

Detection of Mycoplasmalike Organism Antigens from Aster Yellows-Diseased Plants by Two Serological Procedures

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ABSTRACT

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Mycoplasmalike organism (MLO) antigens were detected by enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (IEM) in preparations partially purified from 5-g samples of aster (*Callistephus chinensis*) plants experimentally infected with aster yellows agent. The preparations containing MLOs at estimated concentrations as low as 4 $\mu\text{g/ml}$ gave a positive reaction by ELISA (minimum absorbance value at 405 nm was >0.1), but the minimum concentration needed to trap the MLOs on the antiserum-coated grids in IEM tests was about 1.25 $\mu\text{g/ml}$. The MLOs were also detected by IEM in preparations from six experimentally infected host plant species, and the

concentrations of MLOs in various species was estimated to vary from 80 to 20 $\mu\text{g/g}$ of leaf tissue. The MLOs from aster yellows and clover phyllody-diseased plants appear to be antigenically indistinguishable. Partially purified preparations from 5 g samples of leaves infected with either peach-X or clover yellow edge agent did not give a positive reaction in ELISA or IEM tests with the antiserum against the MLOs from aster yellows-diseased plants. Also, the MLOs in preparations from aster yellows-diseased leaves were not trapped on grids coated with the antiserum against *Spiroplasma citri* in IEM tests.

Mycoplasmalike organisms (MLOs) have been etiologically implicated in ~ 100 diseases of a variety of plants (9). These usually are differentiated by symptomatology, host range, and vector-pathogen relationships, but these methods are time consuming and

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are not suitable for epidemiological investigations. Immunological methods for detecting MLO-antigens in plant extracts have not been fully utilized because antisera are difficult to prepare. Successful *in vitro* cultivation of *Spiroplasma citri* has facilitated the production of an antiserum against this plant mycoplasma (1,15) and enabled the use of sensitive serological techniques (eg, immunosorbent electron microscopy [IEM] and enzyme-linked immunosorbent assay [ELISA]) for rapidly detecting that pathogen in extracts of diseased plants and in pure cultures (5-7).

Earlier we prepared an antiserum against the MLOs purified

from aster yellows (AY)-diseased plants and studied the reactions of cytoplasmic and membrane antigens of the organisms in agar gel double diffusion tests (11).

This paper reports detection of the MLO-antigens by ELISA and IEM in preparations partially purified from infected leaves and estimation of relative MLO concentration in various host plants infected with AY-agent.

MATERIALS AND METHODS

Diseased plants. The AY agent was maintained in aster (*Callistephus chinensis* Nees) plants through inoculation by infective vector leafhoppers *Macrostelus fascifrons* (Stål) (13). Preparations for serological tests were obtained from plants of the following species experimentally infected with the AY agent: aster, celery (*Apium graveolens* L.), periwinkle (*Catharanthus roseus* L.), oat (*Avena sativa* L. 'Random'), wheat (*Triticum durum* Desf. 'Ramsey'), ladino clover (*Trifolium repens* L.), and red clover (*T. pratense* L.). Experimentally infected aster plants were used to study clover phyllody (14), celery for peach-X (4), and ladino clover for clover yellow edge (3). Insect rearing and studies of the vectoring of the AY agent by leafhoppers were carried out in artificially lighted (10,000 lux, 16-hr day) rooms at ~24 C.

