

Effects of Dark Treatment on the Ultrastructure of the Aster Yellows Agent in Situ

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ABSTRACT

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Effects of dark treatment on the ultrastructure of mycoplasma-like organisms (MLO) in aster yellows-affected plants were investigated. After a 7-day dark treatment, some MLO containing low-electron-density material with "inner bodies" or with a membrane-bounded vacuole were found in the cytoplasm or in the lumen of a sieve-tube element; some were irregular in shape and had electron-dense cytoplasm or

ring forms outlined with double unit membranes in necrotic cells. Examination of serial sections suggested that the inner bodies were completely separate MLO trapped in invaginations in other MLO, and that the ring form derived from invagination of electron-dense cytoplasmic bodies. The dark treatment increased the prevalence of invaginations in both the so-called normal and abnormal bodies of MLO.

Additional key words: mollicutes, mycoplasma-like organisms, dark treatment, degradation.

In recent years, natural *in vitro* degradation of mycoplasma-like organisms (MLO) has been studied by electron microscopy of thin-sectioned host tissue, and the following speculations about its causes have been presented (3, 4, 5, 6): (i) that MLO age naturally, (ii) that MLO are altered by the changing physiology of diseased host cells, (iii) that nutrient availability to individual MLO declines with overcrowding, and/or (iv) that accumulated metabolic toxins of MLO become limiting. Degradation of MLO due to certain artificial treatments, such as chemical and heat treatments, also has been reported (7, 10, 11). Similar observations were made with animal-infecting mycoplasmas. Morphological changes reported include bulbous membrane-bound projections (8), formation of a variety of vesicles and granular bodies (9), and formation of a vacuole or trilayered compound membranes (1, 2) in older cultures of MLO or during their death phase. However, at this time, the sequence of events in the natural degradation of mycoplasma is not understood.

The present study describes the changes in the ultrastructure of MLO associated with their degradation in infected plants kept in darkness at 26 C for 7 days.

MATERIALS AND METHODS

Infected plants.—Thirty healthy 2-mo-old aster plants (*Callistephus chinensis* Nees) of the same growth stage, were exposed for 3 days to about 250 six-spotted leafhoppers (*Macrostelus fascifrons* Stål) that had been fed for 7 days on aster plants infected by the Alberta

isolate of aster yellows agent (Chen and Hiruki, *unpublished*). Then the plants were kept in a growth incubator maintained at 26 C with a light intensity of 10,800 lx (1,000 ft-c) until symptoms were developed.

Dark treatment.—Infected plants were subjected to dark treatment after 3-day, 7-day, and 14-day periods of typical vein-clearing, an initial symptom of aster yellows. In each infection stage, six diseased plants were selected; three plants were kept in total darkness for 7 days; the remaining three plants were kept in an incubator with light at 10,800 lx as controls. Similar light and dark treatments were applied to the same number of healthy control plants.

Electron microscopy.—One-half of a leaf showing typical aster yellows symptoms was sampled from each diseased plant before and after the treatments. Similar portions were obtained from both diseased and healthy control plants. Vein tissues were excised and fixed with 3% paraformaldehyde-glutaraldehyde in 0.2 M Millonig's buffer (pH 7.4) for 3 hr. After being rinsed in the same buffer solution at 4 C for overnight, the materials were postfixed with 2% osmium tetroxide 1% sucrose in 0.2 M Millonig's buffer (pH 7.4) for 4 hr. They were embedded in Araldite after dehydration. Thin-sections were stained with uranyl acetate and lead citrate. Micrographs were taken with a Philips Model 200 electron microscope operated at 60 or 80 KV for magnifications ranging $\times 700$ -19,400 and were enlarged photographically as required.

RESULTS

The dark treatment produced etiolation in most plants, including the healthy control. Starch grains were found in

