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# Evaluation of Dot-Immunobinding Assay and Rapid Immunofilter Paper Assay for Detection of Cymbidium Mosaic Virus in Orchids

I Wayan GARA, Hideki KONDO and Takanori MAEDA,

Dot-immunobinding assay (DIA) on nitrocellulose membranes and rapid immunofilter paper assay (RIPA) were examined for their usefulness in the detection of cymbidium mosaic virus (CyMV) in orchids. The minimum detection levels of CyMV by these methods were 100 ng/ml in purified preparations and at a $10^{-4}$ dilution of extracts from infected leaves of orchids could be detected by these methods. Although DIA took 5 to 6 hours for the detection of the virus, it was a reliable method for diagnosis of a large-number of samples. On the other hand, RIPA, which enabled detection of CyMV within a few minutes with sensitivity similar to that of DIA, will be suitable as a rapid and handy tool for virus disease diagnosis in orchids. Moreover, by RIPA, we could detect CyMV and odontoglossum ringspot virus (ORSV) simultaneously form doubly infected plant.

**Key words :** Serological detection, Cymbidium mosaic virus, Orchid

## INTRODUCTION

Cymbidium mosaic virus (CyMV) is the most prevalent virus causing serious damage in orchids worldwide (Zettler *et al.* 1990, Lawson 1995). It occurs wherever orchids are cultivated as ornamentals and has been detected in most orchid genera (Wisler 1989, Inouye 1990, 1992).

Effective control of viral infection in orchids depends on selecting and propagating virus-free plants and eradicating diseased plants (Wisler 1989). CyMV can be successfully controlled by strict application of sanitation programs (Hu *et al.* 1993). Thus, an accurate diagnosis of CyMV in orchids is essential for disease managements. Indexing for CyMV infections in

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orchids is usually done by bioassay, electron microscopy and/or serological test (Lawson 1995, Kondo *et al.* 1996). Enzyme-linked immunosorbent assay (ELISA) is frequently employed for detection of CyMV (Hu *et al.* 1993). Recently, dot-immunobinding assay (DIA) (Yoshikawa *et al.* 1986, Powell 1987, Somowiyarjo *et al.* 1989, Hsu *et al.* 1992) and rapid immunofilter paper assay (RIPA) (Tsuda *et al.* 1992, 1993) are applied to detect some plant viruses. Here, we describe the applicability of DIA and RIPA for detection of CyMV in orchids.

## MATERIALS AND METHODS

1. *Antiserum production*

The CyMV isolate used for antiserum production was isolated from *Vanda* orchid and purified from infected *Tetragonia expansa* leaves (Gara *et al.* 1996). Antiserum was produced by injecting a rabbit four times intramuscularly with 0.5 ml of purified virus (1 mg) mixed with 0.5 ml of Freund's incomplete adjuvant at two-week intervals. The rabbit was bled ten days after the last injection. Antiserum to ORSV was from our own stock (Kondo *et al.* 1996). Antisera were cross-absorbed with host protein before use and antibodies (IgG) were purified from antisera by using an Ampure PA Kit (Amersham).

2. *Dot-immunobinding assay*

A dot-immunobinding assay (DIA) used in the present study was basically described by Powell (1987). The nitrocellulose membrane sheet was first immersed in Tris-buffered saline (TBS, 0.02 M Tris-HCl and 0.5 M sodium chloride, pH 7.5) before spotting antigen samples prepared by grinding infected leaves in TBS. The membrane was placed in the blocking solution of TBS containing 1% bovine serum albumin (TBS-BSA) for 30 min and incubated with respective antiserum diluted 1 : 2000 for 1 hr. After three successive 10 min washing in TBS-Tween, the membrane was incubated in a solution of anti-rabbit IgG-alkaline phosphatase conjugate. The membrane was washed four times in TBS-Tween and incubated for 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 0.1 M Tris, 0.1 M sodium chloride and 0.05 M magnesium chloride, pH 9.5). A positive reaction was indicated by the development of purple spots on nitrocellulose membrane.