Test procedures and preparation of antisera. The antiserum against MLOs from plants infected with the AY agent was prepared as described earlier (11) by using preparations purified from aster plants. The ELISA technique was as originally described by Engvall and Perlmann (8) and later modified by Voller et al (16). The enzyme substrate, *p*-nitrophenyl phosphate, at 0.7 mg/ml, was used in all ELISA experiments. IEM technique, previously termed "serologically specific electron microscopy," was basically the same as described by Derrick and Brlansky (7). Nickel grids (400-mesh)

coated with a 0.5% Parlodion solution (Mallinkrodt Chemical Works, St. Louis, MO 63134) and carbon were floated on antisera diluted in PBSS (0.1 M potassium phosphate buffer containing 0.15 M NaCl and 3% sucrose, pH 8.0) for 1 hr at 37 C in a moist chamber. The grids were then thoroughly washed by first dipping and shaking in PBSS, followed by floating in the same buffer for 15 min, and finally by rinsing with distilled water. They were then floated on preparations from diseased or healthy leaves for 90 min at 37 C in a moist chamber and washed as above. The grids were positively stained with 1% aqueous uranyl acetate for 10 min and rinsed with distilled water. A set of duplicate grids was prepared for each sample and examined in a Philips EM300 electron microscope.

Test antigens. Partially purified antigenic preparations from diseased plants were obtained by following the early steps of the MLO purification procedure (10,12) with modifications to suit extraction of the organisms from 5 g of leaves. Young leaves from plants of various species were collected 1 wk after the appearance of disease symptoms. After infiltration of the leaves with Mg-buffer solution containing 0.1 M MgCl₂ and 0.3 M glycine at pH 8.0 under vacuum for 30 min, they were macerated in a mortar, the juice was squeezed through two layers of cheesecloth, its volume was adjusted to 15 ml with Mg-buffer solution, and the sap was centrifuged at 3,000 g for 15 min. The supernatant solution was adjusted to pH 8.0 and centrifuged again as above. To the supernatant solution, 0.8 g of activated wood charcoal powder (Darco G-60; Fisher Scientific, Ottawa, Canada) was added. After the mixture had been stirred for 1 min, 0.8 g of Celite 545 was added and the mixture was again stirred for 1 min. The suspension was poured ~1.5 cm thick on a Celite pad (Fisher Scientific) in a 8.5-cm-diameter Büchner funnel. The beaker that had contained the mixture was rinsed with 10 ml of Mg-buffer and this suspension

