Detection of Mycoplasmalike Organisms In Situ by Indirect Immunofluorescence Microscopy

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The authors thank M. H. Chen for his assistance in electron microscopy and T. Tribe for preparing illustrations.

This research was partially supported by the Natural Sciences and Engineering Council of Canada Operating Grant A3840 and by an Alberta Agricultural Research Trust Grant. The senior author was a recipient of an NSERC postgraduate scholarship.

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Accepted for publication 29 January 1986.

ABSTRACT

da Rocha, A., Ohki, S. T., and Hiruki, C. 1986. Detection of mycoplasmalike organisms in situ by indirect immunofluorescence microscopy. Phytopathology 76:864-868.

Mycoplasmalike organisms (MLOs) of Eastern aster yellows (a New York isolate), Western aster yellows (an Alberta isolate), potato witches'-broom and clover proliferation were partially purified from *Catharanthus roseus*. Hand sections from MLO-infected and healthy *C. roseus*, previously treated with individual MLO antisera, were exposed to antirabbit goat immunoglobulins conjugated with fluorescein isothiocyanate and were examined by fluorescence microscopy. MLO-specific fluorescence was detected in those infected with MLOs but not in comparable healthy tissue. This technique is suitable for practical diagnosis of plant diseases associated with MLOs.

Additional key words: Diagnosis, MLO, mollicutes, periwinkle, serology.

The immunofluorescence test has been used in animal mycoplasmology (1,8), plant spiroplasmology (13), and especially in virology (12). There are two ways of performing the immunofluorescence test, the direct and the indirect methods. In the direct methods, the antibody produced against the protein to be detected is conjugated with a fluorochrome, such as fluorescein isothiocyanate (FITC), and the staining is done simply by exposing test specimens to a solution of the labeled antibody. The indirect method is based on the capability of antibodies (immunoglobulins) to serve as immunogens; thus, for example, if a goat is injected with rabbit immunoglobulins, goat antibody against rabbit immunoglobulins can be produced and conjugated with a fluorochrome. The staining is then performed in two stages. First, the test specimens are exposed to a solution of unlabeled rabbit antibody produced against the protein to be detected, and unbound antibody is removed by washing. In the second stage, the sites where the rabbit antibody have attached to the protein are labeled by specific binding with goat fluorescent antibody prepared against rabbit immunoglobulins. The specificity of antigen-antibody reactions, coupled with the relatively short time required to prepare and observe the specimen, makes the immunofluorescence procedure an ideal diagnostic tool for plant diseases associated with mycoplasmalike organisms (MLOs). The purpose of this study was to develop a practical diagnostic method for yellows diseases associated with MLOs that have not yet been cultivated.

MATERIALS AND METHODS

Plants and MLOs. *Catharanthus roseus* (L.) G. Don plants with the following MLO-associated diseases were studied: potato witches'-broom (PWB), clover proliferation (CP) (2,4), Eastern aster yellows (EAY, a New York isolate), and Western aster yellows (AY27, a subculture of an Alberta isolate AP-1) (3,5). Healthy *C. roseus* plants were grafted with scions from *C. roseus* plants with these diseases. As controls, healthy *C. roseus* plants were used as scions for grafting. Plants were maintained at 17 C.

Preparation of immunogen. The membrane fraction from healthy *C. roseus* and from *C. roseus* infected with MLO causing PWB, CP, EAY, and AY27 were prepared for use as immunogens by the modified method of Clark et al (7). All procedures were performed at 0–4 C when possible.

Leaves of *C. roseus* were washed for several hours with running tap water to remove soil and other gross contaminants. The tissue (20 g) was shaken dry, passed through a meat grinder, extracted into ice-cold buffer (1 g/5 ml buffer), and squeezed through cheesecloth. The buffer used was 0.3 M glycine-sodium hydroxide, pH 8.0, with 0.02 M magnesium chloride. The extract was clarified by centrifugation at 2,000 *g* for 20 min. The clarified extract was then centrifuged at 39,000 *g* for 45 min and the resultant large green pellet was resuspended in the original volume of buffer overnight. The suspension was clarified by passing it through Whatman No. 2 filter paper in a Büchner funnel. It was subjected to a further cycle of differential centrifugation and the resulting pellet was resuspended in 1/20 volume of buffer. The suspended material was stored in 0.7-ml aliquots at −18 C and was used as immunogen and antigen in serological tests.

