

Purification of Mycoplasma-Like Organisms from China Aster Plants Affected with Clover Phyllody

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ABSTRACT

Mycoplasma-like organisms (MLO) were purified from China aster (*Callistephus chinensis* Nees) plants infected with clover phyllody agent (CPA). Sections of pellets from purified preparations showed MLO that were very similar to those observed earlier in sections of infected plants. When healthy vector leafhoppers *Macrostelus fascifrons* Stal were injected with the purified preparations, and the injected insects were tested in groups of 10 per plant for their ability to transmit CPA on aster seedlings, 5 out of 50 inoculated plants became infected.

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Since Doi et al. (3) demonstrated the presence of microorganisms resembling animal mycoplasmas in

plants affected with certain "yellows"-type diseases, association of such organisms with at least fifty other plant diseases has been reported (2, 5). Attempts to purify these organisms from infected plants, however, have so far been unsuccessful. We described the occurrence of mycoplasma-like organisms (MLO) in China aster (*Callistephus chinensis* Nees) plants infected with CPA (7) as well as in certain tissues of vector leafhoppers *Macrostelus fascifrons* Stal (8). This communication reports for the first time purification and infectivity bioassays of MLO from CPA-infected aster plants.

All purification procedures were carried out at 5 C. Fifty grams of leaves from 7- to 8-wk-old infected asters, obtained through inoculation by the leafhopper *M. fascifrons* (6), were infiltrated with a solution containing 0.1 M MgCl₂ (Mg-solution) and 0.3 M glycine, pH 8.0 (Mg-solution), under vacuum for 30 min. The leaves were then triturated in a meat grinder, the juice squeezed through two layers of cheese cloth, the volume adjusted to 150 ml with Mg-solution, and the sap was centrifuged at 3,000 g for 15 min (low-speed centrifugation). The supernatant solution was adjusted to pH 8.0 and centrifuged, again at low speed. To the final supernatant solution, 5% (w/v) activated charcoal wood powder (Darco G-60) was added first and after stirring for 1 min., 5% (w/v) Celite 545 was added and the mixture stirred for another 1 min period. The MLO were absorbed from the mixture by passing through a Celite pad (1), about 1.5-cm

thick on a 12-cm diam Buchner funnel, eluted with Mg-solution, and 20-ml fractions were collected. It was important to use only 75 ml of the mixture per pad in order to remove all the green material. Therefore, 150 ml of the mixture was divided into two equal lots and passed through two separate pads. Fractions showing opalescence were taken from both lots, pooled, and centrifuged at 25,000 g for 30 min. (high-speed centrifugation). The resulting pellets were soaked in a small volume of Mg-solution overnight, and next morning the final volume of the suspension was adjusted to 5.0 ml. This suspension was then passed through a Sepharose (2B) column (30 × 2.5 cm), eluted with Mg-solution at a flow rate adjusted to 0.6 ml/min., and 1-ml aliquots were collected. Opalescent fractions appeared in tubes 36-56, but MLO were observed in the electron microscope only in fractions 38-49 which were pooled and centrifuged at the high speed. The pellet was soaked for 3 h in 1.0 ml of Mg-solution, and the suspension was layered on a sucrose density-gradient column (sucrose concns of 5, 10, 15, and 20% in Mg-solution, 6.2 ml of each concn) and centrifuged at 10,173 g for 45 min. in a SW25 rotor of Spinco Model L-1. A diffused hazy zone (visible between 4 and 5 cm from the bottom) was removed, diluted four times with Mg-solution and dialyzed against the same solution overnight. The suspension was then centrifuged at the high speed and the pellet resuspended in 1.0 ml of Mg-solution. Healthy leaves were treated in the same manner as described above, but none of 10 trials produced any pellet.