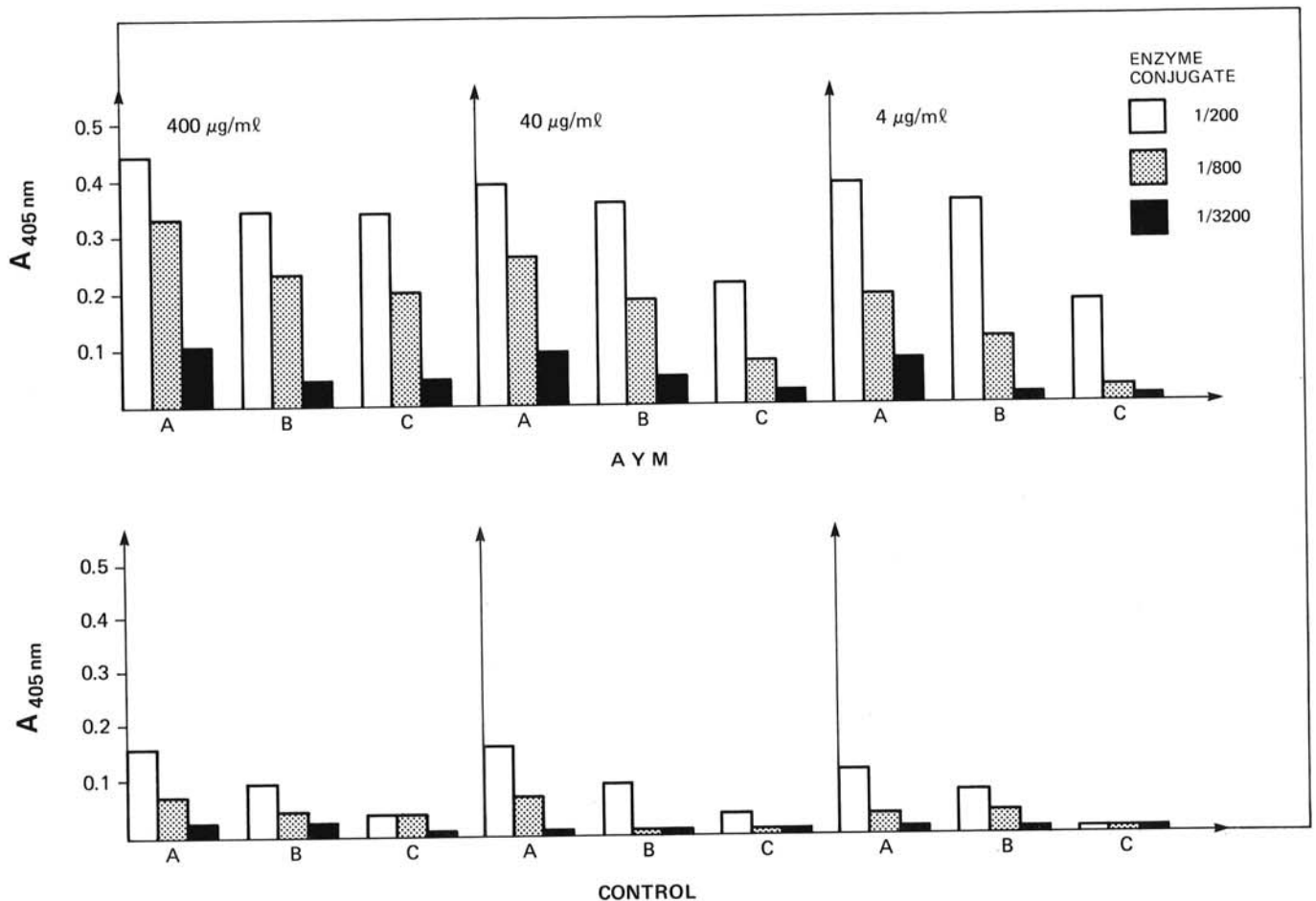


Fig. 1. ELISA absorbance values of partially purified preparations of mycoplasma-like organisms from 5 g of aster leaves infected with aster yellows agent and of controls (preparations from healthy plants) at various concentrations of enzyme conjugate and gamma globulin. A, B, and C represent gamma globulin at concentrations of 10, 1.0, and 0.1 µg/ml, respectively.

