An Improved Method for Serological Detection of Cymbidium Mosaic Potexvirus Infection in Orchids

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ABSTRACT

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Murine hybridomas secreting monoclonal antibodies to Cymbidium mosaic potexvirus (CyMV) were produced by fusion of FOX-NY myeloma cells with immune splenocytes derived from in vivo or from a combination of in vivo and in vitro immunizations. Forty-five CyMV monoclonal antibodies reacted with the homologous antigen, which was trapped by CyMV rabbit antiserum coated microtiter plates. Twenty-nine of the 45 monoclonal antibodies also reacted with CyMV when the antigen was coated directly on microtiter plates. Detection of CyMV in crude sap of infected orchid leaves by immunosorbent electron microscopy was about twice as sensitive as by enzyme-linked immunosorbent assay (ELISA), whereas the sensitivity of dot blot immunoassay was about eight times that of ELISA. CyMV antigen was detected in direct tissue blots on nitrocellulose membranes in 30 leaf blots of 155 healthy looking orchid plants tested. Of six samples that tested positive by tissue-blot immunoassay, three showed A_{405nm} values of less than 0.05 by ELISA, and three gave ELISA A_{405nm} values between 0.15 and 0.2 when assayed at a 1:20 sap dilution. Ten other positive samples gave ELISA A_{405nm} values between 0.2 and 1.0. The remaining 14 samples, however, had ELISA A_{405nm} values of greater than 1.0. All the plants that tested negative with the tissue-blot immunoassay gave ELISA A_{405nm} values of less than 0.06.

Additional keywords: disease-indexing method, immunohistochemical detection, serological diagnosis.

Cymbidium mosaic virus (CyMV), the most prevalent virus in orchids, infects a wide range of orchid genera. Foliar symptoms induced by CyMV infection range from chlorotic streaking to black necrotic spots and necrotic line patterns with sunken patches (2,16). Symptomless carriers are, however, not uncommon (2; this study).

Accurate detection and identification of CyMV infection in orchids are essential elements in a health certification program. Orchid cultivars infected with CyMV often show reduced vigor with flower symptoms as well as foliage symptoms (2,16). Viral infections prevent free movement of orchids around the world. CyMV infections in orchids can be identified by bioassay, serology, or electron microscopy (16). Serological identification of CyMV by enzyme-linked immunosorbent assay (ELISA) is frequently employed. Recently, a tissue-blotting technique for detection of virus and mycoplasmalike organisms in plants was described (18). The method uses direct blotting of a freshly cut tissue surface onto a nitrocellulose membrane followed by incubation with pathogen-specific primary antibodies and enzyme-labeled immunoglobulin-specific secondary antibodies. In this paper, a tissue-blot immunoassay (TBIA) using CyMV-specific mouse monoclonal antibodies is described. The accuracy of TBIA and the sensitivities of ELISA, dot blot immunoassay (DBIA), and immunosorbent electron microscopy (ISEM) for detection and titration of CyMV are compared. A preliminary report was presented (14).

MATERIALS AND METHODS

Virus isolates. Three isolates of CyMV from Thailand and three from the United States (received under permit) were used in these investigations (Table 1).

Virus purification. Purified virus was prepared for each isolate from leaf tissues of a naturally infected *Cattleya* hybrid or inoculated *Datura stramonium* L. as listed in Table 1. Polyethylene glycol (MW 8,000) precipitation and one cycle of differential centrifugation followed by a 10–40% sucrose-gradient centrifugation previously described (5,7) were used for purification. The virus preparation was further purified by equilibrium density gradient centrifugation through 35% cesium chloride at 10 C for 16–18 h. Virus fractions were collected with an ISCO density gradient fractionator Model 640 equipped with UA5 type 6 optical unit (Instrumentation Specialties Company, Lincoln, NE) and dialyzed in phosphate-buffered saline (PBS, 0.02 M phosphate, 0.15 M sodium chloride, pH 7.2). Viral concentrations were determined by using $E^{0.1\%}_{1\ cm,\ 260nm} = 3.0$, an average value of concentrations of potato X, clover yellow mosaic, hydrangea ringspot, and papaya mosaic viruses (3,4,15,19). Purified virus was stored frozen at −70 C.

