

Comparison of Monoclonal Antibodies and Polyclonal Antibodies in Detection of the Aster Yellows Mycoplasma-like Organism

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ABSTRACT

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Monoclonal antibodies (MA) against the aster yellows (AY) agent, a noncultivable mycoplasma-like organism (MLO), were produced by hybridoma techniques. Polyclonal antibodies (PA) were purified from the antisera collected from immunized mice during splenectomy. Both the MA and PA were used in the *in vitro* and *in situ* detection of the AY-agent. In *in vitro* detection by indirect enzyme-linked immunosorbent assay, the MA reacted specifically with the AY-infected plants and differentiated the AY agent from other MLOs. Both the plant antigen-absorbed and the

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nonabsorbed PA, however, cross reacted with plant antigens. In *in situ* detection by immunofluorescent staining, cross- or longitudinal sections of leaf midribs from healthy and AY-infected lettuce were made. The acetone-fixed sections were treated with either the MA or PA, then stained with fluorescein isothiocyanate-conjugated antimouse IgG. MA bound specifically to the AY-MLO in the sieve tubes of diseased plants, while PA treatment showed fluorescence throughout the sections in both healthy and diseased plants suggesting nonspecific binding to cell wall and membrane.

Aster yellows (AY), an economically important disease that affects many crops (21), has been the most widely and intensively studied plant disease of the yellows group. Mycoplasma-like organisms (MLOs) have been implicated as the etiological agents of AY and about 100 other plant yellows diseases since the first report by Japanese scientists (13). Since none of the plant MLOs have been cultivated, the numerous yellows diseases have only been differentiated by indefinite biological properties such as host range, symptomatology, and insect vector relations.

Sufficient quantities of pure AY-MLO have never been obtained for use as an antigen to immunize animals. Although antigen preparations from plants (31,32) were used recently to produce

antisera against the AY agent and other MLOs (8,19,33), the serological data generated were not always convincing. Such polyclonal antisera have relatively low specific titer and exhibit considerable nonspecific reactions because of the presence of antibodies to plant antigens.

The enzyme-linked immunosorbent assay (ELISA) (14) and the *in situ* immunofluorescent test (12) are two highly sensitive serological procedures which have been used for the identification and classification of the cultivable plant and insect mycoplasmas (1,9,28). Serological detection by ELISA, which is widely used for plant viruses (7), has been used to detect *Spiroplasma citri* in extracts of infected plants and insects (1,3,9,28). Archer et al (2) used ELISA to study the distribution of *S. citri* in various tissues of periwinkle. Tully et al (36) studied the serological relatedness of various spiroplasmas by ELISA. Presently, there are very few reported studies of the application of ELISA to plant pathogenic MLOs (8,33).

Immunofluorescence techniques have been used for many years in the cellular location of viral antigens (25-27,30,35), bacterial

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cells (4), and fungal propagules (15). It was first applied to detect the mycoplasma contamination of cell cultures (5,10). It has become a routine serological procedure used specifically for the identification of mycoplasmas grown on agar media (12) or in infected cell cultures (11,18). Yet, it has not been employed in the study of plant and insect MLOs because specific antisera are difficult to prepare.

This paper describes the production of monoclonal antibodies and polyclonal antibodies to AY-MLO and the comparison of these antibodies in the indirect ELISA and immunofluorescence studies for detecting and identifying the AY agent.

MATERIALS AND METHODS

Diseased plants. The AY agent obtained from the diseased lettuce collected from the Rutgers College Farm, New Brunswick, NJ, was maintained in lettuce (*Lactuca sativa* var. *capitata* L.) through inoculation by infective leafhopper vectors *Macrostelus fascifrons* Stål (34). Both AY agent-infected lettuce and periwinkle (*Catharanthus roseus* L.) were used in the ELISA tests. The MLOs associated with aster yellows, ash yellows, paulownia witches' broom, sweet potato witches' broom, peanut rosette, and elm phloem necrosis were maintained in periwinkle, and the maize bushy stunt MLO was maintained in corn (*Zea mays* L.).

