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Use of Gelatin Particle Agglutination Test for the Detection of *Cymbidium mosaic virus* in *Cattleya* Plants

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Gelatin particle agglutination test (GPAT) was used to detect *Cymbidum mosaic virus* (CymMV) in *Cattleya* plants. Gelatin particles were coated with purified anti-CymMV immunoglobulin of 25-100 μg/ml and were subjected to several different concentrations of purified CymMV as well as varying dilutions of orchid leaf extracts. The GPAT detected purified CymMV up to a minimum concentration of 10 μg/ml. CymMV was detected from crude sap extract of infected *Cattleya* leaves and roots up to 1:51,200 and 1:25,600 dilutions, respectively. However, the optimum range of leaf and root sap dilutions was between 50-100. Non-specific reactions were not encountered from any of the healthy orchid plants tested. The entire GPAT process was completed within 2-3 hours. This test was found to be very useful for the detection of CymMV in orchids because it is sensitive, economical, and easy to perform.

Keywords **:** *Cattleya* plants, *Cymbidum mosaic virus*, gelatin particle agglutination test.

Cymbidium mosaic virus (CymMV) is known to be one of the major pathogens causing significant reduction in the growth and quality of cultivated orchid plants in the world (Pearson and Cole, 1986; Pearson and Cole, 1991). Since many orchids are mainly propagated vegetatively from mother plants or through the tissue culture technique, the health of the original plant materials used for propagation is an important component of virus control (Hsu et al., 1992 ; Zettler et al., 1990).

A variety of methods for the identification of CymMV in orchid plants, such as enzyme-linked immunosorbent assay (ELISA) (Hu et al., 1993; Lommel et al., 1982), dot-immunobinding assay (Hsu et al., 1992), tissue blot immunobinding assay (La et al., 1999), and RT-PCR (Eun et al., 2000; Seoh et al., 1988), have been used to assess the virus level of orchid plants. However, these methods generally involve numerous and time-consuming steps for the virus detection. In contrast, microparticle agglutination tests, such as hemmaglutination test (Rajeshwari et al., 1981; Sander et al., 1989), bentonite flocculation (Van Regenmortel, 1982), and latex agglutination tests (Talley et al., 1980) are very rapid, economical, and accurate. In particular, a simplified detection method developed by Ikeda et al. (1984) known as gelatin particle agglutination test (GPAT) has been found to be useful for the detection of *Cucumber mosaic virus*, *Tobacco mosaic virus*, and *Garlic latent virus* (Fukami et al., 1993; Natsuaki et al., 1988). Thus, this study attempted to evaluate the applicability of GPAT to the detection of CymMV in naturally infected *Cattleya* orchids.

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Materials and Methods

Plant and sample preparation. *Cattleya* plants showing mosaic symptom were collected from the orchid farms of the Yonam Junior College of Livestocks and Horticulture in Korea. Leaves and roots were used in the study. Healthy seedlings of *Cattleya* cultivated *in vitro*, which were confirmed CymMV-free by tissue blot immuno-binding assay (La et al., 1999), were used as control. Initial plant sap was prepared by grinding 1 g of leaf or root in 1 ml of 0.1 M phosphate buffer, pH 7.4, supplemented with 0.85% NaCl and 0.1% bovine serum albumin (extraction or gelatin particle dilution buffer) and centrifuged at 7,000 rpm for 3 minutes. The supernatant was used as original extract sap and then serially diluted for GPAT.

Antibody. Polyclonal antiserum against CymMV particle was produced from a New Zealand white rabbit as described by Han et al. (1999). Immunoglobulin G (IgG) was purified by PD-10 column and Protein-A column chromatography (Pharmacia, Uppsala, Sweden) according to the manufacturers' manual. The final eluate was dialyzed through PD-10 column pre-saturated with 0.01 M Na-K phosphate buffer, pH 7.4, containing 0.85% NaCl, and stored at -80°C until use.

Sensitization of gelatin particles. Blue or violet gelatin particles 3 μm in diameter were kindly provided by the Fugirebio Inc. (Tokyo, Japan). Five percent of the gelatin particles were activated with 1% benzoquinone and coated with 25-100 μg/ml of anti-CymMV IgG and stored at 4°C. The IgG-coated gelatin particles (sensitized GP) were diluted to 0.1% with gelatin particle dilution buffer prior to use.