Immunization and cell fusion. For in vivo immunization, three BALB/c mice were each injected intraperitoneally three times with 35 μg of purified CyMV (a mixture of 01, C5, 495, and 875 strains) according to an immunization schedule previously described (11). One week after the third injection, mice were given a fourth injection of purified CyMV in PBS. A single spleen cell suspension was prepared, and cells were fused with FOX-NY myeloma cells (American Type Culture Collection [ATCC], Rockville, MD) 4 days after the last injection.

EL-4 thymoma cells, when stimulated by phorbol myristate acetate (PMA), produce many growth factors that have been shown either to induce proliferation of antigen-activated B cells

or to be essential for the complete development of antibody-forming cells (6,8). Culture filtrate from EL-4 thymoma cells (a gift from Mike Ma of the University of Maryland, College Park) was used in in vitro booster immunization. EL-4 thymoma cells were harvested in log phase growth and cultured at 2×10^6 cells per milliliter in modified complete RPMI (Rosewell Park Memorial Institute) 1640 media (13) with PMA at 10 ng/ml. After 48 h of stimulation of EL-4 cells with PMA, cell-free media were collected by centrifugation and filtered through a 0.22-μm pore size filter. The filtrate was stored in 15-ml centrifuge tubes (10 ml per tube) at −70 C. For in vitro booster immunization, spleens were harvested from mice 1 wk after the third injection as described for in vivo immunization. Single splenocytes were prepared in 24 ml of complete RPMI 1640 media. CyMV (150 μg) and EL-4 culture media (3 ml) were added to spleen cell suspensions. Cells were incubated at 37 C in 6% CO_2 for 4 days before fusion. The procedure for cell fusions was similar to one previously described (9), except the FOX-NY myeloma cell line was used.

Screening for antibody activities. Two types of indirect ELISA were used to identify hybridoma cultures that secreted CyMV antibodies. Before hybridoma culture fluids were added, CyMV antigens were trapped on polyvinyl chloride (PVC) microtiter plates (Becton-Dickinson & Co., Oxnard, CA) either by direct coating at pH 9.6 in 0.1 M carbonate buffer (11) or by incubating a CyMV preparation at pH 7.4 in PBS on PVC plates previously sensitized with rabbit anti-CyMV serum (9) at room temperature for 2 h. Use of either method alone may result in selection of antibodies that are only suitable in one type of assay (10). Alkaline-phosphatase-labeled goat anti-mouse immunoglobulins (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used to detect the presence of CyMV-specific mouse antibodies.

Immunological reagents. CyMV-specific rabbit polyclonal antibodies were obtained from ATCC and were used at a 1:400 dilution. Ascitic fluids containing CyMV-specific mouse monoclonal antibodies (IgG3) that reacted to all isolates listed in Table 1 were prepared at the Florist and Nursery Crops Laboratory in Beltsville. A detailed report on in vivo and a combination of in vivo and in vitro immunizations, generation of hybridoma cell lines, and production and characterization of CyMV-specific monoclonal antibodies will be described elsewhere. Both 2B11G12 and 5D3F5 hybridomas induced ascitic fluids that had antibody titers of 10^{-6} and reacted to all six isolates listed in Table 1. They were used at a 1:1,000 dilution in ELISA, DBIA, and TBIA analyses, and at a 1:200 dilution in ISEM analysis. Alkaline phosphatase-labeled goat anti-mouse immunoglobulins were used at an immunoglobulin concentration of 0.5 μg/ml. All dilutions were made in PBS.

Preparation of samples for tests. Leaf tissues, 1–2 mm immediately adjacent to the cut surface where tissue blots were obtained, were used for ELISA, DBIA, and ISEM analyses. Crude CyMV antigens were prepared by grinding one part of plant tissues into nine parts of PBS. Further dilutions were made in PBS after antigens were briefly clarified by centrifugation at 5,000 *g* for 10 min.

ISEM. Crude sap from CyMV-infected *Cattleya* plants was prepared in PBS. The sap was then diluted in PBS after being clarified by centrifugation at 5,000 *g* for 10 min. Carbon-coated Formvar grids were treated with monoclonal antibodies diluted 1:200 in 0.05 M monosodium-dipotassium phosphate buffer, pH 7.0 (phosphate buffer). Antiserum was incubated for 15 min. The grid was washed with 20 drops of phosphate buffer and was brought nearly to dryness with a filter paper. A drop of the diluted extract was incubated for 20 min on an antibody-sensitized grid. Treated grids were washed with 20 drops of 2% sodium phosphotungstic acid (pH 6.5) and stained for an additional 30 s. Grids were dried and examined with a Philips EM 200 electron microscope at a film magnification of ×18,000. A micrograph was taken of five randomly selected grid openings. Particle counts are based on the average count from two grids.

