Morphology of Filamentous Forms of a Mycoplasmalike Organism Associated with Hydrangea Virescence

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ABSTRACT

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The hydrangea virescence agent was graft-transmitted but not mechanically transmitted in *Hydrangea macrophylla*, the florists' hydrangea. Transmission of the virescence agent was not achieved with the leafhopper, *Macrosteles fascifrons*, or dodder, *Cuscuta campestris*. Mechanical inoculations of healthy hydrangea seedlings with hydrangea ringspot virus failed to induce virescence symptoms. A mycoplasmalike organism (MLO) was observed in ultrathin sections of florists' hydrangea showing flower proliferation and virescence. Spherical and polymorphic mycoplasmalike bodies of varying size and cell content were present in mature sieve cells. The MLO also commonly occurred as branched filaments, beaded filaments, and spherical bodies connected in long chains by a continuous membrane structure. The filamentous forms resembled those observed by Freundt in the culture of the animal pathogen, *Mycoplasma mycoides*. "Octopus-like" structures, consisting of numerous filaments attached to a central body that was devoid of protoplasm, were common in rapidly declining plants.

Additional key words: electron microscopy, hydrangea phyllody, *Hydrangea macrophylla* 'Strafford'.

A green flower abnormality in florists' hydrangea [*Hydrangea macrophylla* (Thunb.) Ser.] was found to be graft-transmissible in 1931 by Muth (18). Similar green flower symptoms have been referred to as hydrangea phyllody, hydrangea proliferation, and hydrangea virescence (2, 4, 17, 18, 25, 27). The causal agent has not been established. Brierley and Smith (4) reported the transmission of aster yellows agent from diseased hydrangea to China aster [*callistephus chinensis* (L.) Nees] with a leafhopper. Mycoplasmalike organisms (MLO's) have been observed in ultrathin sections of diseased hydrangea (17, 25). Welvaert et al. (25) produced green flowers in *Vinca rosea* when a MLO was transmitted from *Hydrangea* to *Vinca* via *Cuscuta subinclusa*. However, hydrangea virescence was also attributed to hydrangea ringspot virus (HRSV) (27).

We have further investigated the etiology of hydrangea virescence and observed the morphology and ultrastructure of the MLO associated with the disorder.

MATERIALS AND METHODS

Approximately 20 1-year-old Strafford hydrangeas with abnormal green flowers (Fig. 1) were obtained in 1973 and 1974 from a New Jersey grower. The severity of the symptoms varied among the plants. Plants with mild symptoms had either normal pink cymes and abnormal pink-and-green cymes, or all green cymes. The florets were full-sized or only slightly dwarfed, the cymes were large, and the foliage was normal. Plants with severe symptoms showed green, often dwarfed, cymes and dwarfed florets with leafy shootlike structures proliferating from the pistils (Fig. 2). In addition to the flower symptoms, these plants had dwarfed leaves with a conspicuous vein yellowing.

We maintained the hydrangeas in the greenhouse during the growing season and placed them in cold storage at 4 C in the dark for 6 weeks during November and December. When returned to the greenhouse after cold storage, the plants rapidly produced new growth and bloomed in 10-12 weeks. The symptom development on each hydrangea was recorded throughout each flowering season until the plants died.

Several times during the experiments, hydrangeas were indexed on *Gomphrena globosa* L. for HRSV and on *Chenopodium quinoa* Willd. and *Nicotiana tabacum* L. for tomato and tobacco ringspot viruses and cucumber mosaic virus.

Graft transmission.—Buds from the vegetative shoots of naturally-infected Strafford hydrangeas were grafted into HRSV-free Strafford hydrangeas and to HRSV-free hydrangeas of the cross Kunnert × Blue Lace Cap.

Insect transmission.—The leafhopper *Macrosteles fascifrons* (Stål.) was tested as a possible vector of the virescence agent. First- and second-instar nymphs from colonies maintained on rye (*Secale cereale* L.) were transferred to the green flowers of diseased hydrangea. After acquisition feeding intervals of 3-7 days, the insects were returned to rye and then transferred to China aster, *Callistephus chinensis* (L.) Nees 'American Beauty' mixed. The insects were then transferred weekly from aster to aster until all insects were dead. Asters were observed 6 weeks for symptom development.

Macrosteles fascifrons (Stål.) known to transmit aster yellows were also placed on four hydrangea plants of the cross Rose Supreme × Sainte Theresa. The hydrangeas were observed for virescence symptoms when they flowered the next year.

In further tests, an extract (1:2, w/v) was prepared from green hydrangea flowers in 0.3 M glycine, 0.01 M $MgCl_2$

