MYCOPLASMA-LIKE BODIES IN FRENCH BEAN, DODDER, AND THE LEAFHOPPER VECTOR OF THE LEGUME LITTLE LEAF AGENT

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Abstract

Mycoplasma-like bodies were observed in the phloem sieve tube elements of French bean (Phaseolus vulgaris L.) and dodder (Cuscuta australis R.Br.) carrying the legume little leaf disease agent. In dodder they occurred in small groups of approximately 5–30, in contrast to the very large numbers in bean. In bean, some of the bodies were located within the sieve plate pores.

Mycoplasma-like bodies were also found in the salivary glands and filter chamber region of the alimentary canals of infective individuals of the leafhopper vector Orosius argentatus Evans. In the salivary glands they were observed in only one of the three types of acini. A technique involving the embedding and sectioning of whole insects for electron microscopy was developed. This avoided leafhopper dissection and allowed the examination of the salivary glands in situ. The technique also had the advantages of being simple and rapid.

Following the earlier discovery of Mycoplasma-like bodies in little leaf-diseased plants, the presence of similar bodies in infective dodder and in leafhopper vectors, and their absence from control material, is interpreted as supporting evidence for mycoplasmal aetiology of the disease.

I. INTRODUCTION

Legume little leaf disease was described by Hutton and Grylls in 1956 and, until recently, was considered to be due to virus infection. Bowyer et al. (1969) and Bowyer and Atherton (1970), in an electron-microscopic examination of diseased plant tissue, observed Mycoplasma-like bodies in phloem sieve tubes, thus providing the first evidence that the disease may be of mycoplasmal, rather than viral, aetiology. Proof of Mycoplasma aetiology requires culture of the legume little leaf agent in vitro and subsequent satisfaction of Koch’s postulates. However, efforts in this direction have so far been unsuccessful.

Further indirect evidence of Mycoplasma aetiology would be provided by the constant association of Mycoplasma-like bodies with diseased plants other than those already studied. Also, Mycoplasma-like bodies would be expected to occur in any dodder or leafhopper capable of transmitting the legume little leaf disease agent.

Since the discovery of structures resembling Mycoplasma in diseased plants by Doi et al. (1967), some 40 plant diseases are now thought to be caused by mycoplasmata (Whitcomb and Davis 1970). However, proof of Mycoplasma aetiology, based on in vitro cultivation of the organisms from diseased plants and satisfaction of Koch’s postulates, has so far been claimed for only three diseases (Hampton, Stevens, * Department of Microbiology, University of Queensland, Medical School, Herston, Qld. 4006.

and Allen 1969; Chen and Granados 1970; Lin, Lee, and Chiu 1970). Many of these diseases are known to have leafhopper vectors and some have experimentally been transmitted by means of dodder (\textit{Cuscuta} spp.). In several cases \textit{Mycoplasma}-like bodies, similar to those seen in diseased plants, have also been found in certain tissues of the leafhopper vectors of the respective diseases (e.g. Maillet, Gourret, and Hamon 1968; Maramorosch, Shikata, and Granados 1968; Hirumi and Maramorosch 1969; Maillet 1970; Nasu, Jensen, and Richardson 1970; Sinha and Paliwal 1970). Dale and Kim (1969) observed \textit{Mycoplasma}-like bodies in dodder parasitizing aster yellows-diseased plants.

Doi \textit{et al.} (1967) suggested that \textit{Mycoplasma}-like bodies pass between the sieve tubes of the phloem by way of the sieve pores, and Shikata and Maramorosch (1969) reported the bodies in the sieve pores of yellows-diseased plants. Electron micrographs purporting to illustrate this phenomenon have been published by Shikata, Maramorosch, and Ling (1969), Sinha and Paliwal (1969), and Bowyer and Atherton (1970).

This paper describes \textit{Mycoplasma}-like bodies (1) in the phloem sieve tubes and sieve pores of little leaf-diseased French bean (\textit{Phaseolus vulgaris} L.), (2) in little leaf-infective dodder (\textit{Cuscuta australis} R.Br.), and (3) in the leafhopper vector, \textit{Orosius argentatus} Evans.

