ISOLATION, PURIFICATION, AND SOME PROPERTIES OF TWO VIRUSES FROM CULTIVATED *CYMBIDIUM* ORCHIDS

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Summary

Two distinct viruses have been isolated from cultivated *Cymbidium* plants in Australia. Each virus isolate has been transmitted to herbaceous plants, has been purified, and high-titre antiserum has been produced. One isolate has been identified as a serotype of tobacco mosaic virus; the other is suspected of being *Cymbidium* mosaic virus and has flexuous rods about 13 by 475 mμ. Electron micrographs of particles of the latter show that they have hollow central canals and consist of helical structures with a pitch of approximately 2·8 mμ. Results indicate that inoculation to herbaceous plants and electron-microscopic examination and serological testing of leaf extracts can be used as methods for the routine indexing of *Cymbidium* plants.

I. Introduction

More than 30 virus diseases of orchids have been described (Jensen 1959). For many of these, the nature of the disease has not been rigorously demonstrated by transmission tests and so far only four diseases have been correlated with particles observed in the electron microscope: (1) *Cymbidium* mosaic virus (CyMV) (Gold and Jensen 1951), having flexuous rod particles about 13† by 475 mμ; (2) *Odontoglossum* ringspot virus (ORSV) (Jensen and Gold 1951), having rigid rod particles about 24 by 280 mμ; (3) tobacco mosaic virus (TMV) (Pérez, Adsuar, and Sala 1956); and (4) *Cymbidium* ringspot virus (Hollings 1963), having polyhedral particles about 30 mμ in diameter. This communication concerns two viruses that have been isolated from *Cymbidium* plants in Australia.

II. Materials and Methods

(*a*) *Plant Material and Sap Transmission of Viruses*

Orchid plants suspected of being infected with virus were donated by growers and were kept in an insect-proof glasshouse. Transmission tests were carried out by grinding small pieces of leaf with 0·1M phosphate buffer, pH 7, in a pestle and mortar and rubbing the extract with the forefinger to leaves of test plants dusted with 500-mesh carborundum.

(*b*) *Purification of Viruses*

Cymbidium leaves were cut into small pieces and homogenized with 0·2M phosphate buffer, pH 7·7, in a Servall omni-mixer (1 ml buffer per 1 g leaf material). The homogenate was strained through cheesecloth and the fibre extracted with an

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† See Section IV, p. 562.

equal volume of distilled water. The pooled liquid was emulsified with an equal volume of chloroform and centrifuged at 1000 *g* for 5 min. The uppermost buffer layer was then removed, centrifuged at 15,000 *g* for 10 min in a M.S.E. 18 high-speed centrifuge (No. 69181 rotor), and the virus was sedimented from the supernatant by centrifugation at 78,000 *g* for 60 min in the Spinco model L ultracentrifuge (type 30 rotor). The pellets were resuspended in distilled water. Two more cycles of alternate slow and fast centrifugation were carried out and the final pellets were resuspended in 0·02M phosphate buffer, pH 7·2. TMV strains U_1 and U_2 (Siegel and Wildman 1954) were purified from infected tobacco leaves by a combination of salt precipitation and ultracentrifugation. Sap was extracted from frozen leaves by grinding in a domestic meat mincer. The extract was strained through cheesecloth, heated at 55–60°C for 10 min, and centrifuged at 15,000 *g* for 10 min. Virus was precipitated by the addition of half-volume saturated ammonium sulphate solution, removed by centrifugation, and dialysed against distilled water. Further purification was accomplished by two cycles of alternate slow and fast centrifugation followed by another precipitation with ammonium sulphate. The final preparation was dialysed against 0·02M phosphate buffer, pH 7·2.

(*c*) *Serological Techniques*

Purified virus preparations emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) were injected subcutaneously into rabbits 1–3 times at weekly intervals followed by 1–3 intravenous injections 1–2 weeks later. Blood samples were taken 1 week after the last injection. Serological tests were carried out by the precipitation tube technique (Matthews 1957) using 0·14M NaCl to make two-fold serial dilutions of antisera or antigen.

Antisera absorbed with homologous or heterologous antigens were prepared by mixing 0·35 ml of antiserum (titre 1/512 to 1/1024) with 2 ml purified antigen preparation (containing 3·4 mg of virus) and 0·15 ml 1·4M NaCl. The mixtures were incubated for 2 hr at 37°C and left for 16 hr at 2–4°C. Precipitates were removed by centrifugation at 1000 *g* for 10 min and the supernatants were used for tests.