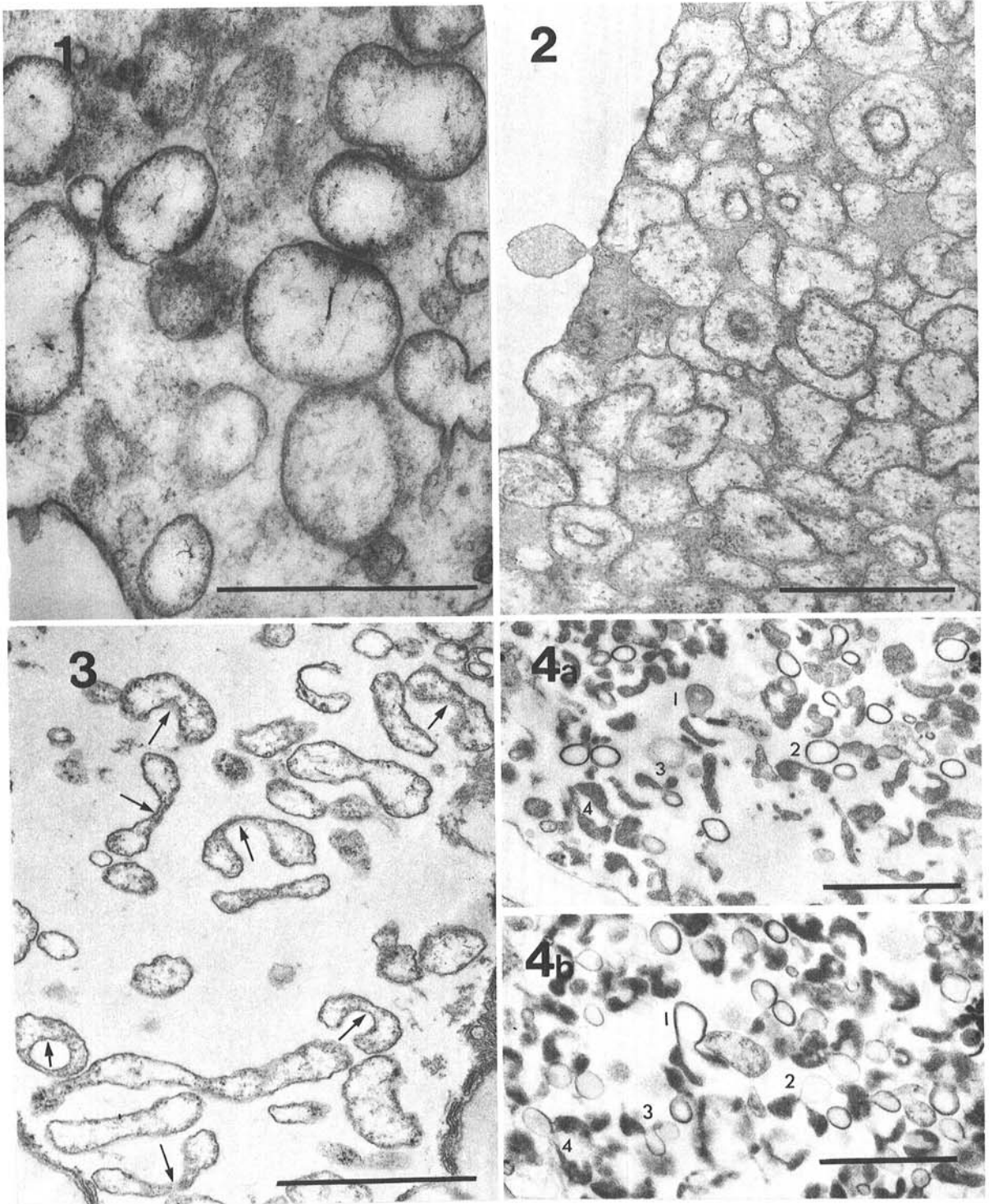


Fig. 1-4. 1) Mycoplasma-like organisms (MLO) in aster. Normal forms consisting of the nucleoid (DNA strand) devoid of membrane; ribosomes of the bacterial type; and the unit membrane. ($\times 42,000$). Bar is $1\ \mu\text{m}$. 2) After a 7-day dark treatment, which started 7 days after symptom development, the cytoplasm of a sieve tube cell of an infected aster plant is packed with MLO containing low-electron-density material. Inner bodies are present in some of the MLO ($\times 28,000$). Bar is $1\ \mu\text{m}$. 3) Various degrees of invagination are evident in some MLO in the large lumen of a sieve tube cell from an aster plant transferred to the dark for 7 days 3 days after symptom development ($\times 32,000$). Bar is $1\ \mu\text{m}$. 4a-b) Successive serial sections of a necrotic sieve tube cell, showing ring-, club-, and dumbbell-shaped forms all of which are associated with high-electron-density bodies. The numbers indicate structural relationships between the two sections, a) and b) ($\times 22,000$). Bar is $1\ \mu\text{m}$.

chloroplasts of parenchyma cells in the vicinity of the vascular bundle in the healthy control plants. Starch accumulation was particularly evident in the diseased control plants. However, no such accumulation was detected in any chloroplasts of the diseased and treated plants nor of the healthy treated control plants. Examination of sectioned phloem cells of diseased control plants revealed so-called normal MLO which represent a simple pleomorphic structure consisting of the nucleoid (DNA strand) devoid of membrane; ribosomes of the bacterial type, 12-13 nm in diameter; and the unit membrane, 8 nm thick (Fig. 1). On the other hand, dark-treated plants at any infection stage contained, in addition to the MLO with low electron-dense cytoplasm (Fig. 2, 3), ring-shaped forms with an empty central space (Fig. 4-a, b; 6). The ratio of the number of sieve tube cells containing abnormal MLO to the number containing normal MLO for the 3- to 14-day infection period was

135/231; that is 36.8% of the total sieve tube cells contained MLO. By contrast, comparable infected samples not subjected to a dark treatment yielded a ratio of 3/190 which is 1.6% of the total cells examined. In general, abnormal MLO were first observed in a group of cells in the area of primary phloem tissue. Overall, in this investigation abnormal MLO rarely were detected in diseased plants not subjected to a dark treatment, and never in healthy plants.