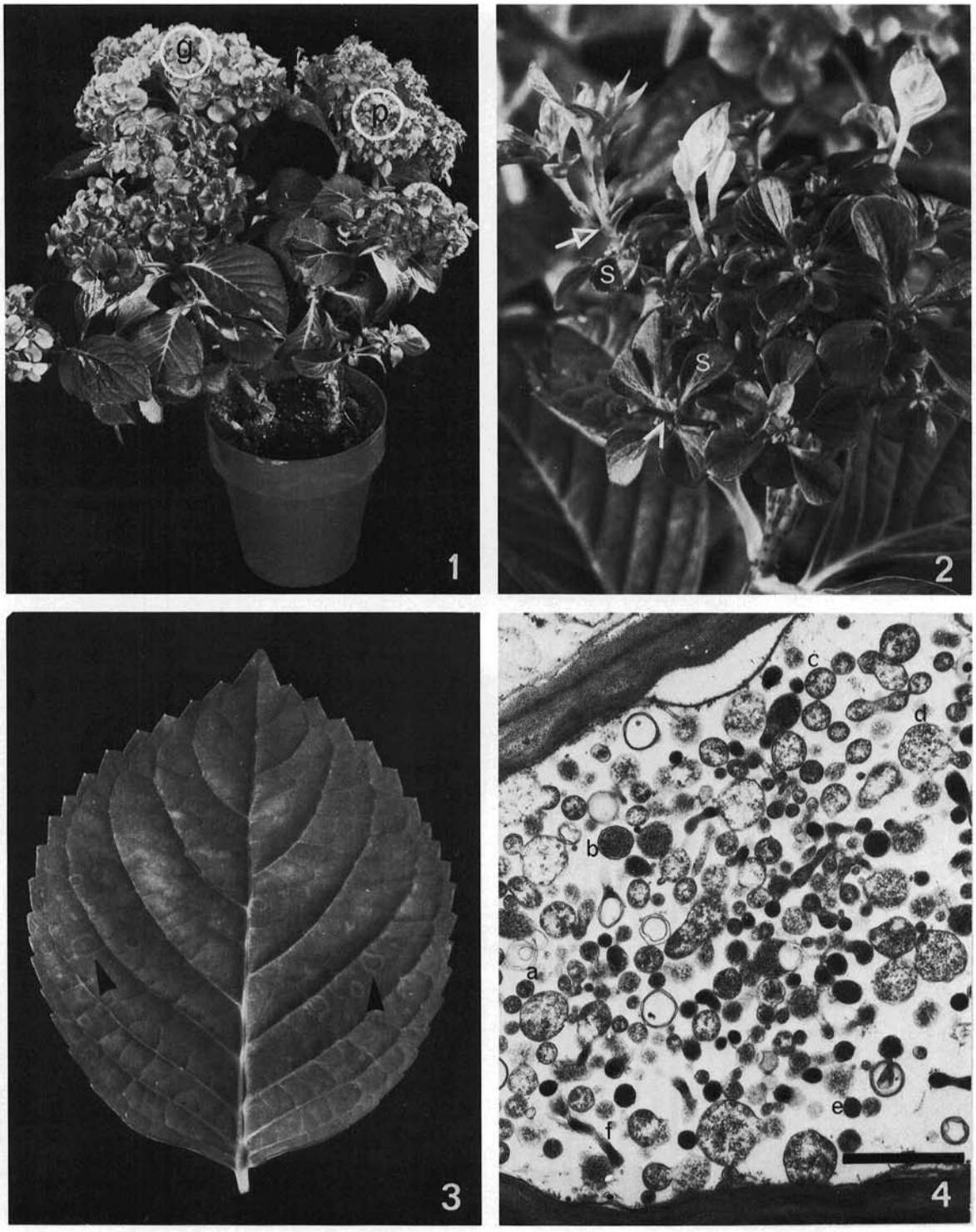


Fig. 1-4. Symptoms of hydrangea virescence and hydrangea ringspot in hydrangea and the mycoplasmalike organism (MLO) associated with hydrangea virescence. **1)** *Hydrangea macrophylla* 'Strafford' affected with hydrangea virescence. Bright green cymes (g) and proliferation of leafy structures from dwarfed florets (p) are typical disease symptoms. **2)** Dwarfed green florets showing various stages of proliferation. Early stage in elongation of the pistil (➢). Branched leafy proliferation (⊸▷) above green sepals (s). **3)** Chlorotic ringspots on the leaf of a hydrangea seedling mechanically inoculated with hydrangea ringspot virus. **4)** Mycoplasmalike bodies (MLB's) in a mature sieve cell of hydrangea affected with hydrangea virescence. The cell contains (a) small spherical bodies, (b) larger spherical bodies with ribosomelike particles and moderately staining cytoplasm, (c) large spherical bodies with threadlike strands, (d) large body with bleb or protrusion, (e) very densely stained small bodies, and (f) filamentous forms. (× 19,000) Bar is 1 μm.

and 0.01 M Na_2SO_3, pH 7.2. The extract was clarified by low-speed centrifugation at 2,000 *g* for 5 minutes. The supernatant solution was injected into leafhoppers that were subsequently tested for their ability to induce symptoms in China asters.

Dodder transmission.—*Cuscuta campestris* Yunck. was maintained on healthy tobacco. Subcolonies on tobacco were attached to infected or healthy hydrangea and then to healthy hydrangea [*Hydrangea macrophylla* (Thunb.) Ser.], China aster [*Callistephus chinensis* (L.) Nees], zinnia (*Zinnia elegans* Jacq.), periwinkle (*Vinca rosea* L.), tobacco (*Nicotiana tabacum* L.), and celery (*Apium graveolens* L.).

Dodder was left on the plants for approximately 2 months before the connections were cut. All plants were observed several weeks longer for symptom development except hydrangeas which were observed through the next flowering season.

