Association of Tomato Ringspot Virus with a Chlorotic Leaf Streak of Cymbidium Orchids

L. M. Goff and M. K. Corbett

Graduate Assistant and Professor, respectively, Botany Department, University of Maryland, College Park, MD 20742.

Portion of a thesis submitted by the senior author in partial fulfillment of the requirement for the M.S. degree.
Scientific Publication No. A2258 and Contribution No. 5252, Maryland Agricultural Experiment Station.
The authors wish to acknowledge the assistance of R. W. Vickery.

Accepted for Publication 28 February 1977.

ABSTRACT

GOFF, L. M., and M. K. CORBETT. 1977. Association of tomato ringspot virus with a chlorotic leaf streak of cymbidium orchids. Phytopathology 67: 1096-1100.

An isolate of tomato ringspot virus was obtained from plants of *Cymbidium* Snowbird 'Jayhurst' that exhibited faint chlorotic leaf streaks. The virus infected a wide range of herbaceous plants and induced symptoms characteristic of viruses of the ringspot group. In crude sap the dilution end point of the virus was between 1:100 and 1:1,000, thermal inactivation was 50-60 C for 10 min, and infectivity persisted for 48 hr at 25 C. Electron microscopy of shadowed leaf-dip preparations and negatively stained purified virus preparations revealed the presence of isometric particles having a diameter of 28 nm. Two light-scattering zones, with estimated sedimentation coefficients of 120 S and 126 S, were obtained after sucrose density-gradient centrifugation of partially purified virus preparations. Infectivity was associated with the light-scattering zones. A third, noninfectious zone, with an estimated sedimentation coefficient of 57 S, was observed in some preparations. Gel double-diffusion serological tests showed that the isolate reacted with antisera to strains of tomato ringspot virus, but not with antisera to tobacco ringspot virus, cucumber mosaic virus, cymbidium ringspot virus, or cherry leaf roll virus.

This paper reports the association of an isolate of tomato ringspot virus with a chlorotic or necrotic leaf streak disease of *Cymbidium* Snowbird 'Jayhurst' and discusses the benefits of including additional hosts for the indexing of orchid viruses.

Virus diseases of orchids have been reported since 1943 (12) and most subsequent studies have associated either rigid or flexuous rod-shaped virus particles with a particular disease. The orchid strain of tobacco mosaic virus (TMV-0) and cymbidium mosaic virus (CyMV) are the most commonly occurring orchid viruses (4).

Two isometric viruses have been reported as disease-causing agents in orchids. Nobrega (14) and Inouye (9, 10) reported cucumber mosaic virus (CMV) in *Dendrobium* orchids. Hollings and Stone (7) isolated a spherical virus, cymbidium ringspot (CyRSV), with a diameter of approximately 28 nm, from cymbidium orchids. This virus reacted homologously, and heterologously in serological tests with a virus from white clover, but did not react with antisera to nine other isometric viruses (8). The physical properties of CyRSV are similar to viruses in the tomato bushy stunt group.

Host range, physical properties, particle size and shape, and serological data, indicate that the virus we have isolated from cymbidiums is different from all previously reported viruses from orchids. Preliminary results have been published (6).

MATERIALS AND METHODS

Source of viruses.—The cymbidium isolate of tomato ringspot virus (C-TomRSV) initially was isolated from leaves of *Cymbidium* Snowbird 'Jayhurst' that exhibited faint chlorotic streaks. Electron microscopy of leaf-dip preparations from similarly infected leaves were negative for rod-shaped virus particles. Bioassay indicated that a mechanically transmitted agent with a wide host range was associated with the diseased orchids. The virus was maintained in plants of *Nicotiana tabacum* L. 'Kentucky 35'.

Experimental host range.—Four to eight plants of each tested cultivar were inoculated with crude sap from infected *N. tabacum* 'Kentucky 35' by the Carborundum gauze-pad method. The plants were indexed 2-3 wk after inoculation on leaves of either 'Kentucky 35' or *Chenopodium quinoa* Willd.