(*d*) *Electron Microscopy*

Drops of virus preparation were placed on copper grids which had been coated with collodion and reinforced with carbon. Excess liquid was removed with filter paper, the grids air-dried at room temperature, and then shadowed with platinum at an angle of 30°. Virus stained with phosphotungstate was prepared by mixing the virus suspension with an equal volume of 2% phosphotungstic acid (neutralized with KOH), pH 7, and applying the mixture to grids as above. Staining with uranyl acetate was carried out by applying a drop of virus suspension to a grid and then floating the grid on 3% unbuffered uranyl acetate solution (pH approx. 4·5). The excess solution was removed with filter paper and the grids were air-dried at room temperature. All grids were examined in a Philips 100B electron microscope.

Lengths of virus particles were measured on large prints by fitting pieces of 1·5 mm diameter plastic tubing along their length. Widths of virus particles were measured on electron micrographs of preparations to which TMV strain U_1 was added as an internal standard.

(e) *Spectrophotometry and Estimation of Virus*

Virus preparations were examined in a Shimadzu QR spectrophotometer using cells of 1 cm path length. Yields of TMV were determined by assuming the absorbancy index of the virus at 260 mμ to be 3·3. The absorbancy index of CyMV was found to be 3·5 from determinations of dry weights of virus preparations.

III. Results

(a) *Disease Symptoms on Orchid Plants*

Cymbidium plants suspected of being infected with virus showed a variety of symptoms but often virus was isolated from symptomless plants. The most common symptoms encountered were those of either necrotic or chlorotic streaks (Plate 1, Fig. 1) and plants thus affected were usually shown to be infected with TMV by methods which are described below. TMV was also recovered from an *Odontoglossum* plant (*Amphone magnifica*) (Plate 1, Fig. 2) with symptoms not unlike those described by Jensen (1959) for ORSV. *Cymbidium* plants were also encountered which were found to be infected with another virus, which is suspected of being, and in this paper will be referred to as, CyMV. Symptoms on these plants were variable but often showed mosaic and necrotic patches (Plate 1, Fig. 3). I have been unable to distinguish TMV and CyMV infection by symptoms on *Cymbidium* plants. Many plants showing symptoms (Plate 1, Fig. 1) similar to those described by Jensen (1959) for CyMV infection were actually shown to be infected with TMV and the presence of CyMV could not be detected.

(b) *Transmission of Viruses from Orchids to Herbaceous Plants*

Virus from infected orchid plants was readily transmitted to some herbaceous plants by sap inoculation. TMV from *Cymbidium* (O-TMV) induced characteristic symptoms on several hosts. On *Datura stramonium* L. (Plate 1, Fig. 4), *Chenopodium amaranticolor* Coste & Reyn. (Plate 1, Fig. 5), *Nicotiana glutinosa* L., and *Gomphrena globosa* L. local lesions were observed 3–6 days after inoculation. No systemic symptoms were produced on any of these hosts. The local lesions on *N. glutinosa* were very much smaller than those produced by common strains of TMV. O-TMV failed to infect *N. tabacum* L. cv. Turkish Samsum. The only hosts on which CyMV produced symptoms were *D. stramonium* (Plate 1, Fig. 6) and *C. amaranticolor* (Plate 1, Fig. 7); lesions were observed 10–14 and 20–24 days respectively after inoculation. Neither of these two hosts became infected systemically.

O-TMV and CyMV can easily be distinguished by the time required for the development of lesions on *D. stramonium* or *C. amaranticolor*. On *D. stramonium* both viruses induce lesions which are not easily distinguished by their appearance but on *C. amaranticolor* they are very distinct. O-TMV lesions are small and grey whereas those of CyMV are large and greenish in colour (Kado and Jensen 1964).