Monoclonal antibodies. A hybridoma clone (3.2G11) produced in our lab (22) secreting specific monoclonal antibodies against the AY agent was used. Six-week-old female BALB/c mice immunized with the AY agent partially purified from the salivary glands of infective leafhopper vectors (*M. fascifrons*) and the murine myeloma cell line P3/NS1/1-Ag4-1 (NS-1) were used in hybridoma production. Hybridoma 3.2G11 was cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum (Gibco Lab., Grand Island, NY). Monoclonal antibodies were harvested from culture supernatants when cell titers reached $5-7 \times 10^6$ cells per milliliter, and were purified by precipitation with 50% saturated ammonium sulfate. The concentrated antibody precipitations were pelleted and resuspended in 1/10 the original volume of phosphate-buffered saline (PBS, pH 7.4) before being exhaustively dialyzed against PBS. These antibodies were further diluted to 50 μ g of protein per milliliter (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, CA) in PBS (pH 7.4) for ELISA tests, and 200 μ g of protein per milliliter for immunofluorescent staining according to our preliminary tests.

Polyclonal antibodies. Antisera were collected from blood when the mice were killed for the production of monoclonal antibodies. Polyclonal antibodies were purified from such antisera by the same method described for the purification of monoclonal antibodies. The polyclonal antibodies were further diluted in the same way as that of the monoclonal antibodies for various tests.

Portions of the polyclonal antibodies of desired dilution (protein = 50 μ g/ml) were further cross-absorbed with healthy plant antigens prepared by two different methods. In the first method, 10 g of healthy lettuce leaf midribs were used to isolate the vascular elements as described by Lin and Chen (22). Vascular preparations were then homogenized in 10 ml of PBS (pH 7.4) with mortar and pestle and clarified by low speed centrifugation at 2,000 g for 10 min. The plant protein was concentrated to 1 ml by precipitation with 50% saturated ammonium sulfate and then extensively dialyzed against PBS (pH 7.4) (24). In another method, the vascular preparations from 10 g of healthy lettuce leaf midribs were fixed in acetone for 30 min and air-dried. The fixed vascular preparations were then ground with a tissue grinder into dry powder. Polyclonal antibodies were cross-absorbed by mixing 1 ml of purified antibody (protein content = 50 μ g) with 1 ml of the concentrated plant protein or the acetone-dried powders prepared from 10 g of leaf midribs. The mixture was incubated overnight at 4 C. The immunoprecipitates were removed by centrifugation at 13,000 g for 10 min with a Fisher model 235B micro-centrifuge (Fisher Scientific, Springfield, NJ). The final protein content of the cross-absorbed polyclonal antibodies was about 15-20 μ g/ml.

Detection of AY agent with indirect ELISA. AY agent for coating ELISA plates was prepared from AY agent-infected

periwinkle and lettuce. Leaf midribs were ground and diluted tenfold (w/v) in coating buffer (0.05 M bicarbonate buffer, pH 9.6), and clarified by low-speed centrifugation at 2,000 g for 10 min. Preparations obtained from healthy lettuce and periwinkle were used as controls. Indirect ELISA with biotinylated antimouse Ig (Vector Laboratories, Burlingame, CA) was used in all of our ELISA studies (23). The 96-well microtiter plates (Vanguard International, Neptune, NJ) were first coated with 100 μ l of coating antigens. In all of our ELISA studies, at least three repetitions were tried each time. Next, the diluted monoclonal antibodies, nonabsorbed polyclonal antibodies, or plant-antigen-cross-absorbed polyclonal antibodies were added. The biotinylated antimouse Ig and the avidin-biotinylated peroxidase were sequentially incorporated before the substrate (*O*-phenylenediamine) was added. Mouse normal serum was used as an antibody negative control. The reaction was stopped with 3 N H₂SO₄, and the plates were read at 490 nm in an ELISA reader (Titertek Multiskan MC; Flow Lab., Inc., McLean, VA).

Serological specificity. Indirect ELISA with biotinylated antimouse Ig was used in the specificity studies. Test antigens were prepared from the healthy and seven MLO-infected plants in the same manner as described previously for the coating antigens. MLO antigens associated with ash yellows, paulownia witches' broom, sweet potato witches' broom, peanut rosette, elm phloem necrosis, and aster yellows were prepared from diseased periwinkle, and that associated with maize bushy stunt were from corn. Preparations obtained from healthy periwinkle and corn were used as controls.

