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Association of a Bacterium-like Organism with Rugose Leaf Curl Disease of Clovers

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Abstract

White and crimson clover plants affected by rugose leaf curl disease showed temporary remission of symptoms when treated with weekly doses of penicillin applied as a soil drench.

A small bacterium or bacterium-like organism was consistently associated with diseased plants of red, white and crimson clover and *Trifolium semipilosum* when sections of diseased tissues were examined by thin-section electron microscopy. No similar bacterial bodies were seen in sections cut from healthy plants. The bacterial bodies were restricted to phloem sieve tubes and phloem parenchyma cells and were irregularly distributed along vascular bundles within infected tissue. Infected phloem cells also contained electron-dense droplet material not seen in adjacent cells.

The bacterial bodies associated with rugose leaf curl measured approximately 0·25 μm in diameter and were 1–2 μm in length, although more elongated bodies also occurred. The organism was bounded by a cell wall and plasma membrane, or a double membrane, both trilaminar in structure and separated by a lightly stained intermediate layer. The cell wall or outer membrane was darker-stained than the inner membrane and was undulating in outline.

Although this organism has not yet been isolated in pure culture, the sensitivity of the pathogen to penicillin suggests that the bacterium-like organism seen by electron microscopy is the causal agent of rugose leaf curl disease.

Introduction

Rugose leaf curl (RLC) disease was fiirst described in plants exposed experimentally to lucerne leafhoppers (*Austroagallia torrida* Evans) collected in the field (Grylls 1954). The pathogen is transmissible by leafhoppers or by grafting (Grylls 1954) to plant species other than those recorded as field hosts.

In Queensland and northern New South Wales, the disease affects newly established pastures of white clover (*Trifolium repens* L.), red clover (*T. pratense* L.) (Grylls 1955), *T. semipilosum* Fres. and *T. burchellianum* Seringe (Grylls and Day 1966). Peanut (*Arachis hypogaea* L.) and lucerne (*Medicago sativa* L.) are also susceptible to the disease (Purss 1962, 1965).

The symptoms of the disease in clover species consist of stunting, leaf curling and twisting, and rugosity of the lamina (Fig. 1). Leaves may also show marginal or more general chlorosis and older leaves frequently develop red pigmentation. The disease is usually most severe in seedling plants and may result in death. However, infected stoloniferous clovers often produce apparently healthy stolons which lead to regeneration of the sward. An unexplained feature of this recovery is that plants which develop from such stolons exhibit some form of induced resistance to reinfection (Grylls and Day 1966; Grylls *et al.* 1972).

RLC disease was originally assumed to be caused by a virus because the pathogen could be transmitted by grafting and by a leafhopper vector. Recently, electron-microscopic evidence has been presented (Grylls *et al.* 1974) of virus-like particles

