Etiology of Flower Necrosis in Cattleya Orchids

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ABSTRACT

Rod-shaped virus particles were partially purified from a *Cattleya* orchid plant showing symptoms of severe flower necrosis. Differential and rate-zonal centrifugation separated three ultraviolet absorbing peaks. Fraction a at the top of the gradient column contained small particulate contaminants, fragments of virus-like rod particles, and a few longer rod-shaped particles. Fraction b contained a high concentration of rod particles. Seventy-four per cent of the particles in this fraction measured 415-445 mμ. Fraction c contained a low concentration of particles 450-475 mμ long. Fractions a and c produced only a few lesions on *Cassia occidentalis,* but fraction b produced many local lesions on this test plant. *Cymbidium* mosaic virus antiserum was incubated with each of the three gradient fractions. No unreacted particles or particle fragments were observed in electron microscope examination of antiserum-absorbed fractions. *Cattleya* seedlings from Bow Bells crosses inoculated with gradient fraction b incubated with normal serum developed flower necrosis. Fraction b absorbed with CyMV antiserum produced no flower necrosis, but flower necrosis was produced by fraction b absorbed only with TMV-O antiserum. Although the orchid strain of tobacco mosaic virus is present in some naturally infected plants with flower necrosis, this virus is not an essential component in the disease reaction. Phytopathology 60:36-40.

Flower necrosis in *Cattleya* orchids occurs in Europe and the United States. Although the disease may be of recent origin, it probably has been present for some time, but was unrecognized because the first symptoms can be confused with senescence of the flowers. An incubation period of 2 to 3 weeks is common between the time the flower opens and the appearance of the first necrosis in the flowers. However, the disease can appear as soon as 1 or 2 days after the flowers open. Brown streaks and spots suddenly appear in the sepals and petals, and necrosis spreads rapidly.

Cattleya flower necrosis is caused by a mechanically transmissible agent; two viruses have been found associated with the disease (8). According to Thornberry et al. (9), the orchid strain of tobacco mosaic virus (TMV-O) and *Cymbidium* mosaic virus (CyMV) produced the disease symptoms in a combined infection of pure cultures of both viruses, but neither virus alone induced the disease. Corbett (3) reported that the flower necrosis described by Thornberry & Phillippe (8) was rare in Florida, although TMV-O and CyMV were isolated singly and in combination from many orchid genera, species, and hybrids. Although both flower and leaf symptoms may be induced by TMV-O infection (3), CyMV causes leaf symptoms in several orchid species and reduces number and size of flowers (2, 6).

Plants of white flowering *Cattleya* crosses with flower necrosis were bioassayed for the presence of TMV-O and CyMV. Both viruses were isolated from some plants, but only CyMV from others. A positive correlation could not always be found between the presence of both TMV-O and CyMV in bioassay and the development of flower necrosis. It was possible that some of the plants were infected with TMV-O, but the virus was not isolated because it was present in such low concentration that it could not be detected in our assay.

Experiments were made to purify and identify the viruses present in *Cattleya* plants with necrotic flowers, and to determine which of these viruses, alone or in combination with other viruses, causes the disease.

MATERIALS AND METHODS.—A rod-shaped virus was purified from the white flowering *Cattleya* 'Angel Island' with flower necrosis. Leaf samples from the source plant were tested on *Nicotiana tabacum* L. 'Xanthi', *Chenopodium amaranticolor* Coste & Reyn., *Gomphrena globosa* L., and *Cassia occidentalis* L. by mechanical inoculation with fresh sap and by the liquid nitrogen extraction procedure described by Thornberry & Phillippe (8). Inoculation tests were performed to determine if CyMV and TMV-O could be detected in bioassay from the Angel Island source plant.