Immunofluorescent staining. Cross and 1-cm-long longitudinal, free-hand sections of leaf midribs were prepared from both AY agent-infected and healthy lettuce. Only the longitudinal sections were first treated with the enzyme solution (1% cellulase, 0.5% pectinase, 0.6 M mannitol, 1 mM CaCl₂, and 0.04% Na₂SO₃, pH 5.5) in a shaker (20 rpm) for 30 min at room temperature and then washed with three changes of PBS at 30-min intervals. The cross- and enzyme-treated longitudinal sections were then fixed with cold acetone for 40 min at room temperature and air-dried. The fixed sections were washed with three changes of PBS at 30-min intervals. Two milliliters of monoclonal antibodies, or nonabsorbed polyclonal antibodies (protein content = 200 μ g/ml), or PBS (Ab control) were then added to the sections and incubated for 4 hr at room temperature. After being washed with three changes of PBS, the sections were incubated in PBS at 4 C overnight. The sections were then stained with 2 ml of fluorescein isothiocyanate-conjugated F(ab')₂ fragment of antimouse IgG (Sigma Chemical Company, St. Louis, MO), diluted 100 times in PBS (pH 7.4) for another 4 hr at room temperature. The sections were washed three times and incubated in PBS for 2 hr before observation under the microscope.

Samples were mounted in PBS and examined by using a Nikon epifluorescence microscope with a HBO 50-W high-pressure mercury lamp, an IF 410-485 excitation filter, a DM 505 dichroic mirror, a 460 auxiliary filter, and a 515 W absorption filter. The main wavelength of such an excitation method and filter combination is 495 nm.

Photomicrographs were made with a 35-mm Nikon automatic photomicrographic system (Microflex AFX) using ASA 400 fast film (Kodak Tri-X Pan). To demonstrate the relationship between the fluorescent staining and the structure detail, comparable pictures of bright-field and fluorescence illumination were taken from the same field.

RESULTS

Detection of AY agent with indirect ELISA. In ELISA, a reaction was generally considered positive if the absorbance value was higher than 0.1, since the normal serum-negative controls yield nonspecific background readings less than 0.1. As shown in Table 1, with the monoclonal antibody, the presence of AY agent in diseased lettuce and periwinkle was clearly demonstrated, and negative results were obtained in healthy plant controls. When nonabsorbed polyclonal antibodies were used, high absorbance

values were obtained in all four antigen preparations, which indicated the presence of antibodies to plant antigens. Such high absorbance readings in healthy plant controls thus hampered the value of the small differences between the healthy and AY agent-infected plant treatments. In both of the cross-absorbed polyclonal antibodies, the differences between the absorbance readings of AY agent-infected plant preparations and healthy plant controls were greater than those in the tests using unabsorbed polyclonal antibodies, but high readings were still obtained in healthy plant controls.

TABLE 1. ELISA reactions (at 490 nm) of aster yellows (AY)-monoclonal antibody and polyclonal antibodies with healthy and AY agent-infected lettuce and periwinkle

| AY agent-infected or healthy plant antigens | Antibody | | | |
|---|----------------|----------------|----------------|----------------|
| | 1 ^a | 2 ^b | 3 ^c | 4 ^d |
| Periwinkle | | | | |
| Healthy | 1.271 | 0.307 | 0.205 | 0.013 |
| AY agent-infected | 1.493 | 0.672 | 0.581 | 0.970 |
| Lettuce | | | | |
| Healthy | 1.531 | 0.359 | 0.219 | 0.007 |
| AY agent-infected | 1.697 | 0.806 | 0.708 | 1.428 |

^aUnabsorbed polyclonal antibodies (protein = 50 µg/ml).

^bPolyclonal antibodies (protein = 50 µg/ml) cross absorbed by ammonium sulfate-precipitated plant antigens.

^cPolyclonal antibodies (protein = 50 µg/ml) cross absorbed by acetone-fixed vascular tissues.