TABLE 1. Isolates, sources, and propagation hosts of Cymbidium mosaic virus

Isolate designation	Original host	Purification host	Geographical source
01	*Oncidium* Golden shower	*Datura stramonium*	Bangkok, Thailand
C5	*Cattleya* hybrid	*D. stramonium*	Bangkok
D	*Dendrobium* sp.	*D. stramonium*	Bangkok
495	*Cattleya* hybrids	*Cattleya* hybrids	Beltsville, MD
875	*Cattleya* hybrids	*Cattleya* hybrids	Beltsville
P	*Phalaenopsis schilleriana*	*D. stramonium*	Gainesville, FL

ELISA. An indirect ELISA using CyMV-specific rabbit polyclonal antibody sensitized plates was employed. Unless otherwise stated, all procedures were carried out at room temperature for 60 min or at 4 C overnight. PVC microtiter plates were incubated with CyMV-specific rabbit antibodies prepared in 0.1 M carbonate-coating buffer at pH 9.8. After a 30-min incubation with a PBS blocking solution (PBS-BSA, PBS containing 1% bovine serum albumin), various dilutions of 100 μl of antigen preparation were added to each well in duplicates. After being incubated with antigens, plates were washed three times, 3–5 min each, in PBS containing 0.05% Tween-20 (PBS-Tween) and were incubated with CyMV-specific mouse monoclonal antibodies diluted in PBS. The plates were washed again with PBS-Tween before addition of alkaline phosphatase-labeled goat anti-mouse immunoglobulins. After five washes in PBS-Tween, substrate solution containing *p*-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine, pH 9.8, was added, and results were measured at A_{405nm} with an MR 700 Dynatech ELISA Reader (Dynatech Laboratories, Inc., Chantilly, VA) 30 min after addition of substrate.

DBIA. An indirect procedure using alkaline phosphatase-labeled goat anti-mouse immunoglobulins was employed in DBIA. Nitrocellulose membranes, 0.45-μm pore size (Schleicher & Schuell, Inc., Keene, NH), were immersed in PBS before being assembled onto a Schleicher & Schuell Minifold I apparatus. Antigen samples were added, 50 μl to each well in duplicates, while the manifold was attached to a vacuum line. Each well was then rinsed twice with 100 μl of PBS under vacuum. The manifold was disassembled. The nitrocellulose membrane was removed before the vacuum was disconnected. The membranes were placed in the PBS-BSA blocking solution for 20–30 min to block unbound sites and incubated with CyMV-specific mouse monoclonal antibodies. After three successive 5-min washings in PBS-Tween, the blots were incubated in a solution of goat anti-mouse immunoglobulin-alkaline phosphatase conjugate. The blots were washed five times in PBS-Tween and incubated 5 min in a substrate solution (14 mg of nitroblue tetrazolium and 7 mg of 5-bromo-4-chloro-3-indolyl phosphate in 40 ml of substrate buffer containing 0.1 M Tris, 0.1 M sodium chloride, and 5 mM magnesium chloride, pH 9.5). Reactions were terminated with a solution containing 10 mM Tris and 1 mM ethylene-diaminetetraacetic acid (EDTA), pH 9.5. A positive reaction was indicated by the development of purple on the blots. Results were scored semiquantitatively according to dilution endpoints of the colors that developed.

TBIA. The same indirect immunological procedure used in DBIA was utilized for detection of CyMV by TBIA. Preparation of tissues for TBIA was previously described (18). Nitrocellulose membranes with tissue blots were first immersed in the PBS-BSA blocking solution for about 20–30 min with gentle shaking, followed by a brief rinse in PBS-Tween. The blots were incubated with CyMV-specific mouse antibodies. After three successive washings in PBS-Tween, blots were incubated with alkaline phosphatase-labeled goat anti-mouse immunoglobulins. Before the addition of substrate solution, the blots were washed again five times in PBS-Tween with gentle shaking. Results were determined qualitatively according to the development of purple on the blots.

