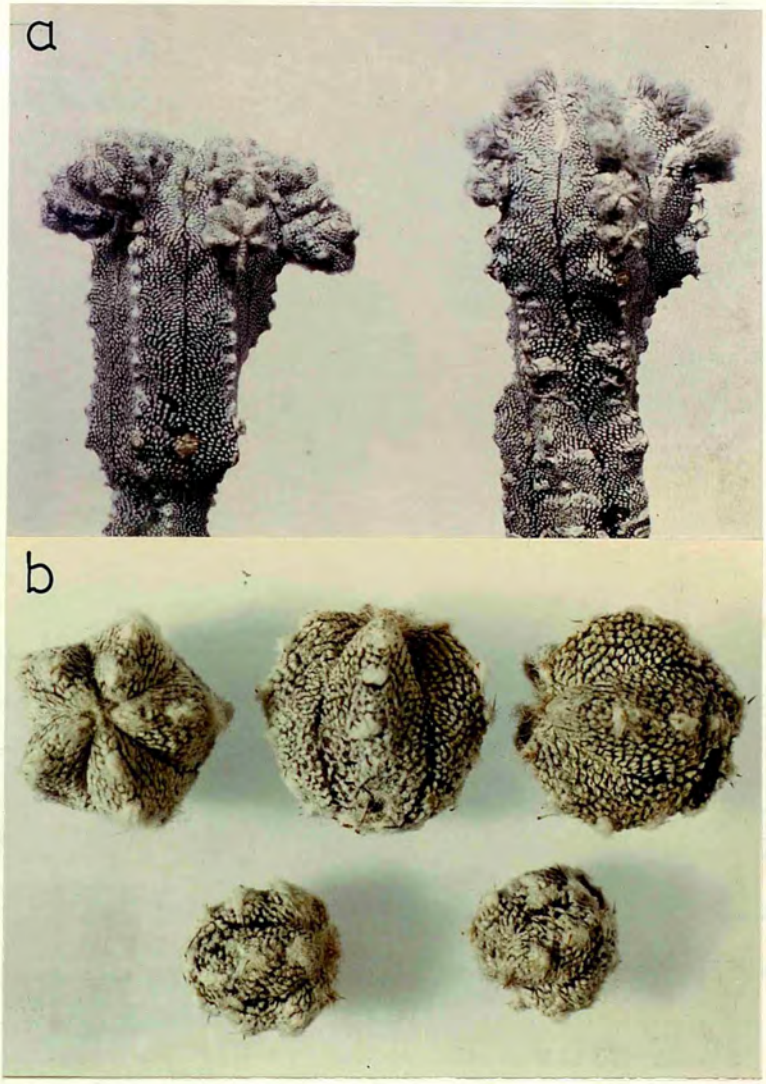
Constant uprooting caused damage to the roots so a method of water culture was developed to avoid the difficulty and to enable a long, continuous chemical treatment to be given. As some chemicals can be either absorbed onto soil particle or inactivated in soil,

FIGURE III.3

a. On the left is the Astrophytum myriostigma plant treated with oxytetracycline hydrochloride by stem injections. This is compared with the untreated plant on the right.

b. Some buds were removed from the plants above and left to root. the size difference is more noticeable in this photograph showing buds from the treated plant above and untreated below.

Figure III.3



water culture allows treatment with these chemicals.

Plants 10 cm high used for these experiments were removed from their pots and the roots washed clean of soil. Trays of Hoagland's water - culture solution (table III.2) were covered with a double thickness of muslin. The roots of the plants were pushed through cuts in the muslin and the stems supported with cotton wool.

TABLE III.2

Hoagland's solution:-

1 litre	distilled water
1.18g	$Ca(NO_3)_2\ 4H_2O$
0.51g	KNO_3
0.49g	$MgSO_4\ 7H_2O$
0.14g	KH_2PO_4
0.005g	Ferric tartrate
1ml	Arnons trace element solution

Arnon's trace element solution:-

250ml	distilled water
2.864g	boric acid
1.810g	$MnCl_2$ (hydrated)
0.222g	$ZnSO_4$
0.008g	$CuSO_4$

Effects of Oxytetracycline Hydrochloride and Carbendazim

Tomlinson et al (209) reported the effects of carbendazim on two virus diseases of lettuce and that treatment with this chemical successfully masked the yellowing symptoms of the two diseases but did not reduce the virus titre in the infected plants.

It was decided to test the effect of carbendazim on CP - infected plants and also to compare it to the effects of oxytetracycline hydrochloride. Although carbendazim can be watered onto the soil around the plants to be treated tetracyclines are adsorbed onto soil particles. In order therefore to apply both chemicals equally in an active form they were administered as a root soak in water culture

solution.

Thirty healthy V. rosea seedlings were grafted with CP - infected cuttings and left for one week to allow the grafts to establish. The plants were then transferred to water - culture solution as described above.

Ten control plants were maintained in standard culture solution. Ten were treated with oxytetracycline hydrochloride by adding the chemical to the culture solution to give 100 ppm of antibiotic. Carbendazim was added to the culture solution for the other ten plants at a concentration of 0.1g per plant (209). Thirty healthy V. rosea seedlings were also kept in water culture and groups of ten were treated as above. The solutions were renewed after one week and all the plants were potted into soil after two weeks of treatment.

Results

None of the healthy V. rosea plants treated by root soak with tetracycline or carbendazim showed any signs of adverse effects. It was therefore concluded that the chemicals were not toxic to any marked extent at this level. Keeping the plants in water culture allowed continuous treatment with chemicals and did not cause the extensive root damage encountered in the cactus experiments.

Two months after the experiment tetracycline - treated plants appeared perfectly healthy (fig III.4a and b). The plants continued to flower normally for five months. After this time some flower - greening was noticed on these plants and then CP symptoms rapidly became severe. The two - week treatment had prevented the plants from becoming infected from the grafts for several months but the effects had not been permanent. It was assumed that the MLO were still present in small numbers, possibly in the grafts, and were able to multiply up and infect the plants when the tetracycline was no

FIGURE III.4

Vinca rosea plants two months after treatment with carbendazim or oxytetracycline hydrochloride.

a. Tetracycline-treated infected plant flowering normally (cf. b.)

b. Untreated healthy plant.

c. Carbendazim treated infected plant. One green, leaflike flower is just visible (arrow) showing that there is no symptom remission (cf. d.).

d. Untreated infected plant showing no coloured flowers. One small, very leaflike flower can be seen (arrow).

Figure III.4

longer present.

In the experiments using carbendazim the infected plants all showed severe CP symptoms throughout the experiment (fig III.4c and d). It therefore appears that carbendazim has little or no effect on the symptoms of CP in V. rosea.

Effects of Chloramphenicol and Experimental chemicals 115799 and 130261 on MLO in plants.

Similar experiments to those described above were set up to test the activity of a further three chemicals against CP and to see if the appearance of symptoms could be correlated with the presence of MLO in the plants.

Oxytetracycline hydrochloride was again used as well as the antibiotic chloramphenicol which has been shown to have some activity against mycoplasmas (149) and the two experimental chemicals 115799 and 130261.

Grafted plants were left for three weeks for the infected grafts to become fully established. The plants were then transferred to water culture containing the chemical treatments. Ten infected plants were used for each treatment and ten as controls. Five healthy plants were also given each treatment. All chemicals were used at a concentration of 100 ppm. Because the toxicity of the experimental chemicals was unknown the treatment was only continued for one week before the plants were re-potted.

For six weeks following the root soak treatment the plants were checked for the appearance of symptoms. At the end of this time the plants were examined for the presence of MLO in the phloem using Dienes stain as described in section IV - Light Microscope Investigations.

Results

The oxytetracycline hydrochloride treated plants were flowering

normally after 6 weeks. Using Dienes stain no MLO could be detected in the phloem of grafted stock plants. However the scion contained large numbers of MLO and, as it had grown considerably since being grafted, there must have been intimate contact with the vascular system of the stock plant.

The antibiotic treatment seems to have protected the stock plant from infection but did not kill the MLO in the infected scion. It is possible that the MLO in the scion had been reduced by the chemical treatment but had built up again by the time the staining test was carried out.

All oxytetracycline treated plants began to show symptoms about three months after the end of treatment. This was earlier than in the previous experiment but may have been a result of the shorter exposure to chemical treatment. The longer time allowed for the grafts to establish before treatment may also have affected recovery by allowing more complete interconnection of the vascular systems of the stock and scion permitting the disease to spread further before being affected by the antibiotic.

Plants which were treated with chloramphenicol and chemicals 115799 and 130261 were found to contain large numbers of MLO in the phloem of both stock and scion. The flowers produced were green with leaflike petals indicating that the chemicals had little or no effect on the disease in these plants.

One plant treated with 115799 flowered normally but was found, using Dienes stain, to contain MLO in the stem of the scion and also at the base of the main stem of the stock. All the other plants treated with this chemical showed disease symptoms. As MLO usually spread slowly from the top of a grafted plant and this plant contained MLO in the base of the stem the disease organisms had clearly moved

from the graft through the vascular system of the stock plant. For some reason the MLO had not influenced the reproductive primordéa so allowing the production of normal flowers. During the course of these studies it was found that uninfected branches occasionally occur on heavily infected plants so it is assumed that a similar effect occurred here. The distribution of MLO presumably comes about by movement through the phloem and may therefore depend on 'source - sink' relationships.

IV LIGHT MICROSCOPE INVESTIGATIONS

The most reliable method for demonstrating the presence of MLO in plants is electron microsccpy of the phloem tissue. Because this is an elaborate and expensive process it has long been realised that a quicker, more simple method is needed. Some light microscope staining techniques have been used to distinguish MLO - infected material frcm healthy (66, 74, 81, 109, 182, 183) although many of these techniques involve fluorescent microscopy using relatively complex chemicals, staining procedures and equipment. Ideally as simple a technique as possible is required using inexpensive equipment and chemicals and demanding little technical skill. Such a technique would allow for cheap, rapid and reliable diagnosis of MLO even under field conditions by unskilled personnel.

With a view to developing such a technique, studies were made of a number of fluorescent and non-fluorescent stains. The experiments using fluorescent stains will be described first followed by descriptions of experiments using less elaborate visible light microscopy.

Tissue preparation

Most staining reactions were originally tested on hand - cut stem or root sections. Sections were cut using a single - edged razor blade, the tissue being supported in preserved carrot if necessary.

If more precise or uniform sections were needed a simple freezing microtome was used to cut regular sections approximately 100 µm thick. This method was also useful for cutting thin stems and roots which could not easily be sectioned by hand.

In some experiments root squashes were used instead of sections. This technique was devised since cactus stem sections were difficult to cut and examine because of the sticky mucous present in the cells.

It was decided to examine roots where problems of mucous secretion would be minimal but as the roots were too small to allow easy cutting squashes of the soft young roots were used. Pieces of root about 1cm long were placed on glass slides and squashed gently under a fairly thick (grade 1) coverslip. Mature parts of such root squashes showed clearly defined xylem and this could be used as a 'marker' to locate the phloem. In many instances distinct sieve plates could be observed. Where possible stem sections were also used as stained areas could be more easilyidentified in transverse sections.

Fluorescent staining

The following stains were tested: 1. Aniline Blue, 2. Methyl-umbelliferones, 3. Europium chaelate.

1. Aniline Blue

It has been reported that aniline blue can be used to show a difference between healthy and MLO - or Spiroplasma - infected plants (66, 81). To test this a 0.1% solution was made up in 0.07M K_2HPO_4 pH 8 as described by Granet et al 1975(81). Hand - cut sections of CP - infected V. rosea plants were stained for 10 minutes. Stained sections were either examined under a G and S conference microscope using blue light and a yellow eyepieces filter or under the Vickers M41 Photoplan set up as described later for 4 - methylumbelliferone staining.

Results

In repeated experiments no difference was found between healthy and infected material stained with aniline blue. This contrasts with the success reported by some other researchers. Granet et al used V. rosea plants infected with Spiroplasma citri and reported differences between healthy and infected tissue. Their positive results may indicate that either the stain is specific for some MLO, but not CP,

or the reaction is dependent on the host physiology.

2. Methylumbelliferones

A relatively new fluorescent compound, 4 - methylumbelliferone has been used to detect animal mycoplasma (18). There are no published accounts of methylumbelliferones being used to detect plant MLO. The umbelliferone molecule is linked to a radical to form a non - fluorescent compound. In the presence of suitable enzymes the radical is split off leaving the fluorescent 4 - methylumbelliferone. Early research on these compounds reported chemical methods for producing pure umbelliferone - radical complexes (59, 145, 186) but such compounds are now commercially available using a choice of radicals. The radical used in any experiment is chosen to indicate the presence of a particular enzyme.

To test for phosphatases, 4 - methylumbelliferyl phosphate was selected, and 4 - methylumbelliferyl acetate to test for esterases.

These compounds were chosen not only because they are readily available commercially but also because phloem is known to contain enzymes including phosphatases (56, 126). It seems likely that the presence of large numbers of MLO in phloem cells might influence the activity of these enzymes provided that the levels of endogenous enzymes being tested are low.

Phosphtases show optimum activity in acid (acid phosphatases) or alkali (alkaline phosphatases) conditions so the methylumbelliferyl phosphate was made up at pH 5.3 and pH 9.3 to test for each type of phosphatase. For the acid reaction a 1.0 mM solution of methylumbelliferyl phosphate was made up in McIvan's buffer pH 5.3 and 1% bovine serum albumen was added to stablise the enzyme. The pH 9.3 solution was made up similarly using Tris/HCl buffer and 1% albumen.

4 - methylumbelliferone acetate was only slightly soluble in water. It could be dissolved in a small volume of absolute ethanol but precipitated out when the alcohol was diluted with water. A small amount of the compound was therefore left for 24 hours in a Tris/HCl buffer at pH 7.0 with occasional shaking. The resultant saturated solution was supplemented with albumen.

It was anticipated that in order to allow the umbelliferone substrate to penetrate through the tissues to the phloem a long staining time would be required. Furthermore thin sections might soften and collapse with such treatment making the detection of phloem difficult. It was therefore decided initially to use the root squash technique described earlier. Entry of the substrate into the tissue was facilitated by a vacuum infiltration procedure.

Pieces of healthy and infected root from V. rosea plants were placed in 10 ml of the substrate solution in 50 ml conical flasks. The flasks were evacuated and re-pressurised 3 - 4 times to ensure infiltration. The tissue was then left for 1 hour at room temperature for the reaction to take place. At the end of this time the root pieces were transferred to phosphate buffer at pH 10.4 to stop the reaction and enhance the fluorescence (59). They were then transferred to microscope slides and squashed gently under a grade 1 coverslip.

The stained root squashes were examined by blue light using a Gillet and Seibert (G and S) Conference microscope. The transmitted light was set at maximum and a G and S blue filter placed over the light source. A secondary filter G and S H or K (yellow - orange) was used in the eyepiece.

Similarly treated material was later observed by ultra-violet (UV) light using a Vickers M41 photoplan microscope. Transmitted

light from the UV lamp excited fluorescence and no supplementary filters were used.

Results

a. Alkaline Phosphatases

The test for alkaline phosphatases (pH 9.3) on root squashes showed a difference between healthy and infected tissue. Cortical root cells of all squashes showed some slight fluorescence. The phloem of infected roots however showed up as a very bright row of cells. Using the G and S microscope the intensity of the fluorescing area could be confirmed by removing the blue filter on the light source and observing (and photographing) the same field of view by white light (fig IV.1). The phloem cells of healthy roots also fluoresced more brightly than the surrounding cortical cells but the distinction between phloem and cortex fluorescence was not so obvious as in the infected material (fig IV.2).

These observations suggest that either the MLO contains phosphatase activity so causing the observed increase in fluorescence or that infection increases the phosphatase activity in the phloem cells. Whatever the explanation this method is of some value in confirming the presence of MLO in suspected infected material but is of limited use for diagnosis as a comparison must always be made with similar uninfected material.

b. Acid Phosphatases

There was no apparent difference between healthy and infected plants using the test for acid phosphatases at pH 5.3 with either blue light or UV light. Phloem fluoresced weakly in both tissues. It is known that acid phosphatases occur in plant cells (126) and have been detected mainly in parenchyma and companion cells (56).

FIGURE IV.1

Root squashes of healthy and Clover Phyllody infected Vinca rosea stained with methylumbelliferyl phosphate.

a. Infected root under visible light.

b. Same field as above under blue light. Phloem fluorescing brightly.

c. High power view of fluorescing phloem showing walls and sieve plates fluorescing brightly.

d. Similar area of uninfected root under visible light.

e. Same area as d. under blue light. Vascular tissue is fluorescing but not as brightly as in b.

Figure IV.1

Plant MLO may also contain acid phosphatases but presumably they are not in sufficient quantity to distinguish them from the background enzymes present in the plant and neither do the MLO change the acid phosphatase activity of the host phloem.

c. Esterases

Staining with 4 - methylumbelliferyl acetate resulted in intense fluorescence in all tissues of both healthy and infected roots. Reducing the staining time to as little as 15 minutes did not reduce the intensity of the fluorescence so this method could not be used to differentiate between healthy and infected tissue.

3. Europium chaelate

The effects of another fluorescent stain, europium chaelate, were also tested. The method used was taken directly from that used at I.C.I. Plant Protection, Jealott's Hill, to detect viable bacterial cells in soil samples (4, 194). It has not previously been used on plant MLO.

Europium chaelate forms a complex with the nucleic acid of bacterial cells (4) causing them to fluoresce red and can be easily detected against a green or non-fluorescent background in soil smears. If the nucleic acids of MLO react in a similar way it was thought that they should show up as bright red areas in green or non-fluorescent phloem tissue. Healthy phloem tissue should, on the other hand, show little or no fluorescence.

2 mM europium thenoyltrifluoroacetate was supplemented with a brightener - the disodium salt of 4, 4 - bis (4 - anilino - 6 - bio (2 hydroxyethyl) amino - 5 - triazin - 2 ylamino) 2 - 2 - stilbene disulphonic acid - at a concentration of 25 µM. Chaelate and brightener were shaken in half the required volume of absolute ethanol to dissolve the compounds completely. This solution was then diluted

with an equal volume of distilled water to give a solution in 50% (v/v) ethanol.

It was found that root squashes were too thick to examine under the microscope used with this stain so either hand - cut or 100 µm thick freezing microtome stem sections were used for staining. Sections of both healthy and infected V. rosea stems were stained for one hour then rinsed in 50% ethanol.

Stained hand - cut sections were transferred to acid - cleaned Chance slides of 0.6 - 0.8 mm depth, mounted in 50% ethanol and covered with a Chance no. 0, 19 mm diameter coverslip. These sections were examined immediately.

Microtome - cut sections of healthy and CP - infected V. rosea plants were stained as above and rinsed in 50% ethanol. The sections were then air dried on to Chance slides as above, mounted in Gurr Uvinert mountant and then covered with a coverslip. These preparations were left overnight for the mountant to harden before being examined.

All sections were observed under a Vickers M41 Photoplan microscope using incidence light at a wavelength of 250 nm. The exciter filter combination in the UV emission line was 2 mm UG1 and 2.5 mm BG 38. A dichromatic reflector or interference beam splitter was used in the binocular head of the microscope with a barrier filter (3 mm K Y418) overlaid with a yellow 'Wratten' gelatin filter (Kodak 12). Photographs were taken on a Kodak Ektachrome 200 Daylight film.

Results

Sections were observed at x 200 magnification - the minimum possible using the M41 microscope. Hand - cut sections could be examined at this magnification but only with frequent changes of focus to allow for the variable thickness. As these sections were mounted in 50% ethanol there was also a problem of mountant evaporation and

it had to be constantly renewed.

The microtome - cut sections were used for permanent mounting and photography as they were of uniform thickness and could be held flat under a coverglass. Drying the sections caused severe distortion of the cells and seemed to reduce the brightness of the fluorescence but the phloem could be clearly distinguished and the fluorescence was sufficient to show differences between healthy and infected stems.

Europium chaelate worked very well on V. rosea stem sections and showed a distinct difference between healthy and infected plants. In both types of section most of the tissue fluoresced green but in infected sections areas of the phloem fluoresced a bright orange/red. The fluorescene in preserved, infected sections was a much less bright orange but still distinctive (fig IV.2). Fluorescence appeared to come from the contents of the cells so it is likely that the MLO were fluorescing.

These results are encouraging and numerous trials of the stain on V. rosea plants showed that it could be used reliably to distinguish healthy from CP - infected material. The use of this reagent to test other diseases and its employment to substantiate the work with Dienes stain are described later in this section.

Visible Light Microscopy

The experiments with methylumbelliferyl phosphate (alkaline) and europium chaelate showed that MLO - infected material behaves differently to these stains than does healthy tissue. In order, however, to obviate the use of these relatively complex fluorescent techniques and to avoid the need for expensive reagents, simpler visible light microscope stains were tested as possible diagnostic reagents. To this end the following stains were used:- haematoxylin,

FIGURE IV.2

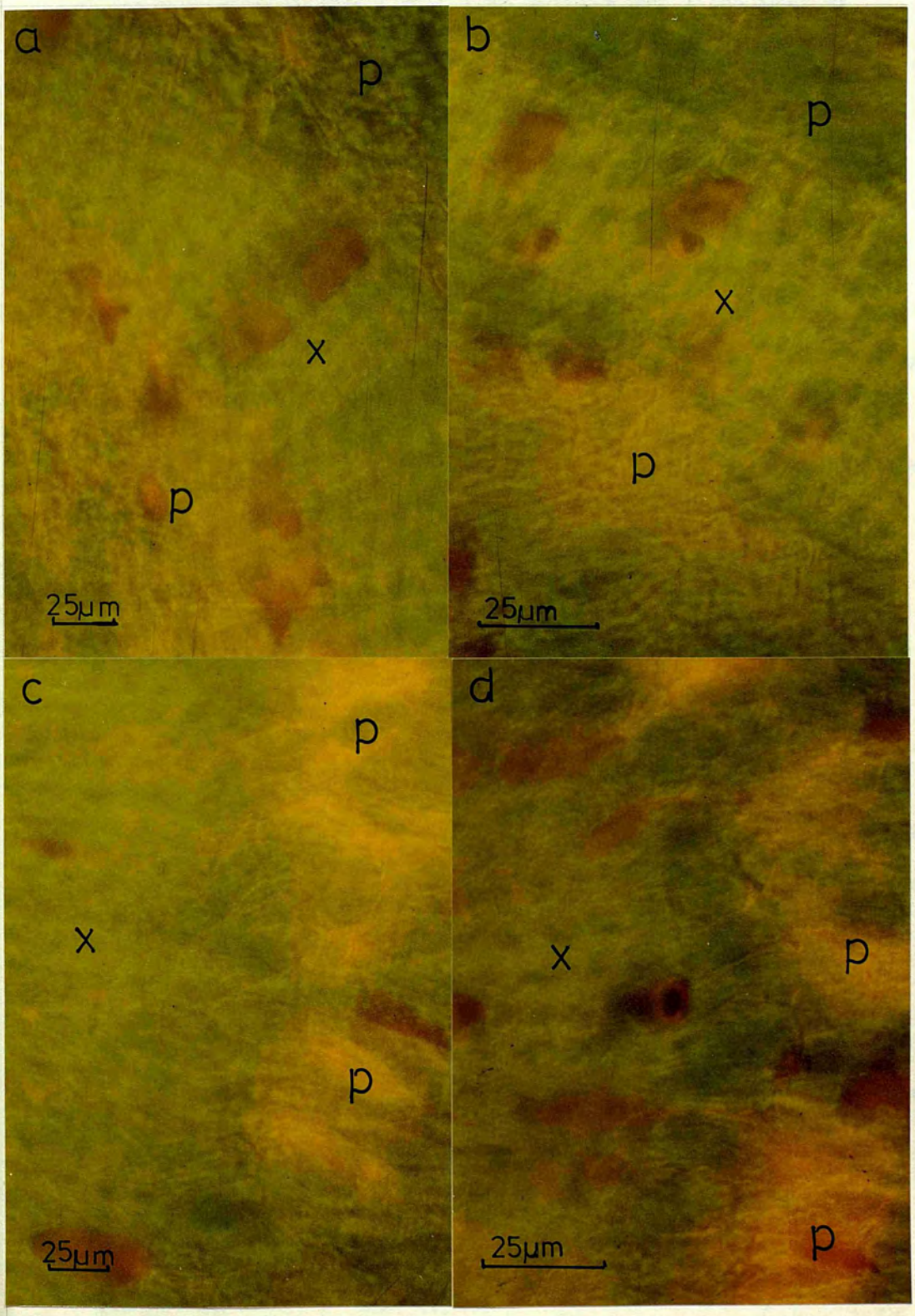
Stem sections of healthy and Clover Phyllody infected Vinca rosea stained with europium chaelate.

a. and b. Uninfected.

c. and d. Infected. Areas of the phloem (p) are a bright orange/yellow.

x = xylem.

Figure IV.2



gentian violet, tetrazolium salts, Feulgen stain and Dienes stain.

No differentiation between healthy and infected tissue could be shown using the first three stains so details are given only of experiments using Feulgen and Dienes stain.

4. Feulgen stain

Feulgen stain has been used to detect MLO in diseased plants (109) but the method described entails a long fixation and wax embedding process before sections can be cut ready for staining. It was therefore decided to try to adapt the Feulgen method used routinely for staining chromosomes in root tip meristems to indicate MLO infection. The stain was tried out on V. rosea and cacti.

With V. rosea hand cut stem sections of healthy and CP - infected plants were fixed for 24 hours in a 3:1 mixture of absolute ethanol and glacial acetic acid. The tissue was hydrolysed for exactly 7 minutes in 1N HCl held at 60°C then transferred to cold Schiff's reagent and left in the dark for one to one and half hours. The tissue was washed in water then examined immediately.

The test was also carried out on cacti but as stem sections of cacti are very difficult to cut and handle the root squash technique was used. Opuntia lanceolata and O. lanceolata cristata plants were tapped out of their pots so the exposed roots on the surface of the soil could be easily removed. Small pieces of root about 2 - 3 cm long were used. The root pieces were fixed and stained as above but using a more usual hydrolisation time of 12 minutes. The root pieces were then soft enough to be squashed on a microscope slide under a grade 1 coverglass.

Results

The method used for root meristem squashes was not entirely suitable for cross - sections of V. rosea stems as the tissue was softened by

the hydrolysing process. Softened sections distorted under the coverslip making interpretation impossible.

After numerous trials on V. rosea stem sections a 7 minute rather than a 12 minute hydrolization time was found to give adequate staining but did not over soften the tissue. Sections stained in this way could be mounted in water, covered with a grade 0 coverslip, and examined directly under a light microscope.

The xylem of all sections of V. rosea stems stained a deep pink with Feulgen stain. In healthy stem sections the phloem remained unstained but in CP - infected phloem there were many areas stained pink (fig IV.3).