(c) *Purified Preparations of the Viruses*

Both viruses were readily purified in high yields by the method already described. These preparations were opalescent, usually very light brown in colour, and exhibited anisotropy of flow. From 100 g of *Cymbidium* leaves infected with O-TMV between 14 and 36 mg of virus was obtained; from those infected with CyMV the yields

TABLE 1

DILUTION END POINTS OF *CYMBIDIUM* LEAF EXTRACTS WITH ANTISERA PREPARED AGAINST O-TMV AND CyMV

Sap was extracted from *Cymbidium* leaves by grinding them in 0·1M phosphate buffer, pH 7·2 (1 g leaves to 1 ml buffer). Extracts were heated for 10 min at 55°C and centrifuged at 15,000 *g* for 10 min

Origin of *Cymbidium* Leaf Sap	Dilution End Point of Leaf Sap (antiserum* to O-TMV)							Dilution End Point of Leaf Sap (antiserum* to CyMV)						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Leaves infected with O-TMV	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Leaves infected with CyMV	±	±	−	−	−	−	−	+	+	+	−	−	−	−
Healthy leaves	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*Antisera were used at a constant dilution of 1/40 in 0·14M NaCl.

were usually considerably poorer, but in one experiment 27 mg of virus was obtained. In one experiment, CyMV was also purified from the corms of an infected plant, but the yield was less than one-fortieth of that obtained from the leaves.

Absorption spectra between 230 and 300 mμ of both O-TMV and CyMV preparations are characteristic of nucleoproteins of relatively low nucleic acid content (Fig. 1). Judging by the electron micrographs (Plate 2, Figs. 1 and 2) the purified preparations of both viruses appear relatively free of contaminating materials.

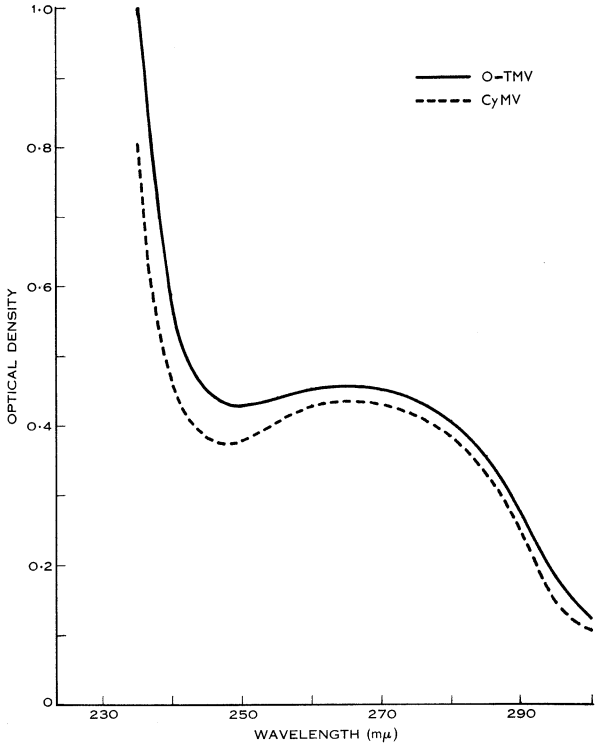

Fig. 1.—Ultraviolet absorption spectra of a purified preparation of O-TMV and CyMV.

It was also found that antisera prepared against these viruses did not give visible precipitates when tested against sap from healthy *Cymbidium* plants by the precipitin tube test (Table 1). Extracts from leaves infected with virus produced visible precipitates (Table 1). It remains obscure why antiserum to O-TMV produced a slight precipitate with sap from a CyMV infected plant (Table 1) as no O-TMV could be detected in this plant by either infectivity tests or electron microscopy. The striking differences in the particle morphology of these two viruses would almost certainly preclude a serological relationship.

(*d*) *Identity of O-TMV*

Electron micrographs of O-TMV preparations (Plate 2, Fig. 1) showed rigid elongate rods similar to TMV in diameter. Particle-length distribution of rods

showed that particles between 275 and 325 mm were the most frequent. The mean length of particles in this size range was calculated to be 296 mμ which is very similar to that of TMV (Brandes and Wetter 1959).

The serological relationships of O-TMV, U_1-TMV, and U_2-TMV were studied by tube-precipitation tests. Antisera were used unabsorbed and also after absorption with homologous and heterologous antigens. The results of these tests are summarized in Table 2 and indicate that all these viruses are serologically related

TABLE 2

PRECIPITATION TITRES OF UNABSORBED AND ABSORBED ANTISERA TO STRAINS OF TMV

Results given are reciprocals of precipitation titres of antisera recorded after 2 hr incubation at 37°C. The value 0 signifies the absence of precipitation when tested at an antiserum dilution of 1/2. In all tests antigen at a concentration of 5 μg/ml was used