Inoculation and detection of CyMV. One-hundred and fifty-five tissue-culture-derived plants of *Cattleya* and *Phalaenopsis* hybrids at the three-leaf stage were obtained from a commercial

TABLE 2. Reactivities and immunoglobulin classes and subclasses of Cymbidium mosaic virus (CyMV) specific murine monoclonal antibodies produced by hybridomas generated from fusion of FOX-NY myeloma cells with BALB/c mouse splenocytes derived from in vivo or a combination of in vivo and in vitro immunization

Immunization	Number of hybridomas	ELISA reaction to CyMV		Immunoglobulin classes and subclasses				
		CyMV direct coating	Rabbit anti-CyMV coating	IgG1	IgG2	IgG3	IgM	Others[c]
In vivo[a]	18	+	+	0	0	10	6	2
	8	−	+	0	0	1	6	1
In vitro[b]	11	+	+	1	0	4	5	1
Assisted	8	−	+	0	0	0	7	1

[a] Mice were injected intraperitoneally four times each with 35 μg of purified CyMV. Fusions were made 4 days after the last injection.
[b] Immune spleen cells from mice injected three times, as described in in vivo immunization were incubated with 150 μg of CyMV in 24 ml of complete RPMI 1640 medium and 3 ml of EL-4 thymoma cell culture filtrate at 37 C in 6% CO_2 for 4 days before fusions.
[c] Classes and subclasses could not be determined using mouse IgG, IgM, or IgA immunoglobulin-specific goat antibodies.

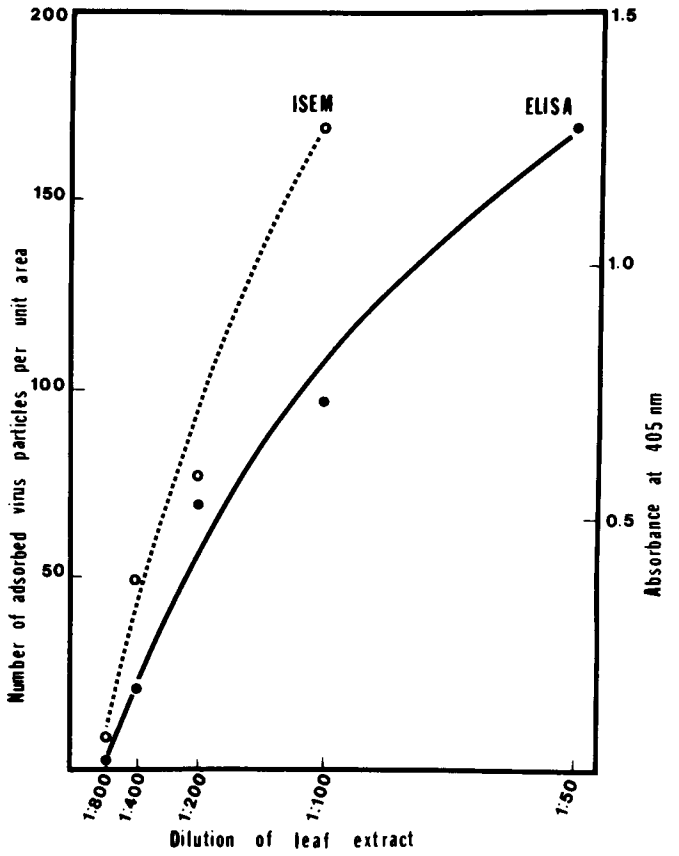

Fig. 1. Assays of Cymbidium mosaic virus (CyMV) antigen in leaf sap of an infected *Cattleya* orchid by immunosorbent electron microscopy (ISEM) and by enzyme-linked immunosorbent assay (ELISA). A serial dilution of CyMV-infected leaf extract was incubated on mouse monoclonal antibody-treated carbon-coated Formvar grids in ISEM analysis, or on rabbit polyclonal antibody-treated PVC plates followed by mouse monoclonal antibodies and alkaline-phosphatase-labeled goat anti-mouse immunoglobulin antibodies in ELISA analysis.