Young root tips of Opuntia lanceolata stained by the Feulgen method had a dark puprple stained meristem and any visible nuclei were pale pink. O. lanceolata cristata had a less densely stained meristem but two pink - stained rows of cells were visible behind the meristem. At x 200 magnification these lines could be identified as the phloem of the young roots.

This much simplified method for using Feulgen stain appears to be just as effective as the complex method of Kartha et al (109).

5. Dienes stain

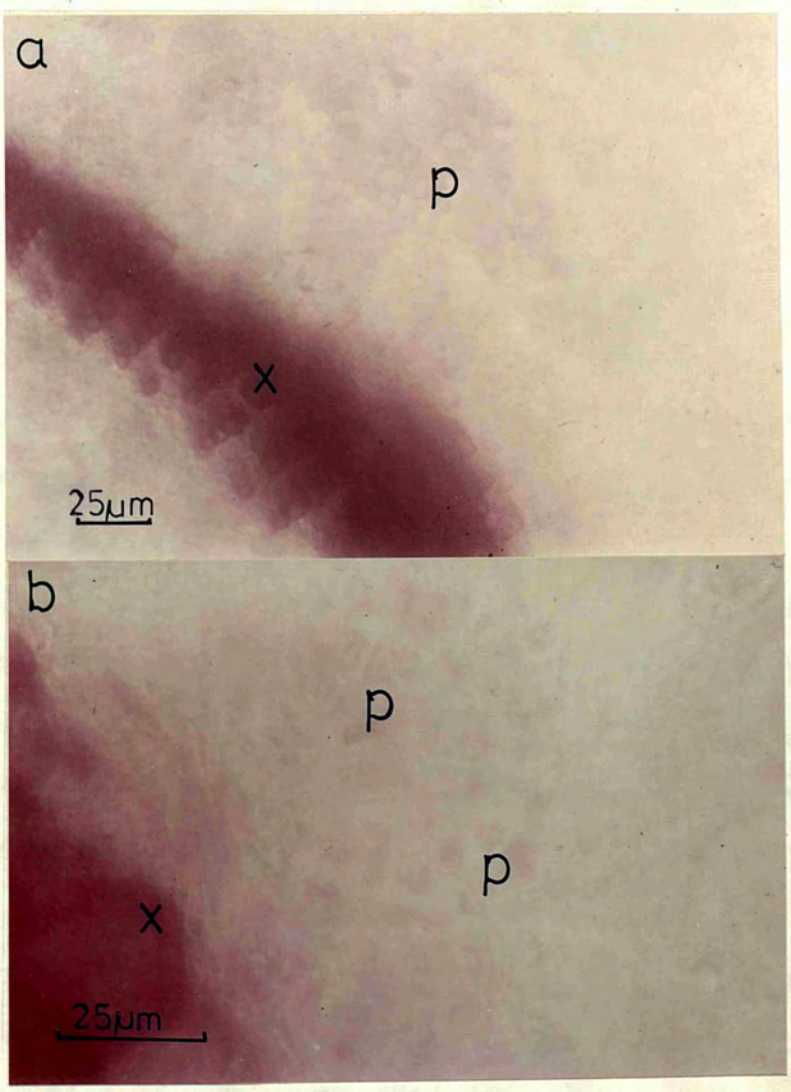
Dienes stain is used routinely to detect mycoplasma colonies on agar (50, 51, 84, 127, 185). As the organisms could concentrate the stain from adjacent agar it was possible that the stain could be used for detecting MLO in plant sections. Experiments were carried out to test this hypothesis.

This method for making Dienes stain was taken from Hayflick (84). Methylene blue, 205g; azure II, 1.25g; maltose, 10.0g; and sodium carbonate, 0.25g were dissolved in 100 ml distilled water. The stain was filtered through a Watman no. 1 filter paper and a series of dilutions (0.5 - 0.1%) were made in water.

FIGURE IV.3

Stem sections of Clover Phyllody infected Vinca rosea stained with Feulgen stain. The xylem (x) is stained a dark pink. The pink stain in the phloem (p) is only feint in these photographs but is easily detected using a light microscope.

Figure IV.3



Thin sections of healthy and infected plant stems were cut by hand into distilled water using a single - edge razor blade. The sections were transferred to stain for 10 minutes after which time the stain was withdrawn and the sections well washed with distilled water. Sections could either be examined immediately or could be left in distilled water for some hours before examination. Several MLO and Spiroplasma diseases were stained in this way. Freezing microtome sections of 100 μm thickness were also used for this staining method as they were more convenient for photography.

Tests were also carried out to see whether disease organisms other than MLO could be stained in this way. Sections from stem and leaf phloem from plants infected with fungi, bacteria and viruses were stained and examined. Table IV.1 gives a list of all the diseases tested.

Freezing microtome sections were cut from stems and roots of CP - infected V. rosea, Rubbery Wood - infected pear and apple, Chat Fruit-infected apple and healthy material of the same species. Half of the sections from each plant were stained with Dienes stain and half with europium chaelate.

Results

After staining with undiluted Dienes stain no differentiation of tissue could be observed in sections of V. rosea as all tissues stained deep blue. In all sections treated with dilute Dienes stain (0.1 - 0.5%) however the xylem was coloured bright turquoise - blue and cortical cell walls stained pale purple - blue. With a 0.2% solution the phloem of healthy stem sections contained many, regularly distributed areas stained a distinct blue (fig IV.4c). These areas could be resolved under higher magnification as groups

FIGURE IV.4

Stem sections of healthy and Clover Phyllody infected Vinca rosea stained with Dienes' stain.

a. and b. Healthy. Xylem (x), phloem (p) and some starch granules (s) are visible.

c. and d. Infected. Areas of the phloem are stained a distinct blue easily distinguishable from the xylem.

Figure IV.4

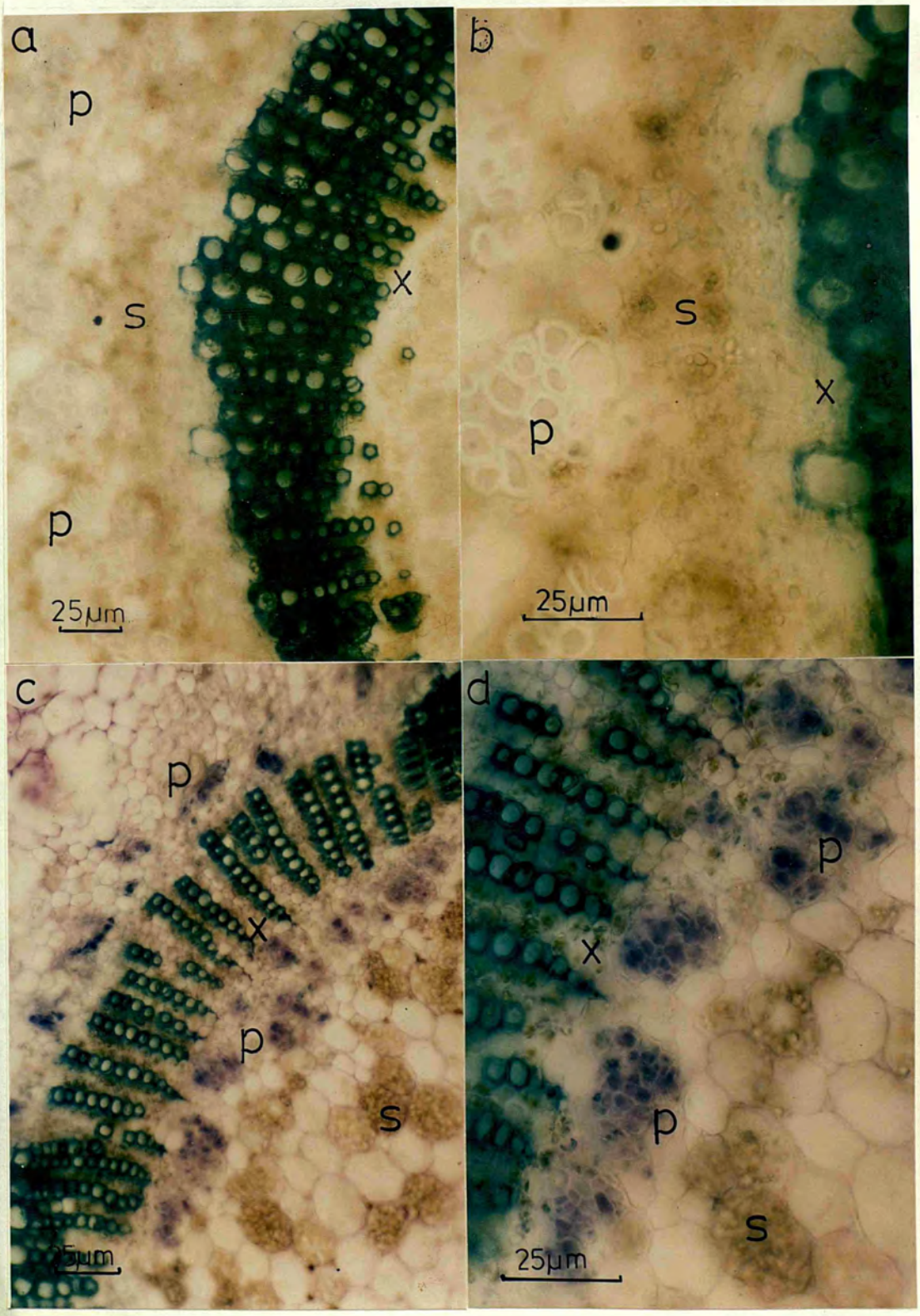


TABLE IV.1

Host	Disease	Aetiological agent	Blue staining reaction in phloem
Vinca rosea	None	-	-
V. rosea	Clover Phyllody	MLO	+
V. rosea	Citrus Stubborn	*Spiroplasma citri*	+
V. rosea	Corn Stunt (CS)	Spiroplasma	+
Clover	None	-	-
Clover	Clover Phyllody	MLO	+
Apple	None	-	-
Apple	Rubbery Wood	MLO	+
Apple	Chat Fruit	Possibly MLO	+ (young shoots only)
Rose	None	-	-
Rose	Flower Greening	MLO	+
Helenium	Flower Greening	MLO	+
Loganberry	Rubus Stunt	MLO	+
Melon	Watermelon Mosaic	Virus	-
French Bean	Tobacco Mosaic	Virus (systemic)	-
Tobacco	Henbane Mosaic	Virus (systemic)	-
Tomato	Bacterial Spot	Bacterium (*Xanthomonas vesicatoria*)	-
Tomato	Bacterial Wilt	Bacterium (*Pseudomonas solanacearum*)	-
Tomato	Potato Blight	Fungus (*Phytophthora infestans*)	-
Grape-vine	Downy Mildew	Fungus (*Plasmopara viticola*)	-
Tomato	Grey Mould	Fungus (*Botrytis cinerea*)	-
Apple	Powdery Mildew	Fungus (*Podosphaera leucotricha*)	-
French Bean	Bean Rust	Fungus (*Uromyces phaseolus*)	-

of sieve elements with blue - stained contents (fig IV.4d).

Application of a 0.2% solution of Dienes stain made it possible to distinguish MLO - infected from healthy tissue in both woody and non - woody plants. CP MLO could be detected in the phloem of leaves as well as in stems.

All MLO and spiroplasma infected plants tested gave similar results to the above. Of the plants containing other pathogens none showed a blue colour in the phloem of leaves or stems (table IV.1).

Three more MLO diseases: mulberry dwarf, jujube witches' - broom and Paulownia witches' - broom have since also been shown to give similar results using Dienes stain (117).

Where alternate sections of stem tissue taken from the same plant were stained with Dienes stain or europium chaelate they showed an exact correlation between the two stains. Only those sections giving a positive reaction to Dienes stain had red fluorescent areas in the phloem when stained with europium chaelate. In this experiment all the infected material gave positive results, the controls remaining negative. Both these stains appear to be reliable methods of detecting MLO in diseased plants. Dienes stain has the advantage of being rapid and employing simple techniques and microscopy.

Disease spread within host plant

Because Dienes stain was so successful and easy to use, its further potential was tested in experiments designed to examine the route and timing of the spread of CP disease through a host plant.

For this purpose young V. rosea plants at the 6 leaf stage and about 10 cm high were grafted at their apex as described earlier with CP - infected scions. Each week after grafting one plant was cut

off just above the lowest pair of leaves. Sections were cut from each internode of the main stem and from each branch. The sections were examined for MLO by staining with 0.2% Dienes stain. Roots were difficult to section so the roots and lower leaves were left in the pot and allowed to grow on in the glasshouse under the same conditions as all the other plants. The resulting "root stock" plants were examined over a period of time for MLO disease symptoms.

Results

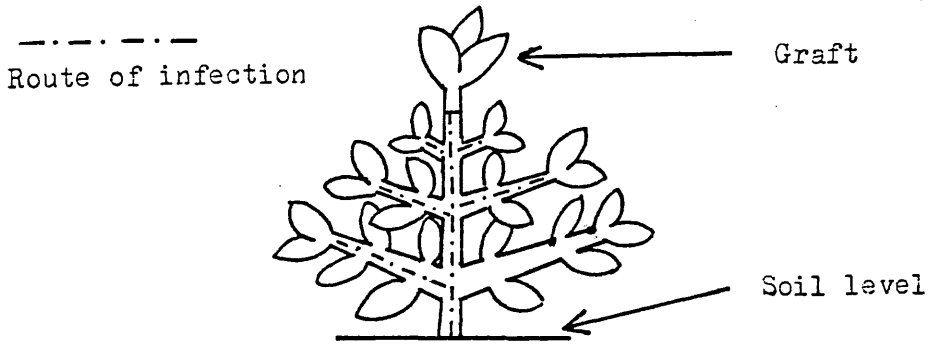
None of the plants tested gave a positive reaction to Dienes stain during the first five weeks after grafting. The "root stock" plants which eventually grew from the roots and stem base were also healthy producing normal coloured flowers. Had any MLO been present in the untested lower parts of the plants the new shoots would have been infected and eventually shown green flower symptoms. Thus MLO had not, during this time, been transmitted from the apical graft to any other part of the plant.

Six weeks after grafting MLO could be detected in the top internode of the stem of the plant tested. The rest of the plant, including the roots, was apparently uninfected as no MLO could be found using Dienes stain and the "root stock" plant was healthy.

After eight weeks MLO could be detected throughout the main stem almost to soil level. The disease organisms had not entered the roots since the "root stock" plant flowered normally. The plants infected by grafting had numerous laterals - two per node - by this eight week stage and were noticeably more bushy than healthy controls. Examination of the laterals in the tested plant showed the MLO had travelled to the apex of each with the exception of one basal lateral (see fig IV.5). These observations show that the main spread of the disease is downwards via the stem vascular system with infection of

the lateral branches. The infection reaches the root system last.

Figure IV.5



This route of infection is similar to that indicated by the appearance of green flower symptoms in the large grafted plants used in transmission experiments. The plants used here had not reached flowering age so the disease could not have been diagnosed from external symptoms. Dienes stain can therefore be used to indicate the presence of the disease organism before the appearance of symptoms.

The time taken for infection of these plants was longer than expected. The reason for this is not known but possibly grafting such young plants by removing the main stem apex reduces the growth rate of the plants and it is likely that the rate of infection is linked to the growth rate. It may also be related to the time taken for conducting tissue, including phloem, to establish and permit passage of disease organisms into the plant.

Only one plant was tested each week and it is to be expected that some variation in the rate of infection will occur between plants. The time scale shown by this experiment is therefore only applicable to the plants used here but the route of infection should apply to all plants infected in the same way.

V ELECTRON MICROSCOPE INVESTIGATIONS

Electron microscope (EM) studies were undertaken in order to describe and compare MLO from different host plants. In addition detailed studies were made of the effect of MLO in Vinca rosea on the structure of corolla plastids during green flower symptom development.

The following plant tissues were sectioned and examined:-

1. Trifolium repens with CP (MW and SD symptoms) - flower stems
2. Vinca rosea with CP - flower stems
3. Cuscuta epiphytum parasitic on CP - infected Vinca rosea - stems
4. Various MLO - diseased plants
   a. Green strawberry - petals and stems
   b. Green Helenium - flower stems
   c. Green rose - petals
5. MLO - infected cacti
   a. Opuntia lanceolata cristata - fleshy stem
   b. C. gronovii parasitic on Opuntia tuna monstrosa - stems
   c. C. europea parasitic on Opuntia tuna monstrosa - stems

The following aspects of MLO infection were also investigated:-

6. Division of MLO
7. Movement through pores
8. Changes in host plant ultrastructure
   a. Inclusion bodies
   b. Starch granules
   c. Plastid development

Plant material was prepared for the EM by fixing small pieces for 24 hours in a 3% solution of gluteraldehyde in Sorensen's buffer pH 7.4. The tissue was then washed in buffer and post - fixed for one to two hours in a 1% solution of osmium tetroxide in veronal acetate buffer followed by a buffer wash.

Fixed tissue was dehydrated in a graded ethanol series, transferred to propylene oxide and finally embedded in Spurr low viscosity resin (199). The blocks were hardened at 60°C for two days.

Blocks were trimmed and sectioned on an LKB III ultratome. Pale gold sections were collected on LKB 400 uncoated grids. To improve resolution sections were stained for 20 minutes with a saturated solution of uranyl acetate in 50% ethanol followed by a two minute stain with lead citrate (170).

Results

1. Studies of MLO in Trifolium repens (EM 1 - 4)

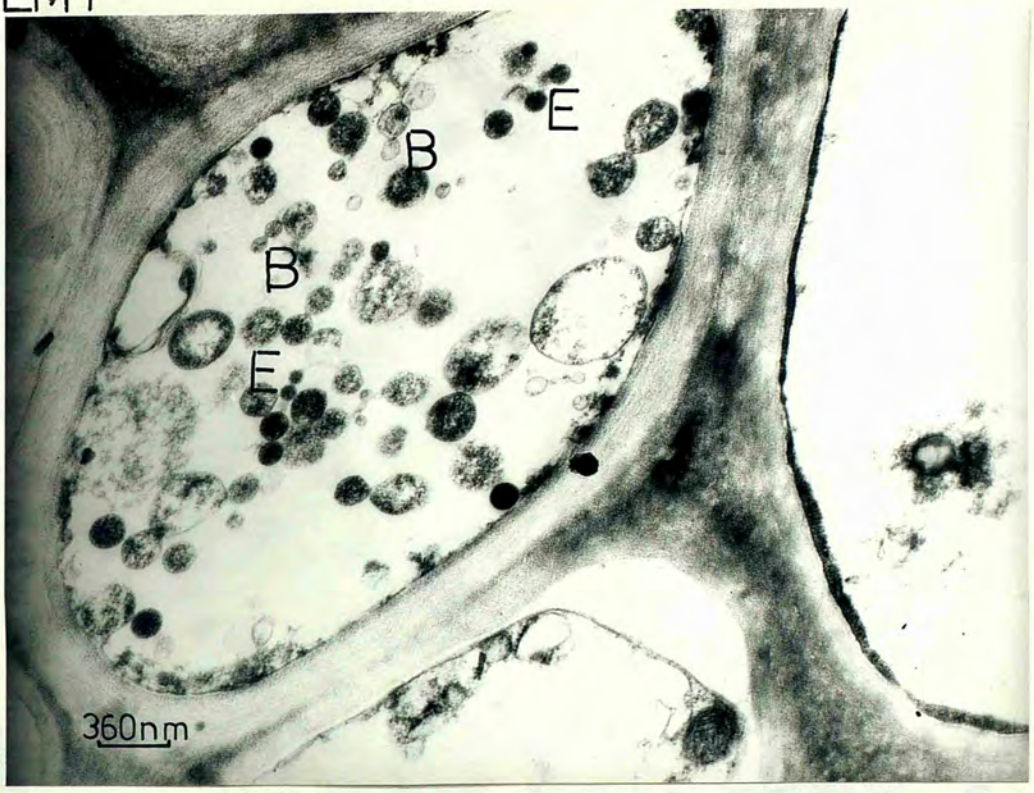
EM 1 is a typical view of a phloem cell to show the SD strain of CP in the phloem of infected T. repens plants. The MLO vary greatly in size from 70 nm diameter to 640 nm diameter having an average diameter of 350 nm ± 300 nm. Each one is surrounded by a unit membrane and some can be seen to contain central stranded material similar in appearance to that described by other workers as DNA. In this section several of the MLO are budding (B) and some of the small membrane - bound bodies with dense contents (E) are possibly elementary bodies.

EM 2 shows phloem cells from the stem of an MW CP - infected clover plant. Some of the cells have normal contents. One cell with an otherwise empty lumen contains 5 distinct MLO. Details of this cell are shown at higher magnification in EM3. The MLO measure 255 to 382 nm in diameter. They have the limiting unit membrane and central DNA - like strands characteristic of MLO. Two appear to have just separated by binary fission and are still joined by a narrow neck of cytoplasmic material. The membrane is continuous round both bodies and the connecting neck (arrow).

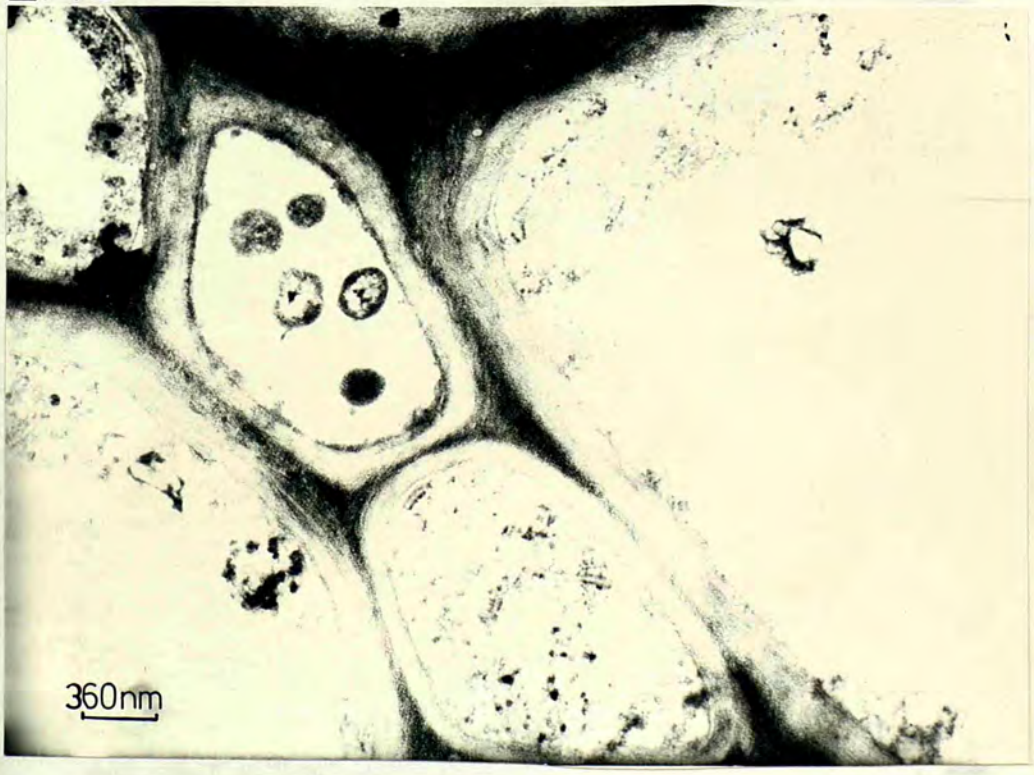
EM 4 This is another phloem cell from clover containing MW MLO. Three separate MLO show characteristic features but most interesting is the chain of MLO across one end of the cell. These may have arisen from successive buds which have not separated completely or from a filament which has constricted into this bead - like arrangement.

Careful examination and measurement of MLO from numerous EM graphs from a number of clover plants has revealed no obvious morphological or fine structural differences between the SD and MW strains of MLO.

