An Immunofluorescence Test for Maize Chlorotic Dwarf Virus

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ABSTRACT

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Maize chlorotic dwarf was diagnosed by indirect labeling with fluorescent antibody. Maize chlorotic dwarf virus (MCDV) was precipitated from clarified extracts with MCDV antiserum and the precipitate was labeled with fluorescein-conjugated rabbit gamma globulin antiserum. Immunofluorescence results were in good agreement with other methods for MCDV diagnosis.

Additional key words: fluorescence microscopy, disease diagnosis.

Diagnosis of animal and human virus diseases by immunofluorescence has been widely reported (3). Fluorescent antibody staining has been used for the detection and localization of plant viruses in infected tissue, but not for diagnosis of plant virus diseases (1). This paper reports an immunofluorescence test for maize chlorotic dwarf virus (MCDV) in plant extracts. Because immunofluorescence test results are available in a short time, the use of immunofluorescence to rapidly diagnose maize virus diseases was tested.

MATERIALS AND METHODS

Infected plants.—Naturally infected corn plants (*Zea mays* L.) were from field plots or plants exposed briefly to field conditions (trap plants) (6) in Portsmouth, Ohio. Inoculated MCDV-infected plants obtained by serial transmission of MCDV in inbred Oh28 with the leafhopper, *Graminella nigrifrons* (Forbes) (7), were maintained in greenhouses at Wooster, OH.

Antisera.—Antisera to MCDV and maize dwarf mosaic virus (MDMV) were prepared in rabbits (5, 11). Lyophilized fluorescein-conjugated rabbit gamma globulin antiserum (anti-rabbit serum) (ICN Pharmaceuticals, Inc., Cleveland, OH 44128) was reconstituted with water and centrifuged at 12,000 *g* for 20 min to remove nondissolved materials.

Sample preparation.—Unless otherwise stated, assays were performed as follows: One g leaf tissue, sliced transversely into 1-2 mm sections, was vacuum infiltrated with buffer. After blotting, the tissue was ground with a mortar and pestle and filtered through 45 μm nylon mesh. The extract was emulsified with 1/2 vol $CHCl_3$ and after low-speed centrifugation (7,500 *g* for 10 min) 0.05 ml of the aqueous phase was placed in a 10 × 75-mm test tube and mixed with 0.025 ml MCDV antiserum. After incubation at room temperature for 1 hr, 1 ml PBS (0.15 M NaCl, 0.01 M potassium phosphate, pH 7.0) was added and the mixture was centrifuged (7,500 *g* for 10 min). The pellet was washed twice by alternate suspension in 1 ml PBS and centrifugation. The final pellet was suspended in 0.025 ml anti-rabbit serum. After 0.5-1 hr at room temperature, 1 ml PBS was added. The contents were again centrifuged and the pellet washed once with PBS. After decanting the supernatant of the final wash, the pellet was resuspended in the small amount of liquid (about 0.025 ml) that remained.

Microscopy.—A 1 μliter droplet of the final suspension was spotted on a glass microscope slide, allowed to dry, and then viewed in a Zeiss PMQ II photomicroscope with HBO mercury lamp epi-illumination at ×31. The BG 12/4 + BG 38 exciter filters and the 53 barrier filter were used. For photographic records, high-speed Ektachrome daylight film (ASA 160) was exposed 20 min and developed at ASA 320; or Tri-X (ASA 400) was exposed 80 sec and developed at ASA 1200.

Fluorometry.—The final suspension, except for the μliter used for microscopy, was diluted with 0.25 ml water and placed in the micro sample holder in the Turner Model 110 filter fluorometer. The exciter filter was #48 and the barrier filter was 2A-12.

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RESULTS

Tissue preparation.—Extracts of plant tissues containing MCDV were concentrated by high-speed centrifugation or PEG precipitation. In preliminary fluorescent antibody tests, significantly more fluorescence was observed in infected samples than in healthy controls. To save time, the test then was attempted with unconcentrated preparations. For maximum virus concentration and, presumably, maximum sensitivity, the volumes of tissue extracts were kept small by grinding with small volumes of buffer or by vacuum infiltrating with buffer and grinding. Figure 1 shows the effect of tissue extraction procedure on test results. Best results were obtained by infiltrating small pieces of tissue. All three buffers gave satisfactory results, but 0.1 and 0.5 M phosphate were better than PBS.

Two washings of the pellet after incubation with MCDV antiserum and one washing after incubation with anti-rabbit serum were necessary to eliminate background fluorescence in healthy controls before viewing them in the microscope. Florescence was usually more intense with unconcentrated than with concentrated preparations. Results with unclarified extracts were variable and complicated by autofluorescence and nonspecific staining.