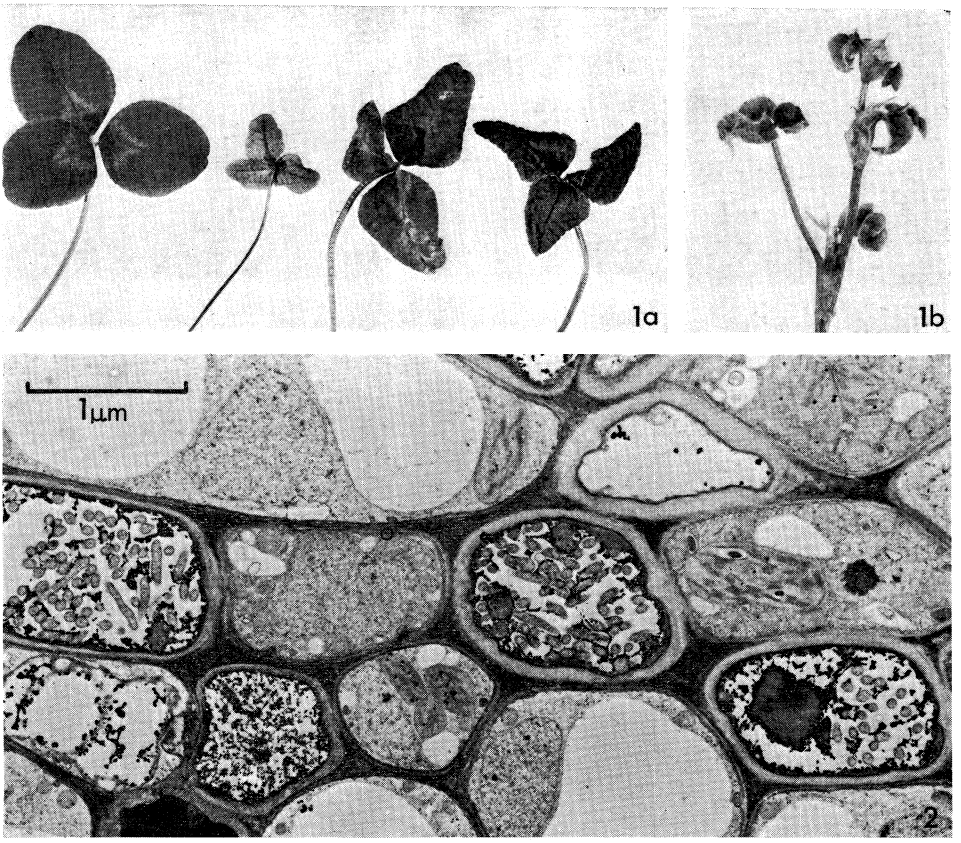
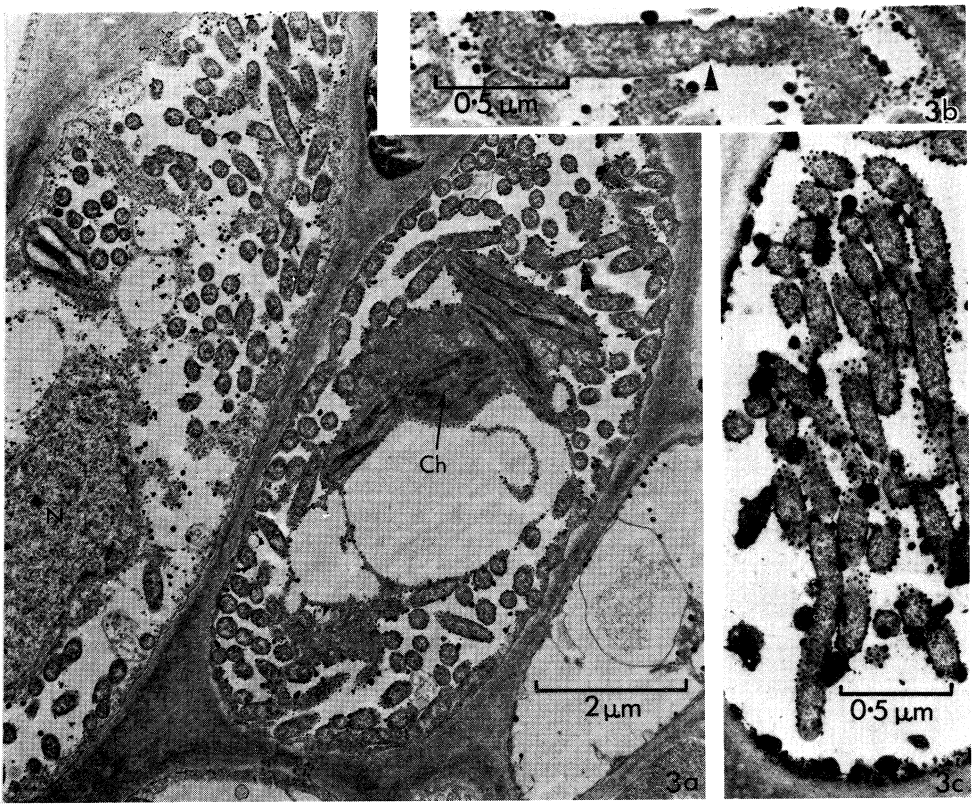


Fig. 1. Symptoms of rugose leaf curl disease in white clover. These consist of leaf curling and twisting, with rugosity of the lamina (*a*) and a reduction in leaf size, and internodal and petiole length (*b*). Healthy leaf on left in (*a*).