Fifty g of leaf tissue were chopped into small pieces with a razor blade and ground in a blender in 200 ml of 0.1 M PO_4 buffer, pH 7.5. The homogenate was squeezed through cheesecloth, and the solution was centrifuged at an average of 5,900 *g* for 15 min. The resulting supernatant fluid was centrifuged at an average of 69,600 *g* for 90 min to concentrate the virus. Pellets were suspended in 0.01 M PO_4 buffer, pH 7.5, and left overnight at 4 C. The resuspended pellets were combined and the mixture was clarified at an average of 5,900 *g* for 15 min. Further purification was accomplished by rate-zonal density-gradient centrifugation in 10-50% sucrose in 0.01 M PO_4 buffer, pH 7.5. Ten ml of 10, 20, 30, 40, and 50% sucrose were hand-layered on the columns and equilibrated 18 hr. Two ml of the partially purified virus preparation were layered on each gradient. The gradient tubes were centrifuged at 23,000 rpm for 2.5 hr in a Spinco 25.2 rotor and fractionated with an

ISCO Model D density-gradient fractionator. Density-gradient fractions were rubbed on *C. occidentalis*, 'Xanthi' tobacco, and *C. amaranticolor* to assay for the presence of CyMV and TMV-O. Each gradient fraction was dialyzed against 0.01 M PO_4 buffer, pH 7.5, and examined in the electron microscope. The particle length distribution in gradient fraction b was determined, and a comparison of particle length was made from dip preparations of leaves from the Angel Island source plant and necrotic flowers of a seedling incubated with fraction b.

Mixing experiments were also made to determine how completely TMV-O could be separated from CyMV in the sucrose density gradient. A sample of partially purified TMV-O was mixed with partially purified CyMV, and the mixture was layered on the sucrose gradient column prepared as described above. Gradient fractions were collected and dialyzed overnight in 0.01 M PO_4 buffer, pH 7.5. Each fraction was assayed on indicator plants and examined in the electron microscope. The particle length distribution in each fraction was determined, and comparisons of particle length were made among the gradient fractions.

All electron micrographs were made from samples stained with 2% phosphotungstic acid adjusted to pH 6.8 with potassium hydroxide. The preparations were mounted on Formvar-coated 400-mesh grids. A drop of stain was added to a drop of the preparation and gently mixed with a micropipette. The excess liquid was removed with a filter paper after 30 sec. Particle measurements were made by comparing the length of the particles with the diameter of untreated 312 mμ and 500 mμ polystyrene latex. The diameters of 312 mμ and 500 mμ latex were compared with each as an unknown. A mixture of latex spheres was included on the same grid and different grids, thus making it possible to account for possible sources of error introduced when comparing virus particle size with the latex on separate frames of film. Measurements were made from projected images with a sprocketed tracing wheel.

Serological absorption tests were made to determine whether unreacted rod-shaped antigen could be detected in fractions a, b, or c incubated with CyMV antiserum prepared to a *Cymbidium* isolate of the virus in The Netherlands. Twofold dilutions of fraction b and CyMV antiserum were made in 0.01 M PO_4 buffer at pH 7.0. The reactants were mixed and incubated for 1 hr at 35 C. The samples were placed overnight at 4 C and centrifuged at an average of 3,000 *g* to remove the precipitates. The treated samples were examined in the electron microscope and assayed on *C. occidentalis*. Controlled absorption tests were made with normal serum.

To determine whether TMV-O related antigens were present in fraction b the following test was performed. A dialyzed sample of fraction b was incubated with TMV-O antiserum according to the procedure described for the CyMV absorption test. Uncentrifuged samples were observed for the presence of attached antibody along the particle surface.

The sedimentation pattern of the virus in fraction b was determined in a Spinco Model E analytical ultracentrifuge equipped with ultraviolet optics, photoelectric scanner, and monochrometer. Wavelength of the recording was 265 mμ. Fraction b samples were reconcentrated by high-speed centrifugation following gradient separation. The suspending buffer was 0.5 M PO_4 at pH 7.5.

Cattleya seedlings from Bow Bells crosses were used in inoculation tests with the three separated gradient fractions. Plants were inoculated by rubbing Carborundum-dusted leaves and wounding the pseudobulb with a razor blade contaminated with the preparation. Most inoculated seedlings had flower buds showing in the sheath, or just emerging from the sheath, and were 1 to 3 weeks from flowering at the time of inoculation. Dip preparations were made from necrotic flowers produced on inoculated seedlings, and the particle lengths were compared with those from leaves and flowers of the naturally infected Angel Island source plant.