^dMonoclonal antibody from hybridoma clone 3.2G11 (protein = 50 µg/ml).

Serological specificity. The monoclonal antibody reacted specifically to AY agent prepared from infected periwinkle. On the other hand, the AY-agent monoclonal antibody did not react with the antigen preparations of healthy plant controls, corn infected with maize bushy stunt, or five other MLO-infected periwinkle plants: ash yellows, paulownia witches' broom, sweet potato witches' broom, peanut rosette, elm phloem necrosis (Table 2). When nonabsorbed polyclonal antibodies were used, high reaction

TABLE 2. ELISA reactions (at 490 nm) of aster yellows monoclonal antibody and polyclonal antibodies with healthy and MLO-infected plants

| MLO-infected or healthy plant antigens | Antibody | | | |
|--|----------------|----------------|----------------|----------------|
| | 1 ^a | 2 ^b | 3 ^c | 4 ^d |
| Ash yellows | 1.263 | 0.281 | 0.218 | 0.013 |
| Paulownia witches' broom | 1.277 | 0.295 | 0.215 | 0.017 |
| Sweet potato witches' broom | 1.232 | 0.276 | 0.183 | 0.002 |
| Peanut rosette | 1.247 | 0.279 | 0.211 | 0.007 |
| Maize bushy stunt ^e | 1.132 | 0.219 | 0.196 | 0.017 |
| Elm phloem necrosis | 1.302 | 0.278 | 0.184 | 0.006 |
| Aster yellows | 1.505 | 0.657 | 0.577 | 0.984 |
| Healthy periwinkle | 1.259 | 0.287 | 0.213 | 0.017 |
| Healthy corn | 1.021 | 0.204 | 0.182 | 0.003 |

^aUnabsorbed polyclonal antibodies (protein = 50 µg/ml).

^bPolyclonal antibodies (protein = 50 µg/ml) cross absorbed by ammonium sulfate-precipitated plant antigens.

^cPolyclonal antibodies (protein = 50 µg/ml) cross absorbed by acetone-fixed vascular tissue.

^dMonoclonal antibody from hybridoma clone 3.2G11 (protein = 50 µg/ml).

^ePrepared from corn; all the others are from periwinkle.