Fig. 2. Bacterial bodies in phloem sieve tube cells of red clover affected by RLC disease.

Fig. 3. Sections of phloem cells containing bacterial bodies in crimson clover affected by RLC disease. The phloem parenchyma cells in (*a*) containing chloroplasts (Ch) and a nucleus (N) appear to be degenerating. The presence of a constriction in one bacterial body (*a*, arrow; *b*) may indicate a body undergoing binary fission. The electron-dense droplet material present in infected cells is seen in (*c*).

Figs 4 and 5. Sections of bacterial bodies showing the cell wall structure and the homogeneous nature of internal contents in phloem sieve cells of crimson clover (Fig. 4) and red clover (Fig. 5) affected by RLC disease. The organism is seen to be bounded by a cell wall or outer membrane (OM) and inner membrane (IM) separated by a space (Fig. 5). The trilaminar structure of the membranes can be seen in the outer membrane (Fig. 4, arrow); the outer membrane or cell wall is also darker-stained than the inner membrane. Ribosome-like particles (Rb) and fibrillar material resembling DNA strands (D) can be seen in Fig. 5.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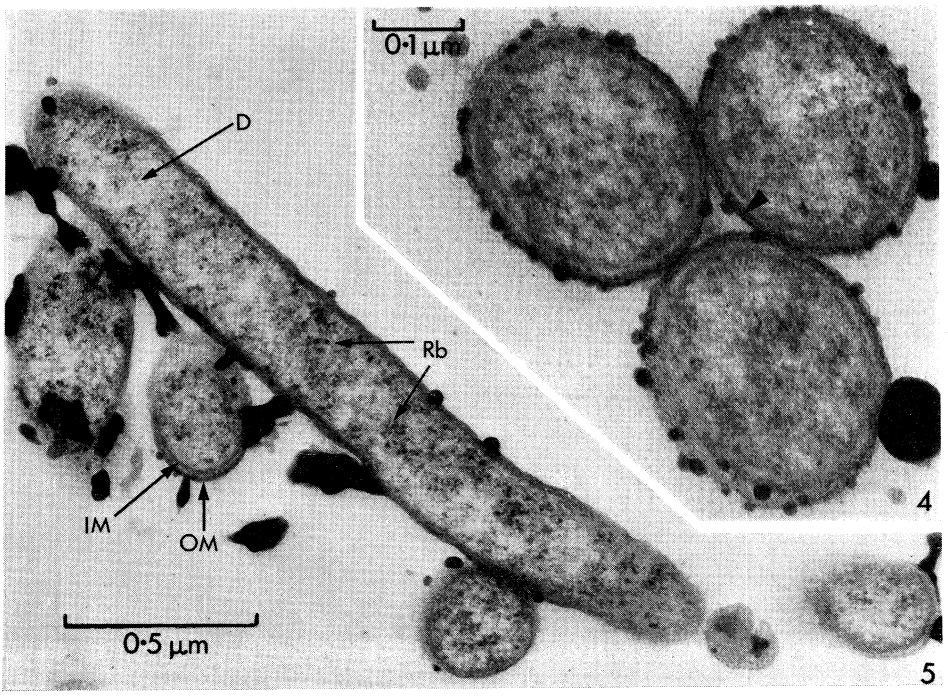
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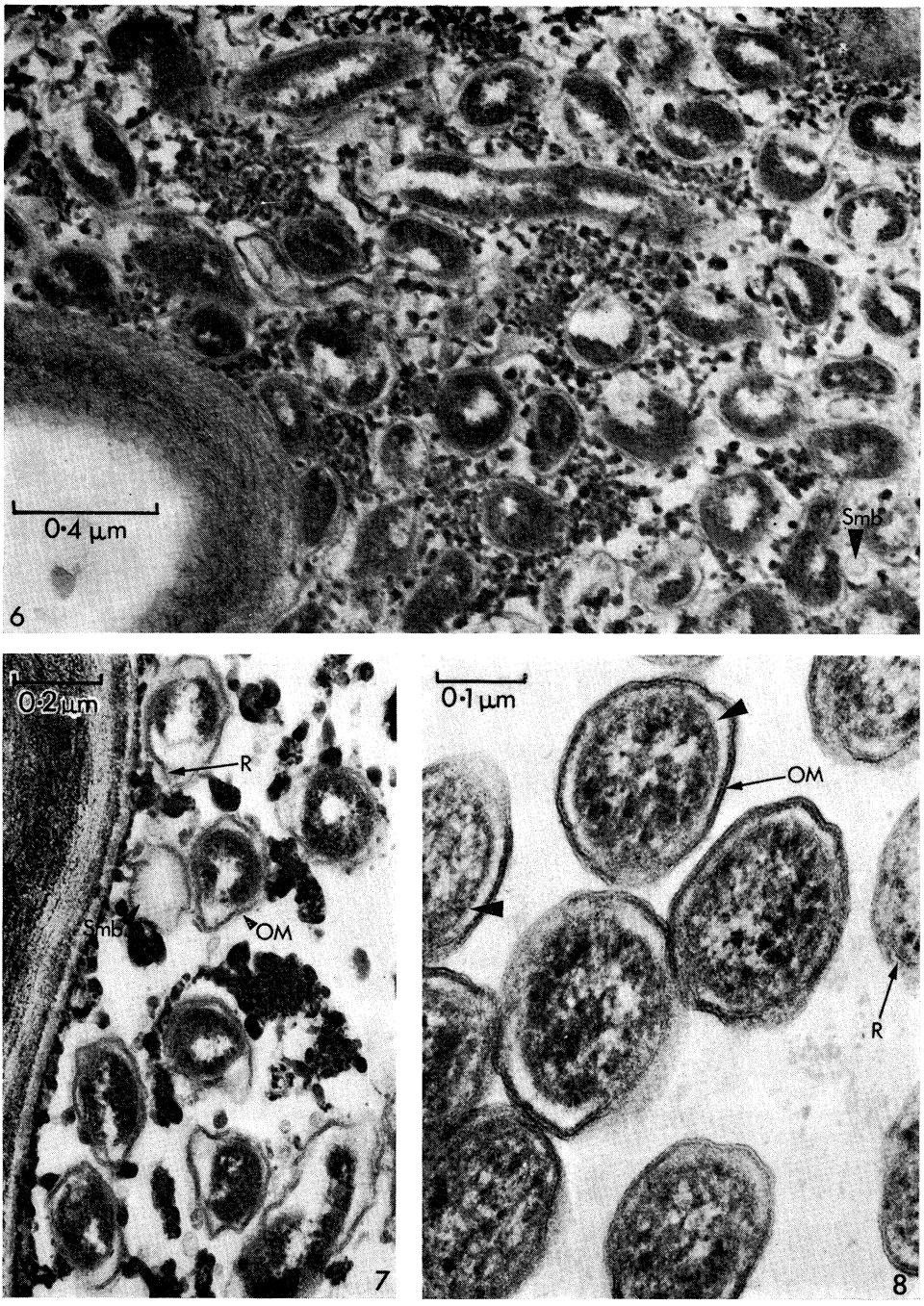