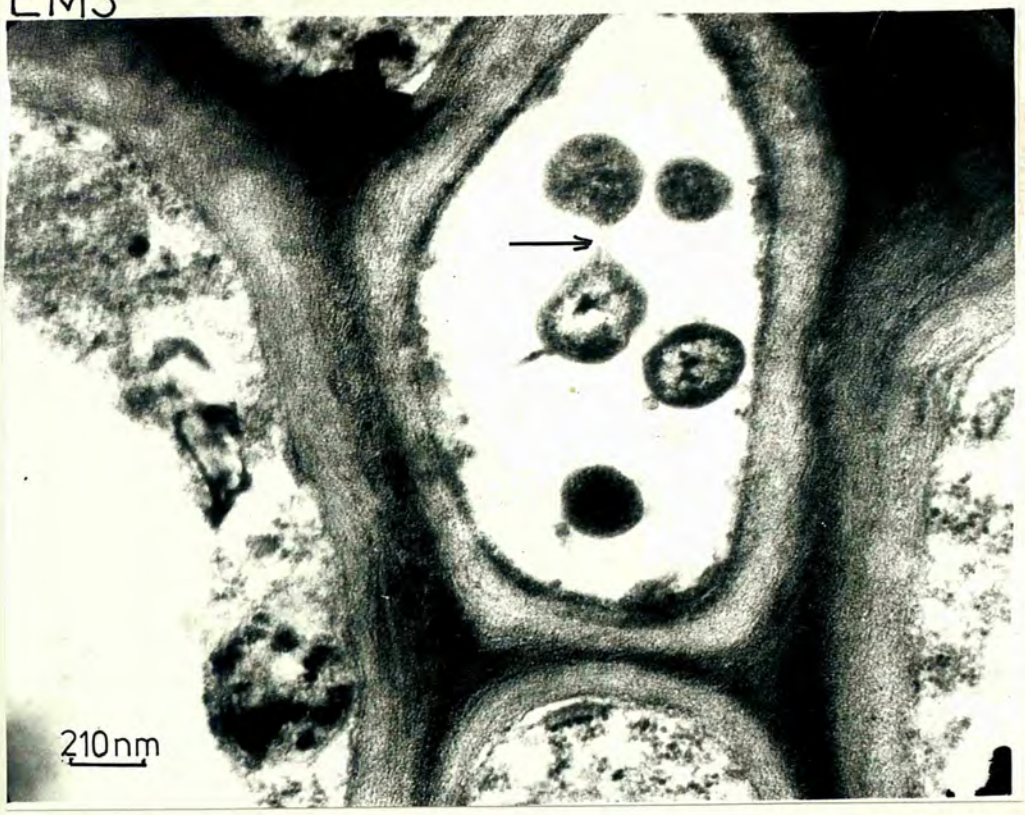
EM1



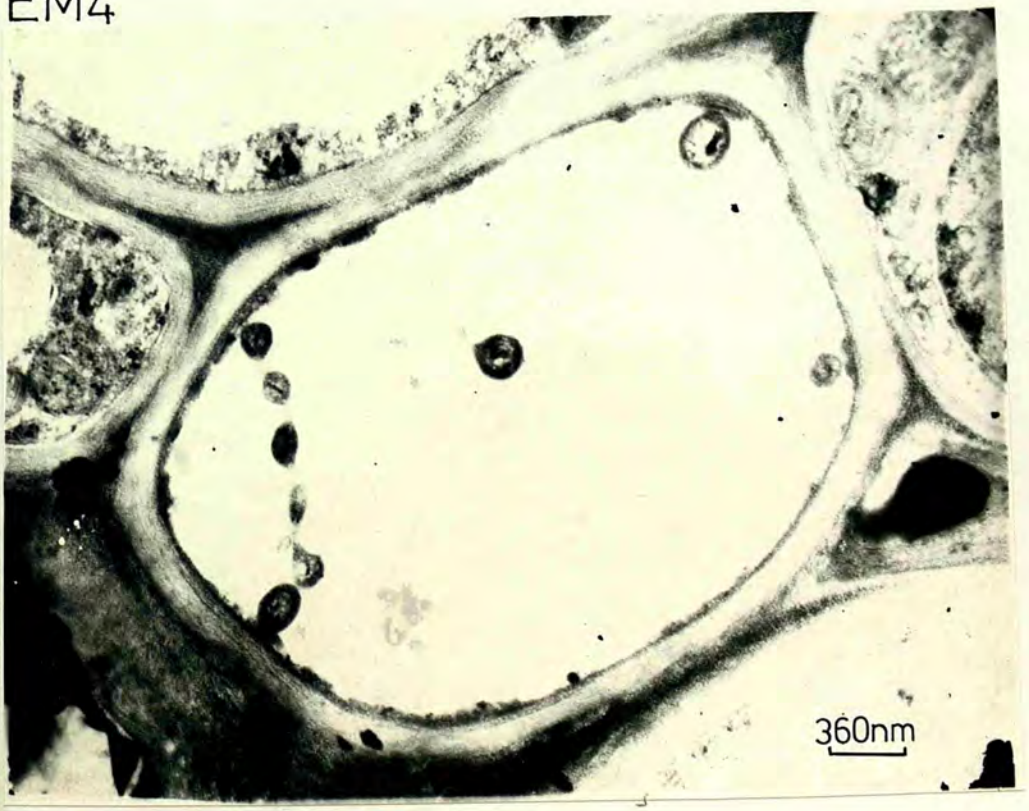
EM2



EM3



EM4



2. Studies of MLO in Vinca rosea (EM 5 - 7)

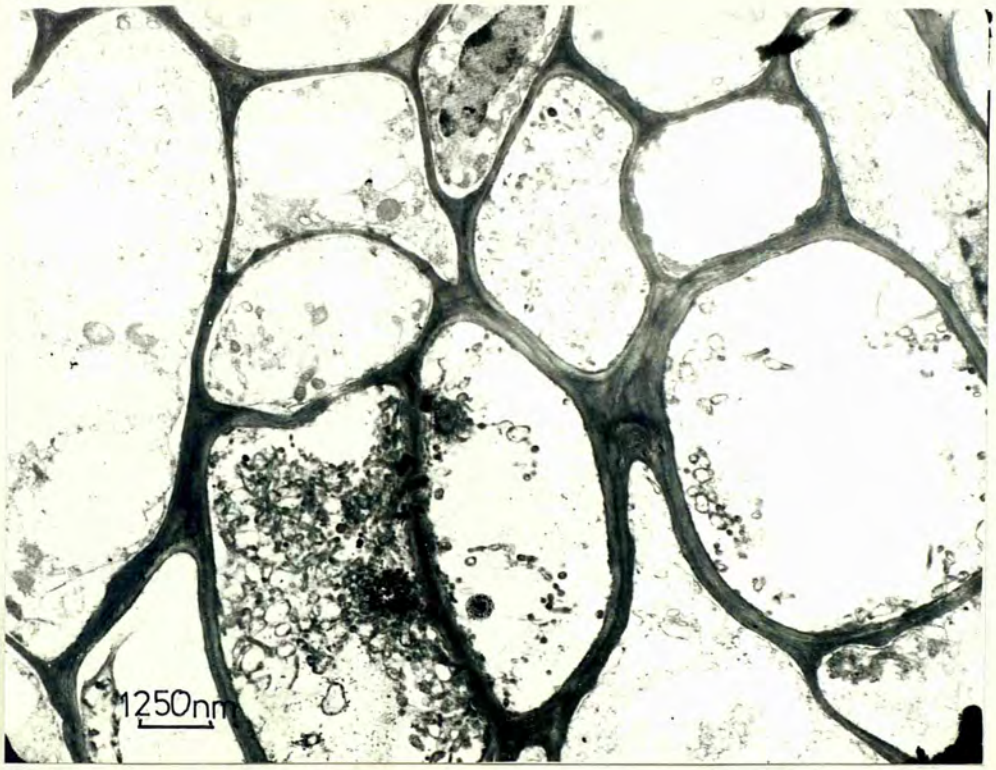
EM 5 A low power view in TS of an area of phloem from an SD CP - infected V. rosea plant showing that many of the cells contain large numbers of MLO.

EM 6 This cell containing large numbers of MLO is adjacent to a sieve plate. The plane of section has cut through some sieve pores (p) but they appear to be blocked with material from the adjacent cell.

EM 7 MW MLO are present in two adjacent phloem cells. Many of them are quite small (71 - 142 nm diameter) although a few larger ones are present (393 nm diameter).

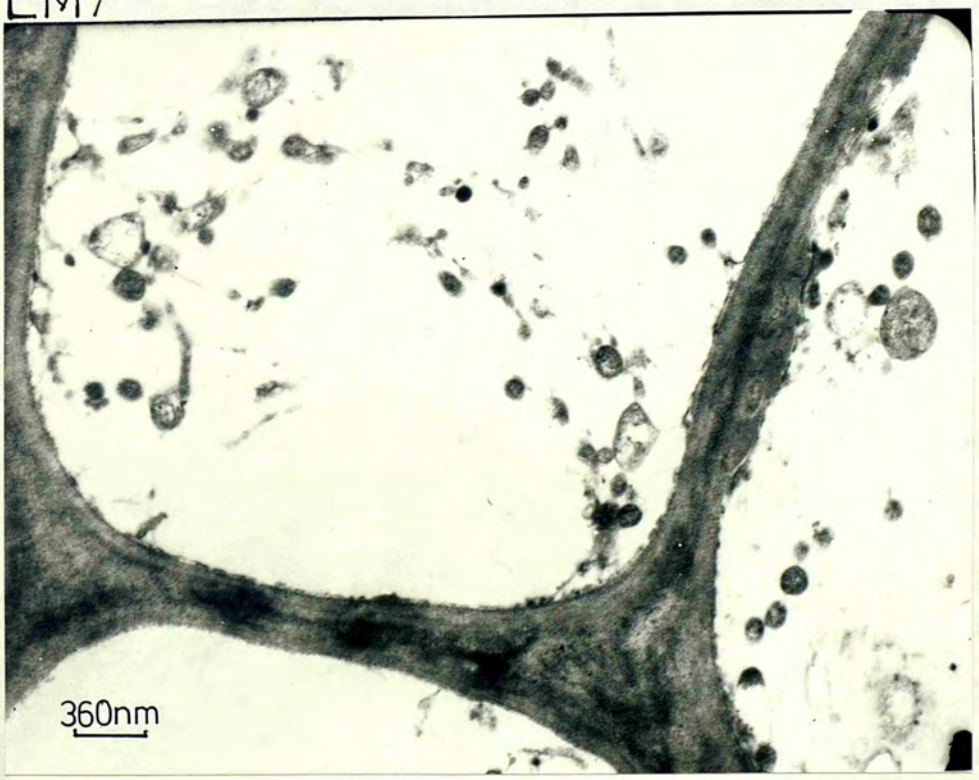
Again no visible difference was found between the two strains of CP in this host plant. There was also no difference in external symptoms.

EM5



EM6

EM7



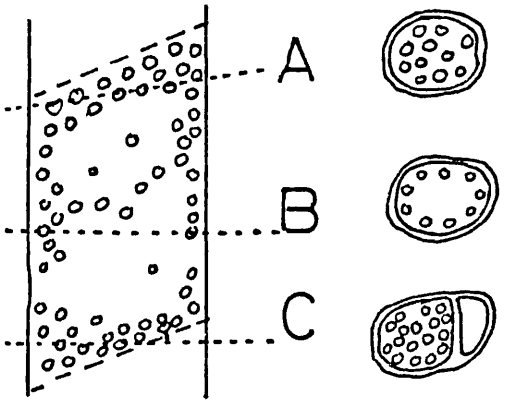
3. Studies of Cuscuta epiphytum parasitic on CP - infected Vinca rosea (EM 8 - 10)

C. epiphytum was used successfully to transmit CP from clover plants to V. rosea (see section I). As MLO only occur in the phloem of diseased plants it is likely that it was transmitted via the phloem of the dodder. The phloem was therefore examined in the EM for the presence of MLO.

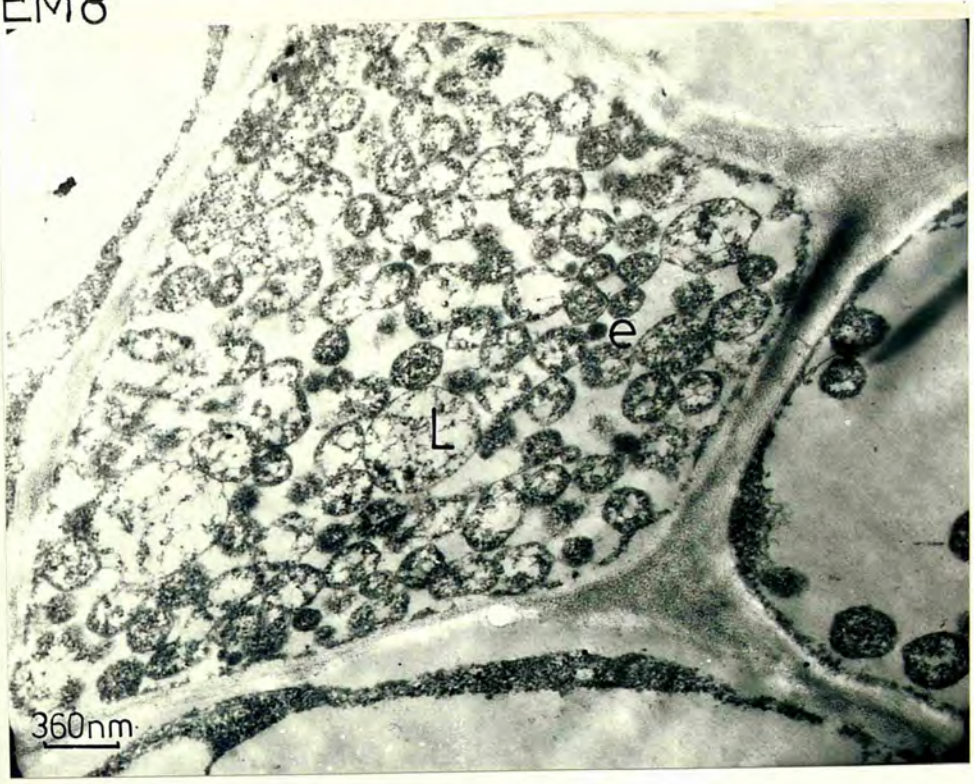
EM 8 This shows a typical section through the stem of C. epiphytum and shows clearly the presence of very large numbers of MLO in the phloem tissue. The MLO sizes range from 86 nm for the elementary bodies (e) to 643 nm for the very large MLO with diffuse contents (L). It is possible that these large MLO are no longer visible as the contents appear to be disrupted.

EM 9 MLO in plant phloem are often found to aggregate near the cell walls and sieve plates (163). This phenomenon has been noticed during this research especially in the occasional LS of cells. The following diagram (fig V.1) indicates how the varying distribution of MLO in electron micrographs might be explained. Sections A and C near sieve plates would show sieve cells "packed" with MLO, whereas sections in mid - portions of the cell (B) would show fewer MLO peripheral in distribution. It is likely that EM 8 section was close to a sieve plate.

Figure V.1



EM8



EM9

EM 10 In this cell the plasmalemma is shown clearly round the cell wall and some MLO occur in the cell lumen. MLO usually occur in sieve elements which are devoid of other contents. In this cell however there is also some fibrous material, possibly P - protein, present in the lumen.

These MLO are indistinguishable from CP MLO in both clover and V. rosea.

EM10

4. Studies of Various MLO - diseased plants (EM 11 - 20)

a. Green Strawberry (EM 11 - 17)

A green flower symptom has been found occasionally in strawberry plants and it has been associated with the presence of MLO in the phloem (38). The origin of the disease is unknown but the symptoms are similar to CP in clover and V. rosea so it is possible that greening in strawberries is caused by CP MLO. Micrographs 11 - 17 show a selection of sections from green flowered strawberry plant stems showing cells containing MLO.

EM 11 and 12 are two low power views of TS of the stem phloem showing the large numbers of MLO typically found in this tissue. The cells have thick nacreous walls and some can be seen with normal contents although many of the sieve cells are densely packed with MLO (S).

EM 13 This is one of the cells from EM 11 at a higher magnification. Some of the large MLO in this section are rather elongated (up to 570 nm long). The shape of some of them showing constricted centres suggest that they are about to divide. The dense, possibly nuclear material, can be seen at opposite ends of some of the MLO (n).

EM 14 The cell in this micrograph is densely packed with MLO and there is dense material visible between the bodies. It is possible that the MLO are close to the sieve plate which would explain this electron - dense background.

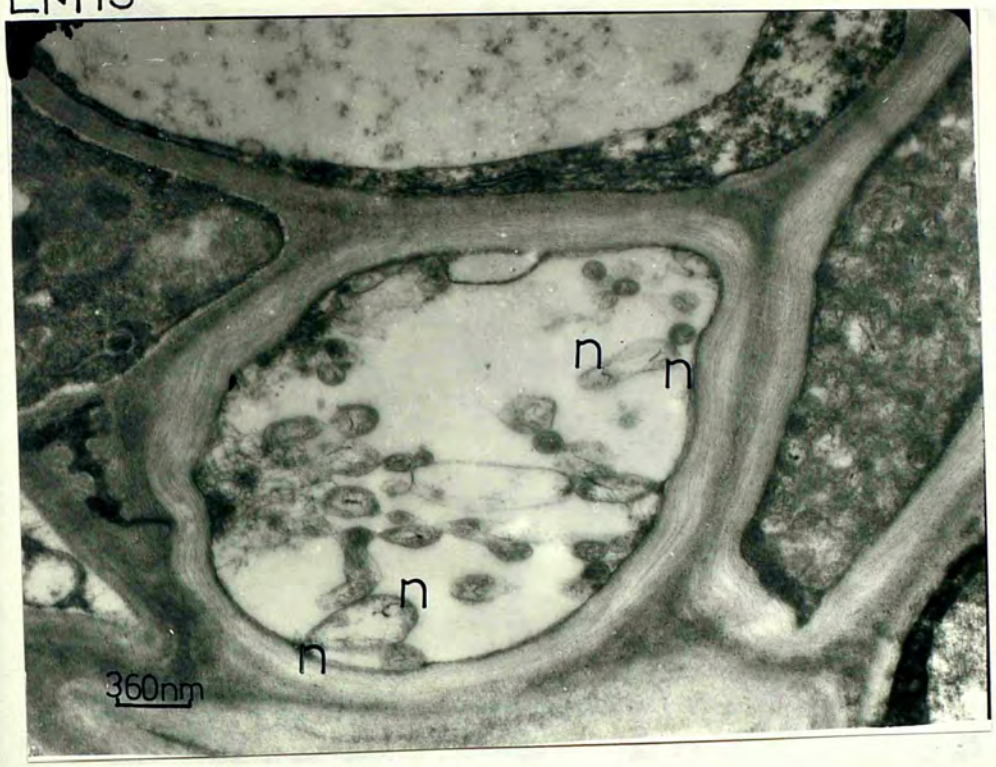
EM 15 This cell is also densely packed with MLO. The nacreous wall thickening (t) of the phloem cells is clearly visible in this section. Some adjacent cells have normal healthy contents including chloroplasts (c) and mitochondria (m).

EM 16 The thick cell wall is again visible round this less densely packed cell. A very large size range of MLO are present in this section

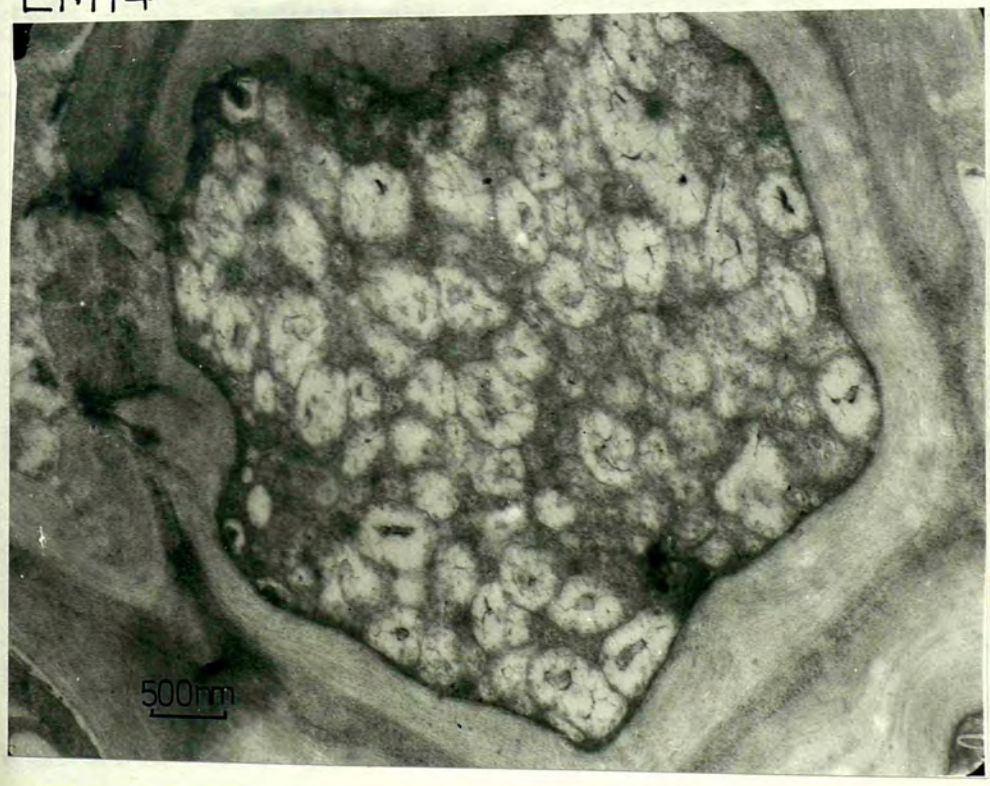
EM11

EM12

EM13

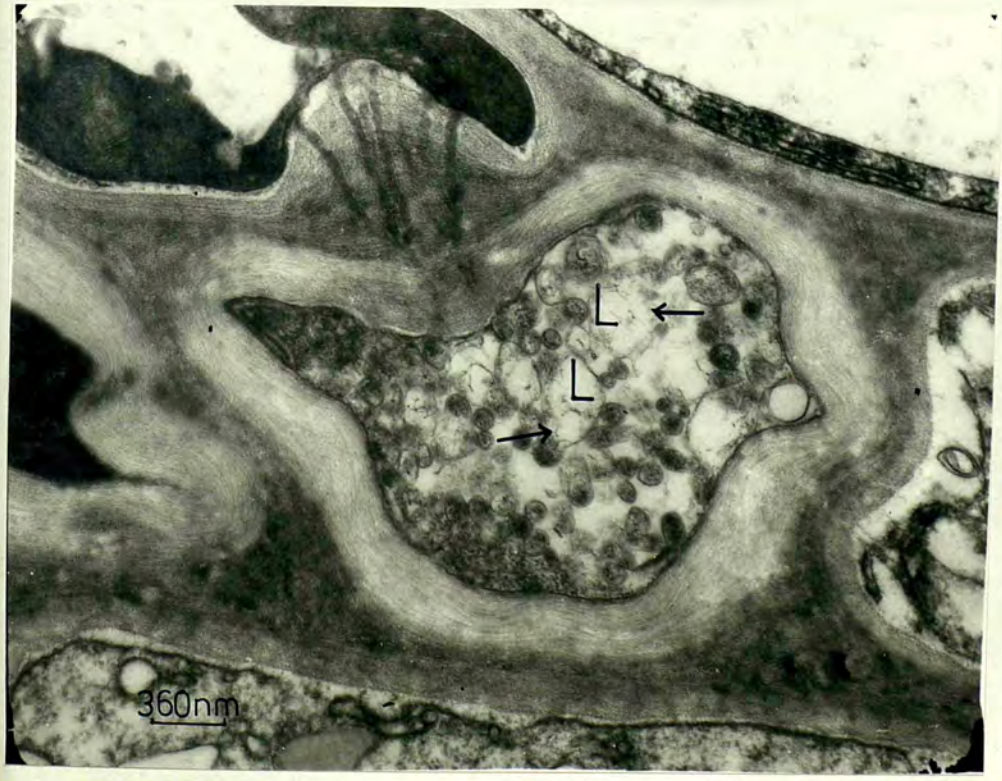


EM14



EM15

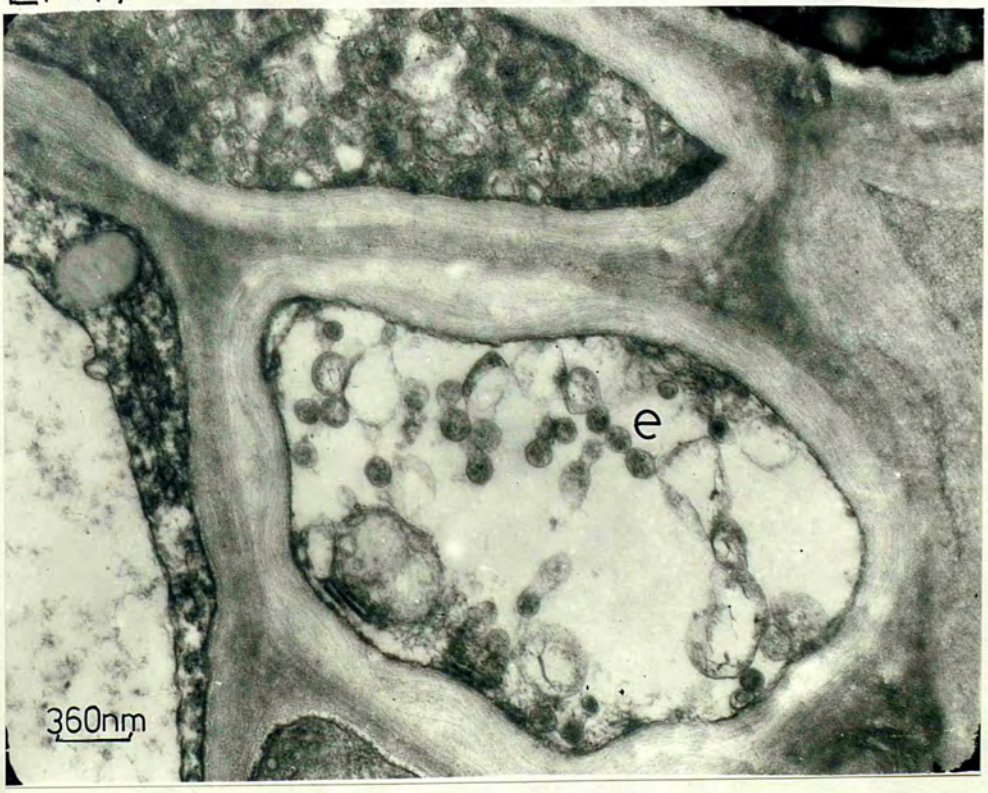
EM16



(143 nm - 500 nm). Some of the very large MLO (L) may be breaking down as the contents are very diffuse and the membrane appears to be disrupted in places (arrows).

EM 17 This cell also contains a wide range of sizes and shapes of MLO. Some dense elementary bodies (e) are visible and appear to be budding off from larger, less dense bodies.

EM17



b. Green Flowered Helenium (EM 18 - 19)

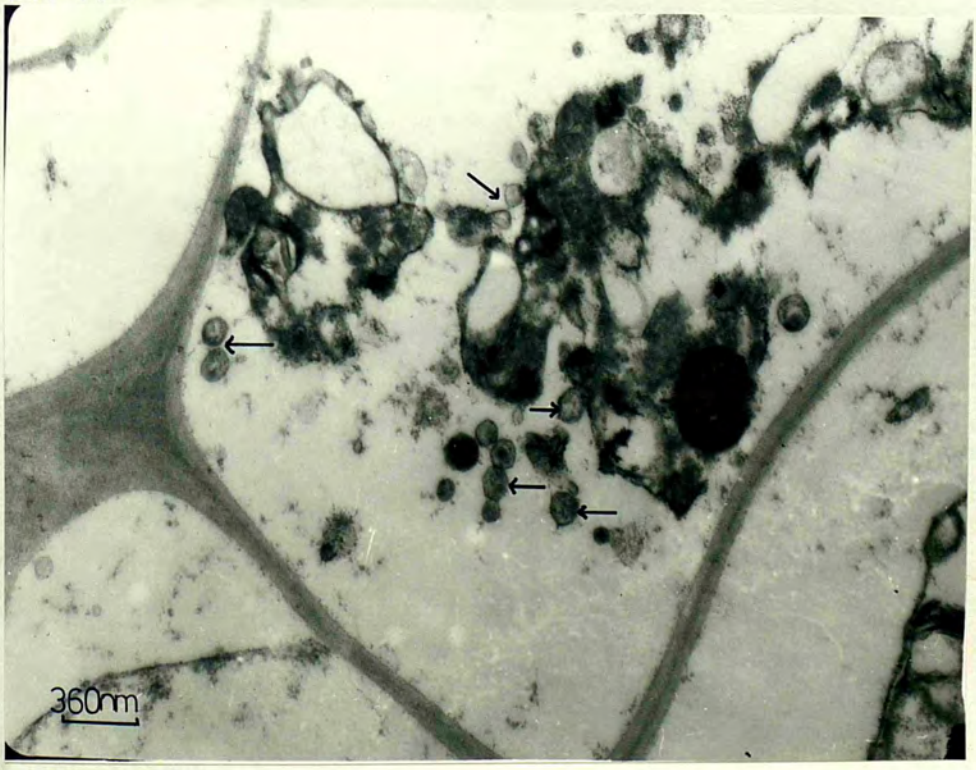
EM 18 This photograph shows a TS through the stem of a green flowered Helenium just below the flower head. The cell in the centre contains large numbers of MLO ranging in size up to about 500 nm. The small ones are too small to measure accurately. The cell on the other side of the sieve plate (sp) has dense contents and may contain some MLO.

EM 19 The MLO in this cell (arrows) seem to be surrounded by membraneous material. This may be the remains of other MLO which have broken down or it may be a plant substance.

Other sections from this Helenium plant show that it did not contain such large numbers of MLO as the clover and strawberry plants also described although the MLO were indistinguishable structurally from those observed in these plants.