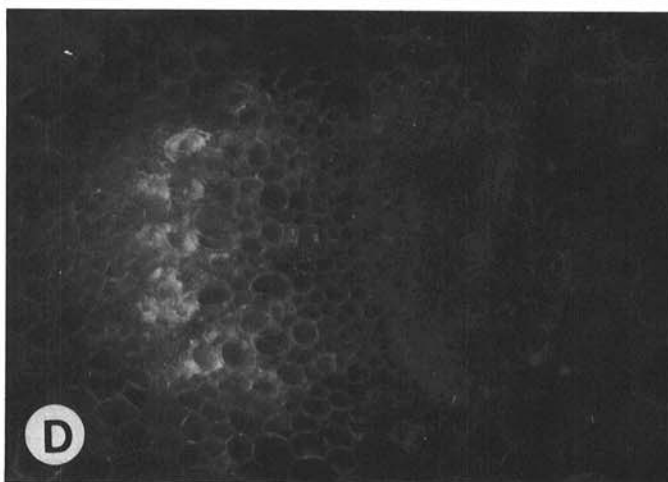
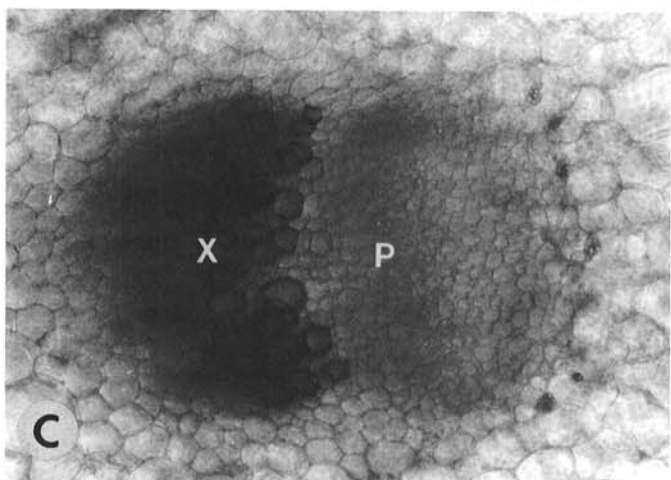
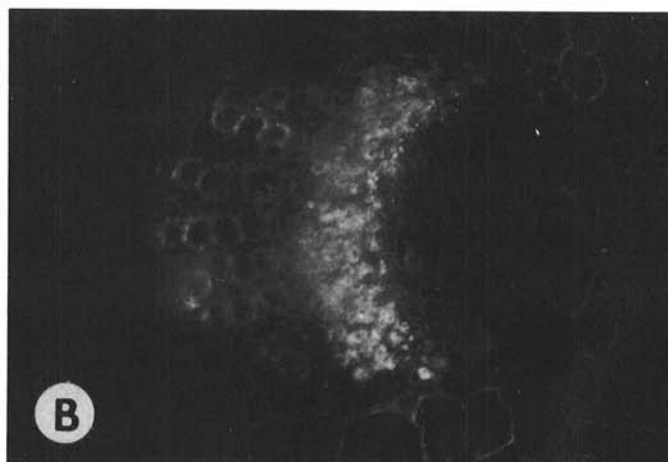
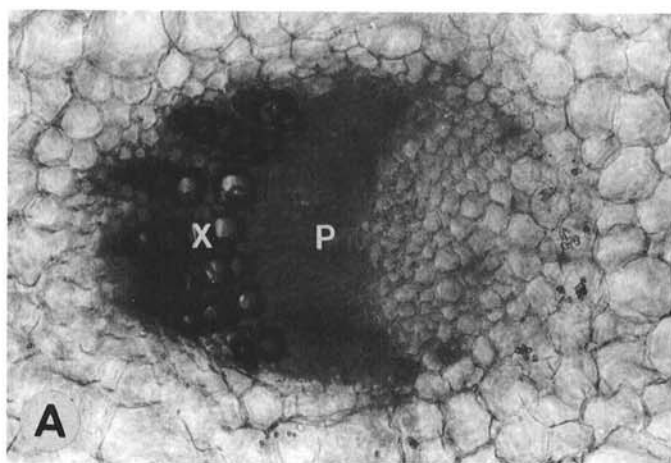


Fig. 1. Immunofluorescent staining with polyclonal antibodies for aster yellows mycoplasma-like organisms. **A and B**, Cross sections of diseased lettuce; **C and D**, cross sections of healthy lettuce. X = xylem, and P = phloem. Pictures were taken of the same field under **A and C**, bright-field and **B and D**, fluorescent microscopy ($\times 150$).

readings were obtained in all diseased and healthy plant antigen preparations. Only a slight difference between preparations from AY-infected and healthy periwinkle plants were observed. In both of the cross-absorbed polyclonal antibody tests, although greater differences could be seen between the AY agent-infected and healthy plant preparations, positive cross reactions in all the nonspecific MLO preparations and the healthy plant controls still existed (Table 2).

Immunofluorescence staining. Under ultraviolet light, both the healthy and diseased sections in PBS-treatment controls (instead of antibodies, PBS was used in the antibody treatment step) produced a brownish and/or greenish autofluorescence of low intensity in xylem areas. When polyclonal antibodies were incorporated, the fluorescein isothiocyanate-specific apple green fluorescence was observed throughout the membrane and cell wall areas in both the healthy and AY agent-infected sections. The entire sieve tube areas in diseased lettuce sections were also heavily stained (Fig. 1). On the contrary, monoclonal antibodies bound specifically to AY-MLO in the sieve tubes of diseased sections, and only in these tissues was the bright fluorescein isothiocyanate-specific apple green fluorescence observed (Fig. 2A and B). No fluorescence could be seen in healthy samples except very slight greenish autofluorescence appeared in the xylem areas (Fig. 2C and D). When longitudinal sections were used, the monoclonal antibodies also specifically bound to the MLOs residing in the sieve tubes of AY agent-infected lettuce sections (Fig. 3A and B). There was no fluorescence in healthy plant controls (Fig. 3C and D).