II. Materials and Methods

(a) Pathogen Strain

The strain of the little leaf agent was the same as that used previously (Bowyer and Atherton 1970). Unless otherwise specified, the leafhopper vector (\textit{O. argentatus}) was used to maintain the disease in \textit{Datura stramonium} L. or to transmit it to other experimental hosts. All experiments were carried out in a glasshouse.

(b) Electron Microscopy

All sections were cut on an LKB ultramicrotome, mounted on carbon-coated copper grids, and stained on the grids with 5\% (0.12M) aqueous uranyl acetate and lead citrate (Reynolds 1963). They were examined in a Siemens 1A electron microscope. Other details are given where appropriate.

(i) Little Leaf-diseased French Bean

Seedlings of \textit{P. vulgaris} cv. Spartan Arrow were caged with a group of leafhoppers previously fed for 28 days on little leaf-diseased \textit{D. stramonium}. Symptoms of little leaf disease appeared in the bean plants after 19–23 days. Segments (6 mm) of petioles bearing typical small leaves were prepared for electron microscopy 14 days after the first appearance of symptoms. The fixation procedure has been described previously (Bowyer and Atherton 1970). Before embedding in Araldite the fixed specimens were trimmed by removing 2 mm of tissue adjacent to each of the two cut ends, thus discarding tissue adjacent to the original cut surfaces.

(ii) Infective Dodder

Dodder seed (\textit{C. australis}) was germinated on moist filter paper. The seedlings were attached to little leaf-diseased \textit{Nicotiana glutinosa} L. plants. Four weeks later, tips of the vigorously growing dodder filaments were removed and attached to \textit{N. glutinosa} seedlings, which developed symptoms of little leaf disease 7–10 weeks later. Tips of the dodder filaments from these plants were then prepared for electron microscopy as previously. Control material was prepared from non-infective dodder grown on healthy \textit{N. glutinosa}.
Fig. 1.—Mycoplasma-like bodies in a sieve tube element of bean. The sieve plate (SP) is pierced by several pores (P), two of which contain Mycoplasma-like bodies (arrows). The diameter of each pore is approximately twice that of the bodies, which thus are not constricted by the pores. ×40,000.

Fig. 2.—A group of Mycoplasma-like bodies in the cytoplasm of a phloem cell of little leaf-infective dodder (C. australis). V, vacuole of host cell. ×4000.
Fig. 3.—Higher magnification of *Mycoplasma*-like bodies in dodder. Note the triple-layered limiting membrane (M) and the central nuclear region consisting of a network of fine DNA-like fibrils (F); the latter contrast with the coarse fibrils of the bodies in Figure 8. R, host cell ribosomes; r, ribosome-like particles in bodies, smaller than those of the host cell. × 40,000.
(iii) Leafhopper Vector

A colony of O. argentatus was established in November 1968. Since then periodic transmission tests have shown that insects from the stock colony are not carrying the little leaf agent. To obtain infective leafhoppers, a group of fifth instar nymphs was caged on a little leaf-diseased D. stramonium plant for 14 days. The insects (now adults) were held for a further 14 days on a healthy plant to allow for completion of the incubation period. Twenty insects were then caged singly on individual D. stramonium seedlings and allowed a 7-day inoculation feed. The 18 surviving insects were then processed as follows: After immobilization in an ice-bath, the wings and legs were removed and the bodies transferred to a drop of cold 0·1 M cacodylate-buffered 4·1 % (0·41 M) glutaraldehyde, pH 7·4 (Sabatini, Bensch, and Barnevet 1963). Using fine needles (entomological “steels”), the dorsal terga were carefully teased apart to facilitate penetration of the fixative into the internal organs. The specimens were then transferred to fresh fixative at 4°C for 2 hr. The fixative was replaced by cacodylate-buffered 0·2 M sucrose, in which the specimens were held at 4°C until transmission results were known. The four proven transmitters were then post-fixed in 1 % (0·04 M) buffered osmium tetroxide (Caulfield 1957) for 1·5 hr at 4°C, dehydrated through a graded ethanol series, and flat-embedded in Araldite. Four leafhoppers from the stock colony were also tested individually for transmission and processed similarly, as controls.