EM18

EM19



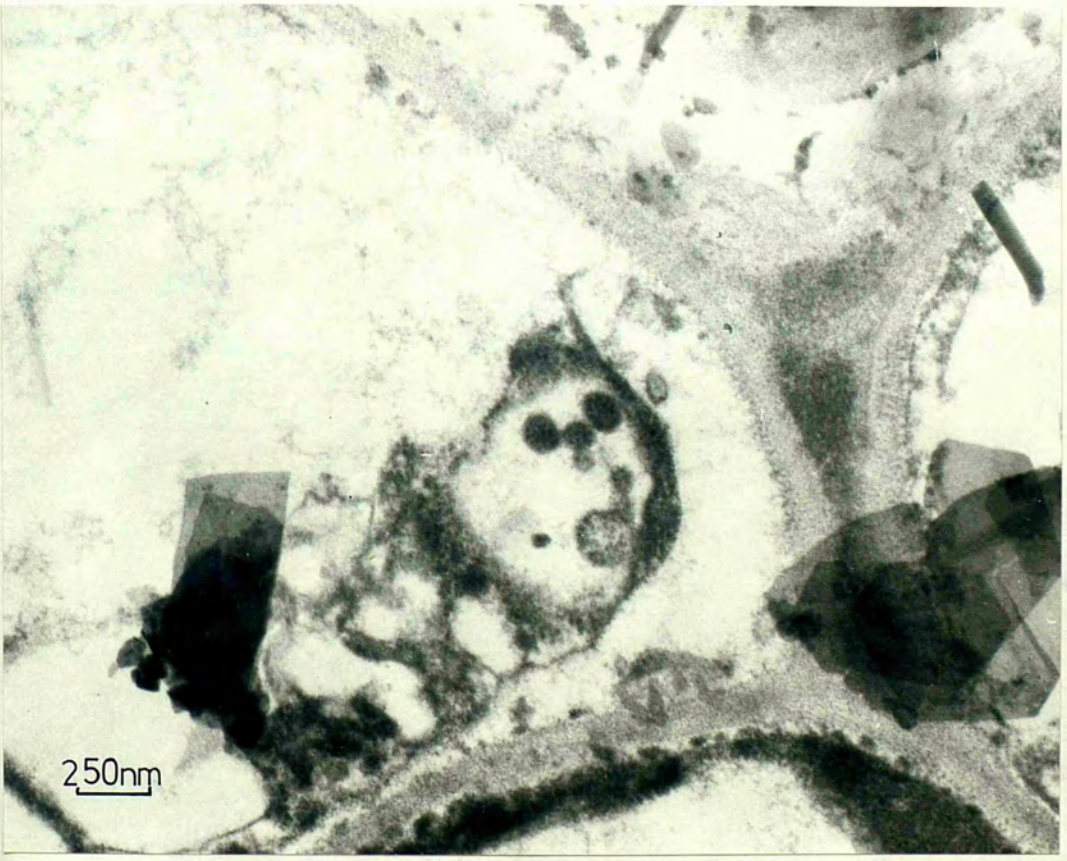
c. Green Rose (EM 20)

EM 20 Cross-section of the petals of a rose with green flowers were examined for the presence of MLO as the stems were difficult to fix and section. The vascular bundles are very small in the petals which may explain why MLO were difficult to find. However, a small group are shown here in the corner of a phloem cell. They are indistinguishable in size and shape from other plant MLO.

Although the MLO in these herbaceous plants show a wide range of shapes and sizes it is not possible to distinguish between those from different plants. It is possible that the same organism is causing the green flower symptom in all the plants but if this is not so the different organisms cannot be identified by electron microscopy.

The MLO shown in these micrographs range in size from 70 nm to 640 nm with a mean diameter of 285 nm.

EM20



5. Studies of MLO in cacti (EM 21 - 36)

a. Opuntia lanceolata cristata (EM 21 - 26)

EM 21 shows a phloem cell at low magnification (x 17,500) containing fairly large numbers of membrane - bound bodies. These are surrounded by a thread - like material which was present in all cells and is thought to be mucous. The presence of this material made it difficult to obtain 'clean' preparations. Although the bodies resemble MLO they are considerably larger in size than those found in herbaceous plants ranging from 300 - 1,000 nm in diameter. The bodies are not mitochondria as no christae were visible and no similar structures were found in healthy O. lanceolata.

EM 22 and 23 At higher magnification these bodies can be seen clearly as can their surrounding membrane. The size range is 428 - 714 nm diameter. Internally they appear to have ribosome - like contents although there is no clear nuclear - like strands visible. There are also no internal membranes or internal extensions of the outer membrane as found in mitochondria.

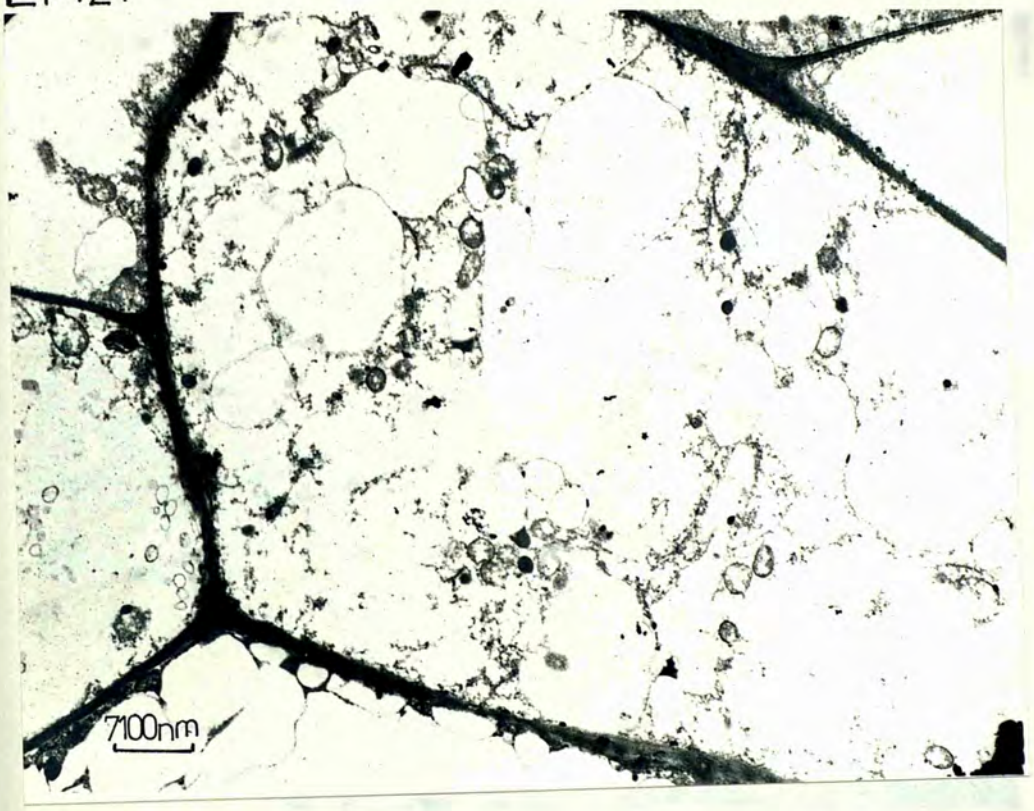
EM 24 shows an elongated body 2,570 nm x 430 nm, very similar in structure to the rounded bodies in EM 23, suggesting that these are membrane - bound structures.

There are no signs of division or elementary bodies in these micrographs but the organisms are not chloroplasts as these structures, from the same plant material, are shown in EM 25 (Ch).

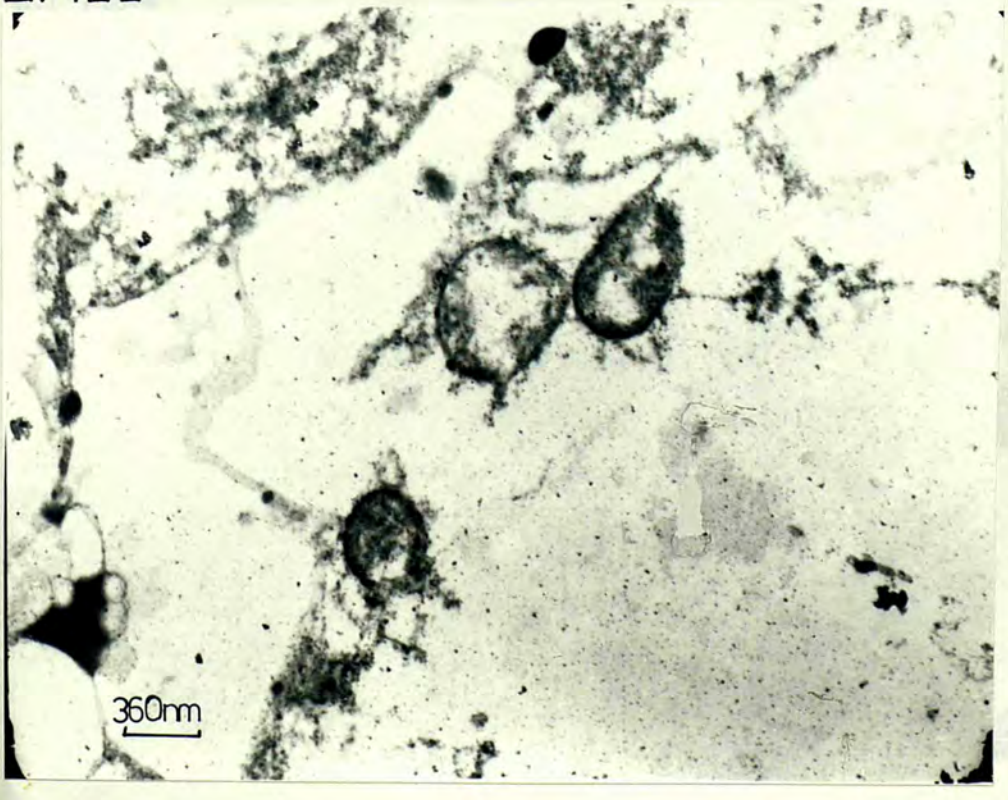
EM 26 shows starch granules surrounded by membranes. The folded appearance is probably caused by poor penetration of the embedding resin. Clearly the bodies shown in EM 21 - 24 are not starch grains.

Although the size of these bodies is much larger than that of MLO in the herbaceous plants investigated during this research and described in the literature, structurally they are similar, having a

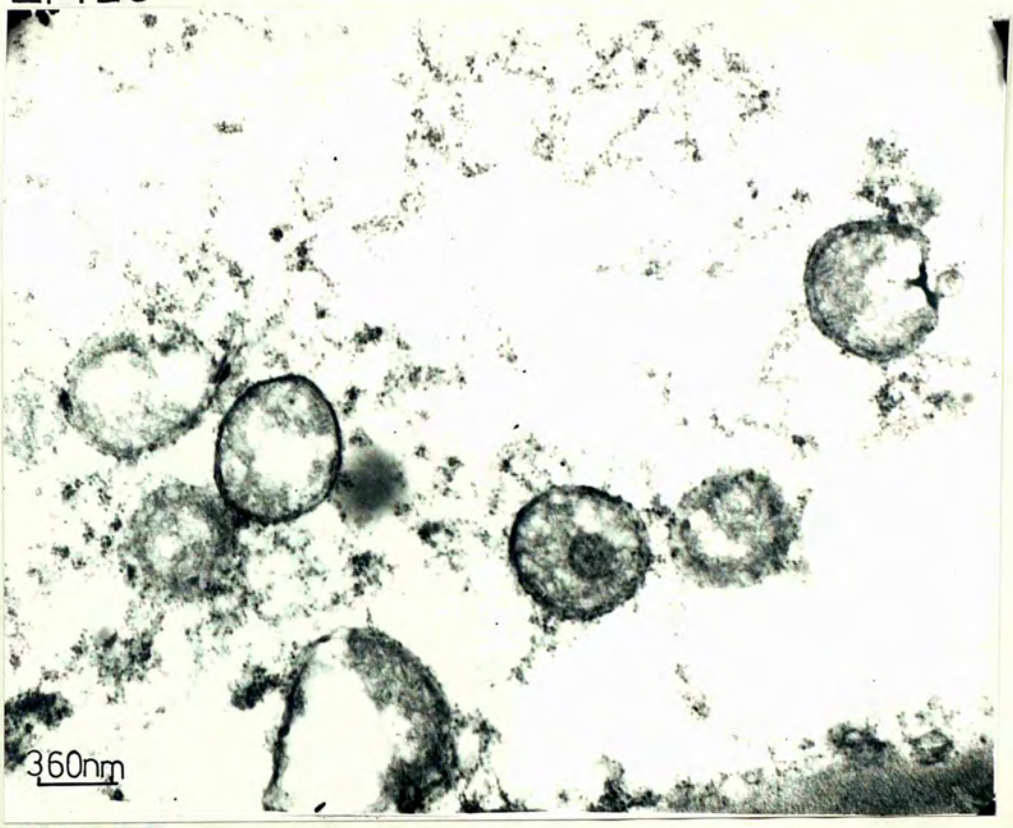
EM21



EM22

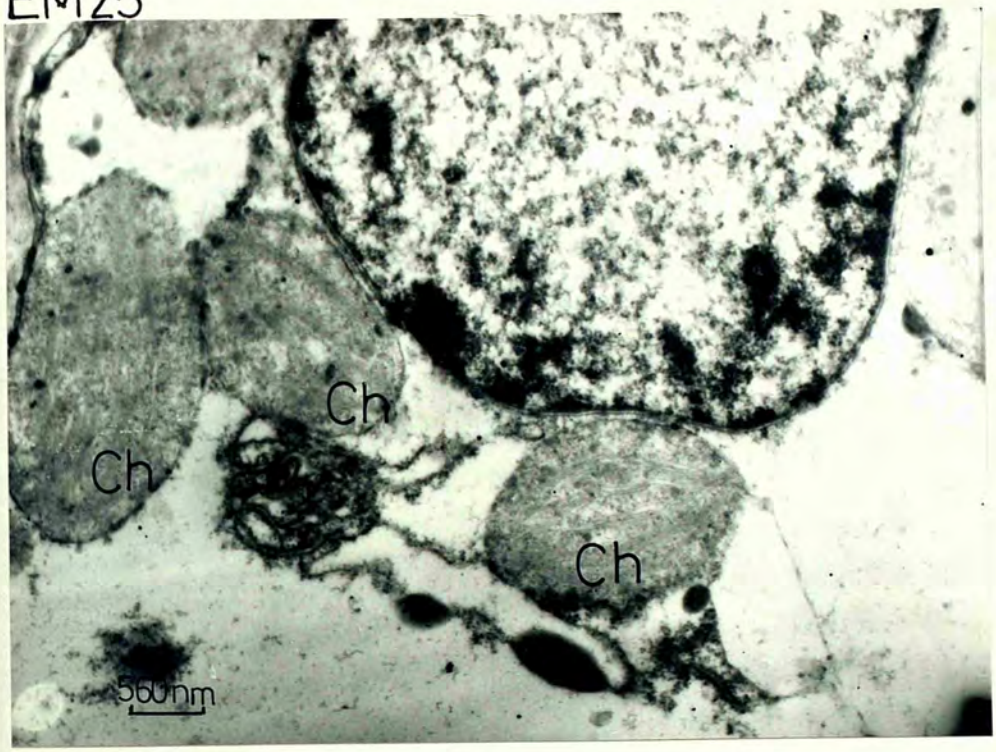


EM23

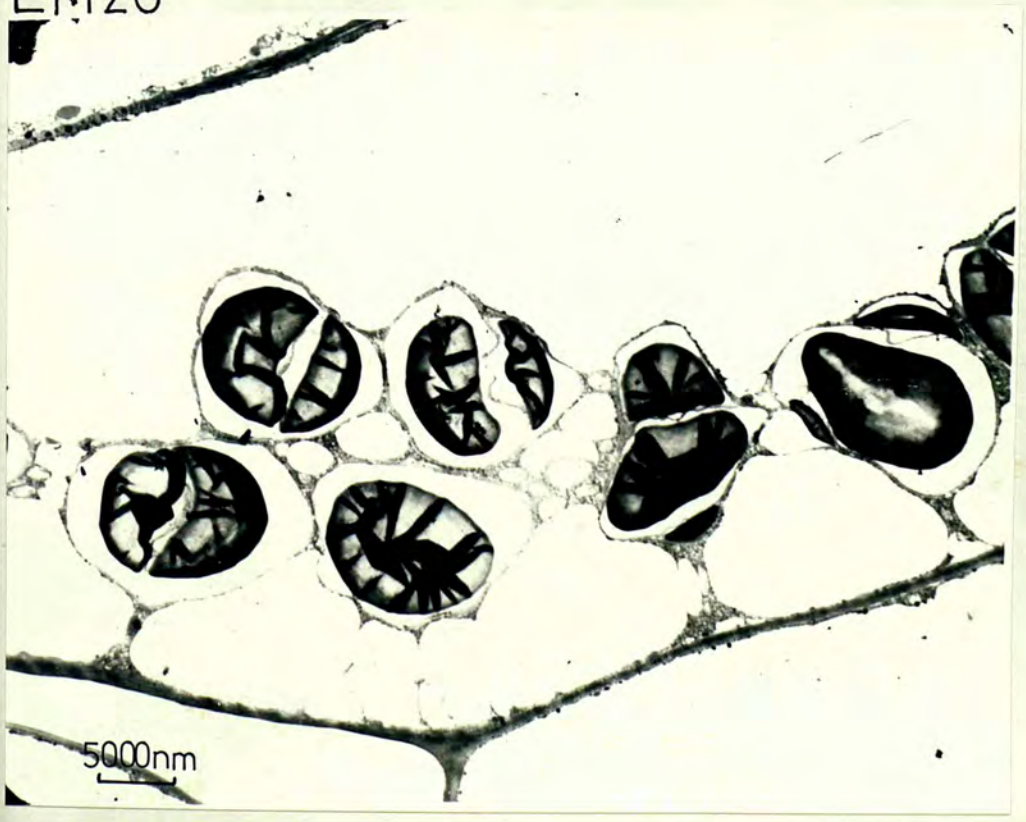


EM24

EM25



EM26



limiting membrane and internal ribosomes. Other workers have also remarked on the large size of presumed MLO in cacti (119, 140, 141).

Cactus tissue is very difficult to fix because of the mucous present in the cells and the widely dispersed nature of the vascular tissue also makes locationing difficult. Since it was possible to establish Cuscuta species on infected cacti it was decided to examine the parasitic plant for MLO. Cuscuta sp were first examined in the EM to establish that no MLO were present. Portions of the MLO - free Cuscuta were then established on Opuntia tuna monstrosa. After the parasite had been established for 2 - 3 weeks on the host plant pieces were examined for the presence of MLO.

b. Studies of MLO from Opuntia tuna monstrosa in Cuscuta gronovii (EM 27 - 29)

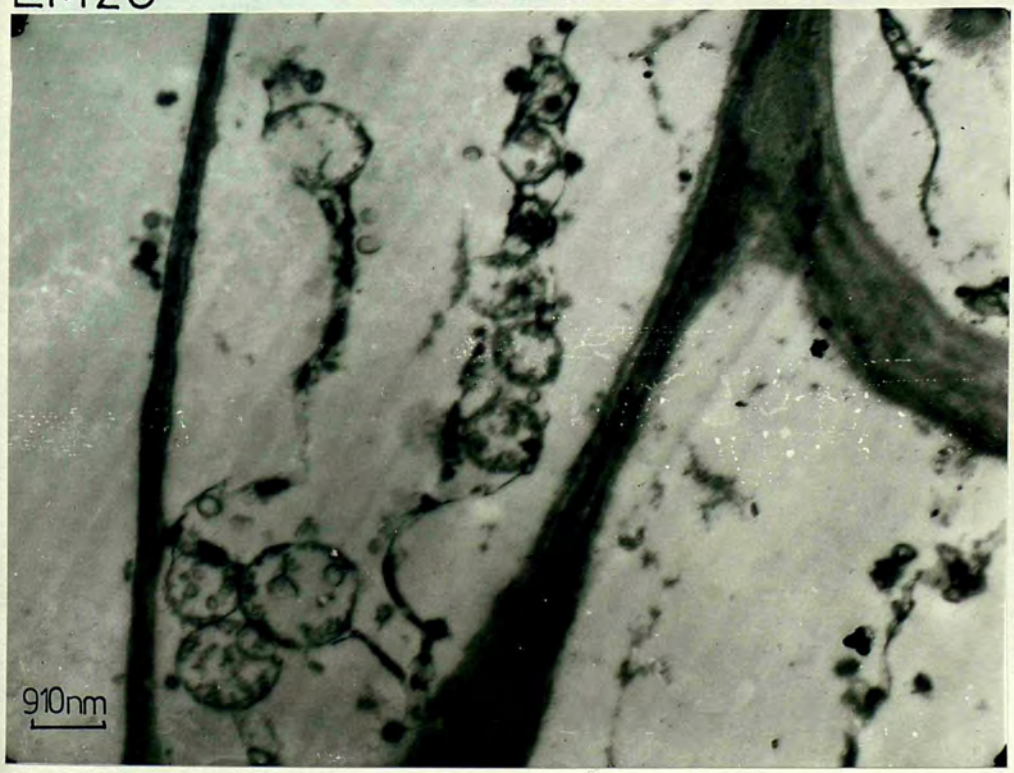
EM 27 This micrograph shows a low power view of the phloem region of a C. gronovii stem that has been parasitic on O. tuna monstrosa. A sieve plate is clearly visible in the centre and MLO are present in several cells (arrows). The whorl-like structures (w) are peculiar to Cuscuta and can be found in healthy plants of this genus. MLO were not found in healthy Cuscuta sp so must have been derived from the infected Opuntia. This technique allows examination of the MLO without interference from host cell mucous.

EM 28 At higher magnification these MLO can be resolved more clearly. They resemble the MLO found in O. lanceolata cristata in both size (545 - 1,455 nm) and structure. Similar bodies were not found in either healthy Cuscuta sp or in C. epiphytum parasitic on CP - infected Vinca rosea.

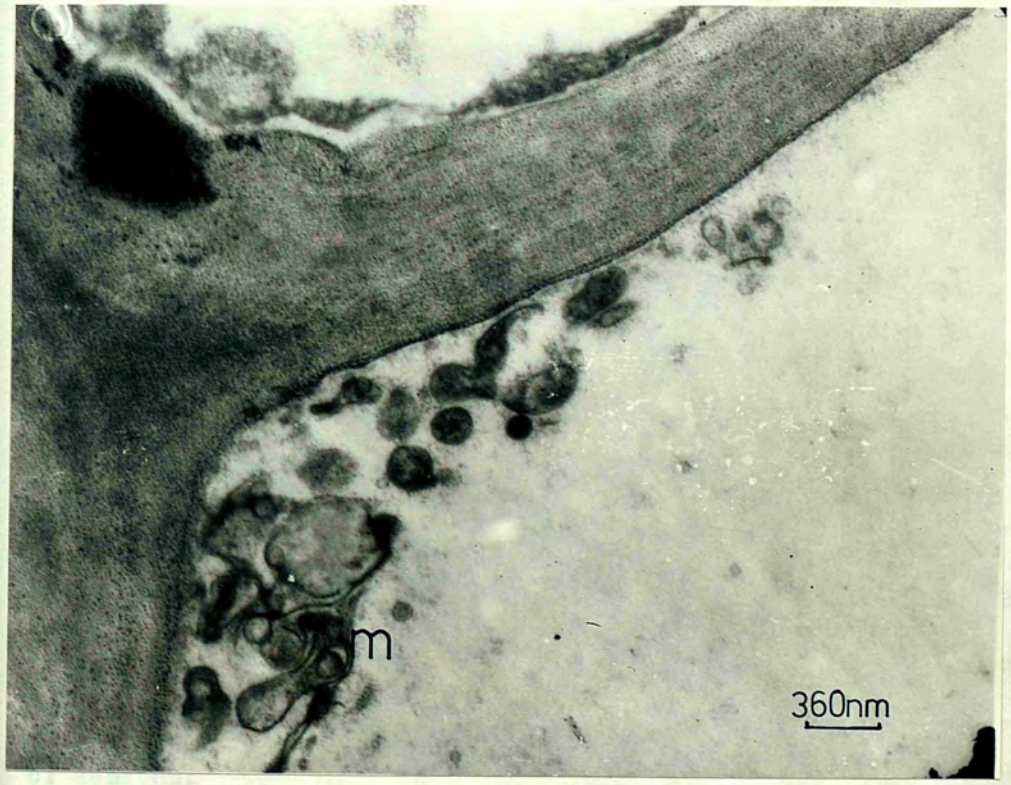
EM 29 Some of the organisms in this field of view more closely resemble MLO from herbaceous plants in size (142 - 285 nm). However they have denser contents than in EM 28 so they may be elementary bodies or MLO just beginning to expand from elementary bodies. The invaginated membranes (m) are probably part of the host cell membrane although they could be confused with closely packed MLO.

EM27

EM28



EM29



c. Studies of MLO from Opuntia tuna monstrosa in Cuscuta europea (EM 30 - 36)

EM 30, 31 and 32 These three micrographs give a low power scan along the length of a phloem element from C. europea parasitic on O. tuna monstrosa. The scan is interrupted between EM 31 and 32 by a grid bar. The phloem cell shows a sieve plate (EM 30) and appears to contain only membraneous material as well as MLO. At one end electron - dense material is tightly packed (EM 32). Some of the rounded structures in this denser end may be mitochondria.

EM 33 is an area of EM 31 shown at higher magnification (x 10,000) and shows some very dense, small, elementary bodies (e) 150 - 200 nm. The larger bodies (400 - 500 nm) can have fairly dense contents (d) or have sparse contents concentrated at the periphery (p).