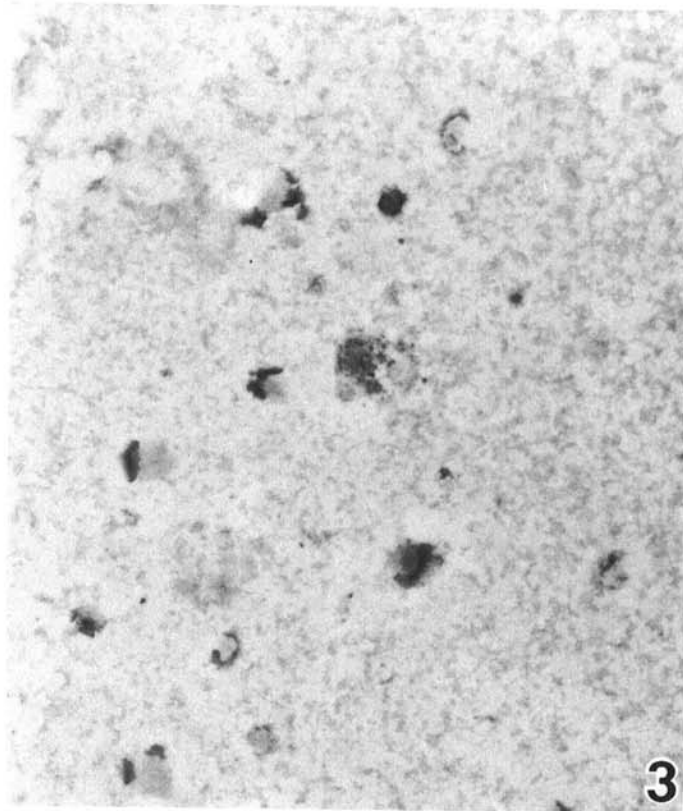
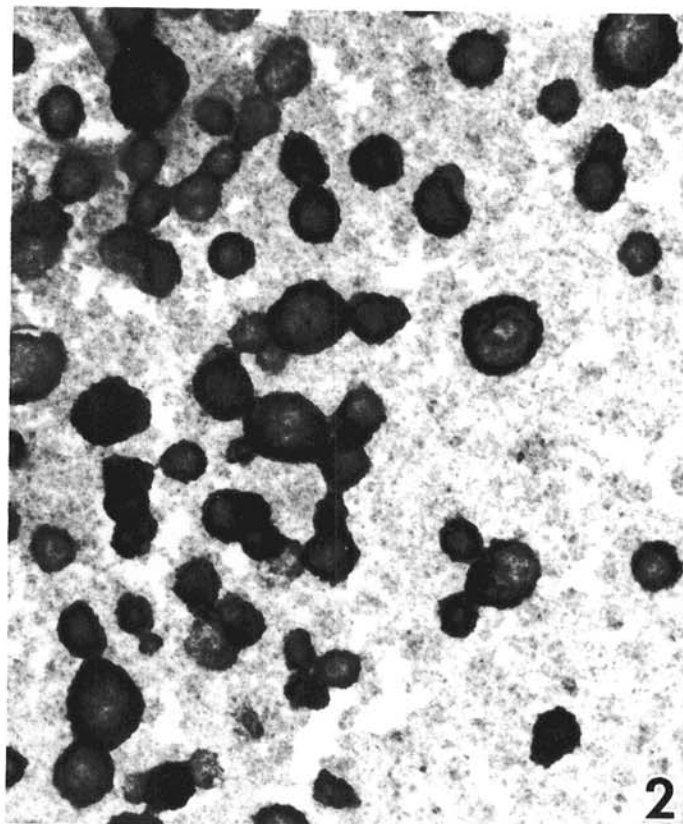
was also poured on the Celite pad. A vacuum was then applied until the pad was almost dry and the clear filtrate was discarded. The adsorbed MLOs on the pad were eluted by adding 40 ml of Mg-buffer and applying a vacuum. The resulting opalescent filtrate was centrifuged at 25,000 g for 30 min, and the small pellet obtained was resuspended in 1 ml of PBST (phosphate buffered saline containing 0.5% Tween and 2% polyvinyl pyrrolidone) for ELISA (5) or in 1 ml of PBSS for IEM (7) tests. Unless stated otherwise, partially purified preparations obtained as above were used as test antigens. Preparations obtained from healthy plant leaves by the same preparative procedures as those used for the diseased leaves were used as controls and tested against the antiserum. All serological tests were repeated five times and the results were reproducible with minor variations.

RESULTS

ELISA tests. Three dilutions each of gamma globulin and of enzyme conjugate were used in an attempt to detect MLO-antigens in preparations from AY-diseased leaves. The approximate concentration of MLOs in partially purified preparations was estimated by its serological titer and comparing it with that of purified preparations containing known freeze-dried weights of the MLOs (11). A reaction was generally considered positive if the $A_{405\text{ nm}}$ value was higher than 0.1 (5). The results showed that absorbance values of preparations from the infected plants were always much higher than those of the control preparations from healthy plants (Fig. 1). Also, no visible color reaction was observed with PBST control preparations and the $A_{405\text{ nm}}$ values obtained were ≤ 0.02 . If the enzyme conjugate at 1/200 dilution was used, a visible color reaction was obtained within 15 min at all concentrations of coating gamma globulin and MLOs tested. However, with enzyme conjugate at a dilution of 1/200 and gamma globulin at 10 $\mu\text{g/ml}$, a weak positive reaction occurred in healthy controls. Reactions were not observed in healthy controls with the higher dilutions of gamma globulin and enzyme conjugate. With the enzyme conjugate dilution of 1/800, positive readings were obtained at gamma globulin concentrations of 10 and 1 $\mu\text{g/ml}$ with all MLO concentrations tested. If 0.1 μg of gamma globulin per milliliter was used, a minimum MLO concentration of about 400 $\mu\text{g/ml}$ was needed to obtain a positive reaction. The enzyme conjugate at a dilution of 1/3,200 was not suitable for detection of the MLO-antigens at any concentration of gamma globulin tested because of very low absorbance values. The results suggest that by using appropriate dilutions of enzyme conjugate and gamma globulin, the MLO-antigens could be detected by ELISA.