DISCUSSION

When conventionally produced polyclonal antibodies were applied to detect the AY agent and other MLOs with ELISA, the

serological data were not always convincing. Sometimes conflicting results evolved in the serological specificity studies from different labs (8,33). These variabilities were probably due to different serological methods and immunization schemes or the use of different animals. Such nonspecific cross reactions often hampered the validity of the interpretation of results such as in the detection of the MLOs in plants or insect vectors. Although Clark et al (8) suggested that using plant protein cross-absorbed polyclonal antibodies might increase the reliability of using PA for MLO diagnosis, the contamination of antibodies to plant antigens in a heterogeneous antibody population was still inevitable. Thus, PA cannot clearly distinguish the diseased plants from the healthy plants.

In the past, MLO-nonspecific fluorescent stains, such as DAPI (4', 6'-diamidino-2-phenylidole) (29) and aniline blue (16,17), have been applied in the detection of MLO agents associated with various yellows diseases. The DNA-specific fluorescent stain DAPI might have produced fluorescing nuclei which would lead to some confusion, especially in plants that were only slightly infected and were colonized by MLOs in the youngest sieve tubes which have not fully developed and were still nucleated. Aniline blue (AB) has been used for the indirect detection of MLOs in the blueberry stunt-infected plants (16) based on excess callose formed in the sieve tubes (17). Studies with the indirect AB techniques could result in false identification, because callose deposits increase as sieve tubes age. Since these two techniques are nonspecific for MLO, their use in diagnosis is limited. In addition, these stains do not indicate whether the fluorescence is due to some other phloem-inhabiting organisms (6). Therefore, until the plant pathogenic MLOs can be cultured, their serological identification must depend on the production of specific antibodies without the production of pure antigens. Highly sensitive serological methods will certainly play an