Properties in crude sap.—Dilution end point, thermal inactivation, and longevity in vitro were determined using crude sap prepared from infected leaves of 'Kentucky 35' harvested 10 days after inoculation.

Purification.—Leaves of *N. tabacum* 'Kentucky 35' were harvested 9-10 days after inoculation with C-TomRSV and frozen. Frozen tissue was homogenized in a Waring Blendor 1:1 (w/v) with 0.05 M citrate buffer (pH 6.5) containing 0.1% thioglycollic acid. After the homogenate was expressed through cheesecloth, glacial acetic acid was added to lower the pH to 4.8. The mixture was placed in an ice bath for 30 min, followed by centrifugation at 10,000 *g* for 10 min. The supernatant

liquid was filtered through Pyrex glass wool and subjected to two or three cycles of differential centrifugation. Pellets resulting from high-speed centrifugation (66,000 *g* for 2 hr) were resuspended in either 0.025 M citrate buffer pH 7.0 or 0.005 M borate buffer pH 9.0.

Further identification of the virus was by sucrose density-gradient centrifugation of 1- to 2-ml samples on gradients of 10-40% sucrose dissolved in a buffer containing 0.01 M KCl, 0.005 M K_2HPO_4, and 0.0005 M KH_2PO_4 at pH 7.7. Centrifugation was at 23,000 rpm in a Beckman SW 25.1 rotor for 2.5-3.0 hr. Zones were removed by syringe, or with an ISCO density-gradient fractionator (Model 640), monitoring absorption at 254 nm. Fractions of 0.5 ml were diluted 1:1 with 0.01 M phosphate buffer pH 7, and assayed by inoculation on leaves of *C. quinoa*.

Estimation of sedimentation coefficients.—The method of Martin and Ames (13) was used to estimate the sedimentation coefficients of C-TomRSV components. Cucumber mosaic virus (CMV) with a reported value of 98 S (5) was included with a partially purified preparation of C-TomRSV on a sucrose density gradient.

Ultraviolet absorption spectrophotometry.—Purified virus was suspended in 0.005 M borate buffer pH 9.0, and the absorption spectrum measured from 230-310 nm using a Zeiss PMQ-II spectrophotometer.

Electron microscopy.—Infected plant tissue was examined for the presence of virus-like particles by chromium-shadowed leaf-dip preparations using a Hitachi HU-11C electron microscope. Negative staining was by suspension in 2% ammonium acetate pH 5.0 with 2% ammonium molybdate pH 7.5. Tobacco mosaic virus was sometimes added and the particle width was used as an internal calibration standard.

Serological tests.—Antiserum to C-TomRSV was prepared in a New Zealand white rabbit by a series of four intravenous and two intramuscular injections of partially purified virus preparations. Freund's complete adjuvant was incorporated with the preparations for intramuscular injections. Ten days after the final injection, the rabbit was bled, the serum fraction was separated and stored at −20 C with Merthiolate added to 1:10,000. Other antisera were obtained as follows: cymbidium ringspot from M. Hollings, Glasshouse Crops Research Institute, Sussex, England; euonymus isolate of tomato ringspot from C. W. Puffinberger, Maryland State Department of Agriculture, College Park, MD 20742; raspberry isolate of tomato ringspot and cherry leaf roll from R. M. Lister, Purdue University, Lafayette, IN 47907; cucumber mosaic from H. E. Waterworth, U.S. Department of Agriculture Plant Introduction Station, Glenn Dale,