Hydrangea ringspot virus.—Two local lesion isolates of HRSV were obtained by mechanically inoculating *Gomphrena globosa* with a sap extract from leaves of HRSV-infected hydrangeas which also showed virescence. Each isolate was increased in *Gomphrena* and mechanically inoculated to five 1-year-old healthy hydrangea seedlings of the cross Kunnert × Blue Lace Cap. Five healthy seedlings were also inoculated with sap from a naturally-infected virescent hydrangea without passage through *Gomphrena*. All seedlings were indexed on *Gomphrena* prior to inoculation and cuttings were taken for controls. Four months after inoculation, healthy and inoculated seedlings were assayed again on *Gomphrena*. The seedlings were observed one year for flower and foliage symptoms.

Electron microscopy.—Sections were made of healthy and infected hydrangeas several times during the study. Plants exhibiting a range of symptoms were included in one sampling. Some plants were also sampled at different times during the growing season. Tissue pieces with vascular bundles were taken from pink, green, dwarfed, and normal-sized florets, from proliferation structures, and from chlorotic, dwarfed leaves. The tissue pieces were fixed for 2 hours in a cold, pH 7.2, phosphate-buffered mixture of 2% glutaraldehyde and 1.5% acrolein. They were postfixed in cold, buffered 1% OsO_4 for 2 hours, dehydrated in a graded ethanol and water series, and infiltrated with Epon 812 using propylene oxide. Sections were cut approximately 60 (pale gold) and 200 (purple) nm thick, and stained with aqueous uranyl acetate and lead citrate.

RESULTS

Symptom development.—The symptoms in the naturally-infected hydrangeas increased in severity and the plants continued to decline. Few plants lasted more than two or three years. Plants with mild flower symptoms and normal foliage at the time of blooming often produced leaves with vein yellowing after flowering. The following year they showed severe symptoms in the flowers and foliage. Plants in advanced stages of decline produced only small clusters of rudimentary proliferating florets without sepals. New succulent branches were not produced and clusters of tiny chlorotic leaflets with vein yellowing formed at the bud positions along the old woody stems. The plants were extremely sensitive to heat and water stress.

Graft transmission.—We were able to produce hydrangea virescence in the two cultivars of hydrangeas grafted with buds from naturally-infected Strafford hydrangeas. Plants developed mild to severe symptoms six weeks to several months after grafting.

Insect transmission.—In 10 attempted transmission tests involving more than 300 asters, no China asters developed symptoms when exposed to leafhoppers previously fed on virescent hydrangea flowers. Leafhoppers injected with the green flower extract also failed to induce virescence symptoms in China asters. No hydrangeas exposed to leafhoppers, known to transmit aster yellows, developed virescent flowers the season following exposure to the insects.

Dodder transmission.—All transmission tests with dodder were negative. However, growth of dodder on healthy and virescent hydrangeas was poor. We cannot conclude that the tested plants are not hosts for the virescent agent.

Hydrangea ringspot virus transmission.—Gomphrena bioassays confirmed that HRSV was transmitted to the 15 mechanically inoculated hydrangea seedlings but no symptoms were observed in that growing season. Chlorotic ring spots formed only on some leaves of some plants after cold storage (Fig. 3). No symptoms of greening or proliferation developed in the cymes.

Electron microscopy.—Mycoplasmalike bodies (MLB's) were difficult to find in plants showing mild symptoms. They were most abundant in proliferations, small green florets, and small chlorotic leaves breaking from lateral shoots of hydrangeas with severe symptoms. The MLB's were found only in mature sieve cells. The MLB's could generally be classified as spherical and filamentous. Both forms were bounded by a trilaminar membrane about 8 nm wide.

The size and cytoplasmic appearance of the spherical bodies varied (Fig. 4). This variation which is described in detail by Hirumi and Maramorosch (9) seems to be a feature common to plant MLO. In general, the small spherical bodies that we observed contained a homogeneous, densely stained cytoplasm and closely packed ribosomelike particles (RLP's), whereas the larger bodies contained a lightly stained cytoplasm, scattered RLP's and DNA-like strands. The large bodies were more polymorphic than the smaller bodies and often had buds or spherical protrusions. Vacuoles, but not small MLB's, were occasionally seen in the larger MLB's. Some bodies, particularly small spheres and filaments, were so dense that the internal structure could not be discerned.

Filamentous forms were unusually abundant in tissues sampled when diseased plants flowered in the spring. Filaments filled some sieve cells (Fig. 5) and commonly occurred with spherical forms in other sieve cells. The filaments were 70-110 nm wide and usually bulged into a rounded or bulb-shaped structure at the tip (Fig. 6). A maximum filament length was not established though a segment of 4 μm was observed in thick sections. Branching of the filaments was also observed. A moderate- to dense-staining cytoplasm and numerous RLP's were evenly dispersed within the filaments. However, within segments of some filaments, dense-staining cytoplasm and RLP's accumulated in oval or