Reliability.—The immunofluorescence test was compared to other methods commonly used for the diagnosis of MCDV including symptomatology, rate-zonal centrifugation (6), and micro agar-gel immune double diffusion (4) (Table 1). Field samples collected for this comparison were beginning to senesce in some instances, and diagnosis by symptomatology was difficult. Rate-zonal centrifugation indicated a very low level of virus in the one infected sample that was negative in the immunofluorescence assay. The use of younger field material would probably be better for the immunofluorescence test. Co-infection with MDMV did not

TABLE 1. Comparison of the immunofluorescence test and other techniques for diagnosis of maize chlorotic dwarf virus

Type of sample		Rate-zonal centrifugation	Agar gel immuno-diffusion	Symptoms	Immunofluorescence Positive	Immunofluorescence Negative	Overall agreement[a]
Field-collected	Pos.	18	18	17	17	1	19/20
(20 samples)	Neg.	2	2	3	0	2	
Inoculated and	Pos.	18 (6 MDMV)[c]	17	18 (7 MDMV)	18	0	29/29
trap plants[b]	Neg.	11(3 MDMV)	12	11 (3 MDMV)	0	11	
(29 samples)							

[a]Compared to the rate-zonal centrifugation assay.
[b]Trap plants were exposed to field conditions for 1 wk and then held in the greenhouse for 2 wk prior to being assayed.
[c]Number of plants which were positive for maize dwarf mosaic virus infection.

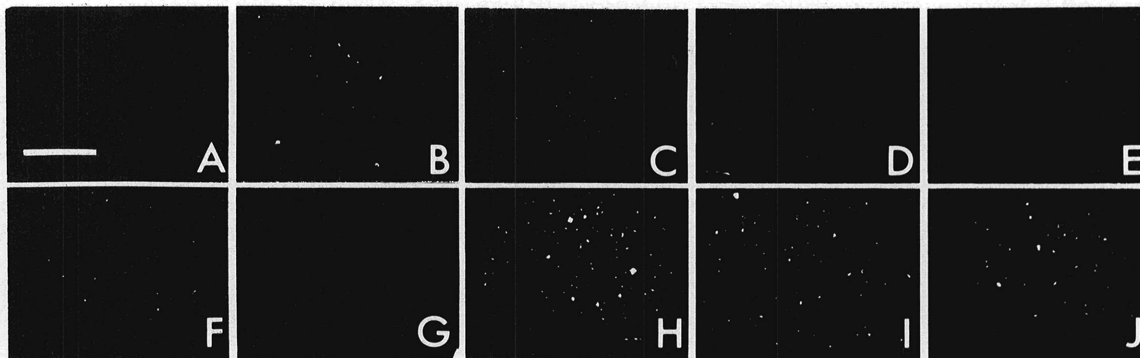

Fig. 1. Micrographs of fluorescence produced from infected tissue subjected to various extraction procedures. Negatives and subsequent contact prints were made from Ektachrome slides. The bar represents 1.0 mm. Figures 1-(A to E) tissue not infiltrated prior to grinding; tissue extracted in:

1-A–0.1 M potassium phosphate buffer ($KHPO_4$), 0.5% 2-mercaptoethanol (2-ME), pH 6.0 (0.1 ml).
1-B–0.1 M $KHPO_4$, 0.5% 2-ME, pH 7 (0.5 ml).
1-C–0.5 M $KHPO_4$, 0.5% 2-ME, pH 7 (0.1 ml).
1-D–PBS (0.1 ml).
1-E–No buffer.

Figures 1-(F to J) tissue infiltrated before grinding with:

1-F–0.1 M $KHPO_4$, 0.5% 2-ME, pH 7; large (2 × 5 cm) pieces of tissue.
1-G–0.1 M $KHPO_4$, 0.5% 2-ME, pH 7; small (1-2 mm transverse slices) pieces of tissue, no clarification.
1-H–0.1 M $KHPO_4$, 0.5% 2-ME, pH 7; small pieces of tissue.
1-I–0.5 M $KHPO_4$, 0.5% 2-ME, pH 7; small pieces of tissue.
1-J–PBS; small pieces of tissue.

There was no fluorescence in the healthy control for each treatment.

affect the immunofluorescence test for MCDV.

Sensitivity.—The sensitivities of the rate-zonal and immunofluorescence tests were compared. Clarified extracts from infected plants were diluted with similar extracts from healthy plants, and the dilution end points for virus detection were determined by immunofluorescence and rate-zonal centrifugation, before and after 16-fold concentration (Table 2). Immunofluorescence was more sensitive for clarified extracts; rate-zonal centrifugation was more sensitive for concentrated virus preparations. A possible explanation for this and for the observation that concentrating the virus did not appreciably increase the sensitivity of the immunofluorescence test was that the immunofluorescence test detected residual, nonencapsidated virus protein in the clarified extract. This protein was subsequently removed by high-speed concentration. Positive immunofluorescence reactions of clarified extracts after removal of virus particles by high-speed centrifugation supported this hypothesis.