In a second experiment rupture of the terga was omitted. In this case the insects were immobilized, then rinsed briefly (5 sec) in 70% ethanol followed by distilled water. The wings and legs were removed and the whole bodies fixed in glutaraldehyde and processed as previously. Of 30 insects tested, three proved to be vectors. Thus a total of seven proven vectors and four non-vector controls were examined.

III. Results

(a) Little Leaf-diseased French Bean

Large numbers of typical Mycoplasma-like bodies were present in many of the sieve tube elements of infected petioles. The lumina of many cells were entirely occupied by the bodies, which were often packed so closely that the membranes of adjacent bodies were in contact, leaving few intercellular spaces (Fig. 1). Numerous sieve plates with sieve pores approximately 350–500 nm in diameter were observed. Most of the pores were “open”, i.e. free of callose deposits, and several pores contained Mycoplasma-like bodies (Fig. 1).

(b) Infective Dodder

Mycoplasma-like bodies, similar to those observed in French bean and other little leaf-diseased plants (Bowyer and Atherton 1970), were present in the phloem sieve tubes of dodder parasitizing little leaf-diseased N. glutinosa. They were observed in small groups (of approx. 5–30) in mature sieve tube elements devoid of cytoplasm, and also within the apparently normal, intact cytoplasm of cells which appeared to be immature sieve elements or possibly phloem parenchyma cells (Figs. 2 and 3). The bodies ranged in size from approximately 125 to 700 nm in diameter. Figure 4 shows a bud-like protuberance at one end of a Mycoplasma-like body. The bud appears to contain an elementary body, characterized by a dense homogeneous internal structure and a diameter of 125 nm. The parent structure has a diameter of approximately 450 nm. Mycoplasma-like bodies were not observed in control dodder grown on healthy plants.

Fig. 4.—Mycoplasma-like body in the cytoplasm of a phloem cell of dodder. Note the small inclusion body (I) within the protuberance of the larger body. The inclusion lacks internal structure and resembles a mycoplasmal “elementary body”; it is bounded by a limiting membrane similar to that of the larger body. The various layers of the two adjacent membranes are distinguishable (arrow). ×120,000.
(c) Leafhopper Vector

Of the seven proven vectors, the salivary glands were examined in four, and the alimentary canals in five; both organs were examined in two of the insects. Starting at the head, a series of transverse sections (approx. 1 μm thick) of each insect were cut, stained with toluidine blue, and examined by light microscopy. This procedure was used to identify the various internal tissues. The papers by Gil-Fernandez and Black (1965) and Forbes and MacCarthy (1969) were helpful for this purpose. The salivary glands were found in the head, approximately 0·25 mm from the anterior end (Fig. 5), and thin sections of this region of the insect were then examined in the electron microscope. To study the alimentary canal, the thorax and abdomen were sectioned and sections of the pro-, meso-, and metathorax and of the first three (i.e. anterior) abdominal segments were examined.

(i) Salivary Glands

Mycoplasma-like bodies, similar to those in little leaf-diseased plants and infective dodder, were present in the salivary glands of three of four proven vectors. The glands consisted of three types of acini, the characteristics of which are illustrated in Figure 6, and may be summarized as follows:

Type A—Numerous large secretion granules; cytoplasm containing abundant, dense, endoplasmic reticulum.

Type B—Relatively fewer secretion granules, smaller than in type A; endoplasmic reticulum not prominent.

Type C—Few or no secretion granules; cytoplasm containing vesicular material not seen in types A and B.

The three types of acini were found consistently in the four proven vectors and in the four non-vector control insects. The Mycoplasma-like bodies present in three of the four vectors were observed only in the type B acini (Figs. 6 and 7). No bodies were found in the salivary glands of the fourth vector.