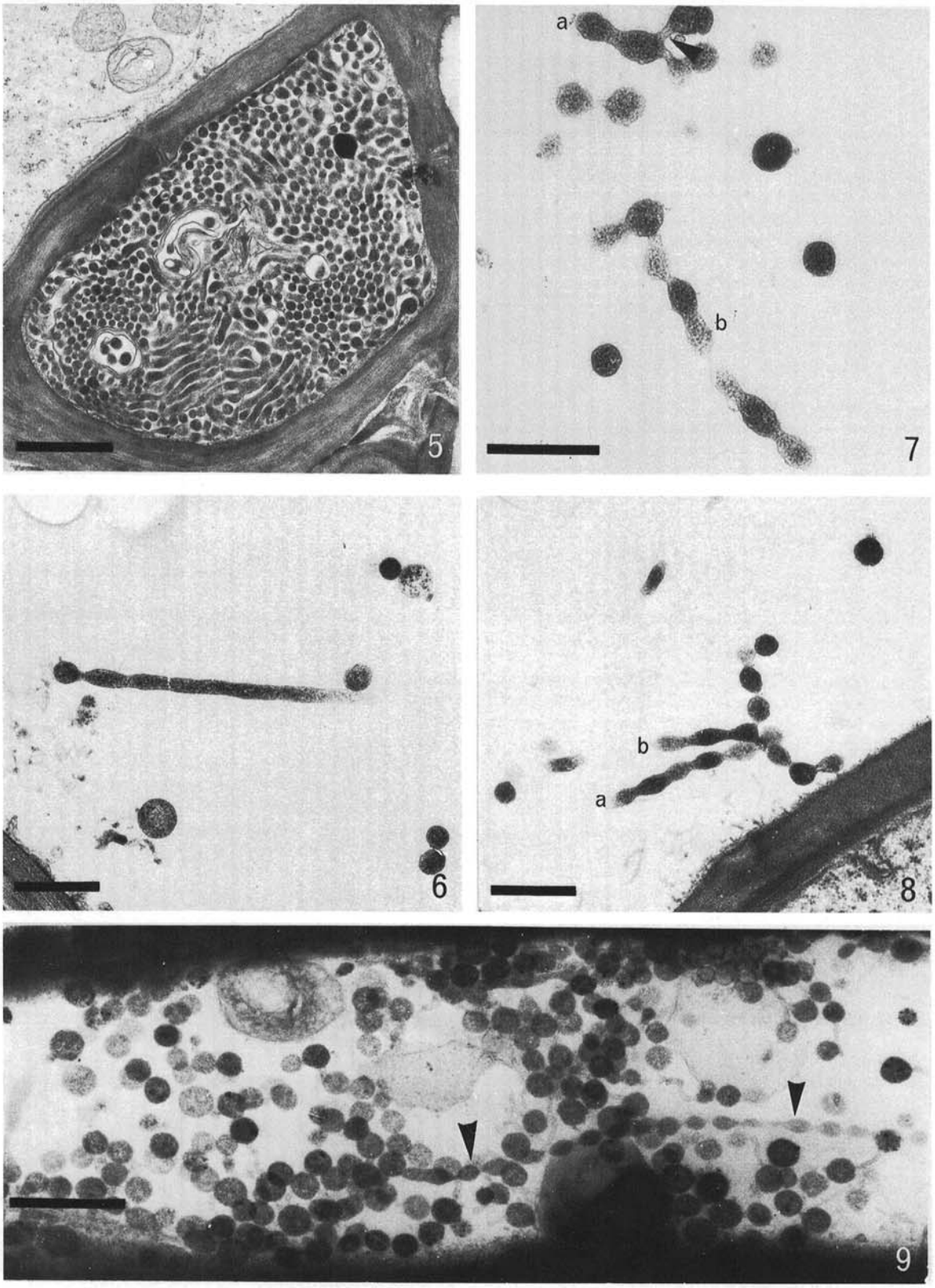


Fig. 5-9. Beaded and unbeaded filamentous forms of the MLO associated with hydrangea virescence. **5)** A sieve cell completely filled with filamentous forms. (× 16,400) Bar is 1 μm. **6)** Segment of a filament showing an even distribution of cytoplasm and RLP's within the filament and a terminal bulb structure. (× 28,100) Bar is 0.5 μm. **7)** Filaments showing stages of beading. The cytoplasm is condensing or accumulating in evenly spaced pockets and the membrane is in the beginning (b) and advanced (a) stages of constriction between the beads. The membrane is continuous over the length of the filament. (►) Elongation or drawing out of a constricted membrane structure which is void of dense-staining matrix and RLP's. (× 37,500) Bar is 0.5 μm. **8)** Unbranched (a) and branched (b) filaments in the beginning and advanced stages of bead or sphere formation. (× 28,100) Bar is 0.5 μm. **9)** Thick section of a sieve cell showing a segment of a beaded filament about 4 μm long. (× 19,200) Bar is 1 μm.

spherical pockets at regularly spaced intervals (Fig. 7, 8). The filament membrane was constricted between the pockets. Various degrees of constriction were observed. Some filaments were slightly invaginated (Fig. 7); other filaments were pinched into chains of discrete oval or spherical bodies with diameters of 100-150 nm (Fig. 8, 9). No RLP's or stainable cytoplasm remained in the 30-40 nm space between the most severely constricted membranes and the constrictions were slightly elongated or drawn out between some of the beads (Fig. 7).

Similar, but more elongated, membrane structures also connected spherical bodies into long chains (Fig. 10). The bodies along a chain were approximately the same size, but their diameter as determined in serial section varied from approximately 150-300 nm in different chains. The length of the connecting membrane structure varied also, but the membrane was continuous around the spherical bodies. The membrane connections and the gradation in size of the spherical bodies suggested that the chains were derived from the beaded filaments. Presumably the spherical bodies might be liberated from the chain by disintegration of the thin connecting membrane strands.