Preparation of antiserum. Antisera to the preparations of the membrane fraction of CP, PWB, EAY, and AY27 MLO-infected and healthy *C. roseus* plants were produced in rabbits. Rabbits were injected at 2-wk intervals with a 1:1 (v/v) mixture of the immunogen and either Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (subsequent injections). One milliliter of the homogenized mixture was injected at subcutaneous, intradermal, and intramuscular sites. Animals were bled at 2-wk intervals, beginning 6 wk after the primary injection. The rabbit was exsanguinated when titers of antisera, determined by double immunodiffusion (11), reached 1/4–1/16. As a control, antigen from healthy plants replaced the test antigen in the center well. After 2–3 days at room temperature, plates were observed for precipitin lines.

To remove the antibodies to healthy plant proteins, the antisera were absorbed with acetone-extracted powder of healthy *C.*

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roseus. For every 10 ml of antiserum, 10 g of healthy leaf material was used for preparing acetone-extracted powder. The healthy leaf material was passed through a meat grinder and extracted in 200 ml of acetone. The extract was filtered through Whatman No. 4 filter paper in a Büchner funnel and the powder was washed several times with acetone (750 ml total) until it was almost colorless. It was placed in a beaker or wide-mouthed conical flask, and 120 ml of 80% ethanol was added. The flask was placed in a boiling water bath and boiled for 15 min. The plant protein powder was then washed with 750 ml of phosphate-buffered saline (PBS), before