(ii) Alimentary Canal

Mycoplasma-like bodies were present in the cells of the filter chamber region of the alimentary canals of the five proven vectors examined, including the one in which they were not found in the salivary glands (Figs. 8–10). They were not found in the alimentary canal posterior to this region, or in the malpighian tubules, fat body tissue, muscle, or nervous tissue.

With respect to internal structure, two types of Mycoplasma-like body were recognized in cells of the leafhopper: (1) bodies with peripheral cytoplasmic zones surrounding electron-transparent nuclear areas, which contained prominent, coarsely stranded DNA-like fibrils (Figs. 7–9); (2) bodies in which the central areas contained

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Fig. 6.—Mycoplasma-like bodies (arrows) in a salivary gland cell of an infective leafhopper. Note the three types (A, B, and C—see text) of adjacent acini, with the bodies present in type B only. SG, secretion granules; VM, vesicular material of type C acinus. ×10,000.
Fig. 5.—Transverse section of the head of *O. argentatus*, showing the salivary gland acini (*SGA*) in relation to the other tissues. *SOG*, suboesophageal ganglion; *ON*, optic nerve; *NS*, nerve sheath; *FB*, fat body; *CE*, compound eye. Section approximately 1 μm thick, stained with toluidine blue. Light micrograph. ×100.
very fine, inconspicuous nuclear threads (Fig. 10). Both types were characterized by pleomorphic morphology, a size range of approximately 125–800 nm, and limiting unit membranes approximately 9 nm thick (Fig. 9). Mycoplasma-like bodies were not observed in any of the four control insects.

IV. Discussion

(a) Electron Microscopy of Dodder and O. argentatus

Relatively few Mycoplasma-like bodies were observed in the phloem cells of infective dodder in contrast to the large numbers of bodies in the sieve tubes of other diseased plants, e.g. French bean (see also Bowyer and Atherton 1970). This may indicate limited multiplication of the agent in little leaf-infective dodder. Dale and Kim (1969) published micrographs of aster yellows-infective dodder, showing large numbers of bodies in the phloem, similar to the numbers seen in aster yellows-diseased tobacco and aster (Worley 1970). The difference between the two results is possibly due to differences in host–parasite relationships of the two systems. Presumably, variation in both the dodder species and the strain of the Mycoplasma-like agent may influence this relationship. The present work involved C. australis, but Dale and Kim did not state the species with which they worked.

An incidental observation made while working with dodder as a transmission agent indicated a physiological difference between infective and non-infective dodder. Infective dodder filaments, when established on healthy seedlings, grew vigorously and remained in the vegetative phase. In contrast, control (i.e. non-infective) dodder began to flower within 2–3 weeks of establishment on new plants. This phenomenon was consistently observed with both C. australis and C. campestris. Helms (1957) reported similar differences between C. campestris grown on plants affected by two different strains of lucerne witches’ broom disease. This effect on the reproductive physiology of infective dodder may be directly associated with the presence of the little leaf pathogen, or indirectly with physiological disturbances in the diseased host plant.

The occurrence of Mycoplasma-like bodies in only one of the three types of salivary gland acini of O. argentatus appears to be the first report of this phenomenon, although its significance is not understood. Previous reports of Mycoplasma-like bodies in the salivary glands of leafhopper vectors have been based on examination of excised glands (e.g. Hirumi and Maramorosch 1969; Sinha and Paliwal 1970). The in situ technique used in the present study allows the various acini to be examined in their natural arrangement. It would be interesting to study the salivary glands of other vectors by this method.

Further advantages of the technique are its simplicity and rapidity, since it avoids the difficult and tedious procedure of leafhopper dissection. It was found that fixation of the insect (after removal of legs and wings) was equally satisfactory with

Fig. 7.—Mycoplasma-like bodies in a salivary gland cell. Note the membrane (arrow) and the prominent nuclear fibrils (F), presumably of polymerized DNA. SG, secretion granule. ×40,000.