The observations of filaments suggested that the organism in hydrangea might be a spiroplasma, but we have seen no spiral or helical filaments in thin or thick sections or in crude sap examined by phase-contrast microscopy.

An additional MLO form was abundant in rapidly declining plants sampled later in the summer (Fig. 11, 12, 13). The structures consisted of numerous filaments extending from the base of an oval or egg-shaped central body and resembled a many-armed octopus (Fig. 12, 13). The central membrane-bound body lacked stainable cytoplasm and RLP's and some measured up to 1 μm in diameter. As many as 12 filaments of varying lengths radiated from one body (Fig. 13-a). The membrane of the body was continuous with the filament membranes; yet unlike the central body, the filaments contained cytoplasm and RLP's (Fig. 13). Some filament extensions were constricted or beaded (Fig. 12-a). The extensions measured approximately 70-200 nm in diameter. Depending on the plane of sectioning, the octopus-like structure appeared as a void vesicle, as a ring with knoblike projections, as a circle of radiating filaments, as a circle of small dense bodies, or as a bleb with one or more filaments (Fig. 11, 12, 13).

Bodies superficially resembling spherical mycoplasma (RMB) were found infrequently in xylem and phloem parenchyma cells (Fig. 14). The bodies were usually in the vascular parenchyma of the green flower proliferations within dilated endoplasmic reticulum (ER) and perinuclear spaces. Similar bodies have been observed in the phloem parenchyma and companion cells of a young leaf of white clover affected by clover dwarf (13). The author (13) considered the bodies to be MLO's. Significant differences in structure were detected in the RMB's and the MLB's in virescent hydrangea (Fig. 15). The RMB's (Fig. 15-a) were surrounded by a membrane, but a trilaminar membrane structure was not resolved; the MLB's showed a clearly defined trilaminar membrane (Fig. 15-b). The RMB's contained RLP's that were often concentrated along the membrane and similar in size to the hydrangea ribosomes; the MLB's contained randomly dispersed ribosomes that were slightly smaller than hydrangea ribosomes. Cells that contained RMB's showed signs of deterioration including extreme dilation of the perinuclear space and ER and evagination of the membranes that were lined with ribosomes. We therefore concluded that the RMB's were probably not MLO's. They may be derived from membrane evaginations and pinched-off cytoplasmic masses. Vesiculations resembling MLO's and formed in the perinuclear space have similarly been attributed to a cytopathic response of the nuclei in pea (*Pisum sativum* L.) to pea enation mosaic virus infection (5).

DISCUSSION

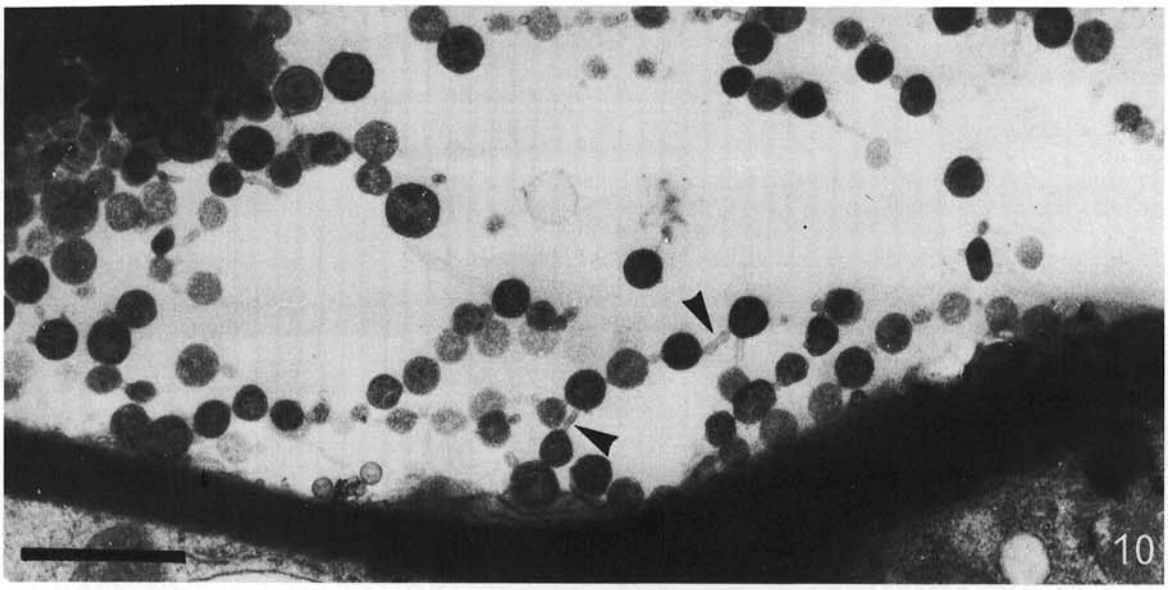
We confirmed the graft-transmissible nature of the hydrangea virescence agent, but were unable to demonstrate a relationship between the disease and aster yellows. Failure to transmit the virescence agent using leafhoppers or dodder may have resulted from the poor survival of the tested vector species on hydrangeas. Transmission of a MLO from virescent hydrangeas to *V. rosea* using *C. subinclusa* (25) indicates that this species of dodder may be a better parasite of hydrangea than *C. campestris*.