RESULTS.—*Purification.*—TMV-O was not isolated from the orchid plant used as a source of virus for purification. Repeated attempts to mechanically transmit the virus by sap and liquid nitrogen extraction failed. In addition, a distinct population of TMV-O particles was not detected in dip preparations from leaves of the Angel Island source plant. CyMV was isolated from the leaves and necrotic flowers of this plant.

Density-gradient fractionation of the partially purified virus from Angel Island resulted in three separate fractions (Fig. 1). Fraction a contained small particle contaminants and their aggregates and some short fragments of rod particles and a few particles 400-500 mμ long. A low level of CyMV activity was associated with this fraction. Fraction b contained the highest concentration of CyMV activity measured by assay on the cotyledons of *C. occidentalis*. Fraction c contained mostly dimers of the 415 to 445-mμ particle lengths in fraction b, and showed a low level of CyMV activity.

The yield of purified and gradient-separated virus from 50 g of *Cattleya* leaf tissue was usually less than 1.0 mg. The ultraviolet spectrum was typical of a nucleoprotein with a low nucleic acid content (maximum at 263 mμ, minimum at 248 mμ; maximum:minimum ratio from 1.1 to 1.25).

Electron microscopy.—Electron microscope examination showed a high concentration of rod-shaped particles in fraction b. Seventy-four per cent of the particles in this fraction measured 415-445 mμ (Fig. 1). These measurements are 30-60 mμ shorter than the normal length reported for CyMV (2, 5). Fraction c from the density gradient contained a few particles 450-480 mμ long and a few 800-900 mμ particles which were apparently dimers of the 400 to 450-mμ length in fraction b. A comparison of particle length from gradient fraction b and a leaf dip from the Angel Island source plant showed that 77% of the particles in leaf dip preparations from Angel Island measured 435-460 mμ (Fig. 1).

Serology.—Gradient fractions a, b, and c incubated with CyMV antiserum failed to produce lesions on inoculated cotyledons on *C. occidentalis*. All the rod-shaped particles remaining in the supernatant of gradient

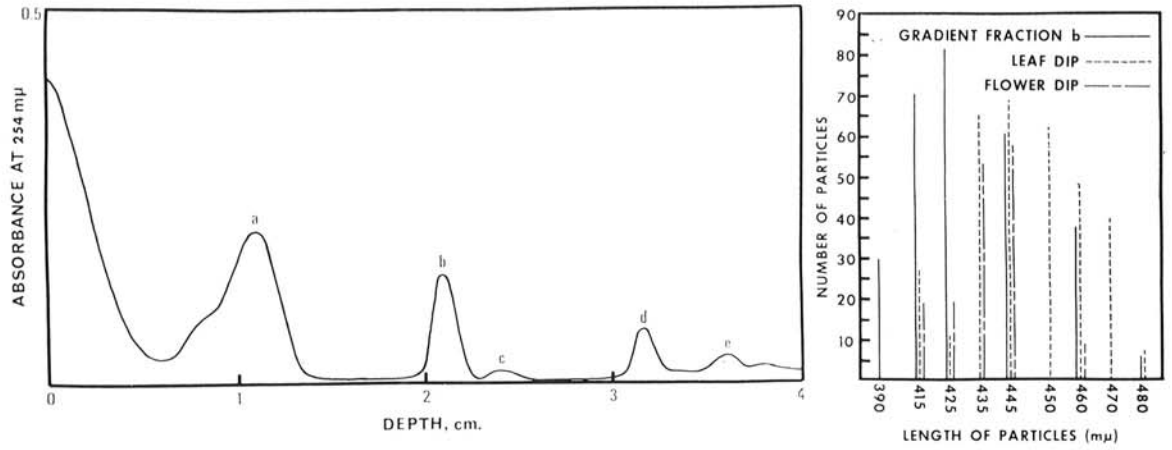

Fig. 1. (Left) Ultraviolet absorbance (254 mμ) of a mixture of *Cymbidium* mosaic virus and the orchid strain of tobacco mosaic virus separated by density-gradient fractionation. (a) Small particle contamination and rod fragments. (b) Most particles 415-445 mμ long. (c) Particles 450-480 mμ and a few particles 800-900 mμ long. (d) Particles about 300 mμ long. (e) Aggregates of 300 mμ particles. **(Right)** Particle length distribution from density-gradient fraction b, a leaf dip from *Cattleya* 'Angel Island', and a dip from necrotic flowers of a *Cattleya* seedling inoculated with fraction b.