IEM tests. Initial experiments were done to determine the highest dilution of the preparation from AY-diseased aster leaves needed to trap the MLOs on grids and the antiserum titer by IEM. The results showed that optimally the minimum concentration required in the preparations to trap the MLOs on the grids was about 12.5 $\mu\text{g/ml}$, and that antiserum titer was between 1/1,600 and 1/3,200. To determine if MLOs are nonspecifically adsorbed, the grids were coated with different dilutions of the preimmune serum and treated with the preparations from infected leaves. The results showed that MLOs were nonspecifically adsorbed on the grids coated with preimmune serum at dilutions of 1/50 or less and MLO concentration of about 40 $\mu\text{g/ml}$. With preimmune serum at 1/100 dilution, only a few MLOs were trapped at all dilutions of the preparations tested. Therefore, in all subsequent experiments, antiserum at 1/100 dilution was used to coat the grids. At this antiserum dilution, numerous MLOs were trapped on the grids. Mostly spherical and ovoid cells, some of which were in chains, were observed in preparations from infected plants (Fig. 2), whereas no such cells were seen in healthy controls (Fig. 3).

The MLOs were also observed in preparations obtained from all other experimentally infected host plant species tested by IEM. Morphologically, these bodies were very similar to those seen in preparations from aster plants. The relative concentration of the MLOs in preparations from different plant species could be calculated from the serological titers by assuming that the minimum concentration required to trap the MLOs was 12.5



Figs. 2-3. 2, Detection of mycoplasma-like organisms by immunosorbent electron microscopy in preparations partially purified from 5 g of aster leaves infected with aster yellows agent. The grids were coated with the antiserum at 1/100 dilution ($\times 46,600$). 3, Partially purified preparation from healthy aster leaves (control) in immunosorbent electron microscopy test carried out in the same manner as for mycoplasma-like organisms in Fig. 2 ($\times 46,600$).

$\mu\text{g}/\text{ml}$. The MLO concentration per gram of aster and celery leaf tissue was estimated to be $\sim 80 \mu\text{g}$; in periwinkle, oat, and wheat, $40 \mu\text{g}$; and in ladino clover, $20 \mu\text{g}$.

Specificity of serological reactions. To determine the specificity of the serological reactions described above, partially purified preparations from 5 g of leaves infected with clover phyllody, peach-X, or clover yellow edge were tested with the antiserum against the MLOs from aster yellows-diseased plants. In ELISA tests, the concentrations of gamma globulin and enzyme conjugate used were the same as employed earlier for AY, and the antiserum dilution 1/100 was used in IEM tests. The preparations from clover phyllody and AY-diseased leaves gave identical serological reactions. However, preparations from peach-X and clover yellow edge-diseased leaves did not give positive reactions either in ELISA or IEM tests. Also, the MLOs in preparations from AY-diseased leaves were not trapped on grids coated with antiserum of *S. citri* in IEM tests.