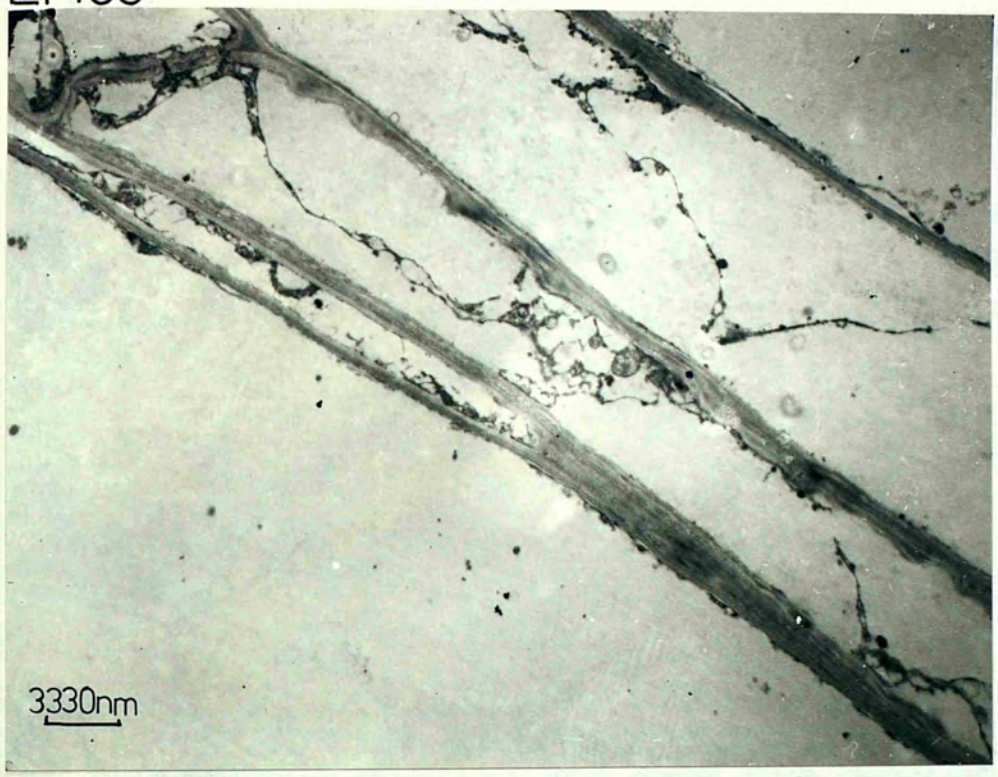
EM 34 shows some clearer MLO which are very similar in appearance to those from herbaceous plants having some fibrous internal structure but they are still large ranging from 255 - 785 nm in diameter. There are no internal membranes as found in mitochondria.

EM 35 This small group of MLO is interesting because one of the organisms is budding to give two elementary bodies (b). There is also a small group of elementary bodies (e). These may be budding themselves or may have budded - off from a larger body which is not in the plane of section.

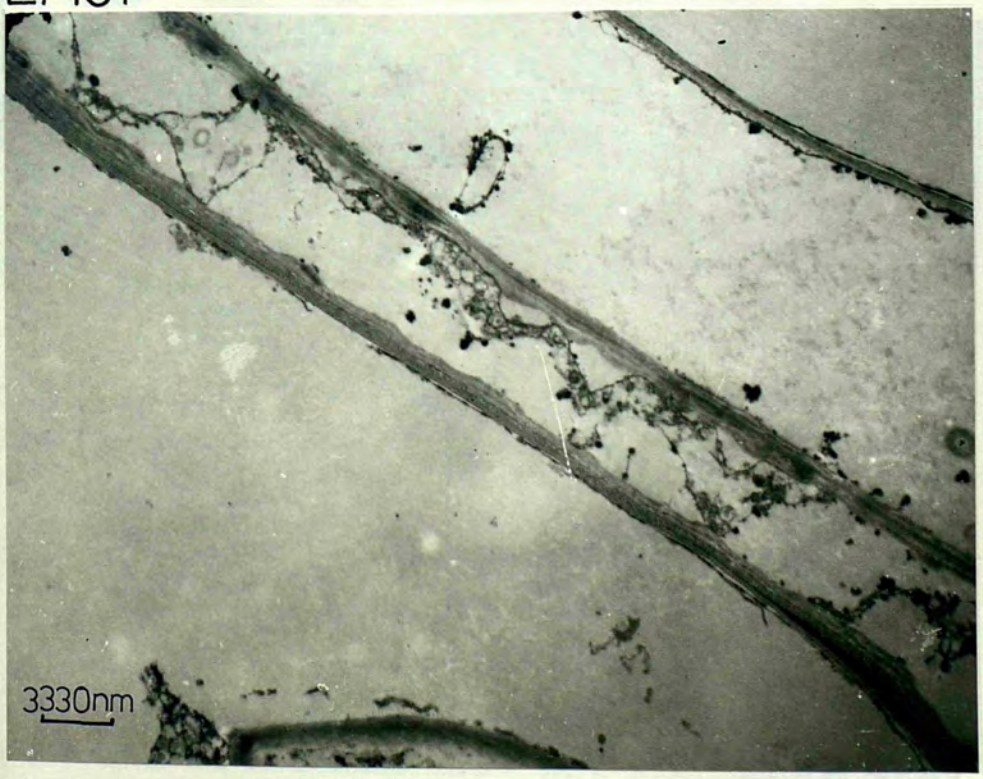
EM 36 This single large MLO (1, 143 nm x 714 nm) shows the distinct membrane - bound structure with granular contents and central, dense, DNA - like material typical of plant MLO. The central constriction (c) may indicate that the cell is about to divide.

The MLO found in Cuscuta sp were structurally indistinguishable from those found in cactus phloem. However, these MLO differ greatly in size from those found in herbaceous plants and Cuscuta sp parasitic on these

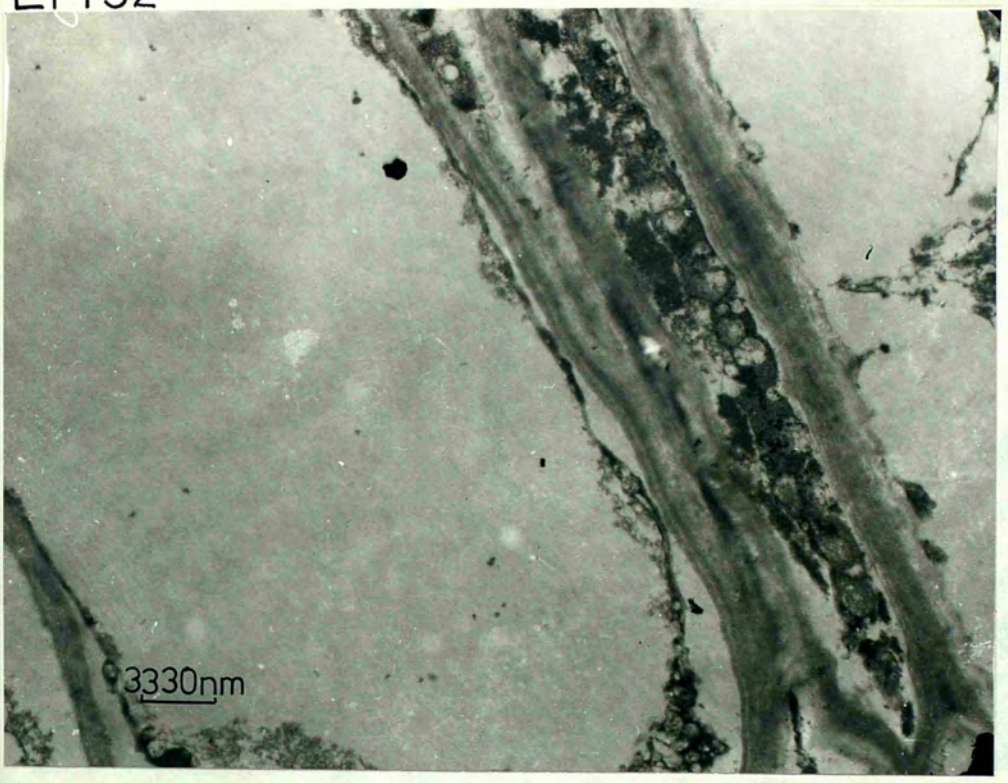
EM30



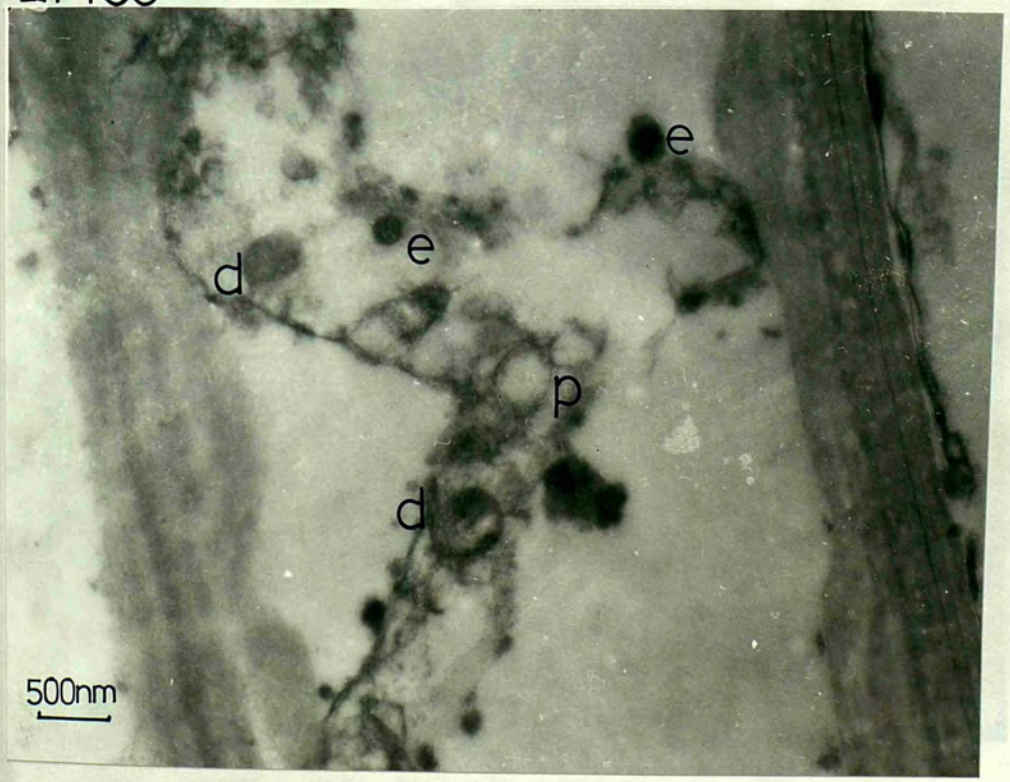
EM31



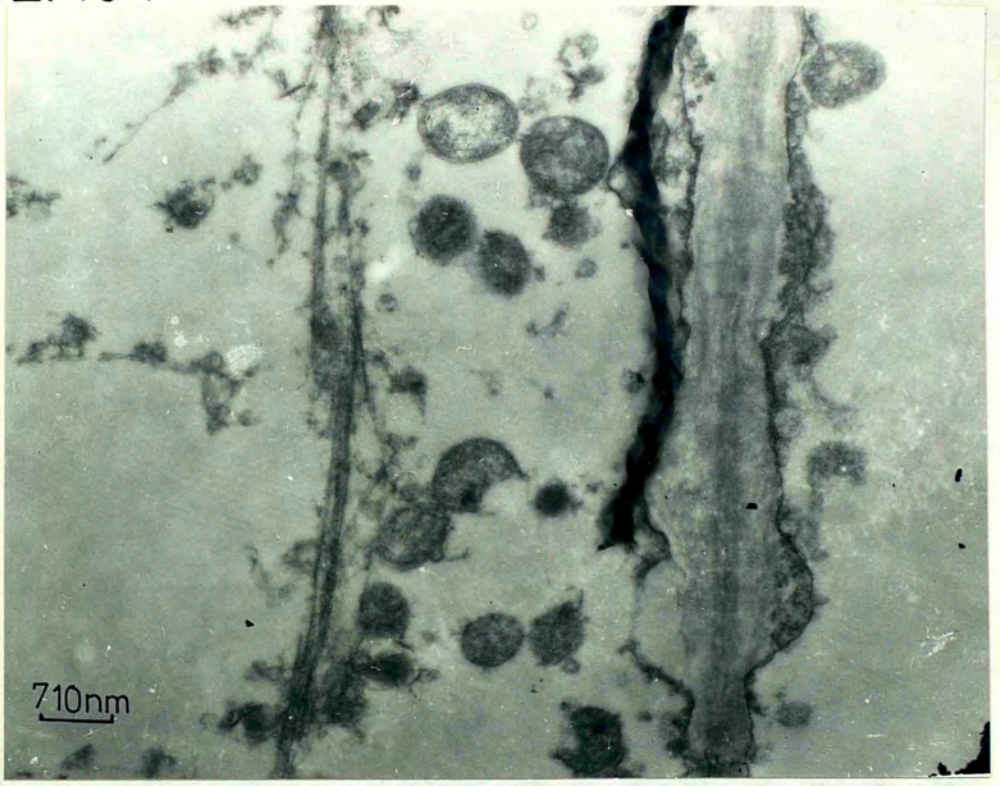
EM32



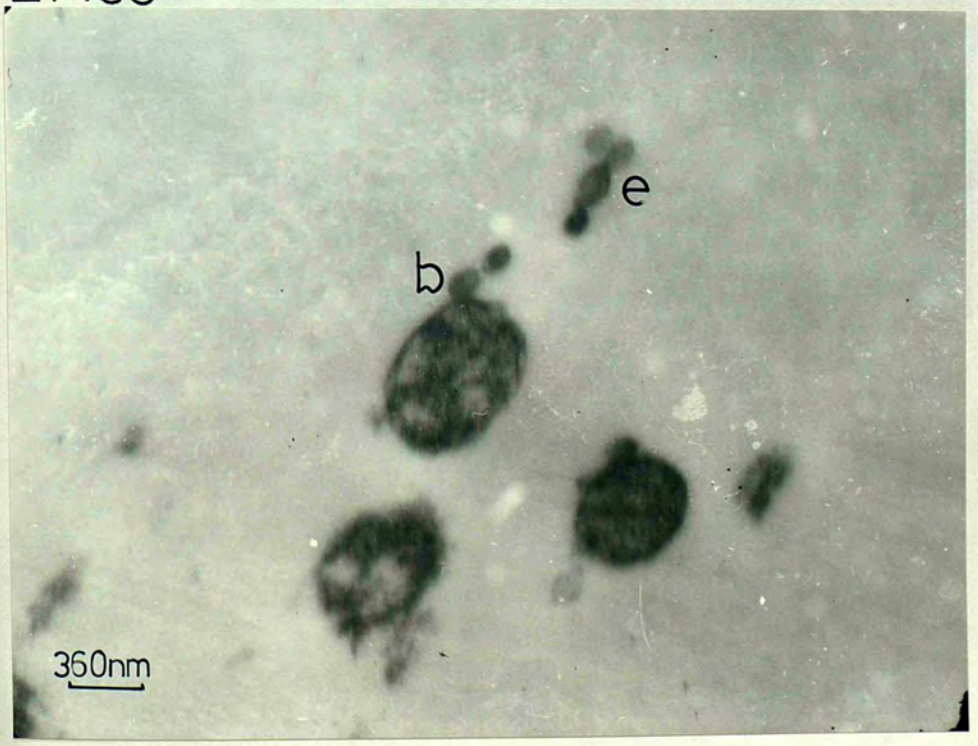
EM33



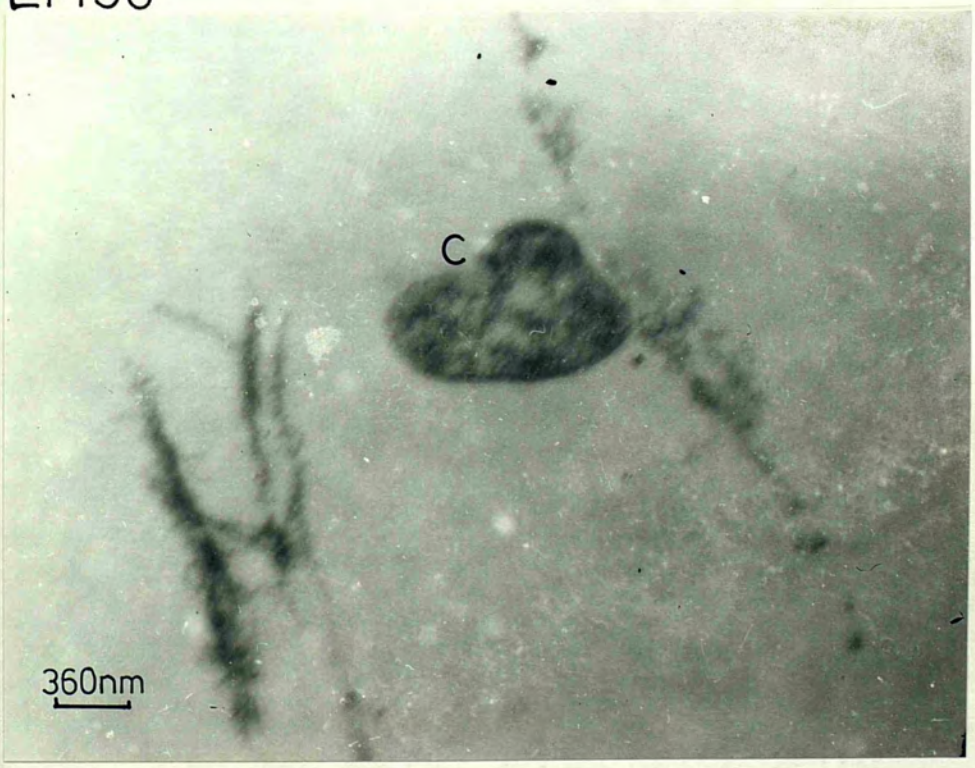
EM34



EM35



EM36



plants. It is therefore most likely that these MLO in Cuscuta were acquired from the host cactus. As the organisms were of the same size and appearance in both species of Cuscuta parasitic on the same cactus it would appear that the dodder does not affect the MLO.

The size variation between MLO of cacti and herbaceous plants is not altogether surprising since it has been noticed by other researchers (119, 140, 141). Until MLO can be cultured and comparisons made between different MLO in vitro the reasons for this size difference can only be speculative.

6. Division of MLO (EM 37 - 41)

The method by which MLO divide in plants is still not understood. The successful culture of the organism *in vitro* will help to establish details of replication *in vivo*. Several suggestions have been put forward as to the life cycle of these organisms (128, 162, 192) but the only agreement is that it is complex or, at least, difficult to elucidate.

One of the suggested methods of reproduction is the production by budding of a small or elementary body. In EM 37, a section from *C. gronovii* attached to CP - infected *V. rosea* evidence of such budding can be seen (b). It is thought that the elementary bodies are formed within the parent MLO prior to budding. Also shown here is a budding cell (B) where the 'bud' is much larger and approaches the size of the parent. This is probably binary fission (see below).

EM 38 (tissue as in EM 37) Another suggested method of division is by binary fission (b) where one large body divides into two equal 'daughter' cells. It is possible to see that if this dividing cell had been sectioned at an oblique angle it could very closely resemble the large bud (B) in EM 37. One body is also beginning to constrict at the centre (c) and it can be clearly seen to have two areas of dense material - possibly DNA - which have separated into the two ends of the cell ready for division.

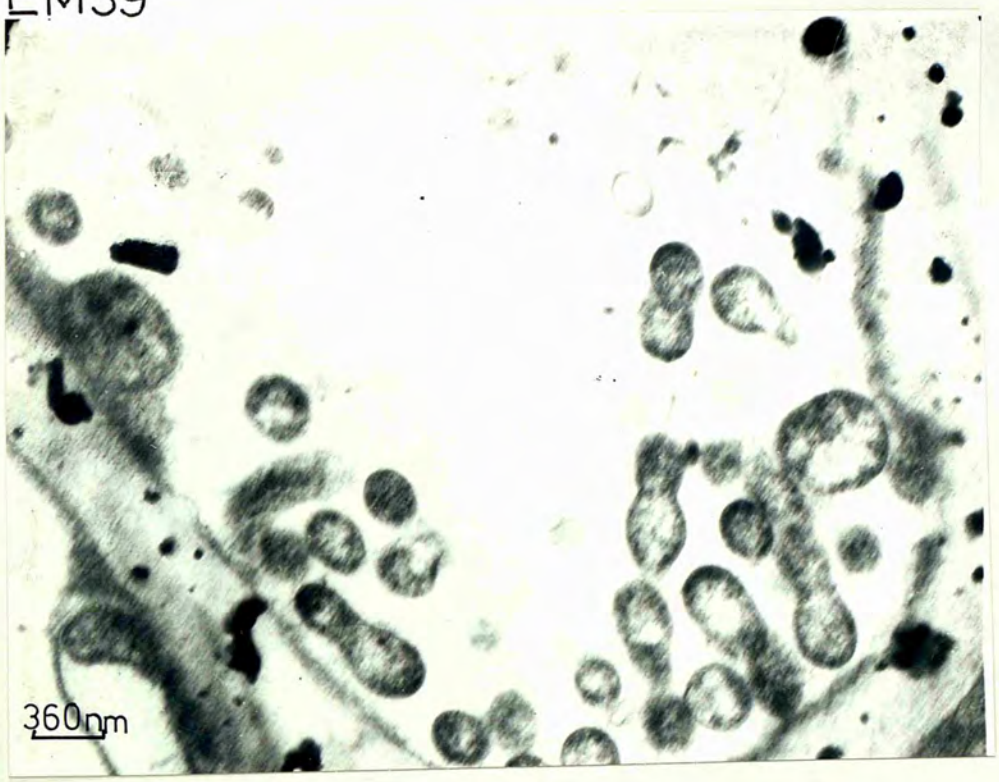
EM 39 A large portion of the MLO shown here are apparently dividing by binary fission as most of them are constricted at the centre and elongated as if the separating portions are moving, or being pulled, apart.

EM 40 shows yet another aspect of division - the formation of filaments of elementary bodies (F). These filaments could either be formed from successive buds which do not separate completely from the 'parent' body or from a long filamentous body which constricts to form

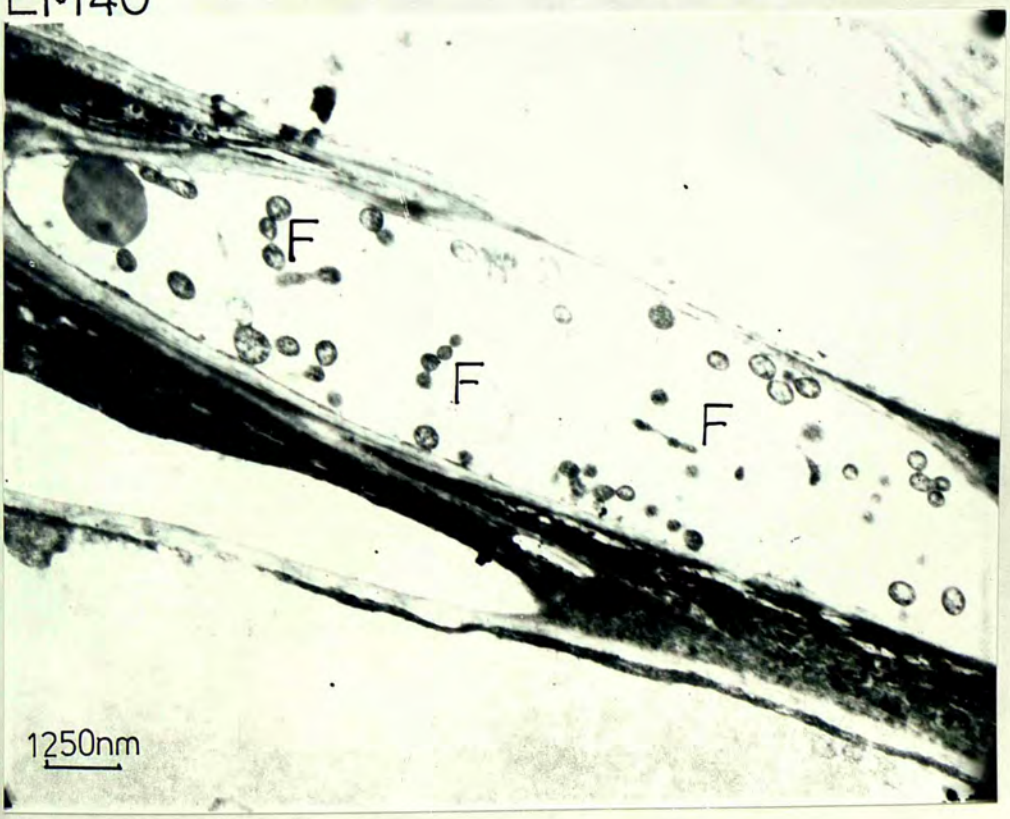
EM37

EM38

EM39



EM40

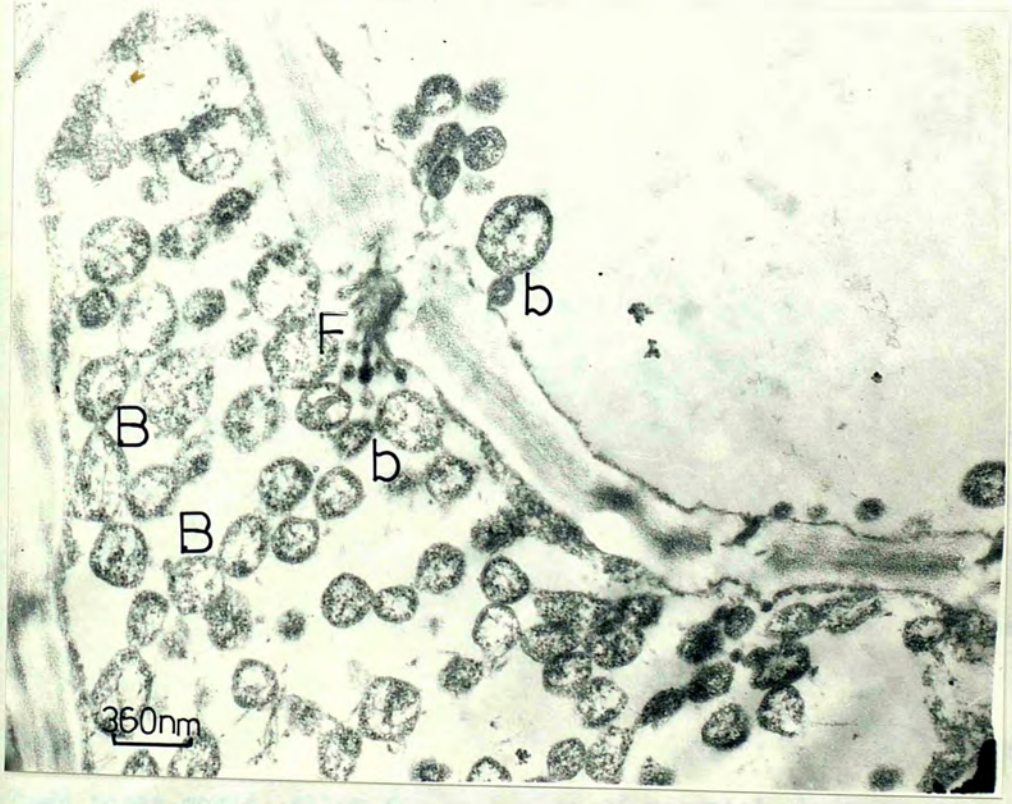


a chain.

EM 41 (CP - infected V. rosea). This cell full of MLO appears to contain all three types of replication i.e. budding (b), binary fission (B) and filaments (F).

It seems likely that several types of replication occur during the life cycle of MLO but, as mentioned above, their importance cannot be assessed merely from sections of fixed material. If the organisms are eventually cultured it will be interesting to see if there are variations in replicative stages and, if so, what factors bring about such variations. It would also be interesting to see whether the small, dense elementary bodies have a specific role to play in the survival of the organism.