3. *Rapid immunofilter paper assay*

The rapid immunofilter paper assay (RIPA) was carried out essentially

as described by Tsuda *et al.* (1992, 1993). Three kinds of latex beads (Japan Synthetic Rubber, Co., Ltd.) were used: non-dyed (white color) for solid phase and dyed latex (pink color for CyMV and red color for ORSV) for tracer. To coat the latex beads with antibody, the white latex was diluted to 0.5% and pink or red latex to 1% concentration with TBS and mixed with the same volume of IgG solution. The mixtures were incubated at room temperature for 2 hr with shaking. The latex beads coated with antibody were washed three times by centrifugation at 8,000 $\times g$ for 10 min with TBS-BSA for white latex, and centrifugation at 15,000 $\times g$ for 15 min for pink and red latex beads. To assay the RIPA, 10 $\mu$l of white latex beads coated with antibody was applied at 1.5 cm from the lower end of 8 $\times$ 0.5 cm strip of Whatman glass filter paper (GF/A) and then filter paper was dried at room temperature. For sample preparation, the crude sap or purified virus was diluted in 0.1 M phosphate buffer, pH 7.0 containing 0.1% 2-mercaptoethanol, 0.01 M disodium ethylenediaminetetraacetate, 0.1% BSA and 0.15% polyvinyl pyrrolidone. The filter paper strip was dipped in the leaf extracts or purified virus preparations for 1 min and then in pink or red latex for 2 min. The positive reaction was indicated with appearance of pink band for CyMV and red band for ORSV on the immobilized white latex zone.

## RESULTS

The specific antiserum produced against CyMV had a titer of 1 : 1024 in a microprecipitin test and was used in DIA and RIPA procedures to detect CyMV in orchids.

The sensitivities of DIA and RIPA were compared for detection of CyMV. CyMV could be readily detected both in purified virus preparations and in crude sap of infected orchid leaves by DIA on nitrocellulose membrane (Fig. 1-A, B). The minimum detection levels of CyMV by DIA were 100 ng/ml in purified preparations and at a $10^{-4}$ dilution of extracts from infected leaves. When this test was applied to detect CyMV in some orchids (*Cattleya*, *Cymbidium*, *Dendrobium*, *Phalaenopsis* and *Vanda*) (Fig. 2-A), the purple spots were developed on nitrocellulose membrane for infected samples, but not in the healthy control or very faint spots remained due to the green pigment in the preparations. Of the 18 samples assayed by the DIA, CyMV was detected in 10 samples of orchids surveyed (Fig. 2-B). The virus particles were detected from all positive samples by immuno-electron microscopy, but not from negative samples.

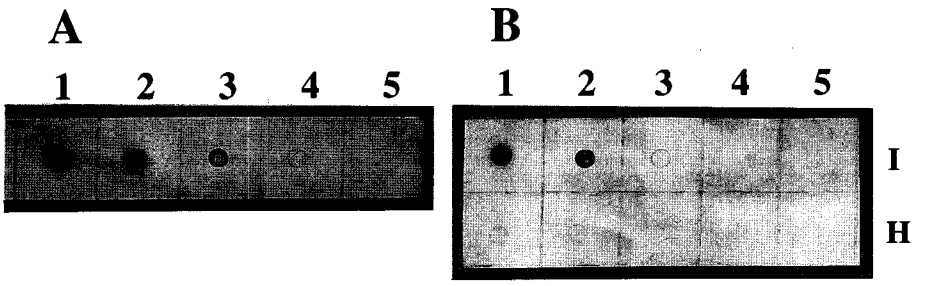


Fig. 1. Detection limit of CyMV by DIA. (A) Purified virus preparations of 100 (1), 10 (2), 1 (3), 0.1 (4) and 0.01 $\mu$g /ml (5). (B) Leaf extract at serial dilutions of $10^{-2}$ (1), $10^{-3}$ (2), $10^{-4}$ (3), $10^{-5}$ (4) and $10^{-6}$ (5). Infected (I) and healthy (H).

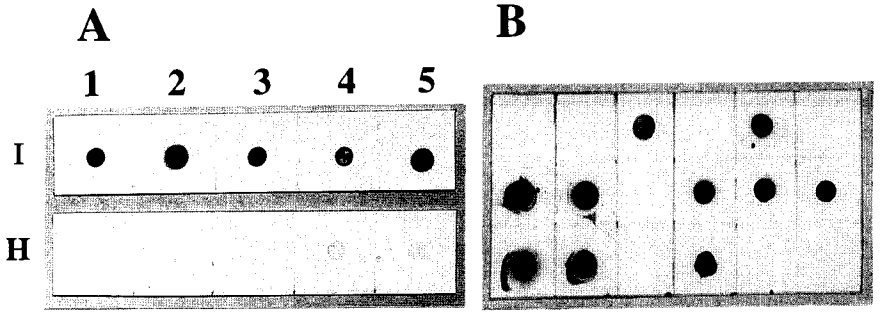


Fig. 2. (A) Detection of CyMV in leaf extract ($10^{-2}$) of various orchids by DIA. *Cattleya* (1), *Cymbidium* (2), *Dendrobium* (3), *Phalaenopsis* (4) and *Vanda* (5). Infected (I) and healthy (H). (B) Randomize detection of CyMV in leaf extracts ($10^{-2}$) of some orchids by DIA.