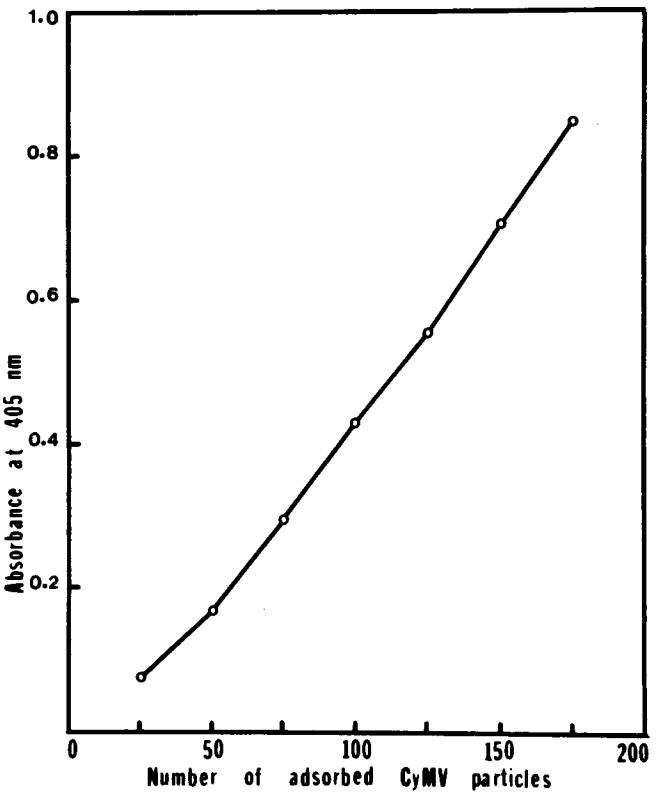

Fig. 2. The relationship between the number of absorbed Cymbidium mosaic virus (CyMV) particles observed by immunosorbent electron microscopy and A_{405nm} values obtained in enzyme-linked immunosorbent assay. The relationship curve was generated from the results in Figure 1.

supplier. They were examined to be rod-particle-free by the leaf-dip procedure (16). One-half of the plants of each genus were inoculated with purified CyMV (01 isolate) through wounding by being pricked with a needle or a razor blade. The remaining plants served as controls. All plants were tested by ELISA and TBIA for CyMV infection 8 mo after inoculation.

RESULTS

Forty-five hybridoma cell lines secreting CyMV antibodies were established from immune splenocytes by hybridization with FOX-NY myeloma cells in four separate fusions (Table 2). Monoclonal antibodies produced by these cell lines all reacted with viral antigens trapped by rabbit anti-CyMV serum-sensitized PVC ELISA plates. Monoclonal antibodies produced by 29 of the 45 hybridoma cell lines also reacted with CyMV on antigen-coated PVC ELISA plates; the remaining 16 monoclonal antibodies did not react with CyMV on antigen-coated plates. The majority of hybridomas secreted immunoglobulin M (IgM); one-third of hybridoma cell lines secreted immunoglobulin G3 (IgG3). Only one cell line secreted immunoglobulin G1 (IgG1). Five cell lines produced monoclonal antibodies that were neither IgG nor IgM.

In ISEM using monoclonal antibody-coated grids, the dilution endpoint for detection of CyMV in crude sap from infected orchids was about 1:800 (Fig. 1), whereas the dilution endpoint of the same sap with the same monoclonal antibodies was about 1:400 by ELISA on PVC microtiter plates (Fig. 1). The relationship between the number of CyMV particles observed by ISEM and the A_{405nm} values obtained by ELISA is presented in Figure 2. The dilution endpoint of the same sap sample as observed by DBIA on nitrocellulose membranes was 1:3,200 (Fig. 3). The apparent positive spots on the membrane with control samples