Capacity.—About 30 immunofluorescence tests could be done per day by one person. By contrast, about 18 tests/day/person can be done by rate-zonal centrifugation.

Speed.—An attractive feature of the immunofluorescence test is that results are available in 4 hr. The rate-zonal test requires about 2 days. The micro agar gel diffusion test requires about 3 days.

Specificity.—No visible fluorescence for the following treatments showed that the immunofluorescence test was specific for MCDV: (i) PBS substituted for MCDV antiserum, (ii) MDMV antiserum substituted for MCDV antiserum, (iii) MDMV extract substituted for MCDV extract, and (iv) healthy extract substituted for MCDV extract.

DISCUSSION

Since the primary antibody (anti-MCDV) was not labeled, the procedure is referred to as the "indirect" or "sandwich" method (10). A "direct" assay with conjugated virus antiserum should work. However, greater sensitivity (up to 12-fold) (2, 8) and the applicability to other viruses (if rabbit-prepared antisera are available) makes the indirect method more versatile. Of course, analagous systems with other animal species are possible.

Unexpectedly, the assay worked better on nonconcentrated virus extracts than on concentrated extracts. Although the dilution end points were similar for both types of extracts (Table 2), with concentrated extracts, the precipitate was sometimes flocculent and the fluorescence diffuse. With unconcentrated extracts, the precipitate was usually granular and the fluorescence more intense making the results easier to interpret. Nonencapsidated viral protein in the nonconcentrated extracts probably contributed to the difference.

Although higher magnifications were tested, ×31 was best because the entire spot could be seen in the microscope field and background fluorescence was not visible. Attempts to quantitate the assay by fluorometry were not successful. Also, quantitation of the fluorescence by microscopy would probably be difficult because antisera titers vary widely and antigen-antibody complexing is largely undefined.

Theoretically, two or more viruses could be assayed simultaneously, if the antiserum to each virus were prepared in different animal species and the corresponding gamma globulin antisera were each conjugated with a different fluorochrome. However, it would seem more practical to test extracts for different viruses separately.

The immunofluorescence test gave a positive reaction with all infected plants which were in a suitable condition. The positive results obtained even after dilution of infected extracts (Table 2) indicates that plants with lower virus concentrations than encountered here could be diagnosed correctly. If negative results were still questionable after immunofluorescence testing, the more sensitive, but more laborious and time-consuming, rate-zonal test could be done on concentrated extracts.

The enzyme-linked immunosorbent assay (ELISA) has recently been adapted to the detection of plant viruses (9). Although direct comparisons of ELISA and the immunofluorescence test were not made, the relative merits of the two procedures can be discussed. The advantages of ELISA are extreme sensitivity, ability to quantitate virus, and the applicability to viruses for which conventional serological techniques cannot be used. However, the procedure is relatively complex and enzyme-linked anti-

TABLE 2. Relative sensitivities of immunofluorescence and rate-zonal centrifugation for detection of maize chlorotic dwarf virus[a]

Sap extract		Experiment 1	2	3	4	5
Nonconcentrated	Rate-zonal	1:2[b]	1:2	1:5	1:2	1:2
	Immunofluorescence	1:2	1:5	1:20	1:10	1:10
Concentrated	Rate-zonal	1:50	1:50	1:50	1:20	1:20
	Immunofluorescence	1:5	1:5	1:20	1:10	1:20

[a]Independent experiments were done at different times on different tissues. For tests on nonconcentrated extracts, healthy and infected tissue was infiltrated and clarified as in Materials and Methods. Healthy and infected extracts were combined in various proportions; 0.05 ml was assayed by the immunofluorescence test and compared to a rate-zonal test in which extract from a total of 1 g tissue (infected + healthy) had been layered on the gradient. In assays of concentrated preparations, clarified extracts were concentrated 16-fold by centrifugation and assayed with volumes identical to those used for testing the clarified extracts.

[b]Ratio of amount of clarified extract from infected tissue to total amount of clarified extract. For rate-zonal centrifugations, the ratios are the lowest at which a virus peak was evident after scanning the gradient at 254 nm and a full-scale range of 0.2 OD units. For immunofluorescence tests, the ratios are the lowest at which there was significant fluorescence.

serum to each virus must be prepared. The immunofluorescence test is simpler and commercially available labeled antiserum may be used. The immunofluorescence test should be suitable for virus detection in many instances in which very high sensitivity and quantitation are not needed.

The immunofluoresence test was useful for routine assay of MCDV because of its speed, reliability, and sensitivity. Preliminary results suggested that the procedure works for MDMV also. This procedure should be widely applicable in the diagnosis of those viruses for which specific antisera can be prepared.

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