fractions a, b, and c showed a reaction with CyMV antibodies. The coating of rods with antibodies and rod clumping, noted by Ball & Brakke (1), were present in these preparations. Particles in fraction b were clumped, and antibodies were present along the surface of the particles (Fig. 2-A). Rod-shaped particles in the preparation that were shorter than 400 mμ also reacted with the antiserum (Fig. 2-A). None of the rod-shaped particles in fraction b, including those less than 400 mμ, reacted with TMV-O antiserum (Fig. 2-B). No globulin attachment was detected along the surface of rod particles in fraction b that were incubated with normal serum (Fig. 2-C).

Sedimentation and mixing experiments.—The sedimentation coefficient of the virus in fraction b was 116S (Fig. 2-D). The boundary was broken, and there was some aggregation in the form of dimers produced when fraction b was reconcentrated by a second high-speed centrifugation at an average of 69,600 *g* for 90 min prior to the analytical centrifugation.

Experiments were performed to determine whether TMV-O could be completely separated from CyMV in a mixture of the two viruses in sucrose gradient columns. CyMV was completely separated from TMV-O with no detectable TMV-O activity in fraction b assayed on *C. amaranticolor* and Xanthi tobacco. Only one local lesion was produced on Xanthi tobacco from gradient fraction c in several experiments. Fractions d and e produced high lesion counts on inoculated leaves of both test species.

Seedling inoculations.—*Cattleya* flowers showed symptoms as early as 6-8 days after they opened when inoculations were made with a preparation containing 20 μg/ml of virus from fraction b (Fig. 3-A, 3-B). Seedlings inoculated with 10 to 100 μg/ml of virus from fraction b showed sunken necrotic spots on inoculated leaves (Fig. 3-C). Plants inoculated with preparations containing 5 to 10 μg/ml of the purified virus usually showed the first necrotic symptoms in the flowers several days after the first symptoms appeared in plants inoculated with higher concentrations of virus.

Results of seedling inoculation experiments have shown that CyMV is rapidly transported through the inoculated plant. The virus moves not only into the fully formed bud above an inoculated pseudobulb, but also moves rapidly out of the inoculated pseudobulb into adjacent parts of the plant. In one test, the virus moved out of the inoculated pseudobulb into an adjacent noninoculated pseudobulb which had a fully opened flower at the time of inoculation. Necrosis was produced in the flower that was fully open at the time of inoculation.

Seedlings inoculated with gradient fraction b absorbed with CyMV antiserum did not produce flowers with necrosis, and rod-shaped virus particles were not recovered from flowers. Those inoculated with gradient fraction b absorbed with TMV-O antiserum showed symptoms of necrosis in the flowers, and rod-shaped virus particles with a predominant length of 415-445 mμ were recovered from the flowers (Fig. 1).

Natural infection.—Observations from naturally infected lavender-flowered *Cattleya* cultivars were made in order to determine whether both CyMV and TMV-O are necessary to induce flower necrosis. It has been previously reported that TMV-O produced color breaking in the flowers of a lavender-flowered *Cattleya* hybrid (3). We have observed plants for the presence of color breaking and necrosis in the flowers for 2 years. Some plants have produced both color breaking and necrosis, and CyMV and TMV-O have both been isolated. Other plants have produced only necrosis, and only CyMV has been isolated from them.

Since some lavender-flowered *Cattleya* hybrids can be infected with TMV-O and produce symptoms of color breaking in the flowers only after many months (3), it is possible that both viruses could be present, yet no color breaking would appear. However, some plants of a cultivar have shown flower necrosis and no color