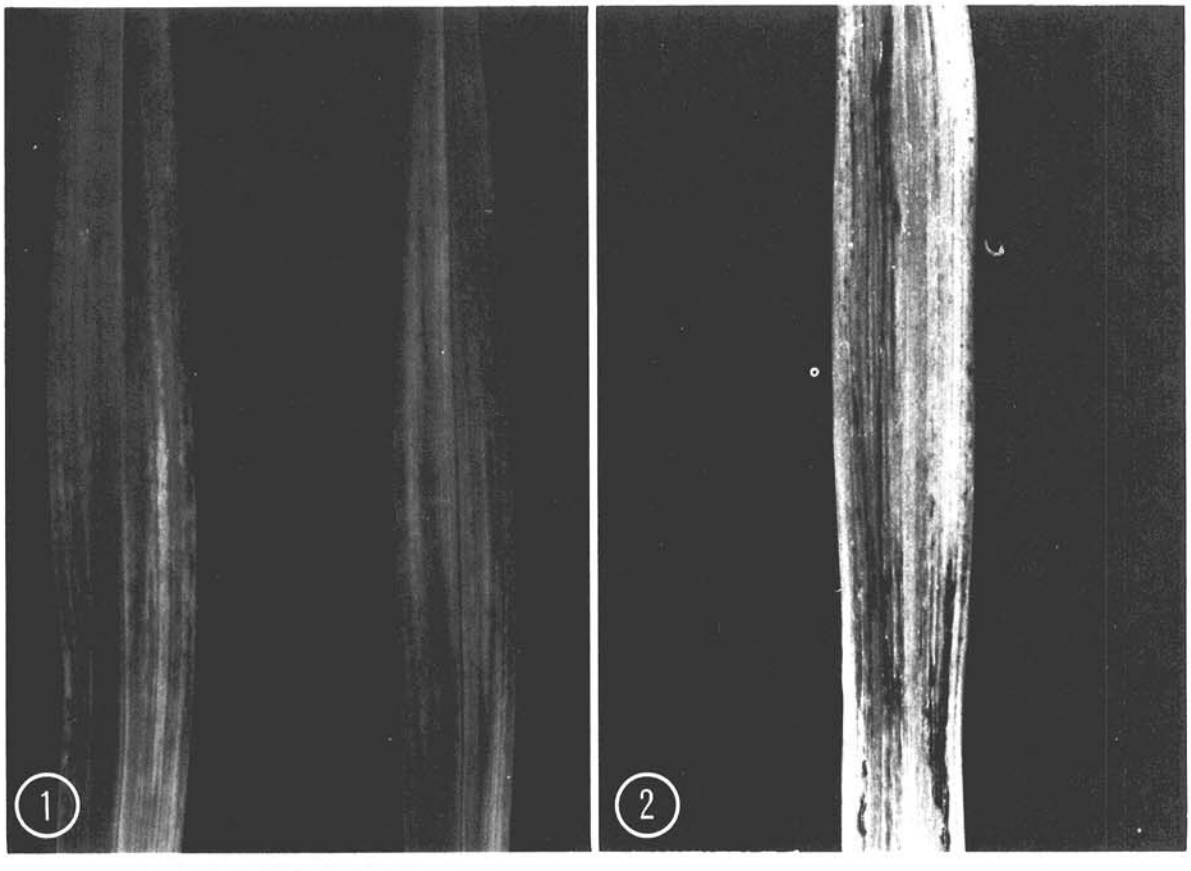


Fig. 1-2. 1) Chlorotic and 2) necrotic streaks in leaves of *Cymbidium* Snowbird 'Jayhurst' infected with the cymbidium isolate of tomato ringspot virus.

MD 20769; and tobacco ringspot (PVAS-45) and tomato ringspot (PVAS-15) from the American Type Culture Collection.

For all serological agar gel double-diffusion tests, 0.5% Oxoid Ionagar No. 2 in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride was used, with 0.1% sodium azide as a preservative.

RESULTS

Symptoms in naturally infected cymbidiums.—The cymbidium isolate of tomato ringspot virus was isolated from three symptomless plants of *Cymbidium* Snowbird 'Jayhurst' and from seven plants exhibiting a very faint chlorotic mottle (Fig. 1). All *Cymbidium* Snowbird 'Jayhurst' plants used in this study were obtained from a parent plant in Australia by tissue culture techniques. Advanced symptoms included basal necrotic streaking (Fig. 2), premature abscission of the younger leaves from the pseudobulb, and a general reduction in vigor. Flowering did not occur during the course of this study.