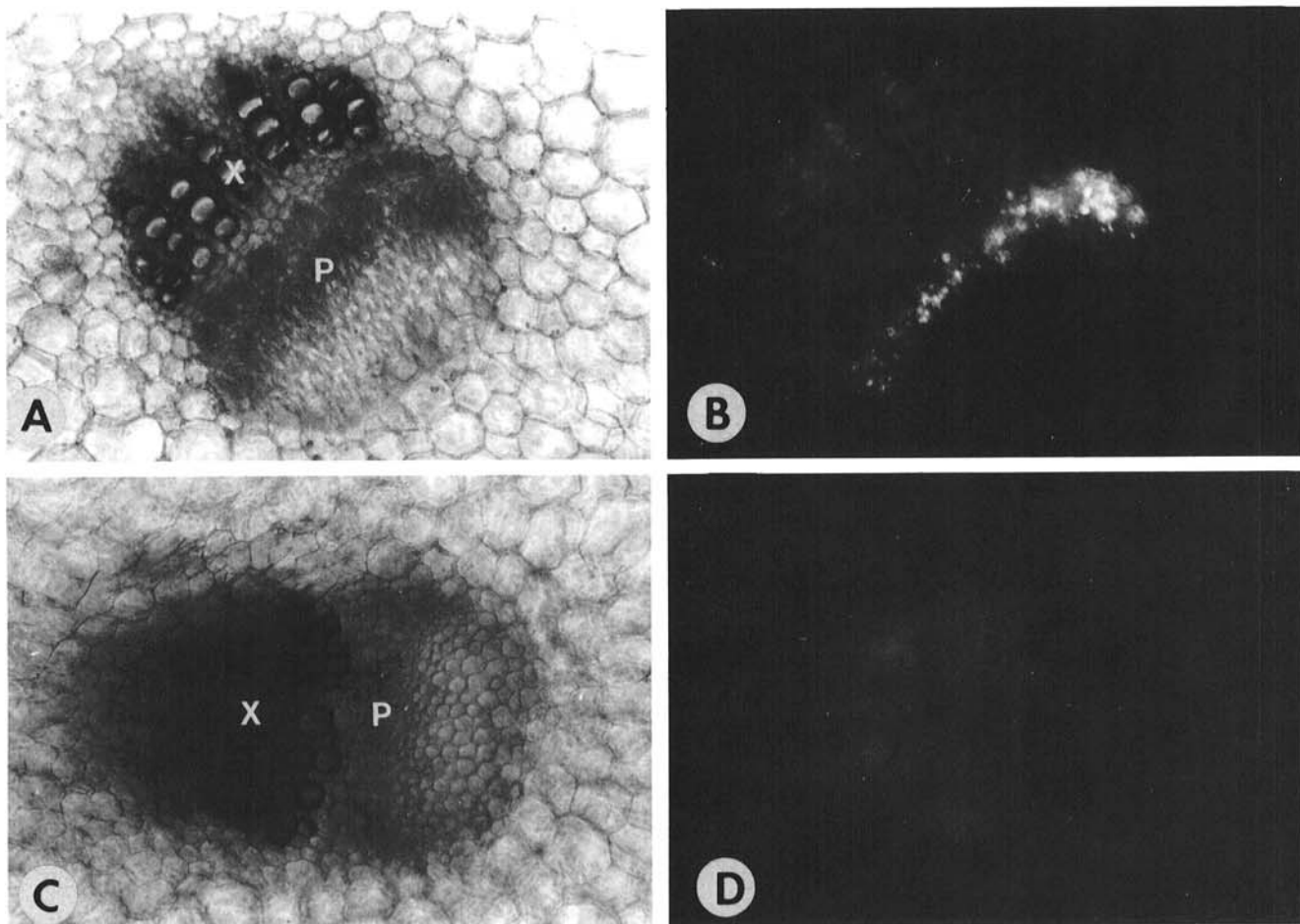


Fig. 2. Immunofluorescent staining with monoclonal antibody for aster yellows mycoplasma-like organisms. A and B, Cross sections of diseased lettuce; C and D, cross sections of healthy lettuce. X = xylem, P = phloem. A and C, Bright-field and B and D, fluorescent microscopy ($\times 150$).