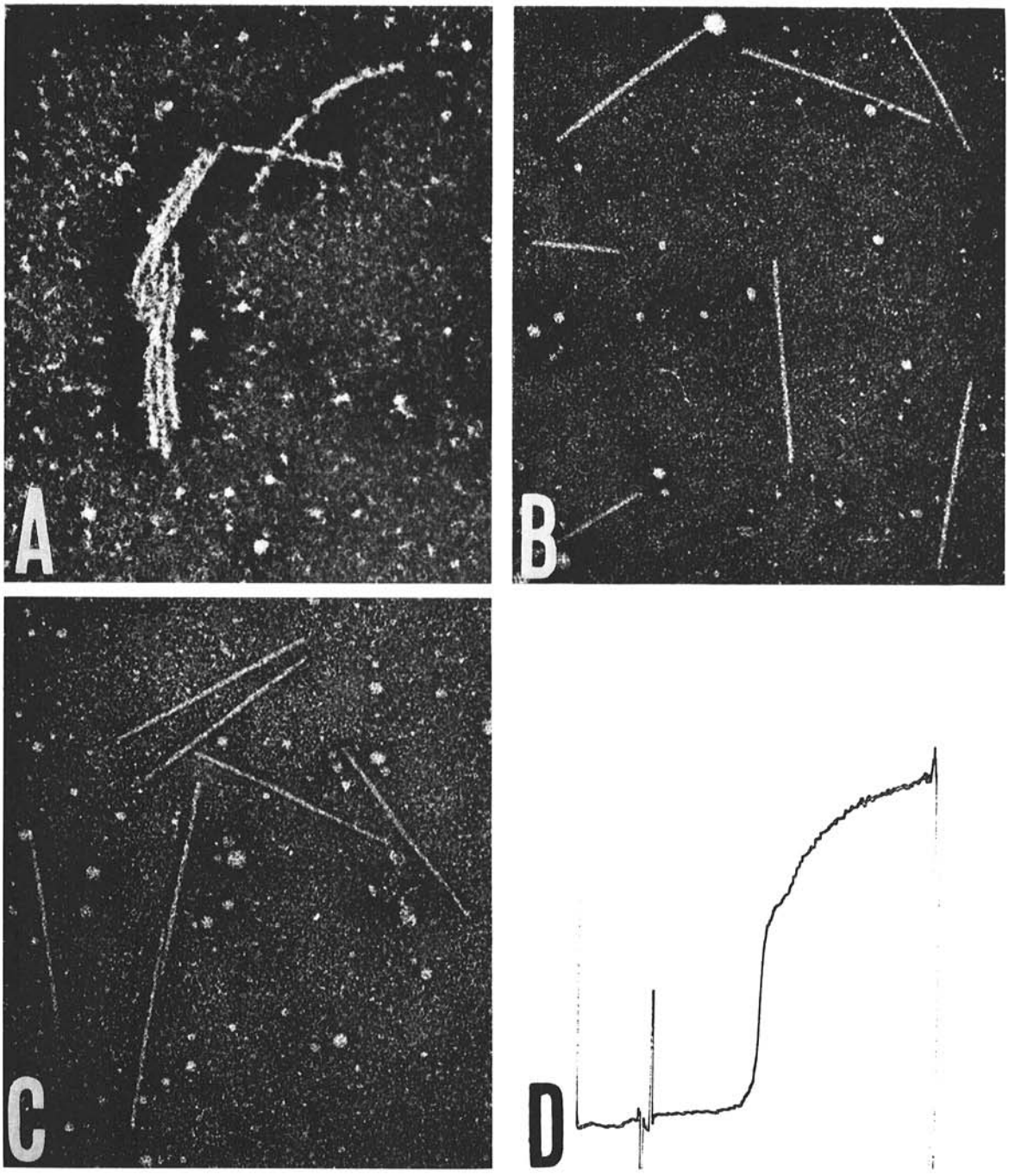

Fig. 2. Electron micrographs of density-gradient fraction b incubated with **A)** CyMV antiserum; **B)** TMV-O antiserum, and **C)** normal serum. Specific antibody attachment to the particles is present only in A (enlarged about 61,600 times). **D)** Ultraviolet sedimentation pattern of gradient fractionb recorded about 8 min after reaching a speed of 23,150 rpm at a temperature of 20.4 C. Sedimentation from left to right.