GPAT procedure. All tests were carried out as follows: Dilutions (25 μl) of *Cattleya* leaf or root extracts were added into wells of

polystyrene microtiter plate with the U-shaped bottom and then 0.1% sensitized GP was equally added. The plate was shaken for 1 minute on a PMX-01 shaker (Fugirebio Inc., Tokyo, Japan) and kept at room temperature for 90 minutes. The results were read as described by Ikeda et al. (1984).

Results and Discussion

Optimum IgG concentration for GPAT. To determine the optimum IgG concentration for sensitizing gelatin particles, 5% gelatin particles (GP) were coated with purified IgG adjusted to 25, 50, 75 and 100 μg/ml concentrations. Each of the sensitized GP was added to serial dilutions of crude leaf sap of CymMV-infected *Cattleya* plants. CymMV was detected in all leaf sap dilutions of 10^3 or less regardless of IgG concentrations except at 25 μg/ml; however, non-specific agglutination occurred in GP dilution buffer at 100 μg/ml of IgG (Table 1). This result was similar to that of Natsuaki et al. (1988) who reported that IgG concentration of 50 μg/ml was adequate enough to sensitize GP. This indicates that the optimum concentration of IgG could be 50 μg/ml, since this concentration had the same detection limit as the higher concentrations and no non-specific agglutination in both GP dilution buffer and healthy leaf extract was found.

Sensitivity of GPAT. To evaluate the sensitivity of GPAT for the virus detection, 5% GP was coated with equal volume of purified IgG of 50 μg/ml in concentration. A series of the purified CymMV dilutions were tested using three replicates. The original CymMV concentration was 1 mg/ml. As shown in Fig. 1, positive reactions occurred up to 10^5 folds of dilution. This indicates that GPAT could detect 10 μg/ml of purified CymMV. Compared with the ELISA test, which is used for detecting plant viruses (Lommel et al., 1982), this result suggests that GPAT could have similar sensitivity in detecting the virus.

Table 1. Optimum concentration of anti-*Cymbidium mosaic virus* Immunoglobulin G (IgG) for sensitization of gelatin particles

Conc. of IgG (μg/ml)	BC[a]	Reciprocal dilution of leaf extract[c]				
		10	10^2	10^3	10^4	10^5
25	–[b]	+	+	±	±	–
50	–	+	+	+	±	–
75	–	+	+	+	±	–
100	±	+	+	+	±	–

[a] Buffer control (0.1 M Na-K phosphate buffer, pH 7.4 containing 0.1% BSA and 0.85% NaCl).
[b] One gram of *Cattleya* leaf infected with *Cymbidium mosaic virus* was ground in 1 ml of antigen dilution buffer and centrifuged at 7,000 rpm for 3 min. The supernatant was diluted accordingly in the same buffer used for buffer control.
[c] +: positive, –: negative, ±: intermediate.

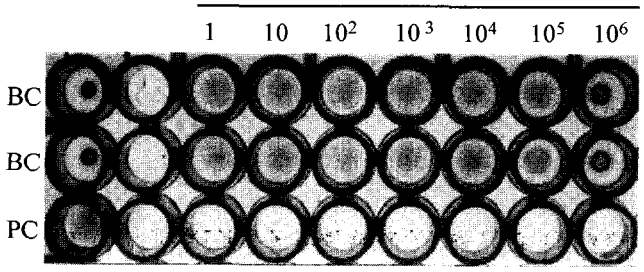

Fig. 1. Sensitivity of gelatin particle agglutination test for the detection of purified *Cymbidium mosaic virus* (CymMV). GP was sensitized with 50 μg/ml of anti-CymMV IgG and then mixed with serial dilutions of 1 mg/ml of purified CymMV. The reaction was read at 90 min. BC: buffer control, PC: positive control (CymMV infected leaf sap).

Dilution range of leaf and root extracts for GPAT. Forty-five out of 150 *Cattleya* plants found positive for CymMV were previously selected by tissue blot immuno-binding assay (La et al., 1999) and used for GPAT in this study. The extracts of leaves and roots from these plants were tested to determine the optimum dilution range for the detection of CymMV by GPAT. All samples showed positive reactions to CymMV in 10-100 and 10-400 dilutions of leaf and root extracts, respectively (Table 2). The maximum dilution limit for detecting CymMV by GPAT was $2^9 \times 100$ for leaf and $2^8 \times 100$ for root. Although non-specific agglutination

Table 2. Determination of optimum leaf and root sap dilutions for the detection of *Cymbidium mosaic virus* (CymMV) in *Cattleya* plants by gelatin particle agglutination test