DISCUSSION

The results show that both ELISA and IEM techniques can be used for detecting MLO-antigens in partially purified preparations. Although the IEM method appears to be less sensitive than ELISA, it has an advantage that the diverse morphological features of the MLOs can be seen in positive serological reactions. It is time consuming to obtain the purified MLO preparations required to prepare the antiserum (11), but once the serum has been produced, the antigens could be detected within a few hours in partially purified preparations from 5-g samples of infected leaves. It should be noted, however, that MLOs found associated with clover phyllody and aster yellows diseases cannot be differentiated by these serological methods. To differentiate these pathogenic agents, it will still be necessary to depend on symptomatology and vector-pathogen relationships (2). It appears that peach-X and clover yellow edge can be serologically distinguished from AY. Our results do not exclude the possibility, however, that negative serological reactions obtained under our experimental conditions could have been due to lower concentration of MLOs in the extracts of plants infected with peach-X or clover yellow edge.

Our results suggest that aster and celery plants infected with the AY agent contain higher concentrations of the MLOs than do the other plants tested. Success in obtaining purified MLO preparations may, therefore, depend on the selection of appropriate host plants, especially since $\sim 80\%$ of the organisms are lost during the purification procedure (11).

LITERATURE CITED

1. Bové, J. M., Saglio, P., Tully, J. G., Freundt, A. E., Lund, A., Pillot, J., and Taylor-Robinson, D. 1973. Characterization of the mycoplasma-like organism associated with "stubborn" disease of citrus. Pages 462-470 in: *Mycoplasma and Mycoplasma-like Agents of Human, Animal and Plant Diseases*. Vol. 225. K. Maramorosh, ed. The New York Academy of Sciences, New York, NY.
2. Chiykowski, L. N. 1973. The aster yellows complex in North America. *Proc. North Central Branch Entomol. Soc. Am.* 28:60-66.
3. Chiykowski, L. N. 1981. Association of mycoplasma-like organisms with clover yellow edge disease. *Can. J. Plant Pathol.* 3:139-144.
4. Chiykowski, L. N., and Sinha, R. C. 1982. Herbaceous host plants of peach eastern X-disease agent. *Can. J. Plant Pathol.* 4:8-15.
5. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.
6. Clark, M. F., Flegg, C. L., Bar-Joseph, M., and Rottem, S. 1978. The detection of *Spiroplasma citri* by enzyme-linked immunosorbent assay. *Phytopathol. Z.* 92:332-337.
7. Derrick, K. S., and Brlansky, R. H. 1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. *Phytopathology* 66:815-820.
8. Engvall, E., and Perlmann, P. 1972. Enzyme-linked immunosorbent assay. Quantitation of specific antibodies by enzyme-linked anti-immunoglobulin in antigen coated tubes. *J. Immunol.* 109:129-135.
9. McCoy, R. E. 1979. Mycoplasmas and yellows diseases. Pages 229-259 in: *The Mycoplasmas*. Vol. 3. Plant and Insect Mycoplasmas. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
10. Sinha, R. C. 1974. Purification of mycoplasma-like organisms from China aster plants affected with clover phyllody. *Phytopathology* 64:1156-1158.
11. Sinha, R. C. 1979. Purification and serology of mycoplasma-like organisms from aster yellows-infected plants. *Can. J. Plant Pathol.* 1:65-70.
12. Sinha, R. C. 1983. Physical techniques for purification of mycoplasmas from plant tissues. In: *Methods and Mycoplasmaology*. J. G. Tully and S. Razin, eds. Academic Press, New York. (In press).
13. Sinha, R. C., and Chiykowski, L. N. 1967. Initial and subsequent sites of aster yellows virus infection in a leafhopper vector. *Virology* 33:702-708.
14. Sinha, R. C., and Paliwal, Y. C. 1969. Association, development, and growth cycle of mycoplasma-like organisms in plants affected with clover phyllody. *Virology* 39:759-767.
15. Tully, J. G., Whitcomb, R. F., Bové, J. M., and Saglio, P. 1973. Plant mycoplasmas: serological relation between agents associated with citrus stubborn and corn stunt diseases. *Science* 182:827-829.
16. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). *J. Gen. Virol.* 33:165-167.