Antiserum to	Antigen Used for Absorption*	Antigen Used for Testing:		
		O-TMV	U_1-TMV	U_2-TMV
O-TMV	—	32	0	8
	O-TMV	0	0	0
	U_1-TMV	8	0	4
	U_2-TMV	32	0	0
U_1-TMV	—	4	128	8
	O-TMV	0	8–16	0
	U_1-TMV	0	0	0
	U_2-TMV	0	2	0
U_2-TMV	—	4	16	32
	O-TMV	0	0	2
	U_1-TMV	16	0	0
	U_2-TMV	0	0	0

*Absorption of antisera was carried out as described in Section II(*c*). This involved a dilution of the original antiserum by approximately 1/9.

but can be distinguished as different serotypes of TMV. Dudman (1965) has recently reported that U_1-TMV and U_2-TMV belong to different antigenic groups. Whereas U_1 antiserum reacted with O-TMV, the reverse reaction (O antiserum with U_1-TMV) did not take place. This was true for a wide range of antiserum to antigen ratios and also when tests were carried out by the gel-diffusion method (Crowle 1961), indicating that these virus strains are only remotely related.

(*e*) *Morphology of CyMV*

The morphology of CyMV is quite distinct from that of O-TMV (Plate 2). This virus is a flexuous rod which appears thinner than O-TMV in electron micrographs of shadowed preparations (Plate 2). In order to obtain accurate width measurements of CyMV, a purified preparation was mixed with a preparation of U_1-TMV and was

stained with phosphotungstic acid for electron microscopy. Electron micrographs of these preparations showed both types of particles (Plate 3, Fig. 1) and measurements disclosed that the ratio of the width of CyMV to that of U_1-TMV is about 0·86. Thus if we assume TMV rods to be 15 mμ wide then the width of CyMV is 13 ± 1 mμ. These micrographs also show that CyMV particles have a central hollow core which is smaller in diameter than that of TMV.

Electron micrographs of CyMV preparations stained with uranyl acetate showed the flexuous rods to have a helical structure (Plate 3, Fig. 2) with a pitch of approximately 2·8 mμ. This figure was obtained from regions of virus rods where the helical arrangement was well resolved, and measurements were made relative to the width of the particles.

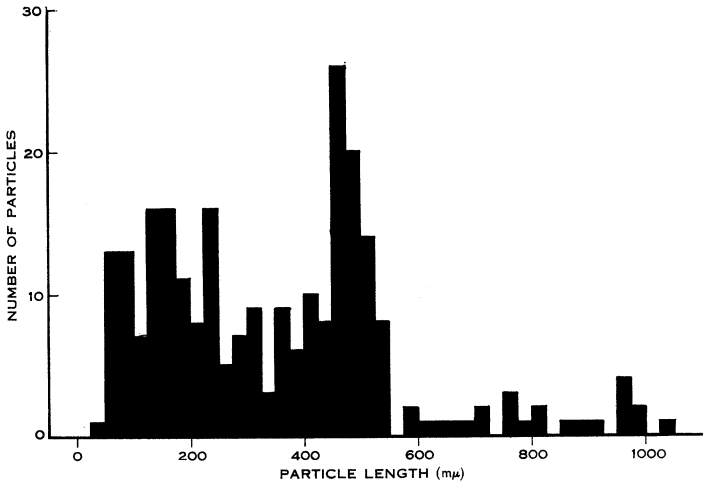

Fig. 2.—Distribution of particle lengths of CyMV rods. Virus prepared by one sedimentation in the ultracentrifuge.

The length distribution of CyMV which had been sedimented only once (Fig. 2) showed it to be polydisperse. However, there appears to be a maximum in the number of particles of length between 450–500 mμ. The arithmetical mean of the particles in this size range was found to be 477 mμ. It is concluded therefore that the "normal length" of CyMV as defined by Brandes and Wetter (1959) is approximately 475 mμ.

(*f*) *Detection of Viruses in* Cymbidium *Leaves*

In addition to detecting and distinguishing O-TMV and CyMV in *Cymbidium* leaves by sap inoculation to *D. stramonium* and *C. amaranticolor*, it is convenient to use either serological or electron-microscopic methods. Small samples of *Cymbidium* leaf, homogenized with 0·1M phosphate buffer, pH 7·2 (1 g leaf to 1 ml buffer), and clarified either by heating at 55°C for 10 min or by emulsifying with an equal volume of chloroform, were centrifuged at 1000 *g* for 10 min. Serological precipitin tube tests of extracts from O-TMV infected leaves gave dilution end-points of at least 1/8 and often in excess of 1/128, whereas those from leaves infected with CyMV

lay between 1/4 and 1/64. When clarified leaf extracts from infected plants were applied directly to grids and examined in the electron microscope, characteristic particles were clearly observed and were very numerous in extracts from leaves infected with O-TMV.