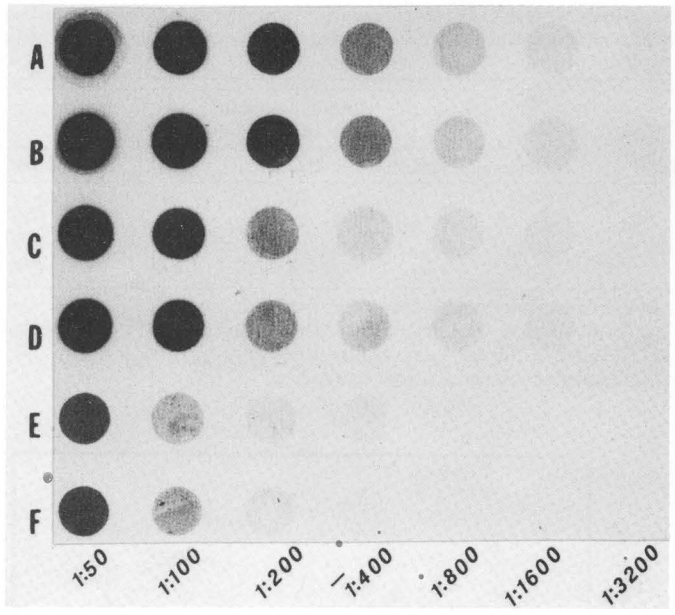

Fig. 3. Titrations of Cymbidium mosaic virus (CyMV) antigens from CyMV-infected orchid leaves as observed by dot blot immunoassay. Fifty microliters per well of serial dilutions of **A** and **B,** CyMV-infected *Cattleya* leaf extract assayed by immunosorbent electron microscopy and enzyme-linked immunosorbent assay (Fig. 1); **C** and **D,** CyMV-infected *Phalaenopsis* leaf extract; and **E** and **F,** healthy *Phalaenopsis* leaf extract were applied to nitrocellulose membranes. CyMV titers were determined by using mouse monoclonal antibodies, followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. The apparent positive spots on the membrane with control samples E and F were due to green pigment in the preparation.

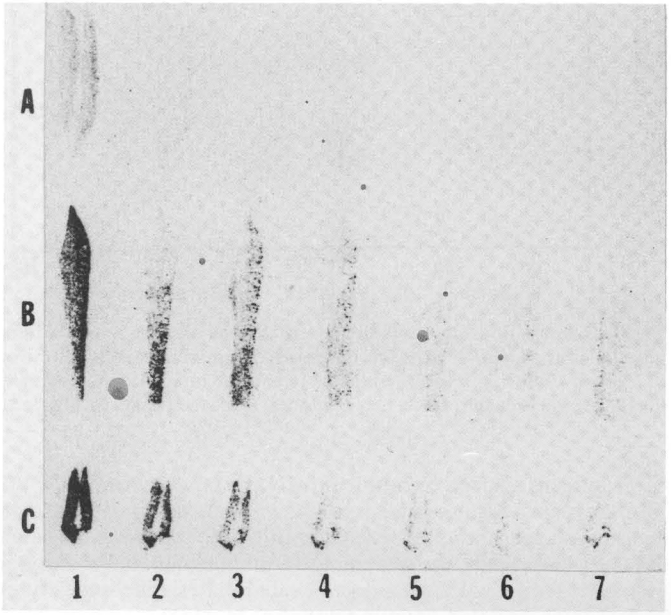

Fig. 4. Detection of Cymbidium mosaic virus (CyMV) antigens in consecutive blots, from left to right, from a single cut surface of leaves of **A,** healthy *Cattleya*; **B,** infected *Cattleya*; **C,** infected *Phalaenopsis* orchids. CyMV antigens were detected by reacting tissue blots with mouse monoclonal antibodies followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. The dark images in healthy control A were attributable to the green of chlorophyll.

in Figure 3, rows E and F, were due to green pigment in the preparation.

CyMV antigens could be readily detected in leaves of infected orchids by tissue blotting on nitrocellulose membranes. CyMV infections in orchids were evident by the development of purple

TABLE 3. Detection of Cymbidium mosaic virus (CyMV) antigens in orchids by enzyme-linked immunosorbent assay (ELISA) and by direct tissue-blot immunoassay (TBIA) using monoclonal antibodies 2B11G12 or 5D3F5

	2B11G12		5D3F5	
Sample	ELISA[a]	TBIA[b]	ELISA[a]	TBIA[b]
1	0.893	+	0.807	+
7	0.964	+	0.881	+
10	0.027	−	0.023	−
12	0.839	+	0.794	+
19	0.008	−	0.002	−
28	0.023	−	0.007	−
30	0.910	+	0.793	+
31	0.058	−	0.030	−
40	0.378	+	0.354	+
42	0.407	+	0.386	+
51	0.238	+	0.260	+
59	0.171	+	0.186	+
60	0.181	+	0.164	+
66	0.152	+	0.097	+
67	0.215	+	0.160	+
68	0.046	+	0.034	+
76	0.209	+	0.204	+
82	0.049	+	0.050	+
83	0.380	+	0.325	+
91	0.030	+	0.020	+

[a] One hundred microliters of leaf extract at a 1:20 dilution was incubated in each well in duplicates in CyMV-specific rabbit antibody-coated polyvinyl chloride plates, followed by mouse monoclonal antibodies and alkaline phosphatase labeled goat anti-mouse immunoglobulin antibodies.