CyMV in orchids could be detected by the RIPA technique as a band produced by an antigen-antibody reaction. By this technique, we could detect CyMV at the minimum detection level of 100 ng/ml of purified virus and at a $10^{-4}$ dilution of crude sap of infected orchid leaves with the naked eye (Fig. 3-A, B). The band became weaker as the concentration of CyMV decreased. Five infected orchids *Cattleya*, *Cymbidium*, *Dendrobium*, *Phalaenopsis* and *Vanda* were tested by RIPA and clear colored-bands were formed for all samples (Fig. 4-A). No positive reaction was produced with the crude sap of healthy plants. Double infections with both CyMV and odontoglossum ringspot virus (ORSV) were demonstrated by the RIPA procedure. The RIPA technique was applicable to detect double infections of CyMV and ORSV in orchids with appearance of two bands on different immobilized zones of glass filter paper (Fig. 4-B).

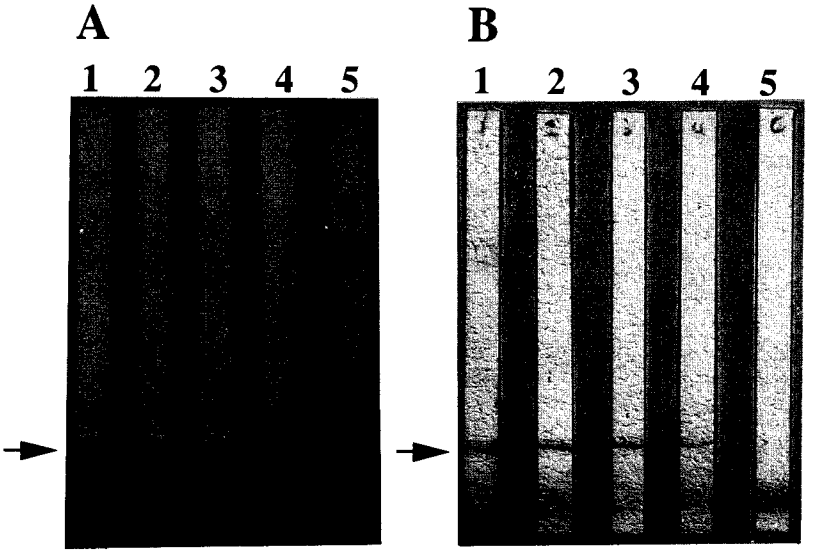


Fig. 3. Detection limit of CyMV by RIPA. (A) Purified virus preparations of 100 (1), 10 (2), 1 (3), 0.1 (4) and 0.01 $\mu$g/ml (5). (B) Infected leaf extract at serial dilutions of $10^{-1}$ (1), $10^{-2}$ (2), $10^{-3}$ (3), $10^{-4}$ (4) and healthy (5). Arrows indicate bands produced by positive reactions.