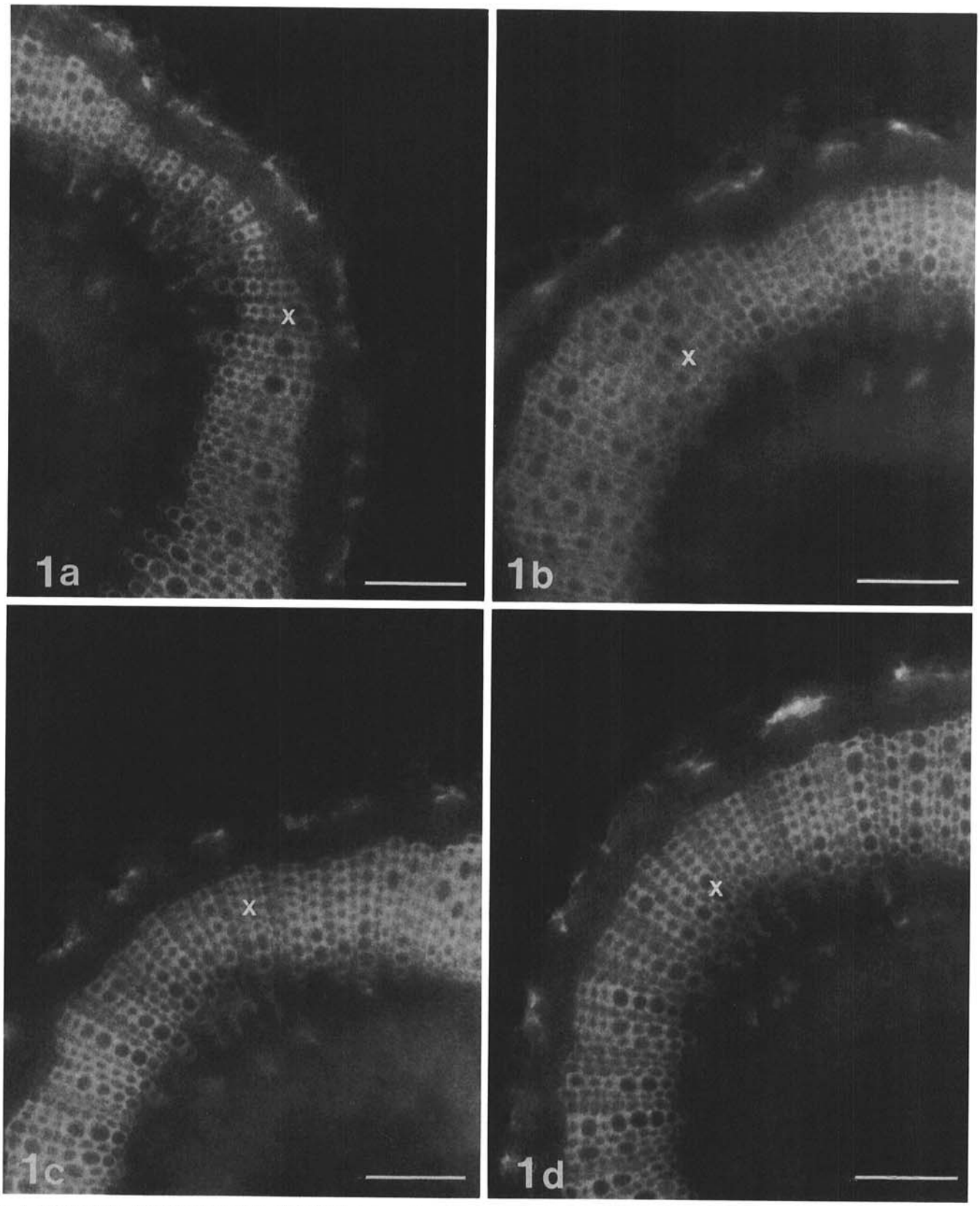

Fig. 1. Micrographs showing the results of indirect immunofluorescence tests on stem cross sections of *Catharanthus roseus* infected with the mycoplasmalike organism of Western aster yellows (an Alberta isolate) reacted with **A,** Anti-Eastern aster yellows serum, **B,** Anti-Western aster yellows serum, **C,** Anti-potato witches'-broom serum, and **D,** Anti-clover proliferation serum. X = xylem. Scale bars = 100 μm.

being placed in a test tube with 10 ml of antiserum and incubated at 40 C for 2.5 hr. The mixture was left overnight at 4 C, then was centrifuged at 17,000 *g* for 15 min. The precipitate was removed by centrifugation, and the antiserum was collected.

The immunoglobulin fraction of the antiserum was precipitated with ammonium sulfate (6). Equal volumes of antiserum and saturated ammonium sulfate were mixed and incubated at room temperature for 90 min. The mixture was centrifuged at 3,000 *g* for 10 min and the precipitate resuspended in 2 ml of 0.005 M PBS, pH 7.5. The suspension was placed in dialysis tubing and dialyzed