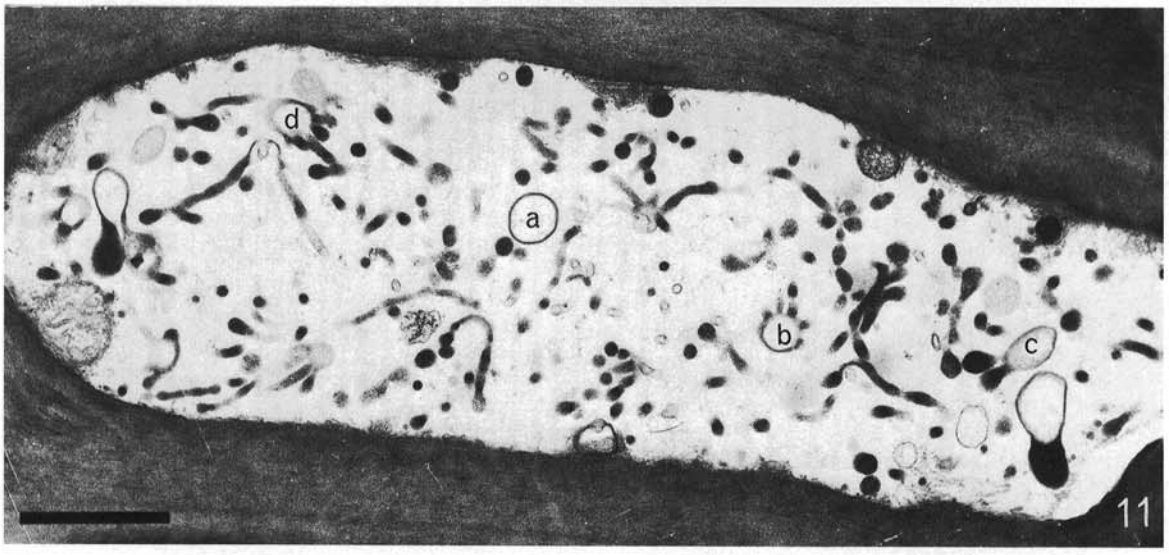
We detected HRSV in most of our naturally-infected virescent hydrangeas, but HRSV did not induce virescence in inoculated seedlings. Our data and the HRSV transmission work of others (3, 10, 20) indicate that HRSV does not induce phyllody or virescence in hydrangea.

Since plant mycoplasmas have not been cultured, it is useful to compare in situ observations of plant MLO with observations of cultured animal mycoplasmas. Filamentous cells are recognized as characteristic of some animal mycoplasmas as *A. laidlawii* and *M. mycoides* (8, 14). Freundt (7, 8) proposed a cycle of growth for *M. mycoides* based on a filamentous form. The process of growth includes condensation of the cytoplasm within the filament into "coccoid" elements or "young elementary bodies", then constriction, drawing out, and finally disintegration of the membrane structure between the formed spherical bodies. Freundt's theory of endomycelial fragmentation has been challenged (14, 16) because the size of the elementary body seems too small (6) to carry a complete mycoplasma genome.

The MLO associated with hydrangea virescence is of interest because the numerous filamentous forms resemble the filamentous forms of some animal mycoplasmas, particularly those of *M. mycoides* as described by Freundt. The filaments in hydrangeas occur in young growing tissue and when the agent is easily graft transmitted. Therefore, the forms may represent the MLO in the log phase and not the decline phase of growth.

Filaments, elongated forms, and cylindrical forms have been recognized in ultrastructure studies of a few plant MLO's (15) as the MLO's associated with peach yellows (12), Western X-disease (19), clover phyllody (11, 23) and aster yellows (9, 26). However, the structures in the micrographs are not identical and their function is not understood. Ultrastructure studies have indicated that filaments may be important in the growth and reproduction of the clover phyllody (11, 23) and aster yellows (9, 26) MLO's. Condensation at the end of the





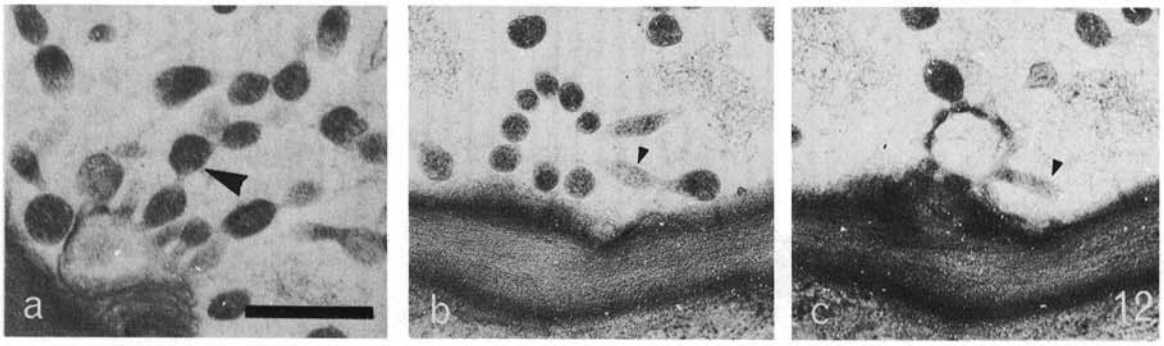
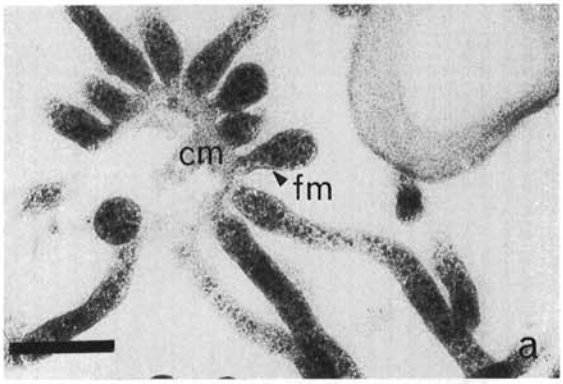