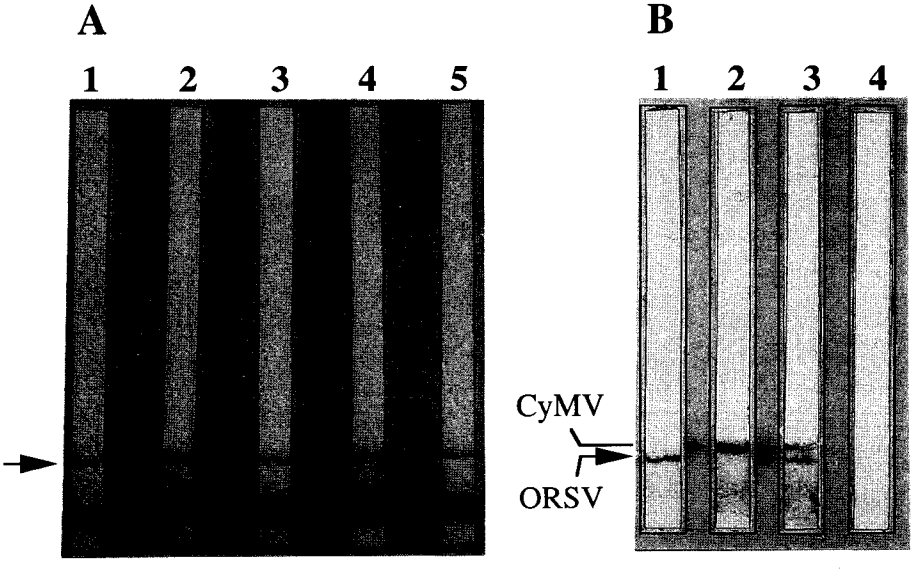


Fig. 4. (A) Detection of CyMV in leaf extract ($10^{-2}$) of various orchids by RIPA. *Cattleya* (1), *Cymbidium* (2), *Dendrobium* (3), *Phalaenopsis* (4) and *Vanda* (5). (B) Detection of single or double infections by CyMV and ORSV in leaf extract ($10^{-2}$) of *Cymbidium*. ORSV (1), CyMV (2), double infections of ORSV and CyMV (3) and healthy (4). Arrows indicate bands produced by positive reactions.

The sensitivity of RIPA was compared with that of DIA in detecting CyMV both in purified preparations and in crude sap of infected orchid leaves (Table 1). The sensitivity of DIA was considered to be similar to that of RIPA for detection of CyMV.

Table 1. Comparison of sensitivity between DIA and RIPA for detection of CyMV*

| Antigen | Detection limit | |
|---|---|---|
| | DIA | RIPA |
| Purified virus | 100 ng/ml | 100 ng/ml |
| Leaf extract | $10^{-4}$ | $10^{-4}$ |

*derived from Fig. 1 and Fig. 3.

## DISCUSSION

CyMV occurs worldwide in a wide range of orchid genera and causes severe damage in cultivated orchids (Wisler 1989, Inouye 1990, 1992). Sensitive and rapid procedures for detection of CyMV are essential for sanitation programs in orchids because effective control of virus diseases in orchids depends on selecting and propagating healthy plants. Bioassay, electron microscopy or serological tests have been used to identify CyMV infection in orchids. ELISA and immunosorbent electron microscopy (ISEM) have been widely used for diagnosing many viruses as sensitive and reliable methods. However, these methods are laborious and time-consuming to analyze a large-number of samples. DIA and RIPA procedures have advantages over ELISA and ISEM, because they do not require specialized equipments and are not time-consuming with considerably high sensitivities.

In this study we examined the usefulness of DIA and RIPA as a sensitive and rapid method for detection of CyMV in orchids. In the limited experiments, DIA was demonstrated to be reliable for detecting the virus in infected plants. No false-positive signals were given from healthy plants, although non-specific reactions were the main problem in DIA as reported by some authors (Hibi and Saito 1985, Powell 1987, Hsu *et al.* 1992). Since many samples can be assayed at the same time after blotting on the membranes, DIA will be a powerful tool for diagnosing CyMV in orchids by large-scale indexing programs.

RIPA was developed for serodiagnosis of plant viruses as easily and simply as pH test paper (Tsuda *et al.* 1992, 1993). RIPA has been applied successfully to detect ORSV in *Cymbidium* orchids (Kawano *et al.* 1994). In

this experiment, CyMV could also be detected in various CyMV-infected orchids and in purified preparations. The sensitivity of RIPA was similar to that of DIA using the same antibody and antigen. Moreover, with RIPA, double infections of CyMV and ORSV in orchids could be easily detected. It will be very useful to diagnosis CyMV in orchids in field survey. RIPA requires no skill or experience and the results can be obtained within a few minutes with the naked eye, so that orchid growers can examine their own plants by themselves.

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# ラン科植物に発生するシンビジウムモザイクウイルスの血清学的検出

イ　ワヤン　ガラ・近藤秀樹・前田孚憲

Dot-immunobinding assay（DIA）法と rapid immunofilter paper assay（RIPA）法の2種の血清学的診断法を cymbidium mosaic virus（CyMV）の検出に適用したところ，両方法とも少なくとも純化ウイルスで100ng/ml，罹病葉汁液では$10^{-4}$希釈まで検出可能であった．DIA 法では検出に 5 ～ 6 時間要したが，本法は多量の試料の診断に適していると思われた．一方，RIPA 法では本ウイルスを DIA 法と同程度の検出感度で数分以内で正確に検出できたことから，本法は今後 CyMV の簡易・迅速診断法として多種属のラン科植物に広く活用できると考えられた．さらに RIPA 法では CyMV と ORSV に重複感染した植物から両ウイルスを同時に検出できた．

**キーワード**：血清学的検出法，シンビジウムモザイクウイルス，ラン科植物