EM41



7. Movement through pores (EM 42 - 44)

It is known that MLO can infect an entire plant fairly rapidly. Plants are usually infected, in nature, via the stylets of an infective vector; the actual point of infection being very small although the pathogen is injected directly into the phloem. The question then arises as to how a relatively large (compared to viruses), intracellular pathogen spreads through the plant without any apparent damage to the host cells.

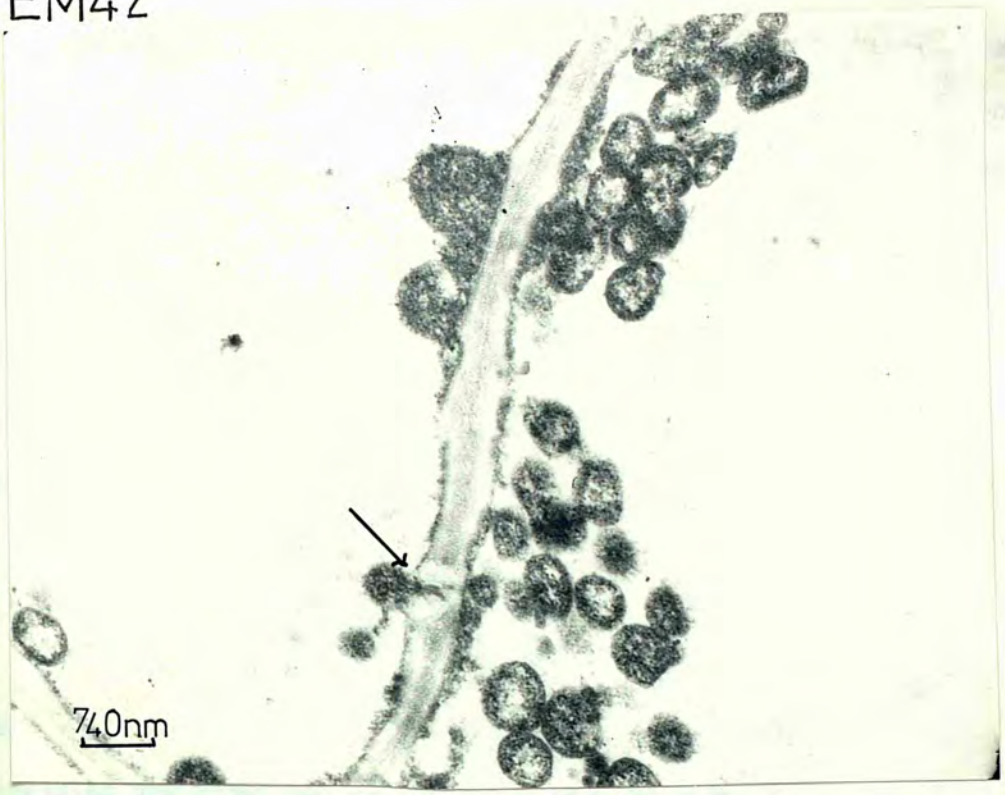
The rate and direction of spread are probably linked both to the flow rate of the phloem sap and to the multiplication rate of the MLO. As the numbers of MLO increase in a cell, the cell will become full so that pressure of numbers will result in the spread of MLO into adjacent cells.

Sieve elements may be either sieve cells with unspecialised sieve areas or sieve tube members with perforated end walls (or sieve plates) connecting the members into a sieve tube. The walls of sieve elements are relatively thick. Sieve plates are so named because, although they are thickened, the thickening contains pits which are continous across the wall to form pores. The phloem sap moves through these pores and they must also be the only way through for MLO (225). There is controversy as to whether pores are open or occluded with P protein. Open pores would allow the mass flow of materials between sieve cells whilst occluded pores could regulate the flow of various materials (157).

Micrographs 42, 43 and 44 show the passage of MLO through sieve pores, and lend some support to the idea of open pores and the mass flow concept of translocation.

EM 42 One MLO in this micrograph is extending into a pore in the cell wall (arrow). It does not appear to go quite through the wall

EM42



EM43

but this may be because the plane of section does not follow the line of the pore.

EM 43 and 44 MLO here also seem to be sending thin protrusions through pores. It is assumed that the MLO would move from the densely packed cells into the less densely packed ones so in EM 43 most of the organisms have moved through the pore leaving a thin projection along their route. In EM 44 one pore is clearly visible (1 - inner and outer diameters marked by small arrows). Close by is an indication of another pore (2) which is just outside the plane of section.

The extreme pleomorphism of these organisms allows the production of thin projections into and through the pores presumably under pressure from the sieve tube contents. It is assumed that the cell contents then move through the projection so that eventually the whole organism has moved through into a different cell.

EM 41 shows how division can be linked to movement. Here a group of filaments of elementary bodies can be seen extending into a large pore. The pore seen here appears to be constricted but this again may be because the plane of section does not go through the widest part of the pore.

The small elementary bodies (70 nm - 90 nm), especially those which form a long filament are ideally suited for transport across sieve plates (pore sizes 90 nm - 105 nm) so that they can continue growth in a new cell. However it has been shown that larger bodies can also pass through pores so the elementary bodies are unlikely to be the only means by which MLO travel along the phloem tissue.

As the MLO have literally to 'squeeze' through the sieve pores it would be expected that the process is rather slow and will depend on pressure gradients in the sieve elements. This is supported by

EM44

observation of longitudinal sections of sieve elements where MLO are often seen concentrated towards one end of the cell. It would also explain the rather slow progress of the disease as described in section IV.

From EM observation it is also seen that MLO do tend to be pushed across from full cells into emptier ones but as they will also move between two relatively empty cells (EM 42) it can be assumed that sap flow is also involved in movement of MLO within the plant.

8. Studies of changes in Host Plant Ultrasturcture (EM 45 - 48)

a. Inclusion bodies

During earlier research on CP it was noted that diamond - shaped inclusion bodies occurred occasionally in CP - infected clover although similar inclusions were not found in healthy material (164).

Similar inclusion bodies were frequently observed in MLO - infected, but not healthy, V. rosea. The rhomboid inclusion bodies shown in EM 45 are typical of those to be found in CP - infected V. rosea and closely resemble those described in earlier studies of clover.

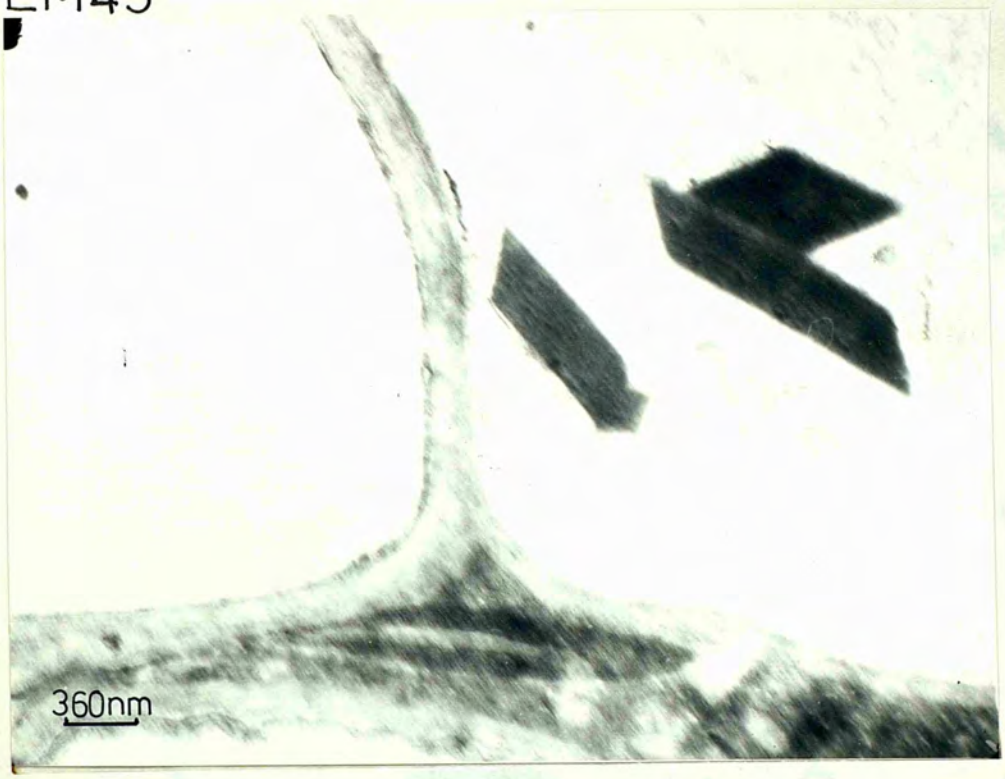
The inclusion bodies found in Cuscuta epiphytum parasitic on CP - infected V. rosea are shown in EM 46, 47 and 48. EM 46 shows an inclusion in an otherwise empty area of phloem cell. In EM 47 and 48 the inclusions are found to be within the cytoplasm and in EM 48 there are abundant MLO in the same cell.

The occurence of these structures only in infected tissue indicates that they result from MLO infection and are not artifacts of EM staining. Inclusion bodies of a proteinaceous nature are commonly found in virus - infected plants (174).

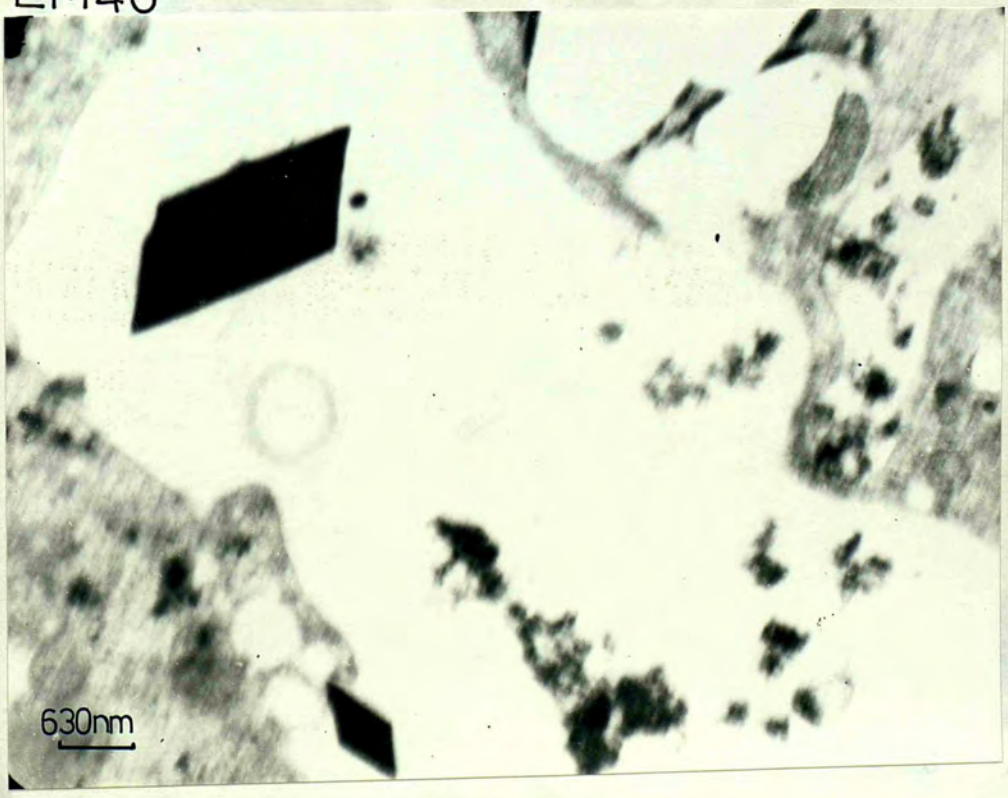
The presence of these inclusion bodies is correlated with MLO infection so their presence in Cuscuta strongly suggests that the parasite acts as a host for the pathogen and not simply as a channel for the transport of MLO. It is unlikely that such large angular bodies could have been transferred in the phloem from the host V. rosea so it seems reasonable to suggest that they were formed after the infection of the C. epiphytum by the MLO.

It is not known why these inclusion bodies are produced. In the case of viruses they are often thought to represent an agglutination of viral protein. With MLO they may be a reaction of the host plant to the disturbance in protein synthesis caused by the presence of the

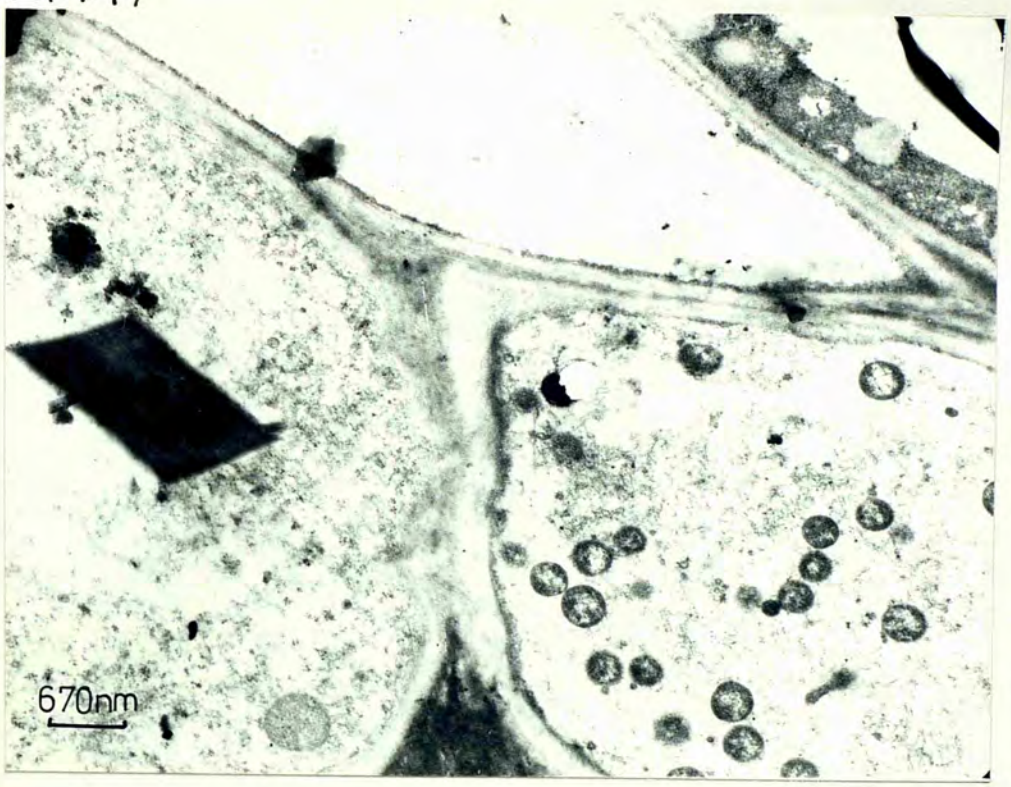
EM45



EM46



EM47



EM48

pathogens or be formed by breakdown products of the MLO.

b. Starch granules (EM 49 - 51)

As mentioned earlier starch granules appear to accumulate in the chloroplasts of MLO - infected material. This phenomenon was found in all the infected plants examined including the cacti. To study these plants further tissue from Cuscuta gronovii, which is more readily fixed and sectioned, was examined. This also allowed a more detailed comparison to be made between healthy and MLO - infected plants of the same species. Micrographs 49, 50 and 51 were obtained from this plant.

EM 49 shows a typical 'cigar' shaped chloroplast from the stem of a healthy C. gronovii. Cuscuta chloroplasts appear not to contain abundant membranes stacked into grana as is found in many herbaceous plants. They may be more similar to the agranal chloroplasts, found in some grasses, which have few grana and contain large starch grains (172). This more simple structure may be associated with the parasitic mode of living or specialised type of photosynthetic activity.

EM 50 and 51 These chloroplasts from CP - infected C. gronovii contain very large starch granules - again reminiscent of graminacious agranal chloroplasts. The starch granules occupy a large volume of the plastids and appear to distort their shape. The internal lamellae present are concentrated mainly at the periphery because the starch fills the centre of the chloroplasts. (In EM 50 some lamellae remain between the starch granules).

This phenomenon does not appear to have been recorded previously in association with MLO diseases although it became very obvious during this extensive EM examination of infected and control material. It should be noted that not all chloroplasts in infected material contained significant amounts of starch but low - power scans of the sectioned material used to locate MLO - containing areas of tissue prior to

EM49

EM50

EM51

photography showed a large proportion of chloroplasts in infected material to be distorted by the accumulation of starch.

This phenomenon may be caused by the chloroplasts being altered slightly by the presence of MLO in the plant so that they store starch. The affected chloroplasts however are not always found close to the phloem so the MLO may be having indirect effects; for example increased photosynthesis induced by petal greening with CP or increased surface area with the cacti may result in build - up of excess starch which is stored in the chloroplasts.

This does not explain the presence of such chloroplasts in dodder which does not have external symptoms. An alternative explanation might be that MLO block or slow down transport of photosynthates via the phloem resulting in their accumulation in chloroplasts - even those remote from the site of MLO activity.

c. Plastid development (EM 52 - 67)

Mycoplasma infection of V. rosea causes the transition of normal coloured flowers into green or virescent flowers. Such flower colour transition no doubt involves the conversion of plastids into chloroplasts. In order to study this phenomenon a series of flowers were collected from one healthy and several CP - infected V. rosea plants which showed a transition from a normal to a green, leaflike flower.

These flowers are shown in figure V.2. The marked areas labelled A - G were cut from the petals and a healthy leaf used as a control. Sections were prepared for electron microscopy as described previously. As a further control a healthy V. rosea plant was kept in a cold room in the dark at 4°C for one week. This plant began to produce small, green - tinged flowers (see section III). Part of a petal from one of these flowers was also examined.

Half of the electron micrographs shown here are low - power views (x 4,375) of each piece of tissue indicating the distribution and relative sizes of the plastids. Each one is paired with a closer view of a plastid (x 17,500) which shows details of the structure.

EM 52 and 53 'A' Healthy petal

EM 54 and 55 'B' Pink area of infected petal

EM 56 and 57 'C' Just green area from same flower as 'B'

EM 58 and 59 'D' Pale green petal

EM 60 and 61 'E' Completely green petal

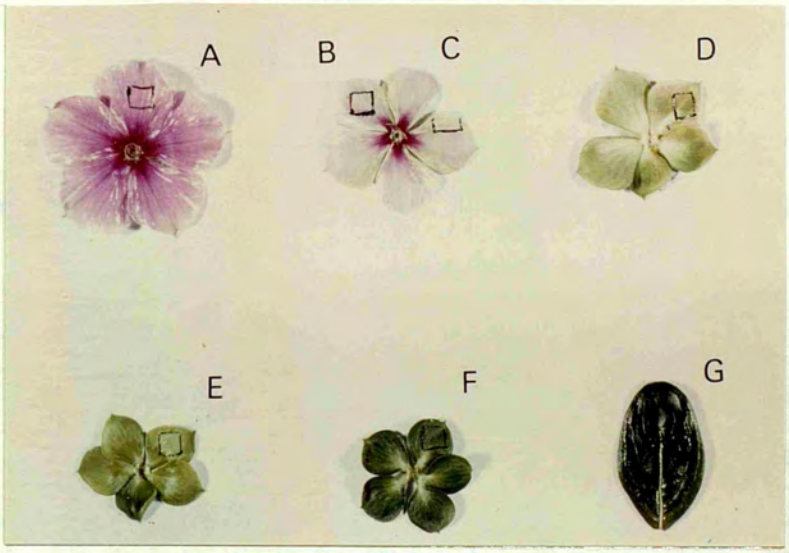
EM 62 and 63 'F' Leaf - like petal

EM 64 and 65 'G' Healthy leaf

EM 66 and 67 Green tinged petal from plant kept cold and dark

In healthy petals plastids occur infrequently and those present contain few or no internal membranes with very little ultrastructure (EM 52 and 53). The succession of micrographs indicate clearly that,

Figure V.2

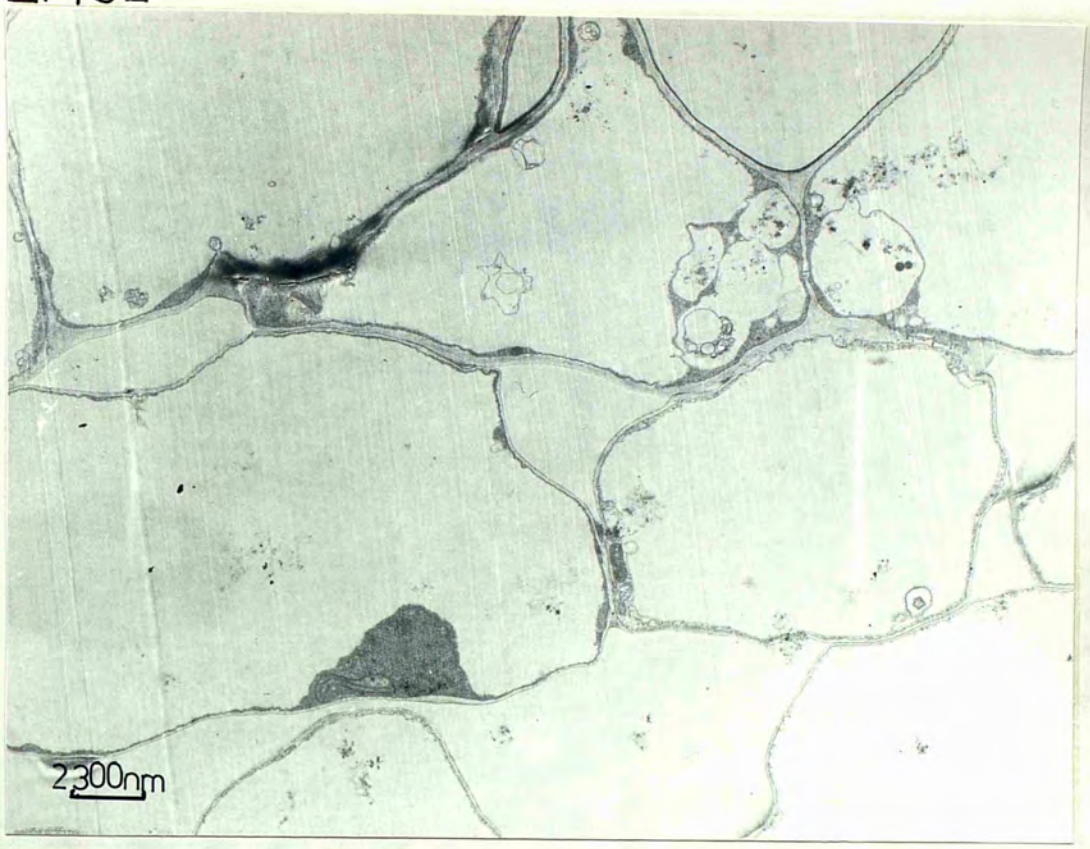


with progressive greening, there is an increase in the number of plastids per cell associated with an increase in the internal membranes. EM 54 and 55, 56 and 57 show early stages in the formation of chloroplasts and the corresponding development of starch granules. The continued development is shown in EM 58 and 59, 60 and 61. Eventually, with complete greening ('F' EM 62 and 63), the plastids are numerous and similar in structure to the chloroplasts of a healthy leaf (EM 64 and 65) except that starch granules are abundant in the infected petal (EM 61 and 62). This supports the results of Gourret (76) and Gourret and Le Normand (77) who have also shown that greening caused the production of normal - looking chloroplasts in Vinca rosea and Cheiranthus allionii.

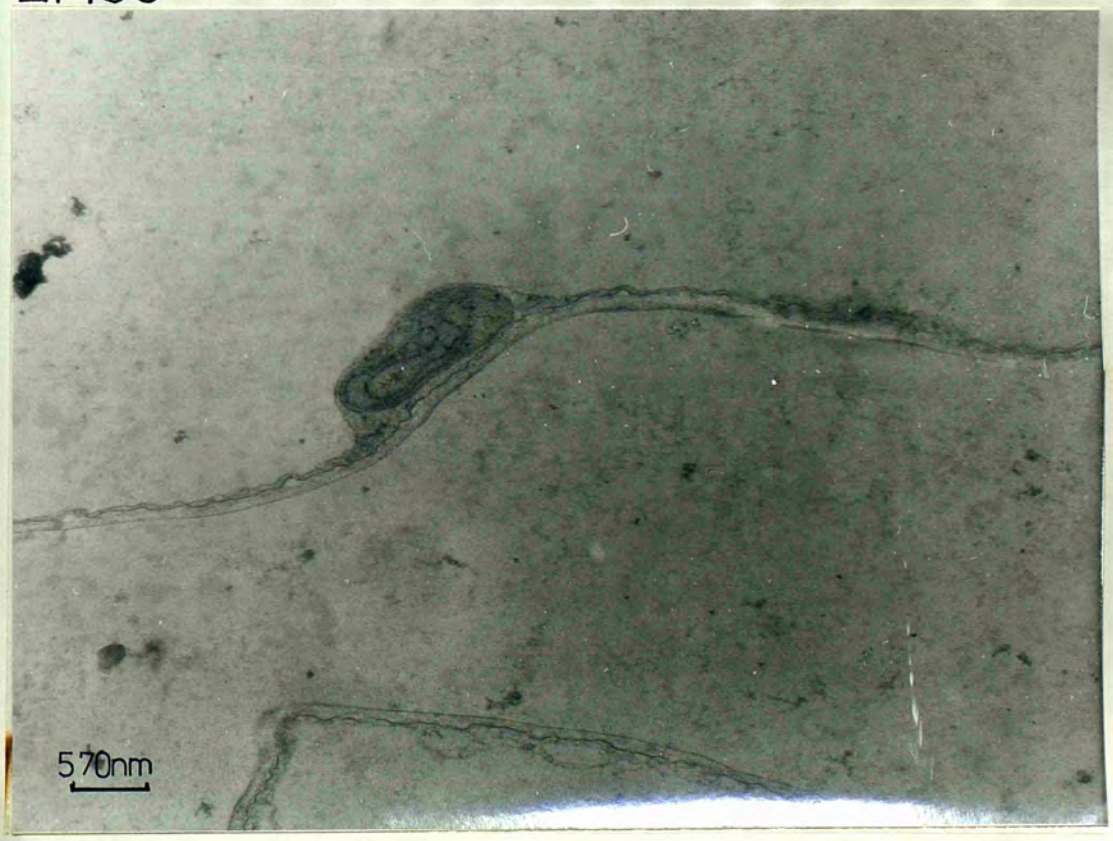
These events can be compared with the reverse effects which occur during leaf senescence (52) and fruit ripening (208) when chloroplast membranes break down and may be accompanied by a reduction in size of the plastids (52).

It is interesting to note that the plant kept in the cold and dark to induce physiological greening contained plastids similar to those in a normal healthy petal. There did not seem to be any major change in the plastid structure although the petals were reduced in size and distinctly green at the tips. Obviously this is a reversible external change only, unlike the symptoms caused by the presence of MLO.