Figs 6–8. Degenerating bacterial bodies seen in compressed phloem cells of crimson clover affected by RLC disease. The inner membrane, still partly visible in Fig. 8 (arrows), contracts and becomes indistinguishable and the bacterial cell contents clump together (Figs 6 and 7). The distorted outer membrane (OM) appears to rupture (R) (Figs 7 and 8). The trilaminar membrane structure (OM) is seen clearly in Fig. 8. Apparently empty, single-membrane bound bodies (Smb) can be seen in Figs 6 and 7.

in the salivary glands of leafhoppers reared on RLC-infected clover and collected in the field. Virus-like particles have also been seen in partially purified extracts from infected red clover but none have been detected in sections of infected plant tissue (Grylls *et al.* 1974).

The present paper describes the occurrence of a small bacterium or bacterium-like organism in phloem tissue of RLC-affected clover species. A brief abstract on this association has been published previously (Behncken 1974).

Materials and Methods

Transmission and Maintenance of the RLC Pathogen

Infected material was derived from diseased white clover and *T. semipilosum* and from *A. torrida* collected from several localities in Queensland. Red and crimson (*T. incarnatum* L.) clover plants were infected by exposure to groups of 1–5 leafhoppers fed on diseased plants. Leafhopper inoculations were done in a growth cabinet at *c.* 27°C, after which the plants were fumigated and moved to an insect-proof glasshouse where they were fumigated at regular intervals. A non-infective colony of leafhoppers was maintained on red clover in a separate growth cabinet.

Antibiotic Treatment

Clonal white clover plants derived from a field-infected plant and crimson clover seedlings inoculated with a leafhopper-derived sample of the RLC pathogen were treated with penicillin in three separate experiments. Antibiotic treatments were carried out with standard sodium penicillin G (Crystapen) containing 1666 i.u./mg.

The antibiotics were applied as a soil drench at a concentration of 500 μg/ml in distilled water and at a weekly dosage rate of 60–100 ml per plant over a period of 6 weeks. Controls consisted of diseased plants treated with distilled water; in some experiments additional controls included healthy plants treated with penicillin or distilled water.

Electron Microscopy

Small pieces of lamina or petiole from leaves of red, white and crimson clover and *T. semipilosum* showing severe RLC symptoms and corresponding samples from healthy plants were fixed in cold 3% glutaraldehyde in 0·1M phosphate buffer, pH 7·2. After fixation for 2–3 h, the specimens were washed several times in buffer and then postfixed overnight in buffered 1% osmium tetroxide. The fixed specimens were stained in 1% aqueous uranyl acetate for 1 h, dehydrated through a graded acetone series, and embedded in Spurr's medium (Spurr 1969). Thin sections were cut on an LKB Ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Siemens 101 electron microscope.

Results

Alleviation of RLC Symptoms by Penicillin

In the initial experiment, five of six penicillin-treated white clover plants showed no symptoms of RLC disease one week after the last application of antibiotic. Symptoms in the sixth plant disappeared in all but a few leaflets on one stolon. Severe RLC symptoms remained in the three control plants except that one plant produced one symptomless stolon. Between 3 and 4 weeks after the cessation of penicillin treatment, severe symptoms developed on all stolons of all treated plants.

In a separate experiment, all of 20 diseased white clover plants treated with penicillin showed complete recovery from RLC disease. The first normal leaves were produced on all plants approximately 4 weeks after treatment began. An equal number of control plants treated with water retained severe symptoms. Reappearance of RLC symptoms on penicillin-treated plants commenced 5 weeks after the last treatment.

Six of 15 diseased crimson clover plants treated with penicillin died before symptom alleviation could be determined but in the remainder new leaves produced after 5–6 weeks of treatment were completely normal in appearance. Symptoms of RLC disease reappeared on seven of the treated plants about 1 month after the last treatment. The remaining two plants died before redevelopment of RLC symptoms could be expected.