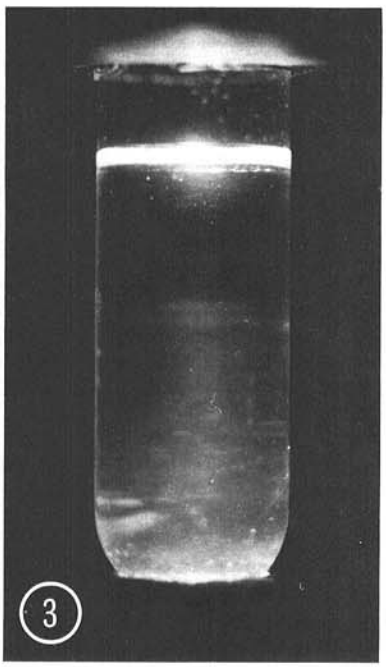
The foliar symptoms associated with C-TomRSV infection in *Cymbidium* are different from late symptoms associated with infections by either TMV-0 or CyMV, but may be confused with the early symptoms induced by either.

Experimental host range.—Plants of 50 cultivars in 28 species of 22 genera in 10 families were inoculated. Infection with C-TomRSV occurred in 33 cultivars of 18 species in 14 genera of eight families.

The following hosts were systemically infected after mechanical inoculation with C-TomRSV: *Gomphrena globosa* L.; *Chenopodium amaranticolor* Coste & Reyn.; *C. quinoa* Willd.; *Zinnia elegans* Jacq. 'Envy' and 'Dark Jewels'; *Cucumis melo* L. 'Rocky Ford'; *C. sativus* L. 'Long Marketer' and 'National Pickling'; *Cucurbita maxima* Duch. 'Early Prolific Straightneck'; *C. pepo* L. 'Sugar and Pie' and 'Small Sugar'; *Phaseolus vulgaris* L. 'Bountiful' and 'Kentucky Wonder'; *Pisum sativum* L. 'Alaska'; *Vicia faba* L. 'Major'; *Vigna sinensis* (L) Savi. 'Black Local', 'Brown Crowder', 'California No. 5', 'Blackeye', and 'Early Ramshorn'; *Phytolacca americana* L.; *Datura stramonium* L.; *Lycopersicon esculentum* Mill. 'Beefsteak' and 'Marglobe'; *Nicotiana rustica* L.; *N. tabacum* L. 'Kentucky 35', 'Samsun NN', and 'Turkish'; *Nicotiana* hybrid (*N. clevelandii* Gray × *N. glutinosa* L.); *Petunia hybrida* Vilm. 'Flaming Velvet', 'Happy Talk', 'Pink Cascade', 'Snowstorm', and 'White Cascade'.

Characteristically, many of these plants developed either chlorotic or necrotic ringspots on inoculated leaves with line-etching or "oak leaf" patterns produced on subsequent growth, and eventual "recovery". Plants of *Gomphrena globosa* inoculated with C-TomRSV were generally symptomless, but virus could be recovered from both inoculated and noninoculated leaves. Local infection without systemic movement of the virus was observed only in *N. tabacum* 'Turkish NN'. *Chenopodium quinoa* developed local lesions, leaf distortion and apical necrosis. The reaction was so severe that plants of *C. quinoa* were used for most bioassays.

The following plants were not susceptible to C-TomRSV by mechanical inoculation: *Vinca rosea* L.;