The higher magnifications revealed that most MLO in phloem cells of dark-treated plants had a vacuole with a single unit membrane and contained an inner body (Fig. 2, 5). These profiles usually occurred when the cytoplasm of sieve cells was overcrowded with MLO. A vacuole-like space with a unit membrane or an invagination usually was observed when MLO occurred scattered in the large vacuole of sieve cells (Fig. 3, arrows). From a serial section (Fig. 5) the inner bodies b and d of main bodies A

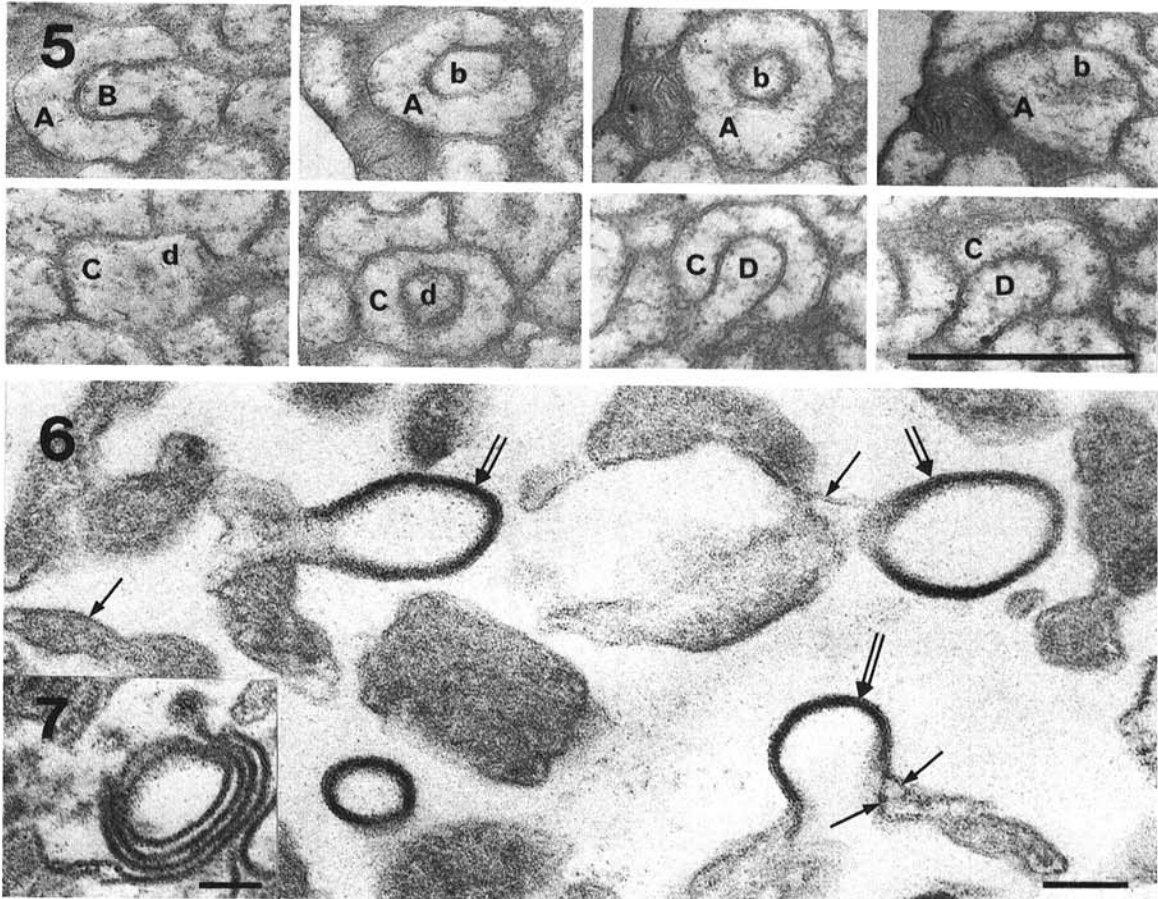


Fig. 5-7. 5) Mycoplasma-like organisms (MLO) in aster. Serial sections showing the relationships of "inner bodies". The arrow indicates the order of sections in each row. The thickness of the sections is 60 to 90 nm. The estimated distance between the first and the last sections of each series is 1.0 to 1.2 μm . ($\times 30,000$). Bar is 1 μm . 6) Numerous irregularly shaped MLO with electron-dense and ring forms with an empty space, observed in a necrotic sieve tube cell of a diseased aster plant subjected to 7 days of darkness 7 days after symptom development. Note dumbbell-shaped form having double membranes (\Rightarrow) which were formed apparently by fusion of two unit membranes (\rightarrow) ($\times 113,000$). Bar is 0.1 μm . 7) A section of a sieve tube cell from an infected and treated aster plant, showing laminated arrangement of double membranes. Seven-day dark treatment ($\times 84,000$). Bar is 0.1 μm .

and C were demonstrated to be not a part of the same body, but merely one part of the other main bodies B and D.

In all the treated plants, several types of abnormal MLO were usually found in both cross-sections and longitudinal sections showing necrosis of vein tissues (Fig. 4-a, b; 6, 7). The morphological features of these abnormal forms can be summarized as follows: (i) irregular form with highly electron-dense cytoplasm, (ii) ring-shaped form with an empty space, (iii) club-shaped form, and (iv) dumbbell-shaped form. At higher magnifications, ring-, club-, and dumbbell-shaped forms of MLO apparently were bounded by double membranes 14-16 nm thick (Fig. 6, double arrows) which consisted of three electron-dense layers and two less-dense intermediate layers. The highly electron-dense bodies were bounded by a single unit membrane (Fig. 6, single arrows). The width of the unit membrane was about 7-8 nm. Apparently, double membranes were formed by the fusion of two single unit membranes (Fig. 6) and, in some profiles of the lamina of double membranes, a myelinlike layer was observed (Fig. 7). Examinations of serial sections from diseased plants which had been subjected to a dark treatment showed that a variety of MLO forms merely resulted from different planes of sections (Fig. 4-a, b). Our critical observations also suggested that the ring- or dumbbell-shaped forms possibly originated by invagination or shrinkage of high-electron-density bodies.

DISCUSSION