For electron microscopy, small droplets of suspensions were placed for 1.0 min. on grids that had been coated with a 0.2% Formvar film stabilized by deposited carbon, then stained with 2% phosphotungstic acid (pH 8.0) for 1.0 min. and examined in a Siemens Elmiskop 1. Preparations purified from infected plants showed numerous pleomorphic bodies resembling MLO (Fig. 1). Many rounded forms of MLO showed small vesicles, up to five on a body, giving an appearance of budding structures. The size of these vesicles ranged from 25 to 40 nm and that of the rounded bodies from 175 to 400 nm in diam. Filamentous forms of MLO were also observed which were sometimes as long as 1,700 nm. Some of these filamentous forms showed beading and varying degrees of branching which resulted in several kinds of configurations. Because PTA-stained solutions may produce artifacts (10), pellets obtained from purified preparations were sectioned and examined (7, 9). A high concn of MLO was observed. These were bounded by a trilaminar unit membrane, contained ribosome-like granules, and some rounded forms showed a nuclear area with DNA-like fibrils (Fig. 2). Small spherical bodies, usually referred to as "elementary bodies," filled with ribosomes were also observed. No other subcellular constituents which could be considered contaminants were observed in the sections. These MLO were indistinguishable from those observed earlier in sections of infected plants (7). During the past year, we have purified MLO from at least 20 batches of infected plants with reproducible results.

Infectivity of purified preparations containing MLO was tested in five experiments. Infectivity bioassays were done by injecting the purified preparations into healthy

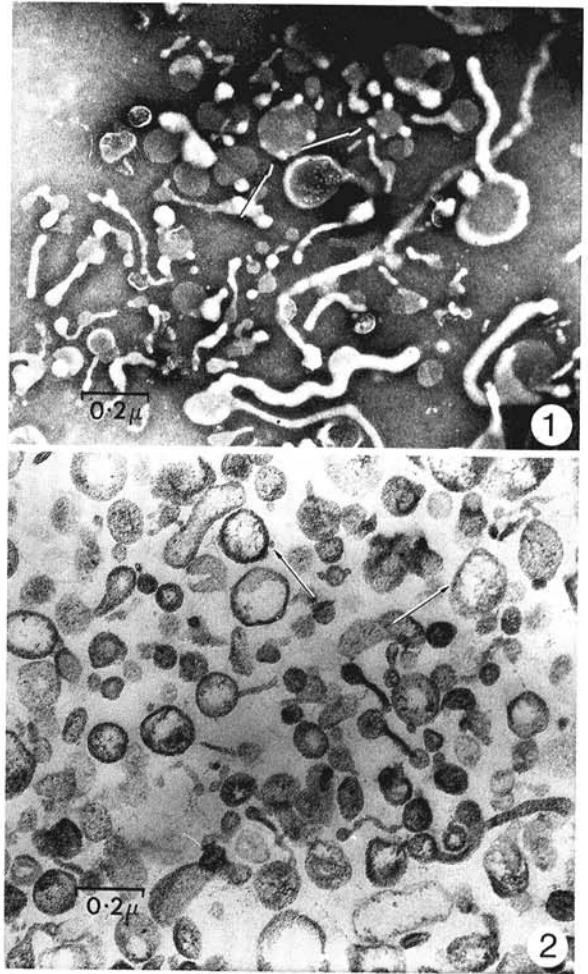


Fig. 1-2. 1) Negatively stained preparations of mycoplasma-like organisms (MLO) purified from clover phyllody-infected plants. Pleomorphic bodies, both rounded and filamentous, can be seen. Some rounded bodies show small vesicles giving an appearance of budding (arrows). 2) Section of purified MLO pellet showing pleomorphic bodies with a unit membrane and ribosome-like granules. A nuclear area with DNA-like fibrils can also be seen in some rounded bodies (arrows).

leafhoppers and then testing them for ability to transmit the CPA to aster seedlings (6). A total of about 500 injected leafhoppers were tested (in groups of 10 per plant), but only 5 of the 50 inoculated plants became infected. This low infectivity is not surprising because extracts of plants affected with "yellows"-type diseases are known to lose most of their biological activity within 24 h at 5 C (4), whereas the purification procedure described here takes about 3 days. Nevertheless, the purified preparations of MLO can be used for biochemical analysis, and for preparing an antiserum. The above results provide further evidence of *Mycoplasma* etiology of clover phyllody disease.

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