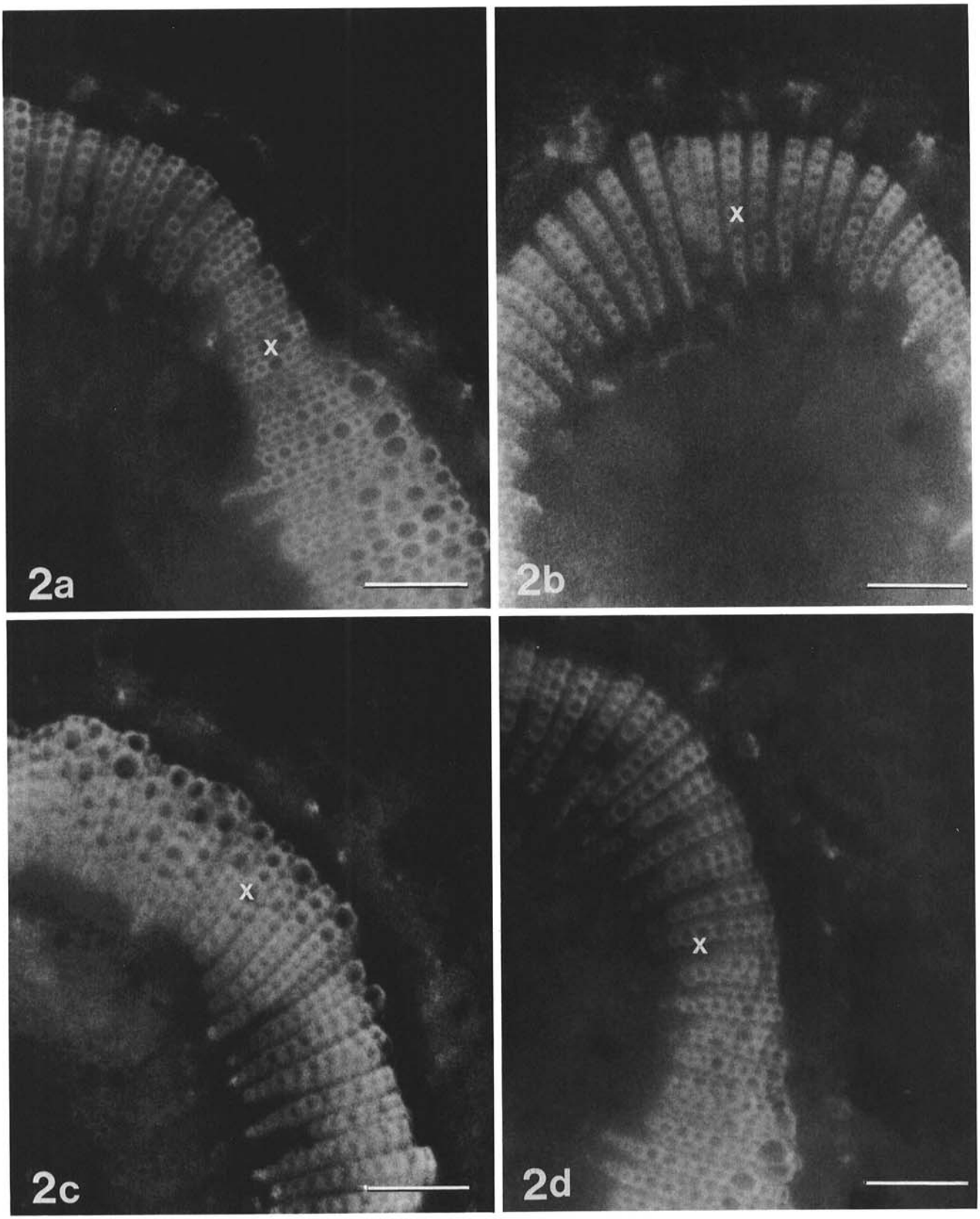

Fig. 2. Micrographs showing the results of indirect immunofluorescence tests on stem cross sections of *Catharanthus roseus* infected with the mycoplasmalike organism of potato witches'-broom reacted with **A,** Anti-Eastern aster yellows serum, **B,** Anti-Western aster yellows serum, **C,** Anti-potato witches'-broom serum, and **D,** Anti-clover proliferation serum. X = xylem. Scale bars = 100 μm.

overnight at 4 C against two changes of approximately 2 L of 0.005 M PBS.

The concentrations of the purified immunoglobulins were determined by measuring the absorbance at 280 nm and using an extinction coefficient of 1.35 $(mg/ml)^{-1}$ cm^{-1} (9). The immunoglobulins were then diluted with PBS to 10 mg/ml and stored in 1-ml aliquots at −18 C.

Indirect immunofluorescence. The indirect immunofluorescence staining procedure described previously (10) was modified to provide a simple and rapid method that is suitable for use with thick plant sections. Free-hand sections from the internode region, 30–60 μm, were cut and placed in 1.5-ml microcentrifuge tubes. Ethanol (200 μl 95%) was added and the sections were fixed for 5 min. The sections were then washed three times with 200 μl PBS. The immunoglobulin, 50 μl (1 mg/ml), was placed in the tube for 15 min at room temperature. The sections were washed as above. Labeled antirabbit goat immunoglobulins (50 μl (100 μg/ml)) conjugated with FITC were added to the sections in the tube and incubated for 15 min at room temperature. The sections were washed as above. The final washing was replaced by 200 μl of the mounting medium, 1:1 (v/v) PBS and glycerol. The sections were mounted on glass slides with coverslips and viewed under a fluorescence microscope.

Immunoglobulins obtained against the membrane fraction of PWB, CP, EAY, and AY27 diseased plants were used to test sections from plants with the four diseases as well as sections from healthy control plants.

Electron microscopy. Stem tissue sections about 1 mm thick were fixed for 3 hr in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. The tissue was washed in the same buffer, postfixed in 2% osmium tetroxide for 3 hr, and subsequently washed in the buffer. The tissue samples were dehydrated in a graded ethanol series and then placed in two changes of propylene oxide for 20 min each. The samples were embedded in Araldite 502 and sectioned 50–90 nm thick with an ultramicrotome equipped with a glass knife. The sections were stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope at 60 kV.