Reciprocal dilution (×100) of plant extract[a]	Positive reaction (%)[b]	
	Leaf	Root
0.1	100.0	100.0
1	100.0	100.0
2	92.9	100.0
2^2	85.7	100.0
2^3	85.7	71.4
2^4	67.9	64.3
2^5	60.7	50.0
2^6	42.9	28.6
2^7	32.1	21.4
2^8	25.0	21.4
2^9	7.1	0.0
2^{10}	0.0	0.0

[a] One gram of orchid leaf infected with CymMV was ground in 1 ml of antigen dilution buffer (0.1 M Na-K phosphate buffer, pH 7.4 containing 0.1% BSA and 0.85% NaCl) and centrifuged at 7,000 rpm for 3 min. The supernatant was diluted accordingly in the same buffer.
[b] Each leaf and root of 45 *Cattleya* plants, which was previously confirmed to be infected with CymMV by tissue blot immuno-binding assay, was tested. The results were presented as percentage.

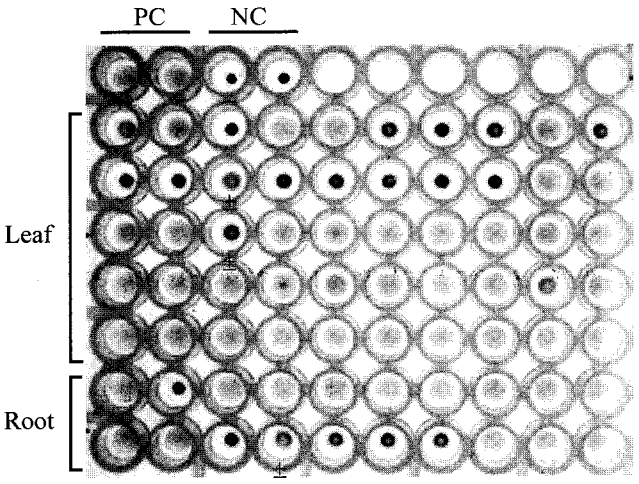

Fig. 2. Application of gelatin particle agglutination test for detection of *Cymbidium mosaic virus* (CymMV) in adult *Cattleya* plants. Gelatin particle agglutination test was carried out with GP sensitized with 50 μg/ml of anti-CymMV IgG and 50-fold dilutions. Samples of leaf extract from CymMV-infected or noninfected plants show positive (+) and negative (−) reactions, respectively. NC: healthy leaf sap, PC: CymMV-infected leaf sap.

occurred occasionally in original and below 10-fold dilution of leaf and root extracts by plant substances, this problem was overcome by further diluting the extract above 10-fold dilution. However, addition of 1% polyvinylpyrolidone or sodium sulfite to the extraction buffer, which was known to be useful for decreasing non-specific reaction in other plants (Van Regenmortel, 1982), showed no distinctive effect on the elimination of non-specific agglutination in highly concentrated leaf and root extracts (data not shown). On the basis of the above results, this study concluded that the optimum dilution range for the detection of CymMV by GPAT in the extracts of both leaf and root of *Cattleya* plants was 50-100 fold.

Application of GPAT for CymMV detection. Accurate and rapid diagnosis of CymMV is particularly important to produce healthy orchid plants (Hsu et al., 1992). For this purpose, the effectiveness of GPAT to detect CymMV was evaluated. Fifty leaves and twenty roots were randomly selected from individual Cattleya plants cultivated in orchid farms. The extract of each sample was diluted by 50 folds and then subjected to GPAT for detection of CymMV. Of the 50 leaf samples, 34 were infected with CymMV and 16 were healthy. Meanwhile, 14 out of 20 root samples were positive for CymMV (Fig. 2). Intermediate reactions, marked with the symbol (±) on Fig. 2, occurred in three leaf extracts and a root extract. However, CymMV was not detected in the same samples after further centrifugation or dilution (data not shown). This result showed that the accurate and successful detection of CymMV in *Cattleya* plants by GPAT could be achieved when both 50- and 100- fold dilutions of the extract were simultaneously subjected to one test. Consequently, clarification and optimum dilution of the extract prior to use were the most important steps to increase reliability of GPAT. Also, since GPAT is a rapid, accurate, and simple procedure for CymMV detection, and neither cause environmental contamination nor require expensive chemicals, this method can be used for the production of healthy orchid plants and plant quarantine.

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