Detection of the Bacterium-like Organism

A small bacterium or bacterium-like organism was found in sections of many of the phloem cells of infected red clover (Fig. 2), crimson clover (Fig. 3), white clover and *T. semipilosum*. In total, bacterial bodies were seen in sections of 15 diseased plants. No similar organism was seen in sections of phloem tissue from several healthy plants of each species.

No virus-like particles, as described by Grylls *et al.* (1974) in leafhoppers or in partially purified plant sap, were seen in any of the sections of these clover species infected with the RLC pathogen derived from either white clover or *T. semipilosum* or from leafhoppers collected from widely separated localities in Queensland.

Distribution of the Bacterium-like Organism

The bacterium-like organism was restricted to phloem tissue and was seen in sieve tube elements (Fig. 2) and, less frequently, in phloem parenchyma cells (Fig. 3*a*). In sieve tubes, most bacterial bodies occurred in mature cells devoid of cell contents and they were usually orientated at random (Figs 2 and 3).

Only some phloem elements in any one infected vascular bundle contained bacterial bodies; distribution along an infected vascular bundle was also irregular and many sections in a series were devoid of any direct evidence of infection. However, infected vascular cells invariably contained a material which bound with the strains to produce very electron-dense droplets. Serial sectioning showed that this dense staining material was specific to infected cells (Figs 2 and 3) and enabled sections to be scanned quickly at very low magnifications.

Ultrastructure of the Bacterium-like Organism

In cross section, the bacterial bodies were more or less round and most were approximately 0·25 μm in diameter (range 0·15–0·35 μm) (Fig. 4). Most were elongate and between 1 and 2 μm in length but some were longer than 3 μm.

No well-differentiated internal structures were observed in the bacterium-like organism although ribosome-like particles and faint strands of material resembling DNA were seen occasionally (Fig. 5). In most bacterial bodies, the distribution of internal contents was homogeneous.

The bacterium-like organism was bounded by a cell wall and plasma membrane, or a double membrane, both trilaminar in structure and separated by a lightly stained intermediate layer (Figs 4 and 5). The cell wall or outer membrane was usually slightly darker-stained and more clearly differentiated and had a width of 5–8 nm. The inner membrane was rarely seen with sufficient contrast for accurate measurement but approximated 5 nm in width. Between the two membranes was a space of about 5 nm but which was up to 15 nm in restricted areas of certain sections.

In longitudinal section, the outer membrane of the bacterial bodies was undulating in outline but without any apparent regularity (Fig. 5). Variation in the width of some of the bacterial bodies when seen in longitudinal profile and tapering at one or both ends indicated that the bodies were not rigid (Figs 3*c* and 5).

No clear evidence of the mode of reproduction of the organism was obtained, although some cells appeared to be constricted as if in the process of division by binary fission (Fig. 3*b*). A clear developmental sequence was not observed. Distorted bacterial forms with distinct separation between the inner and outer membranes, and with contraction of the cytoplasmic contents into darkly stained clumps, were seen most frequently in small compressed phloem cells containing dark granular or fibrous material (Figs 6 and 7). In many of these distorted bacterial bodies the inner membrane was no longer present and the cell wall or outer membrane was ruptured (Fig. 7). Some bacterium-like forms with only an outer membrane and no internal contents were observed (Fig. 7) as well as smaller, single-membrane bound bodies (Fig. 6). Other forms seen in some sections of infected white clover were oval or irregular in cross section, had a space between membranes of up to 25–30 nm, and had more homogeneous cell contents (Fig. 8).

Discussion

The alleviation of disease symptoms in RLC-affected clover plants after treatment with penicillin, and the subsequent redevelopment of symptoms on cessation of antibiotic treatment, suggest that the causal agent of this disease is unlikely to be a virus as has been claimed (Grylls *et al.* 1974). Whether the virus-like particles seen in sections of leafhoppers fed on RLC-affected plants (Grylls *et al.* 1974) are pathogenic to plants or are associated only with the vector leafhoppers is still uncertain. Virus-like particles have been reported previously in sections of several apparently healthy insects (see Richardson *et al.* 1974), including leafhoppers (Lee 1965; Herold and Munz 1967; Ammar *et al.* 1970).