TABLE 1. Indirect immunofluorescence observed with cross-sections of *Catharanthus roseus* stems infected with mycoplasmalike organisms

Plant tissue[a]	Antiserum			
	EAY[b]	AY27[c]	PWB[d]	CP[e]
Healthy	...[f]	...	...	...
EAY	++++[g]	++++	++++	++++
AY27	++++	++++	++++	++++
PWB	+[h]	+	+	+
CP	+	+	+	+

[a] Three stem sections were used in each test.
[b] Eastern aster yellows MLO, a New York isolate.
[c] Western aster yellows MLO, a subculture of the Alberta isolate AP-1 MLO.
[d] Potato witches'-broom MLO.
[e] Clover proliferation MLO.
[f] No specific fluorescence.
[g] Very prominent fluorescence.
[h] Weak, but positive fluorescence.

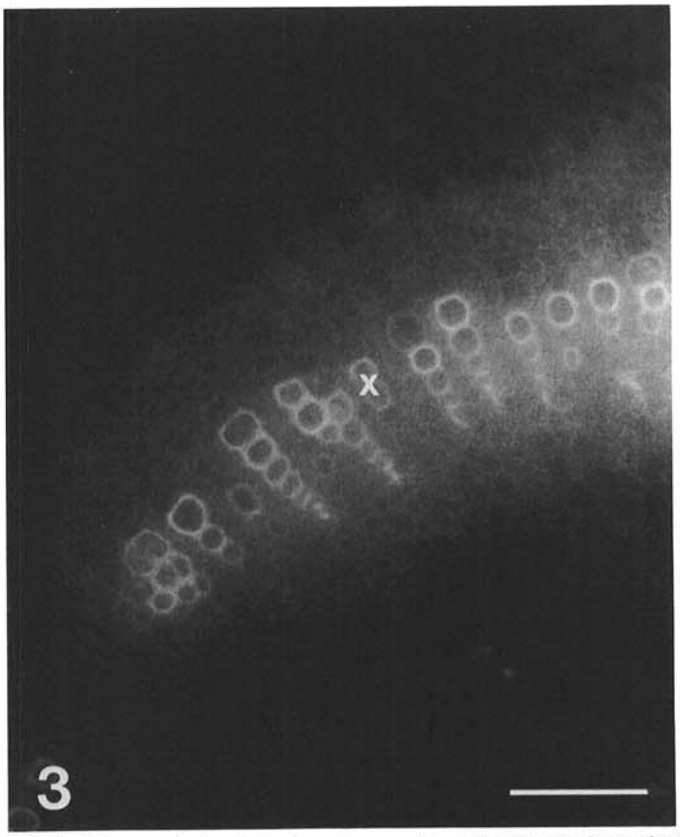

Fig. 3. Micrograph showing a stem cross section of healthy *Catharanthus roseus* tested with anti-Eastern aster yellows, New York isolate serum by indirect immunofluorescence. X = xylem. Scale Bar = 100 μm.

RESULTS

Indirect immunofluorescence. Membrane fraction preparations from MLO-infected plants as immunogens produced antisera with titers ranging from 1/4 to 1/16 in the gel diffusion tests. Indirect immunofluorescence was positive with MLO-infected plant sections and negative with healthy plant sections (Table 1). However, the antiserum did not show specificity for the MLO against which it had been prepared. Four antisera, EAY, AY27, PWB, or CP, gave positive results with plant sections infected with EAY, AY27, PWB, or CP-MLOs (Figs. 1 and 2). In all cases fluorescence was stronger with EAY and AY27 plant sections than with PWB and CP plant sections. Healthy plant sections tested with antisera against EAY, AY27, PWB, and CP showed no fluorescence in the phloem region (Table 1, Fig. 3).

Electron microscopy. MLOs were observed in thin sections of diseased plant tissue from shoots that had given positive results in the indirect immunofluorescence test. No MLOs were observed in the healthy control.

DISCUSSION

The indirect immunofluorescence technique was modified for use with thick sections of *C. roseus* in this study. In a standard method used in the medical field (10), the unknown antigen is smeared on a glass slide and the primary and secondary antisera are added directly on the slide. In another immunofluorescence procedure to detect clover yellow mosaic virus inclusion bodies in cowpea, epidermal strips are peeled from the leaf and attached to glass slides with Haupt's adhesive (11). In our study, in which practical application of the technique is of major importance, free-hand sections were cut, but these were too thick (30–60 μm) and therefore too heavy to allow adhesion on a glass slide by Haupt's adhesive. In addition, thorough washing of the slide would wash off the tissue sections. Therefore, the sections were placed in microcentrifuge tubes and all solutions added to and removed from the tube with Pasteur pipette. Advantages of this method were the assurance that the sections were totally immersed in the solutions, and the use of smaller volumes of antiserum (50 μl each time). Kawamura's procedure (10) required the smear to be covered with solutions, i.e., a considerably larger volume would be required. We also found that incubation times could be shortened in the present procedure, taking just 3 hr for the processing of five different tissues with four antisera (i.e., 20 tests), whereas Kawamura's method requires incubation times of 30–60 min each and washings of 15 min each.