Fig. 8.—Mycoplasma-like bodies in an intestinal cell of O. argentatus. The bodies are not sharply differentiated from the host cell cytoplasm, but the limiting membranes of some are evident (arrows). Note the coarse central fibres (F) and the presence of the bodies within a cytoplasmic vacuole (V). ×40,000.
Fig. 9.—Higher magnification of one of the bodies in a leafhopper gut cell, illustrating all the structural characteristics of a Mycoplasma cell: the limiting membrane (M), the central DNA-like fibrils (F), and the peripheral cytoplasmic region containing ribosome-like granules (r). ×120,000.
or without rupture of the terga. By omitting this step each insect could be processed in a few minutes. Thus, when only a small proportion of insects are vectors (e.g. approx. 10–20% in the case of O. argentatus as a vector of little leaf disease), the large numbers which must be tested and prepared for electron microscopy do not present a problem.

Attention has been called previously to the presence of two types of Mycoplasma-like bodies, with respect to details of internal structure, in the cells of an insect vector (Granados, Maramorosch, and Shikata 1968; Granados 1969). In work with little leaf, the type characterized by prominent DNA-like nuclear fibrils was present in five of the seven leafhoppers examined and in most of the diseased plants (Figs. 7–9). The second type, characterized by very fine inconspicuous fibrils, was less common, but was seen in the other two leafhoppers, in some diseased plants, and in some infective dodder (Figs. 1, 3, and 10). Both types, the significance of which is not understood, have been described in Mycoplasma hominis (Anderson and Barile 1965). The recent work by Biberfeld and Biberfeld (1970) indicates that in M. pneumoniae the age of the cell markedly influences its ultrastructural details.

The presence in infective dodder and in transmitting leafhoppers of Mycoplasma-like bodies similar to those in little leaf-diseased plants provides further circumstantial evidence for their being the causal agents of the little leaf disease. It has now been established that there is a constant, specific association of the bodies with all components of the “little leaf complex”, that is, the diseased plants, the leafhopper vector, and infective dodder. Mycoplasma-like bodies have never been found in corresponding control material. The response by little leaf-diseased plants to tetracycline therapy (Bowyer and Atherton, unpublished data) further supports this direct cytological evidence for mycoplasmal aetiology of the disease.

(b) Mycoplasma-like Bodies in Sieve Pores

Recent electron-microscopic studies of phloem tissue in plants indicates that cutting of sieve tube elements causes a strong hydrostic flow, which results in cellular components such as starch grains being drawn through the sieve pores (Anderson and Cronshaw 1969). This effect may be apparent in cells located within 1 mm of the cut surfaces of the tissue. It seems possible that Mycoplasma-like bodies could also be drawn into sieve pores during preparation of diseased plant material for electron microscopy. In published micrographs, the constriction of the bodies and their apparent orientation toward the sieve pores (Gourret and Maillet 1969; Bowyer and Atherton 1970) suggest that the bodies may have been drawn into the pores by hydrostatic flow.

In the present work, the specimens of little leaf-diseased bean were trimmed after fixation in order to avoid tissue adjacent to the original cut surfaces and so minimize the chances of basing results on possible artefact. In Figure 1, Mycoplasma-like bodies are located within large sieve pores, and there is no evidence of “flow” of the bodies into the pores. The diameters of the bodies are less than those of the pores,

Fig. 10.—Mycoplasma-like bodies in a gut cell of another leafhopper. In contrast to those in Figure 8 they do not have prominent nuclear fibrils. Note the body (arrow) which appears to be dividing. R, host cell ribosomes. × 40,000.
which therefore should not prevent passage of the bodies between adjacent sieve elements. Numerous similar examples of *Mycoplasma*-like bodies within sieve pores were also observed and they support the concept that movement of the bodies through the pores is involved in the systemic infection of the plant (Shikata and Maramorosch 1969; Bowyer and Atherton 1970). It is considered that, compared with previously published examples, Figure I is a superior illustration of *Mycoplasma*-like bodies within sieve pores.

V. Acknowledgments

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VI. References


Mycoplasma-like bodies in Dodder and leafhopper