Abnormal forms of aster yellows agent, previously reported, include certain membrane-bound spaces or vacuoles, large ovoid-shaped bodies containing an empty vacuole, membrane-bounded inclusion bodies, doughnut-shaped bodies, and cytoplasmic condensation in a large spherical body (5). Mushroom- or horseshoe-shaped forms and doughnut-shaped forms containing an inner body also were described and it was suggested that these morphological variations represented the mode of MLO reproduction within the phloem parenchyma cytoplasm (6). In our observations, except for some membrane-bounded forms which were not mentioned in the previous papers, most of the bodies were encountered frequently among the normal forms of MLO in the dark-treated plants. Examinations of serial sections showed that both doughnut-shaped forms and horseshoe-shaped forms could be simply derived from the same body in different planes of the section (Fig. 5). The "inclusion body" or "inner body", and "cytoplasmic condensation" body reported previously (5, 6), were similar to those shown in Fig. 5 in different planes of the same body. We visualize that large MLO bodies can invaginate to form sacs, and other smaller MLO can become accommodated in the sacs. The previous interpretation of these bodies as a form representing a mode of intracytoplasmic replication of MLO (6) could be proven only by evidence for the transfer or exchange of genetic material between MLO.

Our observations indicated that invaginations in so-called normal and abnormal forms of MLO were most prevalent in the sections from the diseased plants which had been subjected to the dark treatment. The presence of membranous laminated structures suggested that at least

two mycoplasmas were involved to form internal bladders (mycoplasma within mycoplasma as seen in Fig. 5) when invagination occurred. The actual mechanisms of or factors involved in causing invagination is unknown. Absence of starch grains in the chloroplasts of aster plants that had been subjected to dark treatment suggested that, as would be expected, photosynthesis was greatly reduced by the treatment. This reduction probably resulted in nutritional deficiencies and drastic physiological changes in the treated plants. The invagination of MLO seen in this study could be due to dynamic changes in infected cells following the dark treatment, including nutritional deficiencies and osmolarity changes in host cells.

The abnormal forms of MLO described here occurred in necrotic cells which seemed to be in an initial stage of their degradation process. Their appearance differed from that of degenerated MLO in diseased plants reported previously (3, 4). However, they appeared similar to MLO seen in the sections of *M. salivarium* and *M. pneumoniae* in respect to terminally bulbous structures and to ring-shaped forms occurring in surface growths of the organisms and in older culture (2, 8). This study demonstrates that examination of serial sections is important in understanding the three-dimensional geometry of MLO.

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