Leaf symptoms of RLC disease resemble those caused by clover club leaf disease in America (Black 1944) and a similar disease of white clover in England (Markham *et al.* 1975). The pathogens of both these diseases have also been shown to be sensitive to penicillin treatment, and sections of infected plants have shown bacterium-like organisms in phloem tissue (Windsor and Black 1973*a*, 1973*b*; Markham *et al.* 1975). These organisms were mostly elongate, of similar dimensions to those seen in RLC-affected plants, and were bounded by two trilaminar unit membranes. This latter property, together with the sensitivity to penicillin, an antibiotic which acts on the bacterial cell wall (Blumberg and Strominger 1974), clearly distinguishes these organisms from the mycoplasma-like organisms found in the phloem of many plants infected by various yellows diseases (Maramorosch *et al.* 1970; Davis and Whitcomb 1971).

In recent years bacterium- or rickettsia-like organisms have been found in the vascular cells of plants affected by several diseases. Some of these organisms have been seen only in xylem cells, as in plants affected by Pierce's disease of grapevine (Goheen *et al.* 1973; Hopkins and Mollenhauer 1973), apple proliferation (Petzold *et al.* 1973), phony peach disease (Hopkins *et al.* 1973; Nyland *et al.* 1973) and ratoon stunt of sugarcane (Maramorosch *et al.* 1973; Teakle *et al.* 1973; Worley and Gillespie 1975). Other similar organisms infect only phloem cells, as in stunting

disease of dodder (Giannotti *et al.* 1970), citrus greening (Laflèche and Bové 1970) and little leaf of *Sida cordifolia* (Hirumi *et al.* 1974). A report of a rickettsia-like organism in wheat showing symptoms of chlorosis and aspermy (Ploaie 1973) did not indicate the site of infection.

The organism seen in RLC-affected plants, like the organisms associated with clover club leaf (Windsor and Black 1973*b*; Markham *et al.* 1975) and citrus greening (Moll and Martin 1974), appears to lack the layer of dense-staining material (R layer) between the inner and outer membranes seen in Gram-negative bacteria and in some xylem-restricted bacterium-like organisms (Mollenhauer and Hopkins 1974). Although the visualization of the R layer of bacteria is at least partially dependent on fixation and staining procedures (Silva and Souza 1973), the absence of an R layer in the clover club leaf and citrus greening organisms in a comparative study with two bacteria (Moll and Martin 1974) suggests that this characteristic may have some taxonomic importance.

In sections of RLC-affected plants most bacterial bodies were seen randomly scattered in phloem cells largely devoid of cytoplasmic material but it is not known if the bacterial bodies are capable of multiplying in such an extracytoplasmic environment. The distorted bacterial forms with contracted internal contents most likely represent a degenerative phase of a developmental cycle. An early event in degeneration of the bacterial bodies may involve the separation of the cell wall and plasma membrane as seen in Fig. 8, followed by the rupturing of the cell wall or outer membrane through which the internal contents escape. The single membrane of some bodies seen in association with such degenerating bacterial bodies may be remnants of the outer membranes; similar small bodies were noted by Windsor and Black (1973*b*) in cells infected with the clover club leaf pathogen. The darkly stained droplet material specifically associated with infected cells is presumed to be an enzymic secretion of the organism; similar material has been seen in association with some phloem diseases (Markham *et al.* 1975) but not in others (L. M. Black, personal communication).

Initial attempts to culture the organism from RLC-affected plants on normal bacteriological media have not been successful (unpublished data); until this can be done it is not possible to assign an etiological role to the organism shown to be associated with RLC disease.

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