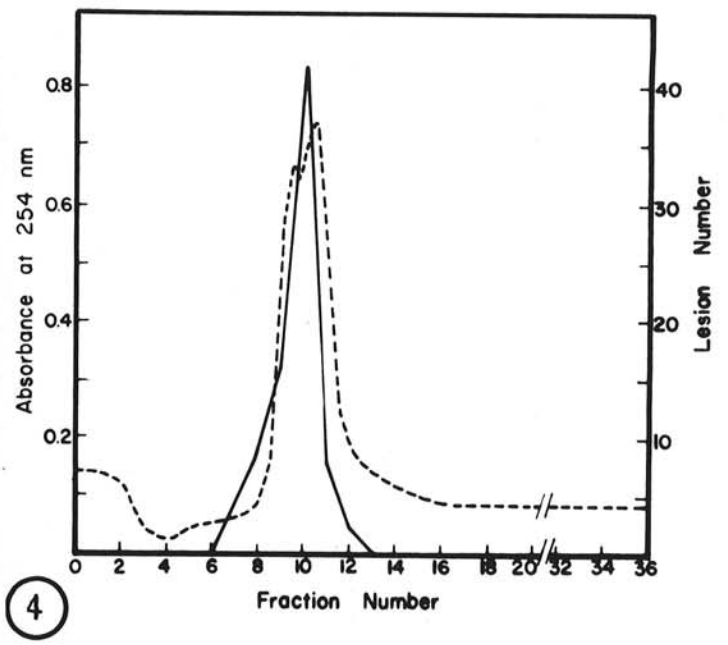


Fig. 3-4. 3) A 10-40% sucrose gradient after rate-zonal centrifugation of 1 ml of a partially purified preparation of the cymbidium isolate of tomato ringspot virus. Centrifugation was for 2.5 hr at 23,000 rpm in an SW 25.1 rotor of a Beckman L2-65 centrifuge. 4) An ultraviolet absorption profile of the cymbidium isolate of tomato ringspot virus in a 10-40% sucrose gradient (Fig. 3). Absorbance was measured at 254 nm in an ISCO UA-2 analyzer attached to a Model 640 fractionator. Absorbance is represented by the broken line and the infectivity of each fraction, as determined in plants of *C. quinoa*, is represented by the solid line.

Dianthus caryophyllus L. 'Mixed Colors'; *Aster* sp. 'Azure Blue'; *Zea mays* L. 'Golden Bantam'; *Cassia occidentalis* L.; *Glycine max* Merr. 'Harosoy'; *Phaseolus vulgaris* L. 'Black Valentine' and 'Pinto'; *Pisum sativum* L. 'Wando'; *Capsicum annum* L. 'California Wonder' and 'Yolo Wonder'; *C. frutescens* L. 'Tabasco'; *Datura metel* L.; *Lycopersicon esculentum* Mill. 'Rutgers'; and *Nicotiana glutinosa* L. To date, 25 cymbidium plants inoculated by Carborundum gauze-pad method or by leaf and pseudobulb cuts with a scalpel dipped in inoculum have not shown symptoms of infection and the virus has not been recovered.

Properties of C-TomRSV in crude sap.—Crude sap from infected leaves of *N. tabacum* 'Kentucky 35' was infectious at a dilution of 1:100, but not at 1:1,000. The thermal inactivation point was 50-60 C, and at 25 C infectivity was retained for 24 hr, but not for 48 hr. These properties are comparable to those reported for other nepoviruses (1).

Sucrose density-gradient centrifugation.—Two distinct light-scattering zones (Z_1 and Z_2) were obtained when 1 or 2 ml of partially purified virus was subjected to sucrose density-gradient centrifugation (Fig. 3). A third zone (T), broader and more diffuse, which sedimented at a much slower rate [possibly representing virus protein "shells", devoid of nucleic acid (18)] was detected in some preparations. Infectivity was associated with that portion of the gradients containing zones Z_1 and Z_2 (Fig. 4).

The sedimentation coefficients for T, Z_1, and Z_2 were estimated at 57 S, 120 S, and 126 S, respectively. These results are comparable to those reported by Schneider et al. (16) for isolates of TomRSV.

Ultraviolet absorption spectrophotometry.—Preparations of Z_1 and Z_2 from density-gradients, had an absorption maximum at 260 nm, and minimum at 240 nm. The 260:280 nm ratio of both components was 1.8.

Electron microscopy.—Leaf dips from infected cymbidium tissue were negative for rod-shaped particles, but contained some isometric particles.