Fig. 10-12. Chains of spherical bodies and octopus-like forms of the mycoplasmalike organisms associated with hydrangea virescence. **10)** Thick section of a sieve cell showing chains of spherical bodies connected by drawn-out membrane structures (►). (× 21,600) Bar is 1 μm. **11)** Configurations observed when octopus-like structures found in declining plants are sectioned in different planes: (a) cross-section through the central body showing lack of cytoplasm; (b) slightly oblique section through the base of the central body showing the point of filament attachment; (c) longitudinal sections; and (d) oblique sections through the filaments at the base of the central body. (× 19,900) Bar is 1 μm. **12)** a) Longitudinal section of an octopuslike structure showing chains of small spheres attached to the central body. b and c) Serial cross-sections of an octopuslike structure showing the filament arrangement (b) below the central body and (c) at the point of connection to the central body. (►) Portion of the same filament in each section. (× 33,600) Bar is 0.5 μm.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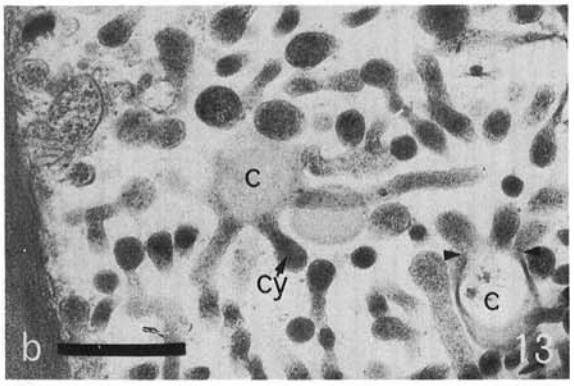
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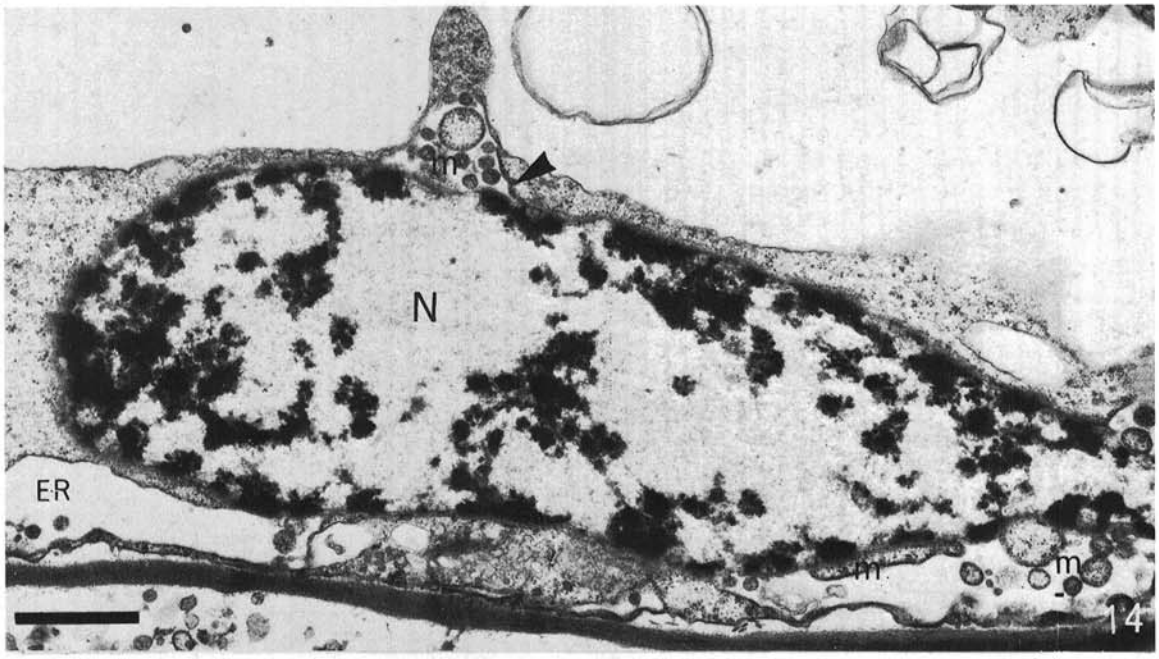
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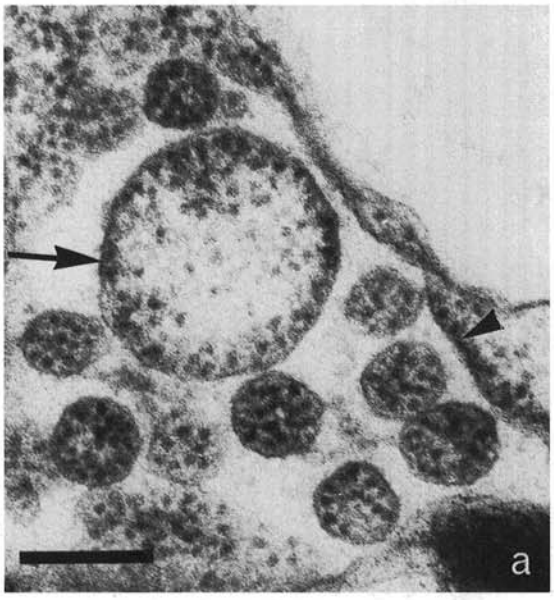
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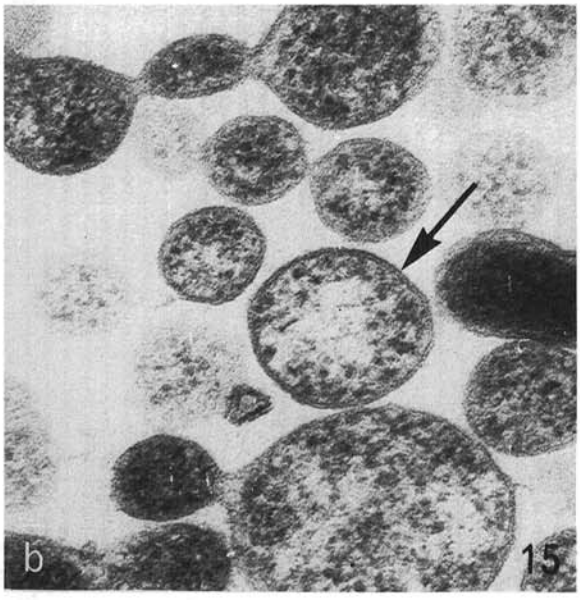
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Fig. 13-15. Octopuslike mycoplasmalike organisms (MLO), bodies superficially resembling MLO, and spherical MLO in hydrangea with hydrangea virescence symptoms. (13-a,b) Octopus-like structures in longitudinal and cross-section. **13-a)** Section showing the continuity of the filament membrane (fm) and the central body membrane (cm), the number and arrangement of filaments at the base of the central body, and the variation in filament length. (× 53,000) Bar is 0.25 μm. **13-b)** The central bodies (c) contain no recognizable cytoplasm or RLP's through the membrane around the central body is continuous with the filament membranes. Filaments contain cytoplasm with RLP's (cy). (▶◀) The plane of the cross-section in relation to the longitudinal section. (× 33,600) Bar is 0.5 μm. **14)** Bodies (m) in the perinuclear space of the nucleus (N) and in dilated endoplasmic reticulum (ER) of a xylem parenchyma cell in a proliferation. Note the ribosomes or RLP's lining the parenchyma cell membranes and the membranes of the bodies, the elongated body (m) at the lower right, and the outer nuclear membrane. (15-a,b) Comparison of **15-a)** bodies in the perinuclear space and **15-b)** mycoplasmalike bodies (MLB's) in a mature sieve cell. Outer nuclear membrane lined with ribosomes (▶). Note the trilaminar structure of the MLB membrane (⟶) which is not seen in the bodies of a). (× 87,400) Bar is 0.2 μm.