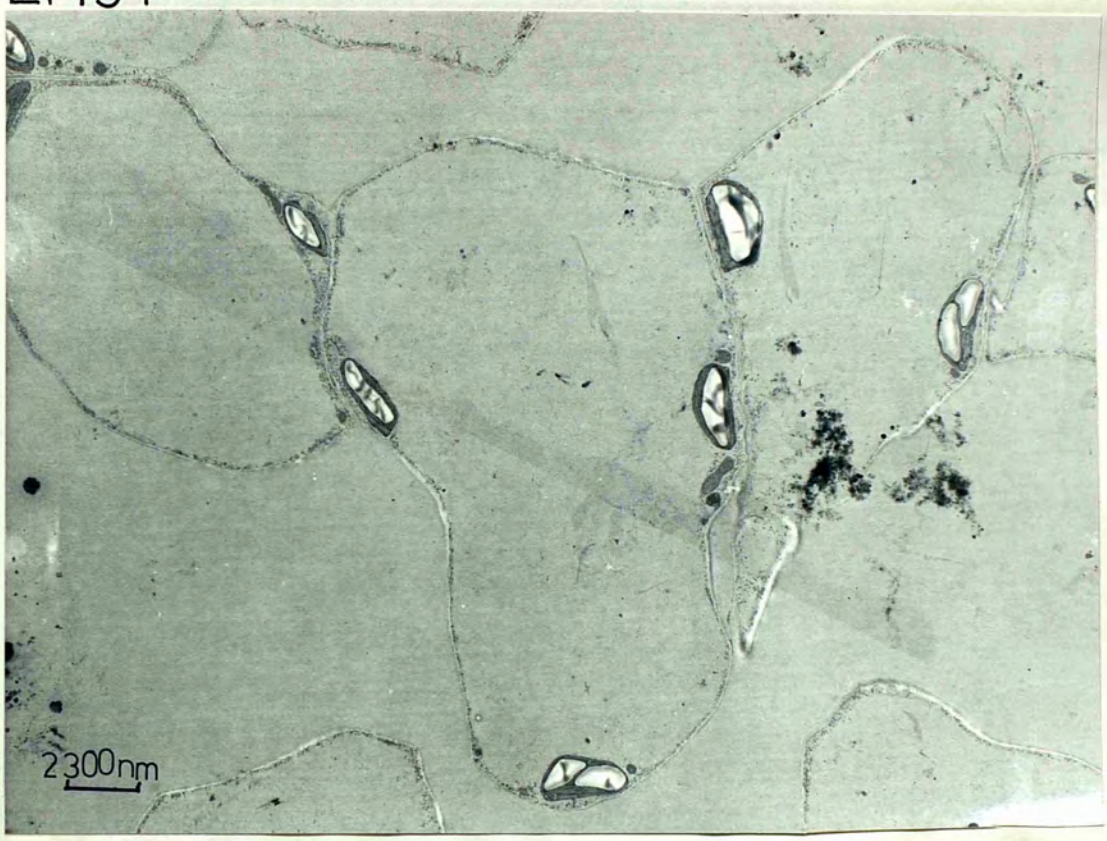
EM52



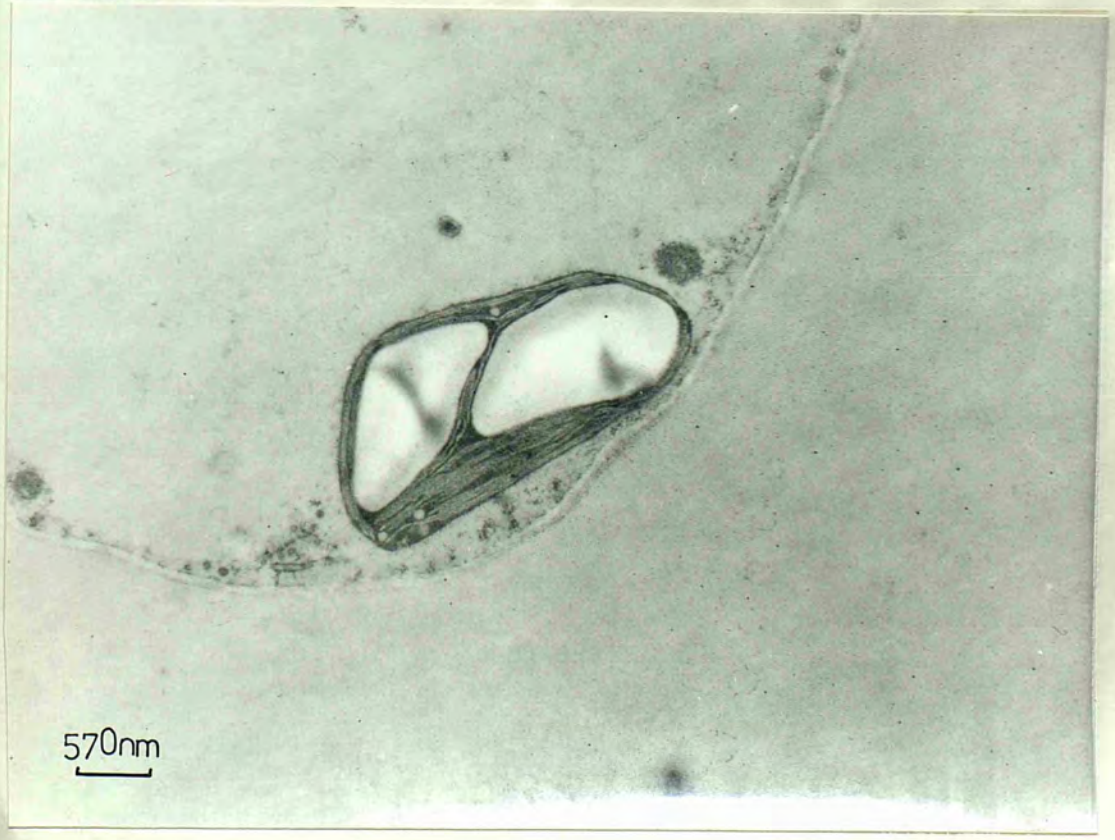
EM53



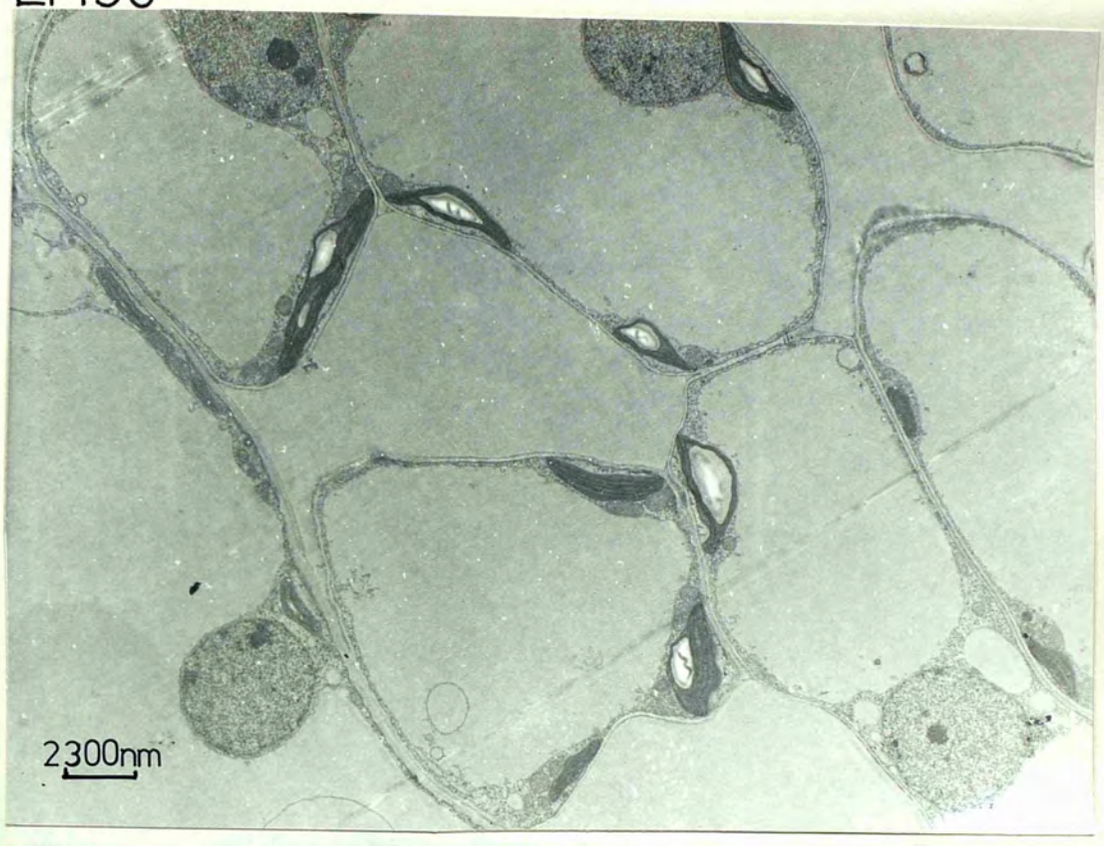
EM54



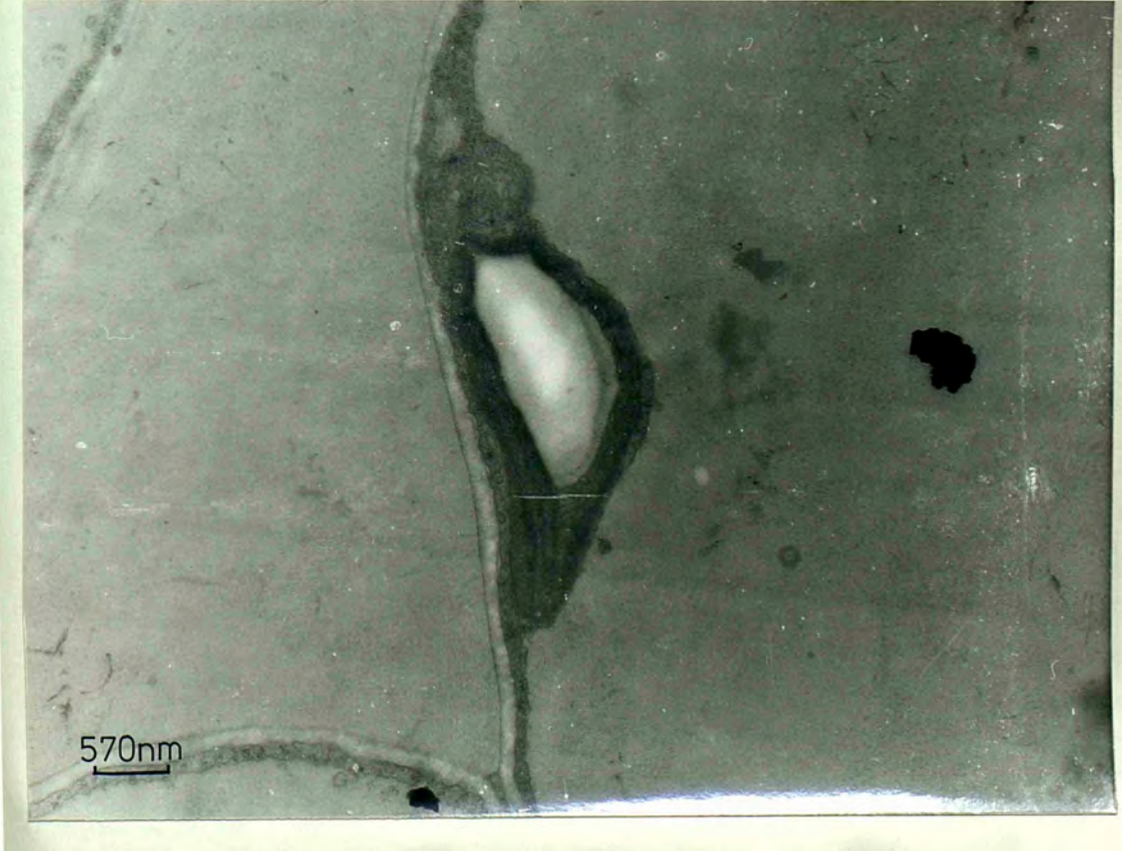
EM55



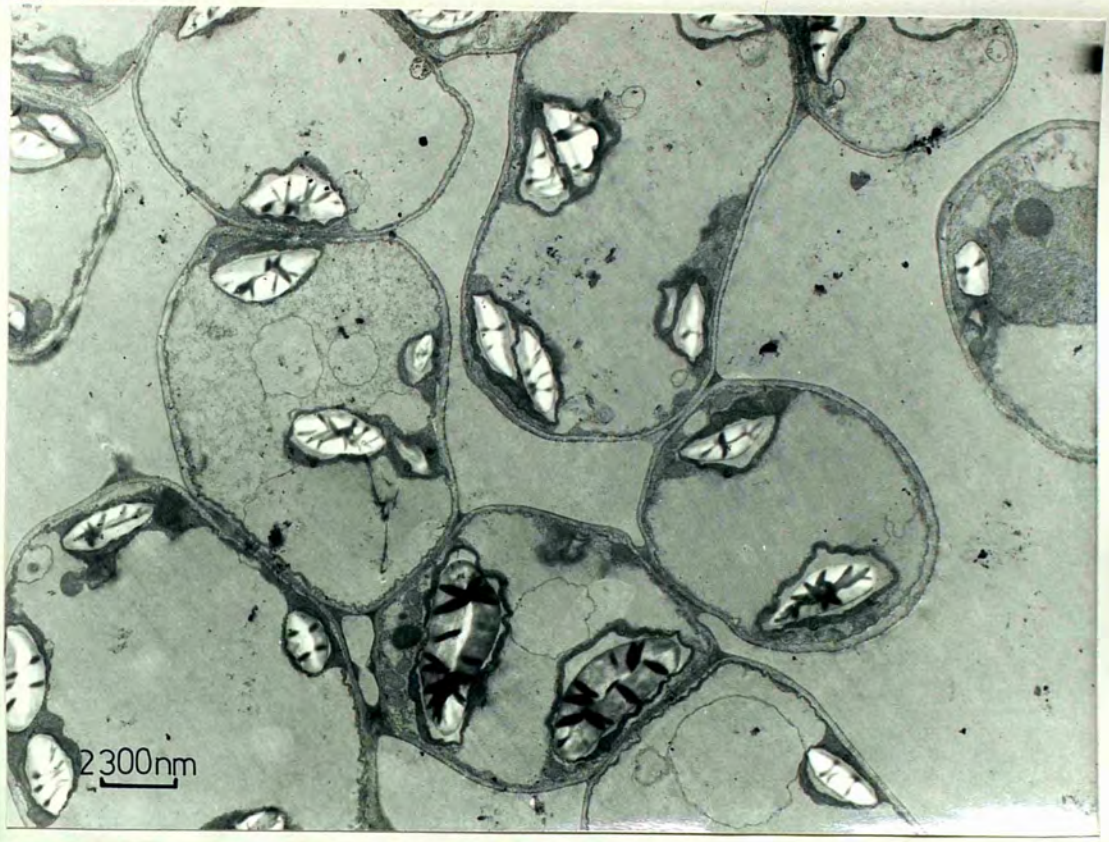
EM56



EM57

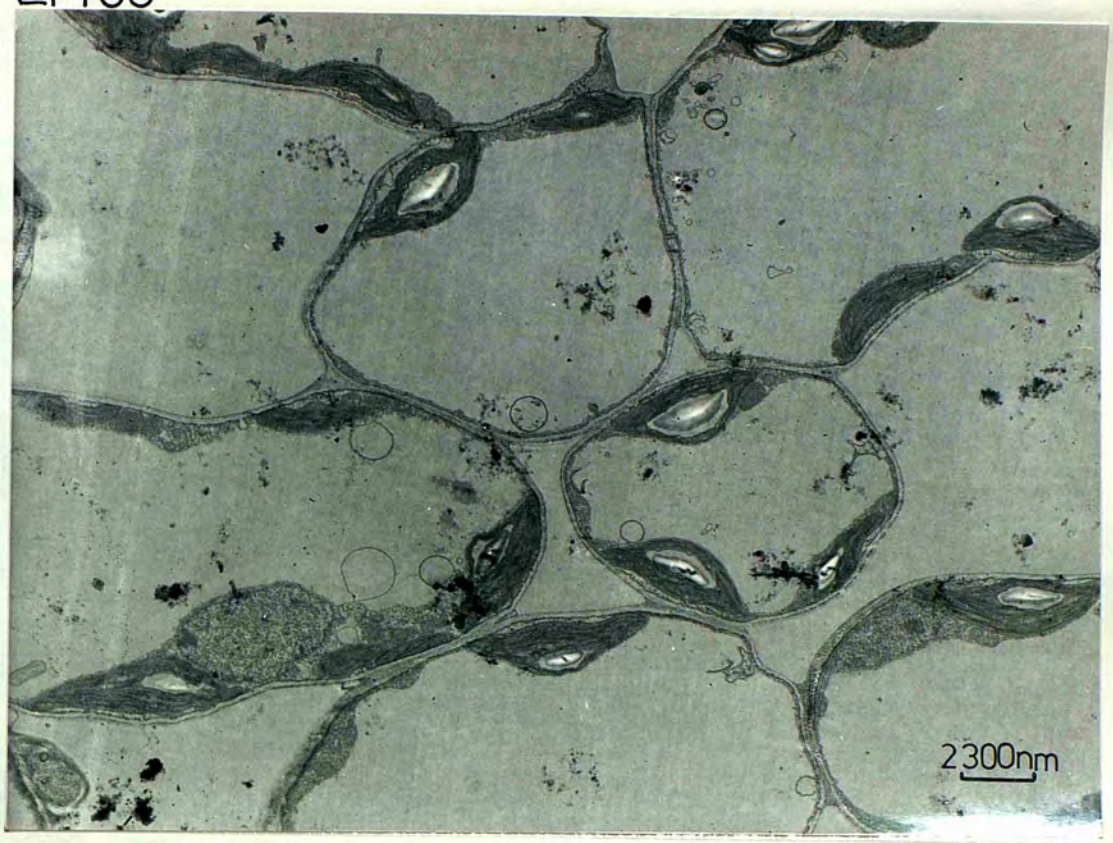


EM58

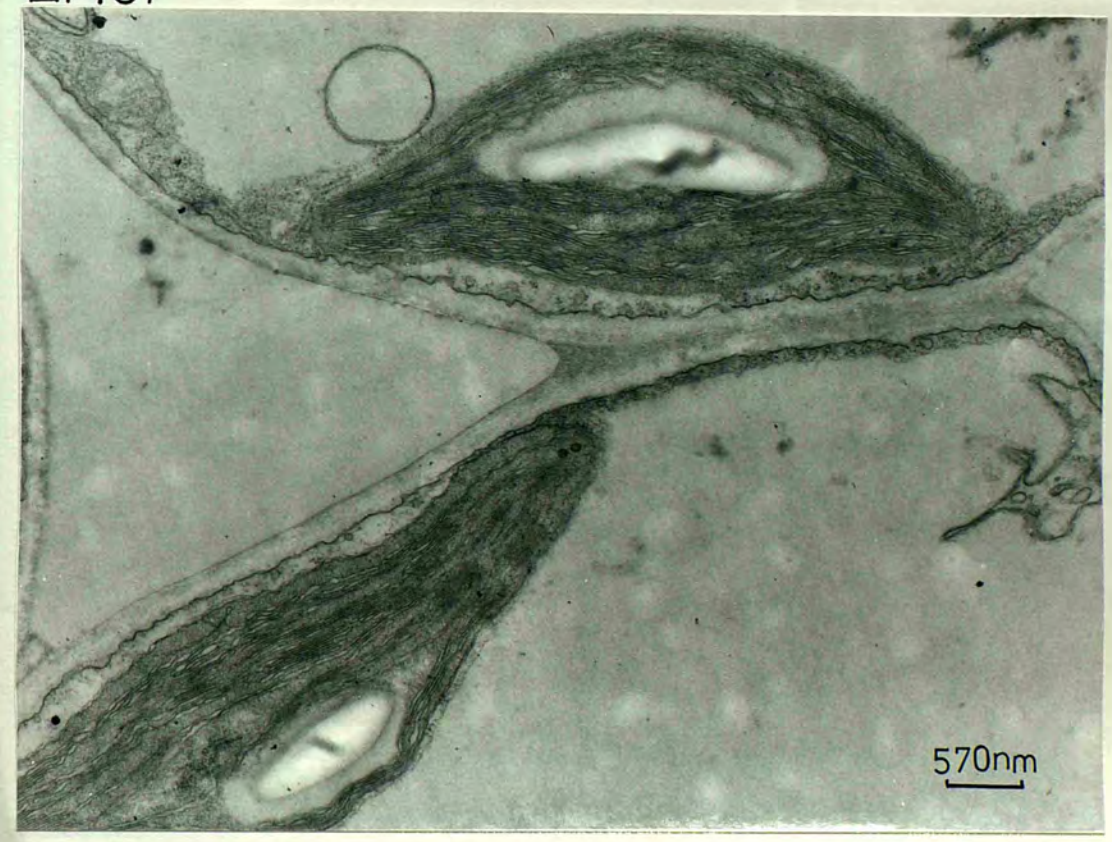


EM59

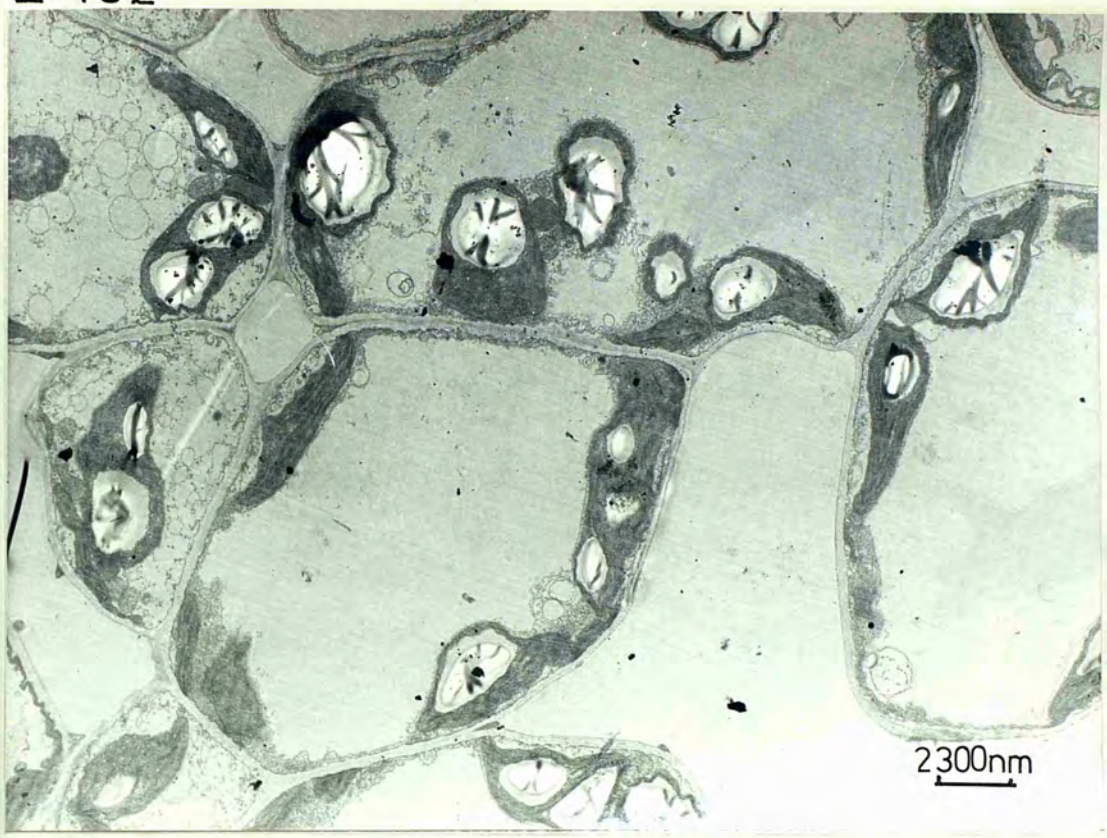
EM60



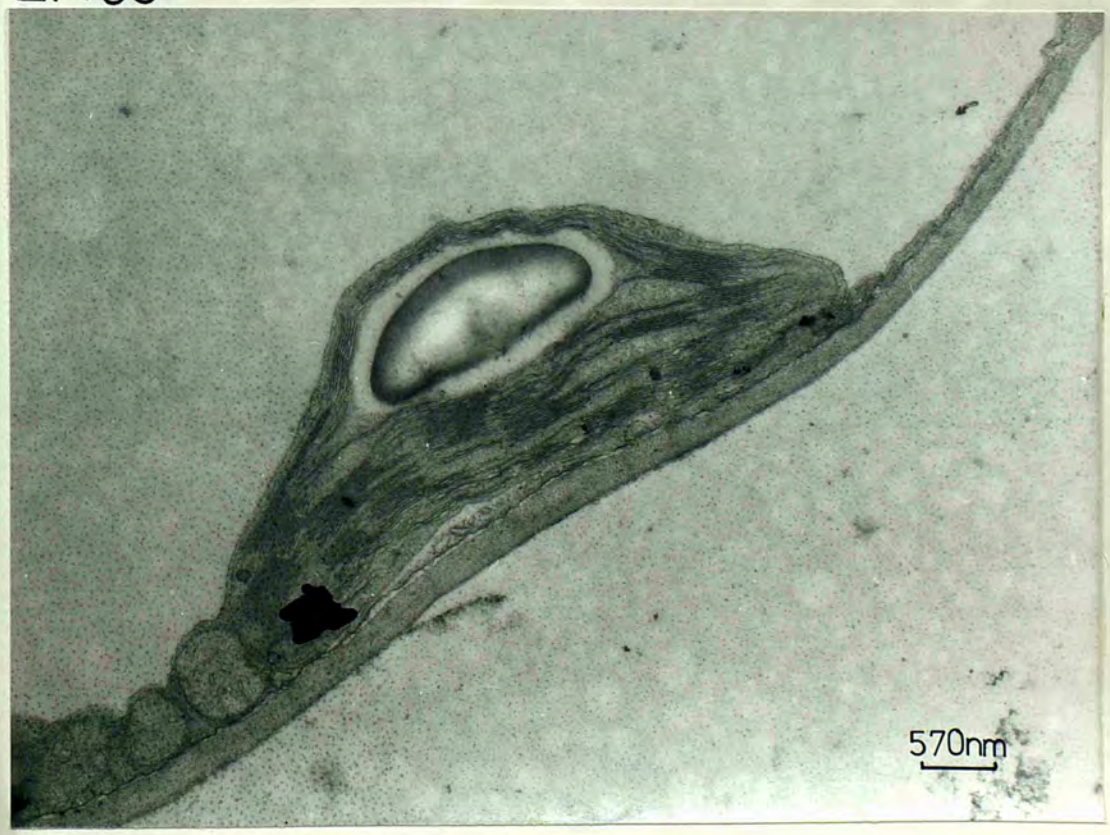
EM61



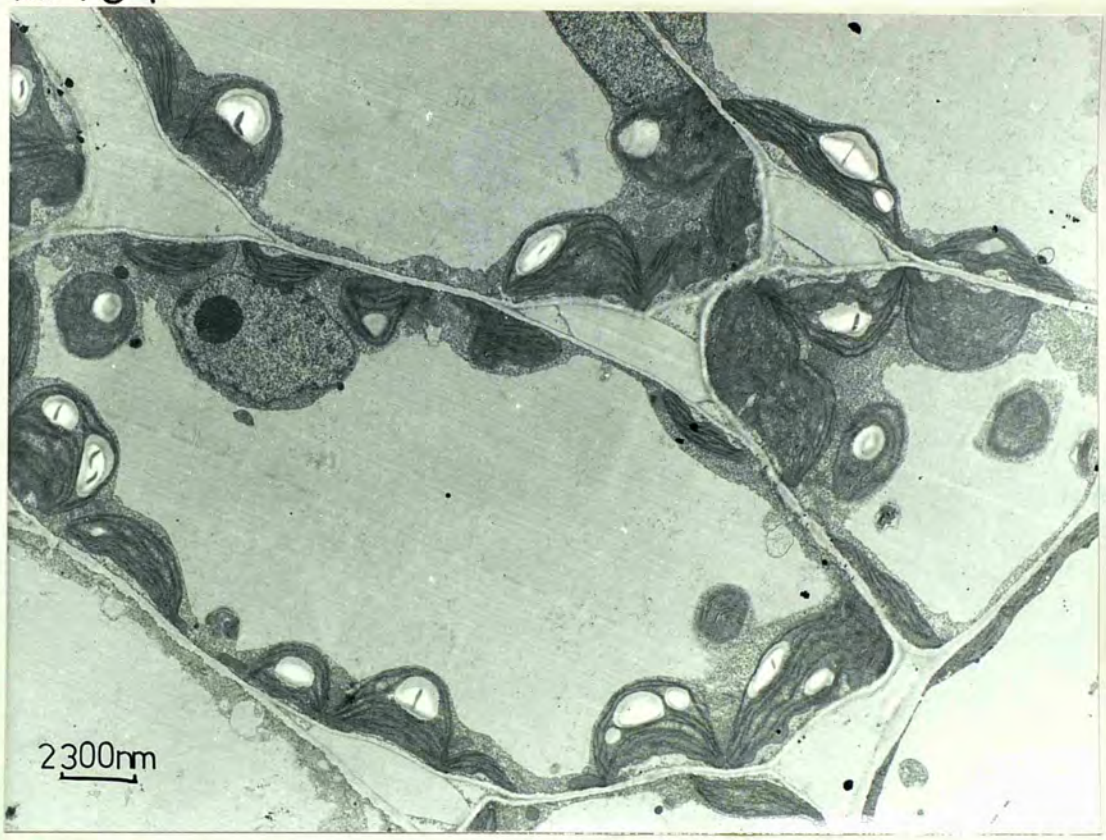
EM62



EM63

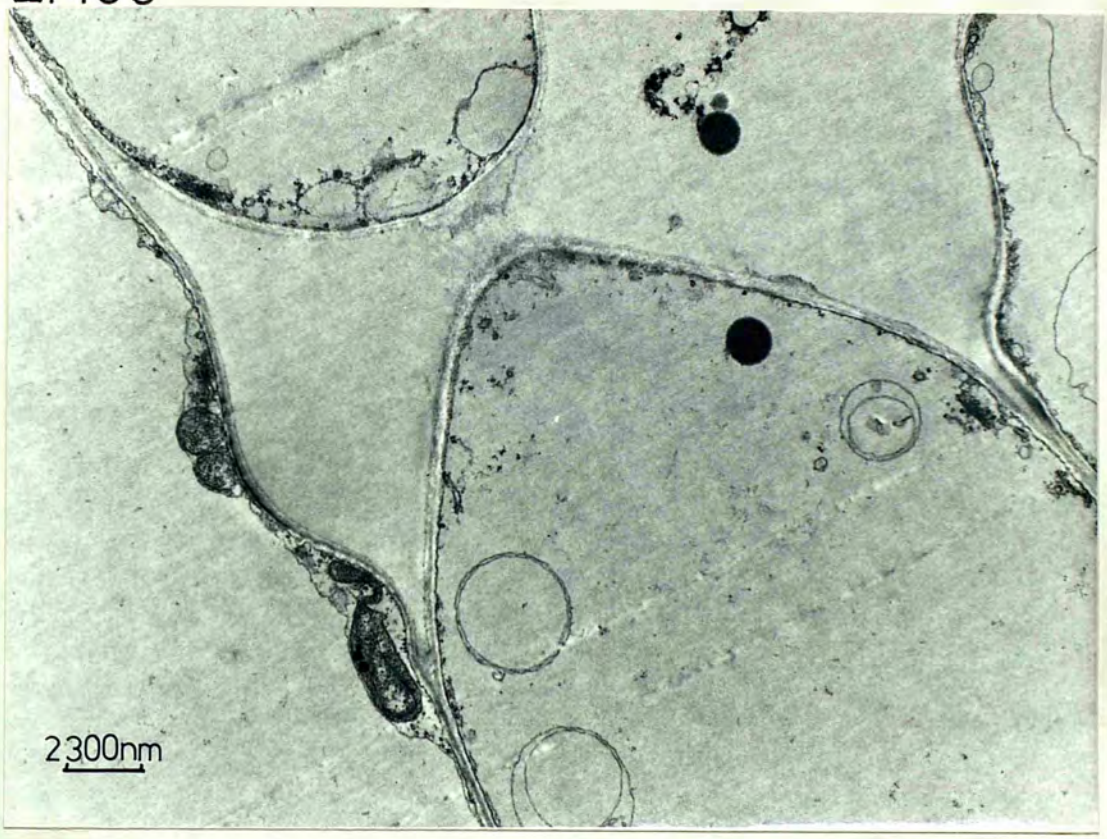


EM64

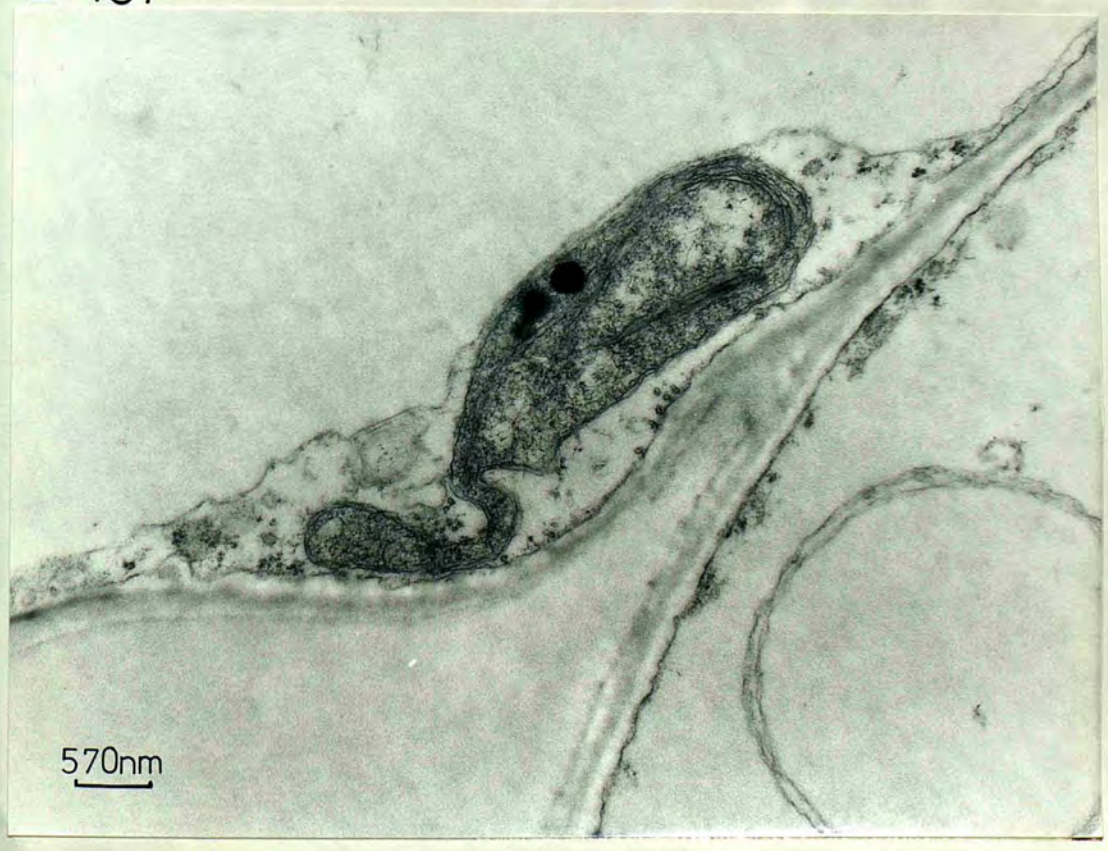


EM65

EM66



EM67



DISCUSSION

Although some discussion of the results of experiments and observations have been made in each chapter of this thesis it seems appropriate to conclude with some general discussion.

In the introduction, attention has been drawn to the fact that many diseases of previously unknown aetiology have now been assumed to arise from infection by mycoplasmas. In the past, prior to the availability of techniques such as electron microscopy, diseases resulting from 'invisible' pathogens were assumed to result from viral infection. In many instances such an assumption has been justified by observation of virus particles in diseased tissue. Where no such particles have been observed doubt still exists as to the causal agent.

The discovery by Doi et al (53) of MLO in plants, particuarly those showing 'yellows type' symptoms, has clarified the situation in many cases. In some instances evidence from symptomatology, sensitivity of the pathogen to tetracyclines and electron microscope studies has shown MLO as the causative agent.

Confusion between diseases of viral and MLO aetiology have come about not only because of symptom similarities and the problems of visualising the disease agent but also because of similarities in transmission. Both virus and MLO diseases can be transmitted by grafting, by insect vectors and by dodders. Many viruses are distinctive in that they can be mechanically transmitted.

A characteristic feature of many yellows diseases is their transmission by leafhoppers whereas relatively few viruses are transmitted by these insects (10). Transmission of viruses by insects is, for the most part, a complex process involving interaction between virus and vector. The inter-relationship is more involved where propagation of the virus in the vector is concerned than where stylet -

borne transmission takes place. It is known that insect transmission of MLO involves propagative transmission with similar complex inter-relationships between vector and pathogen (12, 136).

In this study leafhopper transmission occurred under carefully controlled conditions where insects were only allowed to feed on infected plants. Using more typical wild conditions - where leafhoppers were collected from an area known to contain infected plants - transmission was a rare occurrence. The vector insects need a long feeding period on infected plants to be capable of infecting other plants (31, 190, 220). In the wild they also need to be disturbed in some way to encourage them to move onto a new host plant.

On the other hand, with modern agricultural methods large numbers of plants of the same species are concentrated in one area. Under these monoculture conditions a rare transmission can introduce disease into the crop. The presence of a large insect population will give enough transmission to cause disease problems in the crop.

The experiments described suggest that aphids and whiteflies do not transmit MLO reliably if at all. Aphids are well - known vectors of viruses (160, 173, 206) but there is no evidence that they transmit MLO. The failure of aphids to transmit MLO could be caused by an inability to take up the organism, to transfer it to other plants or by the insects being unsuitable intermediary hosts.

The disease agents transmitted by whiteflies have been assumed to be viruses but only very few have been positively identified as such. Maramorosch (139) points out the possibility of other types of pathogenic organisms being the causal agents. The negative results found here suggest that the disease agents transmitted by whiteflies are not MLO.

Insect transmission is most likely to occur under natural conditions. Other forms of transmission may occur between plants as, for example,

with dodder. Dodder transmission was very difficult to obtain under controlled experimental conditions even though MLO were present in the dodder phloem. This would suggest that if it occurs in the wild it is a very rare event. However rare transmission would be sufficient to perpetuate the disease in the wild especially in plants which spread vegetatively.

The slow build - up of a disease in a wild plant population is advantageous as too rapid a spread could lead to areas of infected plants containing no healthy individuals. In most cases the production of seeds would lead to the perpetuation of healthy plants. Such seeds not only renew host plants for subsequent infection but also allow host plants to survive unfavourable conditions.

With a disease which prevents seed production an unusual event causing the death of the root stock of the plants would result in the death of this host plant. In many MLO - infected plants flowers are sterile so no seeds are produced to survive adverse conditions. Such an event occurred in 1976 in the MW area. All the clover plants along this stretch of embankment died as a result of the Summer drought. Had there been only phyllodied plants present the clover may have been completely eliminated from the area. However, as there were a majority of healthy plants the seeds from these should re-establish a clover population. Infected hoppers from other areas could then introduce the disease back into the area. If a disease organism kills its host plant too efficiently it is of no benefit to the disease organism as it will, in effect, destroy it's own habitat.

CP was found to appear in discreet, isolated patches in wild clover plant populations. The patches remain discreet which suggests a low percentage transmission with infected plants spreading into clumps by vegatative reproduction. If rapid disease spread had occurred most of

the clover plants would have been infected whereas, in the MW area, many of the clover clumps remained healthy.

In view of the relatively complex procedure for maintaining leafhoppers and the unreliable transmission by dodder, graft transmission was found to be a very useful experimental tool for increasing the amounts of infected material. This technique also proved useful in transmission between plants of the same species. If satisfactory grafts can be achieved between unrelated plants it could also be useful to compare diseases from different hosts.

Transmission from host to host and between different hosts may be of importance in establishing the aetiology of MLO diseases. For precise studies of the biology of a given pathogen, in vitro culture allows the more careful control of organism growth and reproduction enabling careful analysis of the conditions for development and assessment of control methods. Numerous attempts have been made to culture plant MLO and the studies recorded here emphasise that this is a very difficult technique. The encouraging results of some of the experiments reported here could not, unfortunately, be reliably repeated. There are several claims that initial plant MLO cultures can be made but not maintained or repeated (29, 57, 71, 168). Clearly some unknown factor is required for sustained culture of MLO.

The culture methods used were suitable for the successful culture of Acholeplasma laidlawii and Spiroplasma citri. However it may be that strains of these two organisms have become adapted to growth in culture. It is known that this phenomenon occurs in myoloma cell lines which become immortal and are used for tissue culture experiments (95). An alternative approach to culture has been to culture leafhopper tissue and use this as a support medium for plant MLO culture (171) but this method has not proved successful.

More sophisticated electrophoresis techniques may be useful to identify proteins present in infected plants as the initial experiment was quite promising. If the method could be linked with the electrophoresis of cultured MLO it could give some insight into the biochemical effects of MLO on host plant cells by showing possible changes in host proteins.

The objectivity of studying plant diseases is largely directed at controlling and, perhaps, eradicating the pathogen so that crop losses may be minimised. Clear understanding of pathogen structure, physiology and life history help in devising methods of disease control. In the absence of precise information about plant MLO their similarities to animal mycoplasmas led to the use of tetracyclines as agents of pathogen control.

MLO infections have been reported in the cactus Opuntia tuna monstrosa (25, 26, 119, 140) and treatment with tetracycline hydrochloride (25, 140) was shown to remit the monstrose or "witches'-broom" symptoms. The use of O. tuna monstrosa and similar abnormal growth forms of cacti for chemotherapy experiments produced inconclusive results and did not encourage the use of cacti as test plants. The suspected remission of disease symptoms by chemicals did not promote rapid growth. The plants remained slow growing and difficult to handle so cannot therefore be used for test purposes. Also, until the MLO involved can be cultured, it is not certain that the micro - organisms found in cacti are related to those found in herbaceous plants.

Applying test chemicals to plants can pose problems if, as with tetracyclines, they are adsorbed by soil. Application by foliage spray is not entirely effective as the chemical may not be adsorbed through the leaves. The technique of water culture for maintaining the host V. rosea plants, linked to grafting for transmission of MLO, provides a

useful experimental tool for applying suspected chemotherapeutants directly on field crops but, given a suitable chemical, could be used to raise disease - free stocks.

A few chemicals other than tetracyclines have been shown to be active against MLO diseases of plants (22, 89, 110, 143, 149, 165, 167, 203, 204). Benelate, a well known fungicide, seems to be effective in curing Sandal Spike (165, 167) but it is not yet known if its effects are permanent. Sulphur drugs have also been shown to have some effect (110) but this is thought to be caused by improving the condition of the plant and not by direct action on the MLO. It would probably be most useful if used in conjunction with active antibiotics.

Many other published results of antibiotic experiments are contradictory and do not indicate any outstandingly effective chemicals.

The use of tetracyclines has one main drawback; they appear only to suppress symptoms and rarely cure the diseases (46). Apart from the dangers involved in the widespread use of antibiotics the temporary effect makes them of little use in the field.

An alternative approach is to control the vectors. In some cases use of insecticides has cut down the spread of disease in the field (146, 180, 181). Insecticide is sometimes applied in conjunction with antibiotics. This slows down the spread of the disease as in the case of Citrus Greening disease. The branches of a citrus tree which are infected with greening are less vigorous than the healthy branches of the same tree. If the spread of the disease can be reduced the vigorous, healthy growth will produce a good crop (181).

Paddick (152, 153, 154, 155) has suggested that systemic insecticides may have a direct effect on MLO. He found that disease spread was reduced much more using systemic rather than contact insecticides. He argues that contact insecticides should be more efficient as they kill

the insects before they feed. Leafhoppers must feed on a sprayed plant and therefore possibly transmit the disease before picking up a lethal dose of a systemic insecticide. He quotes other papers to support his theory (64, 129, 207) although these authors seem to have assumed that systemics are merely more efficient insecticides.