Electron microscopy of purified preparations of C-TomRSV in 2% ammonium molybdate, showed isometric virus-like particles (Fig. 5). Using the width of TMV particles as 18 nm, the estimated diameter of C-TomRSV particles was 28 ± 2 nm.

Serological tests.—The cymbidium isolate of tomato ringspot virus reacted with a 1/64 dilution of homologous antiserum, and with antisera to tomato ringspot, the raspberry isolate of tomato ringspot, and the euonymus isolate of tomato ringspot viruses (15). Reactions were not observed between C-TomRSV and antisera to tobacco ringspot, cucumber mosaic, cymbidium ringspot, and cherry leaf roll viruses.

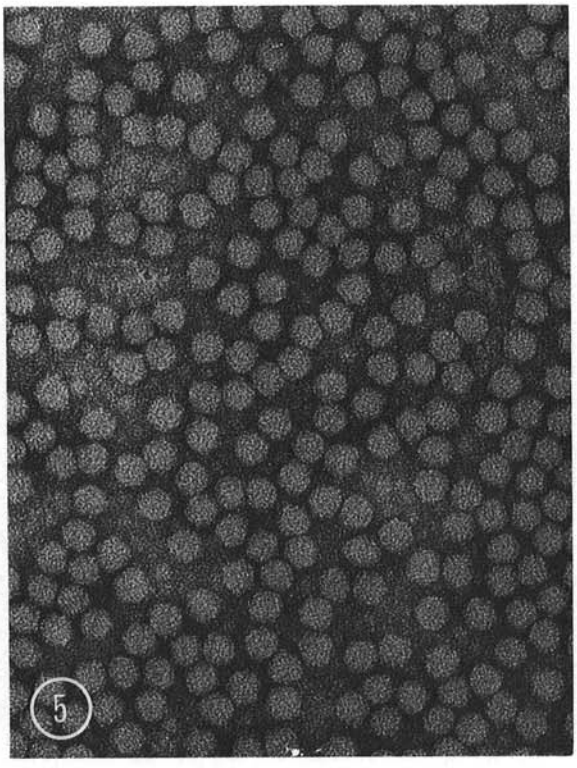


Fig. 5. An electron micrograph of a density-gradient preparation of the cymbidium isolate of tomato ringspot virus negatively stained with 2% ammonium molybdate (× 135,000).

DISCUSSION

Nepoviruses are similar with respect to particle size, physical properties, and symptomatology. Host range studies are of limited value in distinguishing among members of this group and serological tests are the most reliable method for identifying these viruses. In agar gel double-diffusion tests, C-TomRSV reacted only with TomRSV or isolates reported serologically related to it. Reciprocal tests could not be done; the only antigen available was C-TomRSV. Thus, the precise serological relationships of C-TomRSV were not determined, but the virus is at least serologically related if not identical to TomRSV, and hence is a nepovirus.

The symptoms of infection by C-TomRSV in cymbidium orchids are indistinguishable from either CyMV or TMV-0 in the early stages and the reason the virus has not been previously reported is that infected plants may have been discarded in the belief that they were infected with rod-shaped viruses.

Electron microscopy and serological tests are used routinely to screen orchids for the presence of virus (4). Examination of plant material by the leaf-dip method is a reliable and rapid technique for the detection of rod-shaped viruses, but is usually inadequate for the detection of viruses with spherical particles because they are often difficult to distinguish from normal cell components, particularly ribosomes. Serological tests are designed to screen specifically for either CyMV or TMV-0, and thus infection by tomato ringspot virus would remain undetected unless symptoms were severe.

Tests with local lesion assay hosts also are used to screen orchids for viral infection. *Cassia occidentalis* and *Gomphrena globosa* can be used to assay for the presence of CyMV and TMV-0, respectively (2, 3), but not for C-TomRSV because it failed to infect plants of *C. occidentalis* and infected *G. globosa* were symptomless or occasionally gave local lesions that are similar to those of

TMV-0. Plants of *N. tabacum* 'Kentucky 35', *C. quinoa*, or *Vigna sinensis* proved to be satisfactory indexing hosts for C-TomRSV under our greenhouse conditions, and should be included in routine orchid assays for virus infections.