filament to produce a new elementary body was proposed as a means of reproduction for the clover phyllody MLO (23). Like those of the hydrangea MLO, the filamentous forms of the aster yellows MLO in tobacco (9, 26) are compatible with Freundt's proposed mode of filamentous growth and replication. But the filaments in tobacco were reported to form (9) at any developmental stage of the MLO and to reflect growth conditions and the age of the original spherical body from which the filaments emanated. We observed ultrastructure variations in filaments, but our data are not sufficient to confirm a similar development (9) for the hydrangea MLO.

In addition to filamentous forms, we have observed spherical and polymorphic bodies in virescent hydrangea; however, we have made no attempt to classify these forms as elementary, intermediate, or mature forms of a MLO (23). Although we observed protrusions on and constrictions in the large MLB's, this is not adequate proof of budding or binary fission.

The significance of the octopus-like structures was unresolved. They may be analogous to asterodiscules (24). The structures also resemble forms of *M. pneumoniae* released with lipase from organism aggregates (1). Medium composition, the conditions of growth, and the age of the culture influence the morphology of cultured mycoplasmas (1, 8, 21, 22). Plant and environmental conditions may affect the morphology of MLO's in situ. The occurrence of the multifilamentous structures in declining hydrangeas suggests that the forms represented a stationary or death phase morphology of the organism. Although the significance of the bodies is unknown, the large structures may facilitate detection of the MLO in crude sap or culture media.

Our observations of many forms of the MLO in virescent hydrangea indicate that the organism has a complex life cycle. Filamentous forms may constitute an important segment of the growth and reproduction of this MLO as well as that of other plant and animal mycoplasmas. Correlation of the forms observed in situ and in cultures will be necessary to establish the life cycle.

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