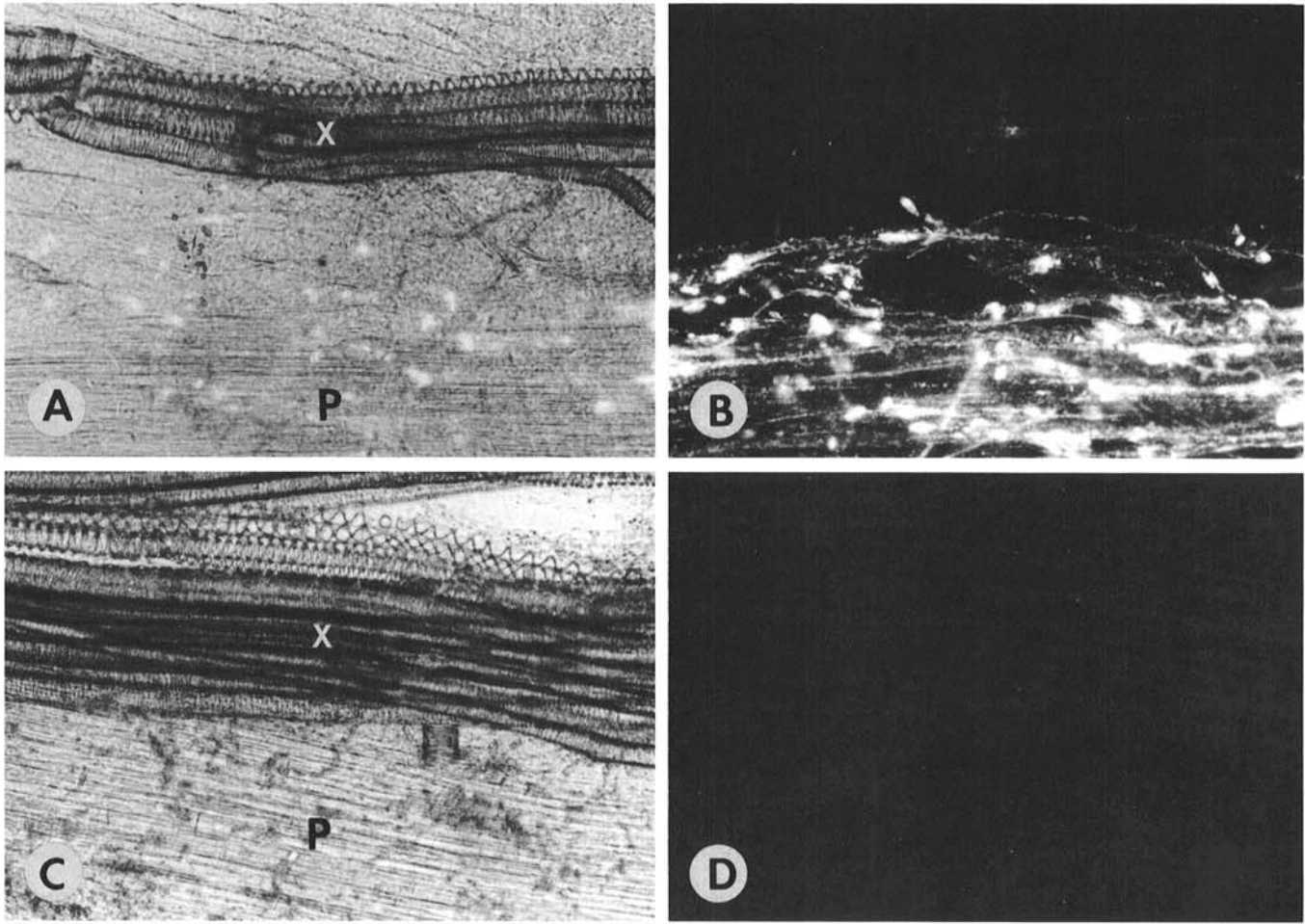


Fig. 3. Immunofluorescent staining with monoclonal antibodies for aster yellows mycoplasma-like organisms. **A and B**, Longitudinal sections of diseased lettuce; **C and D**, longitudinal sections of healthy lettuce. X = Xylem, P = Phloem. **A and C**, Bright-field and **B and D**, fluorescent microscopy ($\times 150$).

important role in diagnosis of yellows diseases. ELISA and fluorescent antibody techniques are two of the most sensitive antigen-binding assays. However, specific antibodies are a prerequisite for using these techniques. Hybridoma techniques (20) can be successfully used to produce specific monoclonal antibodies against the MLOs in spite of the contamination by plant or insect antigens.

The data presented here substantiate the sensitivity of the ELISA and the immunofluorescent staining in studies on plant MLOs and also demonstrated the shortcomings of polyclonal antibodies. Cross-absorbed polyclonal antibodies, when compared to unabsorbed polyclonal antibodies, were able to discriminate more effectively between healthy and diseased plants with ELISA, but the positive reactions to healthy control may still cause some unnecessary confusion for their use in distinguishing serological specificity among various MLOs. In immunofluorescent staining, PA can stain heavily in the phloem areas of diseased plant sections and thus contrast specifically from the results when healthy plant sections were used. It is possible that PA may have specifically reacted to AY agent, but the nonspecific stain on the plant antigens may interfere and easily mislead the interpretation of results. Since the PA reacts to the whole spectrum of AY-agent antigens, there will be a very strong possibility for such PA to cross react to other MLO preparations that share the same common antigens possessed by the AY agent. Once the cross reaction occurs, the accuracy for disease diagnosis will be decreased. The highly discriminatory capacity of MA will be the most important advantage over PA for mycoplasma disease diagnosis, disease forecasting, and epidemiological studies. Our MA reacts positively to several AY agent-infected plants obtained from vegetable fields in southern New Jersey and eastern AY agent-infected asters, but not to other

plants suspected of having AY agent infections. However, because of their high specificity, interpretation of results in taxonomic studies should proceed with caution. MA of different specificity levels must be obtained to understand the array of antigenic determinants of AY-MLO. Such monoclonal antibodies can also be further applied in the purification of MLO antigens or MLOs and thus enable study of the serological relationships among various plant pathogenic MLOs and their taxonomic standing.

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