Less equipment was required with the modified technique. Incubation could be at room temperature, omitting the use of incubators and shakers. Moist chambers were not necessary, as the solutions in the microcentrifuge tubes would not dry, especially when the tubes were capped. Thus, the modifications provided a

method of indirect immunofluorescence for use with thick plant sections that was both rapid and simple.

One advantage of this method is that it is 5 to 10 times more sensitive than the direct method (10), because several molecules of labeled antirabbit immunoglobulin antibody can react with each molecule of rabbit immunoglobulin, thus yielding stronger fluorescence than in the direct method. A second advantage is the small amount of primary antibody required in the test, and a third advantage is that a conjugated secondary antibody such as antirabbit IgG FITC can be used with any primary antibody obtained from rabbit, eliminating the need to conjugate each specific antibody with FITC. Also, several secondary antibody conjugates are commercially available.

Results of this study showed that the antiserum produced was able to detect MLO antigen in diseased plant tissue. All four antisera tested showed similar results with the four MLOs, whereas healthy tissue showed no fluorescence. Therefore, this technique is useful for the detection of MLOs in diseased plants.

LITERATURE CITED

1. Barile, M. F., and Grabowski, M. W. 1983. Detection and identification of mycoplasmas in infected cell cultures by direct immunofluorescence staining. Pages 173–181 in: Methods in Mycoplasmology. Vol. 2. S. Razin and J. G. Tully, eds. Academic Press, New York.
2. Chen, M. H., and Hiruki, C. 1975. Electron microscopy of mycoplasma-like bodies associated with clover proliferation disease. (Abstr.) Proc. Am. Phytopathol. Soc. 2:52.
3. Chen, M. H., and Hiruki, C. 1977. Effects of dark treatment on the ultrastructure of the aster yellows agent in situ. Phytopathology 67:321-324.
4. Chen, M. H., and Hiruki, C. 1978. Occurrence of tubular structures in *Vinca rosea* infected with the Alberta isolate of the aster yellows agent. Protoplasma 95:207-216.
5. Chen, M. H., and Hiruki, C. 1978. The preservation of membranes of tubular bodies associated with mycoplasmalike organisms by tannic acid. Can. J. Bot. 56:2878-2882.
6. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. J. Gen. Virol. 34:475-485.
7. Clark, M. F., Barbara, D. J., and Davies, D. L. 1983. Production and characteristics of antisera to *Spiroplasma citri* and clover phyllody-associated antigens derived from plants. Ann. Appl. Biol. 103:251-259.
8. Gardella, R. S., DelGiudice, R. A., and Tully, J. G. 1983. Immunofluorescence. Pages 431–439 in: Methods in Mycoplasmology. Vol. 1. S. Razin and J. G. Tully, eds. Academic Press, New York.
9. Johnstone, A., and Thorpe, R. 1982. Immunochemistry in Practice. Blackwell Scientific Publ., Oxford. 298 pp.
10. Kawamura, A., Jr., ed. 1977. Fluorescent Antibody Techniques and Their Applications, 2nd ed. Univ. Tokyo Press, Tokyo. 292 pp.
11. Kenny, G. E. 1983. Agar precipitin and immunoelectrophoretic methods for detection of mycoplasmic antigens. Pages 441–456 in: Methods in Mycoplasmology. Vol. 1. S. Razin and J. G. Tully, eds. Academic Press, New York.
12. Rao, D. V., Shukla, P., and Hiruki, C. 1978. In situ reaction of clover yellow mosaic virus (CYMV) inclusion bodies and fluorescent antibodies to CYMV. Phytopathology 68:1156-1159.
13. Williamson, D. L. 1982. Serological characterization of spiroplasmas and other mycoplasmas. Pages 240-267 in: Plant and Insect Mycoplasma Techniques. M. J. Daniels and P. G. Markham, eds. Croom Helm, London.