[b] Tissue blots were incubated in mouse monoclonal antibodies, followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. + Indicates CyMV antigen was positively detected; − indicates no CyMV antigen was detected.

in tissue blots (Fig. 4). Even after seven consecutive blottings from a single cut surface of an infected *Cattleya* or *Phalaenopsis* leaf, the CyMV antigens could be identified in the tissue blots on nitrocellulose membranes. The healthy control in Figure 4 did not develop purple. The apparent dark images of the healthy control were due to the green of chlorophyll.

CyMV was detected in 30 of 155 plants tested by TBIA. Some of the test results are presented in Table 3. Of the 30 plants that tested positive by TBIA, three samples (plants 68, 82, and 91) showed A_{405nm} values of less than 0.05. Another three samples (plants 59, 60, and 66) gave A_{405nm} values between 0.15 and 0.2 when tested at a 1:20 sap dilution by ELISA using monoclonal antibodies 2B11G12 or 5D3F5. Ten other samples showed ELISA A_{405nm} values between 0.2 and 1.0 (Table 3). The remaining 14 plants that tested positive by TBIA, however, had ELISA A_{405nm} values of greater than 1.0 (data not shown). Of all the plants that tested positive, none showed symptoms of CyMV infection. All the plants that tested negative by TBIA gave ELISA A_{405nm} values of less than 0.06, and the majority were less than 0.03 (Table 3) with average readings of 0.027 and 0.019 when monoclonal antibodies 2B11G12 and 5D3F5 were used, respectively.

DISCUSSION

Effective control of viral infection in orchids depends on selecting and propagating virus-free plants and eradicating diseased specimens. Accelerated movement of orchid plants internationally has led to increased spread of orchid viruses. Accordingly, an accurate diagnosis of virus infection is essential for proper disease management. A sensitive and rapid direct tissue-blot immunoassay for identification of CyMV infection in orchids was developed to improve procedures for rapid certification.

Results reported here confirm a previous report that ISEM is more sensitive than ELISA for detecting CyMV infection in orchids (17). ISEM allows direct observation of viral antigens and the antigen-antibody interactions with an electron microscope. Although the procedure was first introduced to virology

in the investigation of tobacco mosaic virus (1), ISEM has not been fully utilized. It is still essentially a research technique, because electron microscopes are not generally available. Our studies also demonstrated that a direct tissue-blot technique for virus detection not only retains specificity and sensitivity but also provides simplicity and accuracy for detection of CyMV in orchids.

A low concentration of pathogen-specific antigen in a test sample is a limiting factor in using ELISA for disease diagnosis in which the causal agent occurs at low concentration in infected plants. Increasing test sample volumes does not proportionally increase the detection limit in ELISA. Also, the volume of a test sample is limited by the size of the well in a microtiter plate. Furthermore, not all viral antigens in test samples are absorbed in double antibody sandwich ELISA during a sample incubation period (12), further restricting the sensitivity of the detection limit by ELISA. In ELISA tests, positive results are routinely indicated by $A_{405\ nm}$ readings greater than two to three times the value of negative controls; if this rule was used in current studies, some infected samples would not have been recognized by ELISA even though CyMV antigen was positively identified by TBIA.

Detection of a low concentration of pathogen-specific antigens in a test sample by DBIA can exceed that of ELISA (12). Antigens applied in DBIA are efficiently bound to membrane supports. A larger sample volume can be used in DBIA than in ELISA with the aid of a manifold under vacuum (13), and the dilution of a test sample is not a limiting factor as it is in ELISA. Rather, it is the total antigen pool applied to the membrane that determines the detection limit in DBIA. It should be recognized, however, that very large volumes of test samples of plant extracts cannot be forced through nitrocellulose membranes.

CyMV antigens can be readily detected in leaves of infected orchids by tissue blotting on nitrocellulose membranes. Viral antigens may be identified by TBIA, although they occur in low concentrations in infected plants and are localized in a few cells in a small area of tissue. Orchid samples that gave negative or marginal positive results by ELISA were identified as positive for CyMV infection by TBIA.

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