Several nepoviruses, including TomRSV, have been reported to be seed transmitted (1), and although there is no report of pollen transmission from plants infected with TomRSV (17), other nepoviruses are pollen-transmitted (11). If C-TomRSV is seed- or pollen-transmitted, the virus may be propagated by growers through seedlings produced from virus-infected parents. In certain hosts, infected seedlings remain symptomless, and the presence of the virus can be detected only by bioassay or serological tests (11).

The importance of infection by C-TomRSV in orchids to the commercial grower or hobbyist is not yet clear. The greenhouse conditions in which infected *Cymbidium* Snowbird 'Jayhurst' orchids were maintained in our experiments were not conducive to flower production, and thus, any effect on flower color, size, shape, or longevity could not be observed. However, chlorotic mottling and necrotic streaking of the leaves detracts from the orchid, and reduced plant vigor would probably result in lower flower yields.

LITERATURE CITED

1. CADMAN, C. H. 1963. Biology of soil-borne viruses. Annu. Rev. Phytopathol. 1:143-172.
2. CORBETT, M. K. 1960. Purification by density-gradient centrifugation, electron microscopy, and properties of cymbidium mosaic virus. Phytopathology 50:346-351.
3. CORBETT, M. K. 1967. Some distinguishing characteristics of the orchid strain of tobacco mosaic virus. Phytopathology 57:164-172.
4. FRANCKI, R. I. B. 1970. Cymbidium mosaic virus. No. 27 *in* Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England. 3 p.
5. GIBBS, A. J., and B. D. HARRISON. 1970. Cucumber mosaic virus. No. 1 *in* Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England. 4 p.
6. GOFF, L. M., and M. K. CORBETT. 1974. A strain of tomato ringspot virus associated with chlorotic mottle of cymbidium. Proc. Am. Phytopathol. Soc. 1:149 (Abstr.).
7. HOLLINGS, M., and O. M. STONE. 1963. Cymbidium ringspot (a previously undescribed virus). Page 90 *in* Glasshouse Crops Res. Inst. Annu. Rep. 146 p.
8. HOLLINGS, M., and O. M. STONE. 1973. Cymbidium ringspot (CyRSV). Page 104 *in* Glasshouse Crops Res. Inst. Annu. Rep. 174 p.
9. INOUYE, N. 1969. Cucumber mosaic virus isolated from Dendrobium. Nogaku Kenkyu 53:49-60.
10. INOUYE, N. 1971. Virus diseases of orchids. V. Symptoms and properties of viruses in Dendrobium. Japn. Orchid Soc. Bull. 17:3-7.
11. LISTER, R. M., and A. F. MURANT. 1967. Seed-transmission of nematode-borne viruses. Ann. Appl. Biol. 59:49-62.
12. MAGEE, C. J. 1943. Orchid mosaic. Aust. Orchid Rev. 8:51-52.
13. MARTIN, R. G., and B. N. AMES. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372-1379.
14. NOBREGA, N. R. 1947. Uma doença de virus em orquidea. Biologico (São Paulo) 13:62-63.
15. PUFFINBERGER, C. W. 1973. A chlorotic ringspot disease of euonymus caused by a strain of tomato ringspot virus. Ph.D. Thesis. University of Maryland, College Park, Maryland. 123 p.
16. SCHNEIDER, I. R., R. M. WHITE, and E. L. CIVEROLO. 1974. Two nucleic acid-containing components of tomato ringspot virus. Virology 57:139-146.
17. STACE-SMITH, R. 1970. Tomato ringspot virus. No. 18 *in* Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England. 4 p.
18. STACE-SMITH, R., M. E. REICHMANN, and N. S. WRIGHT. 1965. Purification and properties of tobacco ringspot virus and two RNA-deficient components. Virology 25:487-494.