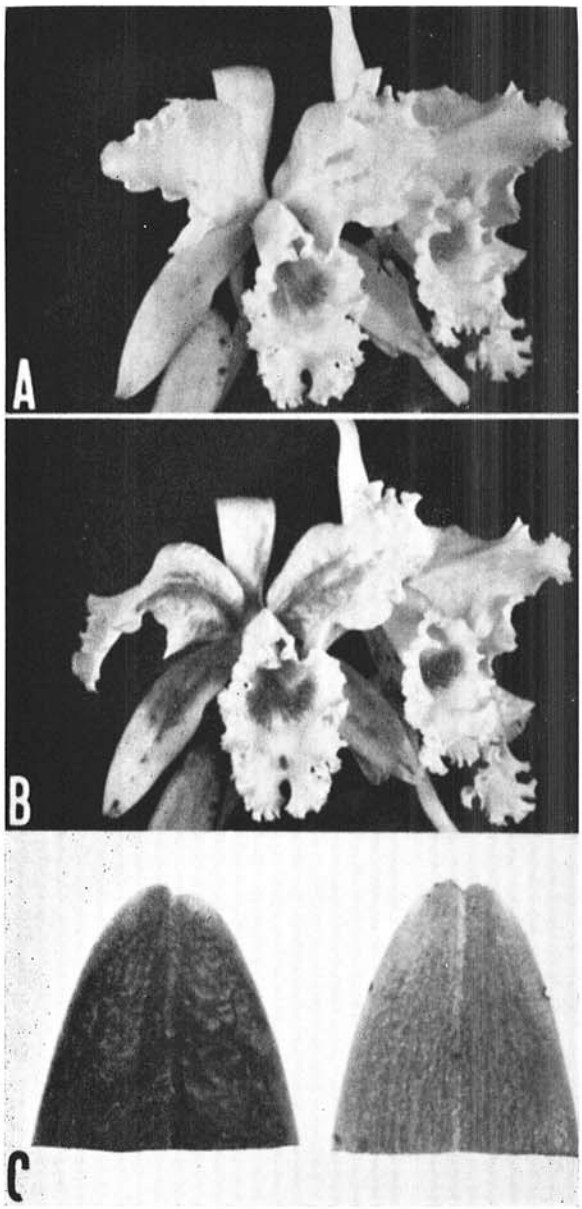

Fig. 3. Flower and foliage symptoms on a *Cattleya* seedling from a cross of cv. Bow Bells inoculated with 20 μg/ml of virus from density-gradient fraction b. **A)** Necrotic symptoms on flowers 6 days after opening. **B)** Flowers shown in A 8 days after opening. **C)** Sunken necrotic spots on the inoculated leaves. (Left) Severe necrosis and death of the leaf. (Right) Mild necrosis with sunken spots.

breaking during 2 years' flowering. Other plants of this cultivar have shown color breaking when infected with TMV-O. This is circumstantial evidence that TMV-O is not involved in the flower necrosis disease.

DISCUSSION.—Flower necrosis in *Cattleya* orchids is induced by a rod-shaped virus identified as *Cymbidium* mosaic virus. The virus produces local lesions typical of CyMV on *Cassia occidentalis*, but 74% of the particles measure 415-445 mμ. This is in contrast to the normal length of 475-480 mμ for CyMV reported by some workers (2, 4, 5, 7).

CyMV alone can induce *Cattleya* flower necrosis. Although most of the rod-shaped virus particles in gradient fraction b were shorter than the normal length reported for CyMV, rod-shaped particles serologically distinct from CyMV were not detected in this fraction. All of the rod-shaped particles in fraction b showed specific antibody attachment along the surface of the rod, and no unreacted particles were detected in samples incubated with CyMV antiserum. *Cattleya* seedlings inoculated with gradient fraction b absorbed with CyMV antiserum failed to produce necrotic flowers.

Results of these tests do not exclude the possibility that fraction b is a mixture of more than one strain of *Cymbidium* mosaic virus with a close serological relationship among the strains. A mixture of strains with similar particle size and density would not have been separated with the procedures used.

None of the rod-shaped particles in gradient fraction b incubated with TMV-O antiserum showed specific antibody attachment along the surface of the rods, and no serological relationship was established between the particles and TMV-O.

Although TMV-O may influence the pattern and intensity of necrotic symptoms in *Cattleya* flowers, it is not a necessary component in the *Cattleya* flower necrosis disease.

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