It could be argued that systemic insecticides which cannot be washed - off the plants by rain remain active longer than contact insecticides. Insect migration into the crop between applications of contact insecticides could therefore account for the increased disease spread. However Paddick's views may be correct and it is possible that systemic biocides may be more useful for combating MLO diseases.

At present the only effective treatment for MLO diseases, as well as some virus diseases, is heat treatment of young plants before planting out in the field. This method is used quite effectively on sugar cane (125) as described in section III.

The identification of MLO as disease agents at present relies on such factors as external symptoms, transmission and remission of symptoms by tetracyclines. It would be useful to develop a technique relying on staining MLO in situ or tissue specifically altered by MLO. Such a technique, particularly if specific for MLO, would be useful as a diagnostic tool.

Several workers have attempted to develop staining techniques but they are usually complex (66, 74, 81, 109, 182, 183). There is a demand for a more simple detection technique and it has been shown here that simple techniques involving Feulgen stain, Dienes' stain, methylum-belliferone - phosphate (alkaline) and europium chaelate can each detect the presence of MLO in infected plants. However Dienes' stain achieves the original aims of the experiments as it is quick, efficient and uses very simple methods and microscopy. Furthermore it appears to be

specific for MLO. Its usefulness was demonstrated by its use to trace the route of infection by MLO from a graft through the stock plant and to confirm the action of chemotherapeutants on the MLO in treated plants.

The more complex europium chaelate stain is as effective as Dienes' stain but is not so simple to use. It may therefore be more useful as a confirmatory technique to back up the quicker Dienes' stain if results are inconclusive.

A specific staining technique is very helpful in detecting MLO in plants but the individual micro - organisms are not visible with a light microscope. Electron microscopy, although involving complex and expensive techniques, is still useful for positively identifying MLO and the effects on the host plant.

All MLO found in herbaceous plants look similar in thin EM sections. Even Spiroplasma citri and the Corn Stunt spiroplasma, which were found to be helical when cultured, show little sign of their spiral morphology in sections thin enough to be examined in the EM. It is possible that some of the green flower diseases examined here are caused by the same, or strains of the same MLO. Other MLO diseases have very different symptoms so it is likely that several species of MLO are involved. Growth in culture would probably indicate growth differences not visible in EM preparations or indicate differences in growth requirements.

The monstrose growth forms of Opuntia tuna and Opuntia subulata have been shown to be induced by MLO - infection (25, 26, 49, 119, 140). It is therefore reasonable to assume that other monstrose forms and the convoluted cristate forms are also caused by MLO infection. The MLO found in the monstrose and cristate cacti which were examined in the EM have some similarities in gross structure to those found in herbaceous

plants. They have a single unit membrane and simple internal structure but they are rather larger (300 - 2,000 nm compared with 50 - 800 nm) and less granular than those in herbaceous plants. The differences may be a reaction to environmental differences but it is likely that they are different MLO. The origins of cactus MLO are not known so transmission to herbaceous plants would be extremely useful for comparisons.

Diseased plants are recognised by external disease symptoms and growth abnormalities. These are a reflection of the internal changes which may be biochemical inbalances or ultra - structural changes. The latter can be observed in the EM and may be observed before the gross changes in the plant are manifested externally. With MLO diseases the internal changes include the accumulation of starch, inclusion bodies and plastid changes. The plastid changes are directly responsible for the colour changes observed in the flowers but can be detected before the flower colour symptoms can be positively identified.

Observation of sub - cellular changes may be useful in the detection of disease prior to major symptom development particularly if combined with light microscopy using such stains as Dienes' stain and europium chaelate. This would then allow for early treatment with, for example, tetracyclines which would probably be more efficient in eliminating MLO from plants at an early stage of infection. Tetracyclines applied after full symptom expression can only cause remission of the disease. Equally, early detection of MLO diseases would allow control of vector insects before the disease becomes established in a crop and possibly before large numbers of insects become infective and subsequently transfer the disease to other plants.

CONCLUSIONS

Working with Clover Phyllody and plant MLO diseases has proved a difficult and testing task. However the following conclusions can be drawn from the research undertaken:-

CP can be transmitted from Trifolium repens to Vinca rosea by the leafhopper Euscelis plebejus. V. rosea is a useful species for maintaining CP MLO under experimental conditions.

No transmission of CP MLO could be achieved with aphids or whiteflies.

Three species of Cuscuta (dodder) were used to study the possible transmission of MLO. EM studies suggest that the MLO use dodders as an alternative host and not just a passive transmission agent although no transmission was obtained.

Some abnormal forms of cacti contain MLO but these are difficult to work with because the conducting tissue occurs in very small, widely dispersed bundles. Mucilaginous exudates also hinder EM preparation. Unfortunately dodders, the most likely experimental transmission agents, do not readily parasitise cacti.

Graft transmission, particularly of CP in V. rosea proved to be a very useful experimental procedure.

Acholeplasma laidlawii and Spiroplasma citri could be readily cultured in vitro but culturing of CP proved to be much more difficult. More experimental work needs to be carried out on this subject.

Using a water culture technique for the root application of chemicals it was possible to control MLO infection of V. rosea with oxytetracycline hydrochloride although its effects were not permanent. Other chemicals were not effective on V. rosea and most chemicals tested were ineffective when used on cacti.

MLO in plants were detected by several fluorescent and non - fluorescent staining techniques. Two new techniques were developed, one

using europium chaelate and fluorescent microscopy, and another using Dienes' stain and light microscopy proved particularly useful. Dienes' stain was subsequently used to distinguish MLO infections from other dieseases and to analyse the passage of MLO through host plant tissues.

Methyl umbelliferones were tested for the first time as possible indicators of plant MLO but results were disappointing.

Electron microscopy was used successfully to detect MLO in phloem tissue but could not be used to distinguish between MLO from different sources. Some size difference was observed however with MLO from cacti compared with those from herbaceous plants and it was particularly valuable to be able to demonstrate these MLO in the monstrose and cristate forms of cacti. It was difficult to fix cactus tissue so Cuscuta parasitic on the cacti was examined.

It was found that electron microscopy could also be used to examine the division and movement of MLO in the host plants and thus give some insight into the mechanisms of infection.

The ultrastructural changes induced by MLO infection include the formation of starch granules, inclusion bodies and plastid changes and these were recognised in the electron microscope.

One further use of the electron microscope was in investigating Cuscuta sp parasitic on MLO - infected plants. As MLO were found in the Cuscuta in large numbers and seen to be dividing and moving through pores it could be shown that the parasite acts as an alternate host for MLO and not just as a passive carrier between hosts.

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Use of Dienes' Stain to Detect Plant Diseases Induced by Mycoplasmalike Organisms

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ABSTRACT

DEELEY, J., W. A. STEVENS, and R. T. V. FOX. 1979. Use of Dienes' stain to detect plant diseases induced by mycoplasmalike organisms. Phytopathology. 69:1169-1171.

Application of a 0.2% solution of Dienes' stain to hand-cut or freezing-microtome sections indicated mycoplasmalike organisms (MLO) in the phloem of infected plants. The stain appeared to be specific for MLO diseases, including those of spiroplasma etiology, and gave no reaction in tissues of plants infected by other pathogens. The procedure is quick and could be of diagnostic value.

The most reliable method for demonstrating the presence of mycoplasmalike organisms (MLO) in diseased plants is electron microscopy of phloem tissue. This elaborate process involves the use of expensive equipment and reagents. The need for a quick, simple method of detecting MLO diseases has long been recognized and for this reason some light-microscope staining methods have been described (5,6,8,10). These often involve specialized fluorescent microscopy or time-consuming embedding procedures. This article outlines a method that is rapid and simple and requires only low-power light microscopy. It involves the use of Dienes' stain, which is widely used to detect animal mycoplasmas (7) and plant spiroplasmas in culture (2).

MATERIALS AND METHODS

Dienes' stain was prepared by dissolving 2.5 g of methylene blue, 1.25 g of azure II, 10.0 g of maltose, and 0.25 g of sodium carbonate in 100 ml distilled water (7). The stain was filtered through Whatman No. 1 filter paper and a series of dilutions (0.5–0.1%, v/v) was made in water.

Trifolium repens L. plants with clover phyllody (CP) mycoplasma were collected from two areas of grassland and were maintained in plots or in the greenhouse. Infected plants have distinctive green, leaflike flowers, unlike the healthy white flowers.

Greenhouse-grown *Vinca rosea* L. plants were originally infected with CP by leafhopper (*Euscelis plebejus* Fallen) transmission from a single CP-infected *T. repens* plant. Stocks of plants were maintained by grafting pieces of infected *V. rosea* plants onto healthy seedlings. Infected plants were identified easily by their green flowers and bushy growth form, since healthy *V. rosea* have white or pink flowers.

Rose (*Rosa chinensis* var. *viridiflora* Dipp.) and *Helenium autumnale* L. plants naturally infected with MLO and showing flower greening were maintained in the greenhouse. Stems of apple with rubbery wood and chat fruit diseases and loganberry (*Rubus loganbaccus* Bailey) canes with Rubus stunt were supplied by Long Ashton Research Station. The John Innes Institute supplied cuttings of *V. rosea* infected with *Spiroplasma citri* and corn stunt spiroplasma.

Sections of healthy and infected stems were cut by hand into distilled water, with a single-edge razor blade. The sections were transferred to stain for 10 min, after which the stain was withdrawn and replaced with distilled water. After being washed in distilled water, the sections either were mounted in water and examined immediately or were left in distilled water for several hours before examination without any obvious detrimental effects. Both longitudinal and transverse sections of stems were examined. Transverse sections were more useful because they could be cut

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