IV. Discussion

Although no detailed survey has yet been carried out as to the incidence of the two *Cymbidium* virus diseases described here, preliminary data indicate that both are widespread in Australia. White and Goodchild (1955) have previously reported the occurrence of CyMV in New South Wales and suggested that other viruses of *Cymbidium* orchids may also be present. In South Australia, it appears that O-TMV is more widely spread than CyMV.

Symptoms of many *Cymbidium* plants found to be infected with O-TMV appeared very similar to published photographs of plants infected with CyMV (Jensen 1959). The possibility that such plants were simultaneously infected with both viruses seems remote, as a thorough electron-microscopic examination of both crude leaf extracts and purified virus preparations from these plants failed to reveal the presence of any particles characteristic of CyMV. This method should detect CyMV even if it was present in concentrations of 1–2% that of O-TMV.

The data reported here indicate clearly that O-TMV is a serotype of TMV and yet that it differs quite markedly from usually encountered strains of that virus as it does not appear to fit either in the U_1 or U_2 group (Dudman 1965). In some respects, O-TMV resembles *Odontoglossum* ringspot virus. The latter virus is reported to have a length very similar to that of TMV but its diameter was thought to be 24 mμ when measured in shadowed preparations (Jensen and Gold 1951). Recently, Dr. C. I. Kado (personal communication) has shown that ORSV is serologically related to TMV, is identical in diameter to TMV, and also shows other properties in common with TMV. Therefore, it would be interesting to compare directly the properties of O-TMV and ORSV.* A strain of TMV has also been reported from *Cattleya* plants (Pérez, Adsuar, and Sala 1956) but no particular disease symptoms could be correlated with infection.

The identity of CyMV has not been fully confirmed but the assumption that it is related to *Cymbidium* mosaic described elsewhere seems justified by its characteristic normal length of 475 mμ. Both Jensen and Gold (1951) and Murakishi (1958) have reported the normal length of CyMV to be 475 mμ. Diameter measurements of the isolate do not agree with those of other workers which has usually been reported as 18 mμ (Jensen and Gold 1951; Murakishi 1958). This discrepancy can probably be explained by the fact that previous measurements have all been made on metal-shadowed preparations which lack precision. My measurements have been made on

Note added in proof.*—Since this paper was submitted a report has appeared (Paul, H. L., Wetter, C., Wittman, H. G., and Brandes, J., *Z. VererbLehre.* **97: 186–203, 1965) presenting physical, chemical, and serological evidence that ORSV is related to TMV. It is concluded that the relationship is as distant as that between the common strains of TMV and cucumber viruses 3 and 4.

phosphotungstic acid-stained preparations with U_1-TMV particles mixed in as a standard and the diameter has been determined as 13 ± 1 mμ. The fact that CyMV symptoms on *D. stramonium* were like those described by White and Goodchild (1955) and those on *C. amaranticolor* like those by Kado and Jensen (1964) would also support my identification of this virus.

In any program for eradicating viruses from *Cymbidium* orchids it would be important to be able to index plants for freedom from infection as very often symptomless plants are infected with virus. Possibly routine serological testing may be the most convenient method by virtue of its speed and the relatively simple facilities that are required.

V. Acknowledgments

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VI. References

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Explanation of Plates 1–3

Plate 1

Fig. 1.—Symptoms on leaves of a *Cymbidium* infected with O-TMV.
Fig. 2.—Symptoms on leaves of *Amphone magnifica* infected with O-TMV.
Fig. 3.—Symptoms on leaves of a *Cymbidium* infected with CyMV.
Fig. 4.—Local lesions on *Datura stramonium* induced by O-TMV, 7 days after infection.
Fig. 5.—Local lesions on *Chenopodium amaranticolor* induced by O-TMV, 7 days after infection.
Fig. 6.—Local lesions on *Datura stramonium* induced by CyMV, 15 days after infection.
Fig. 7.—Local lesions on *Chenopodium amaranticolor* induced by CyMV, 22 days after infection.

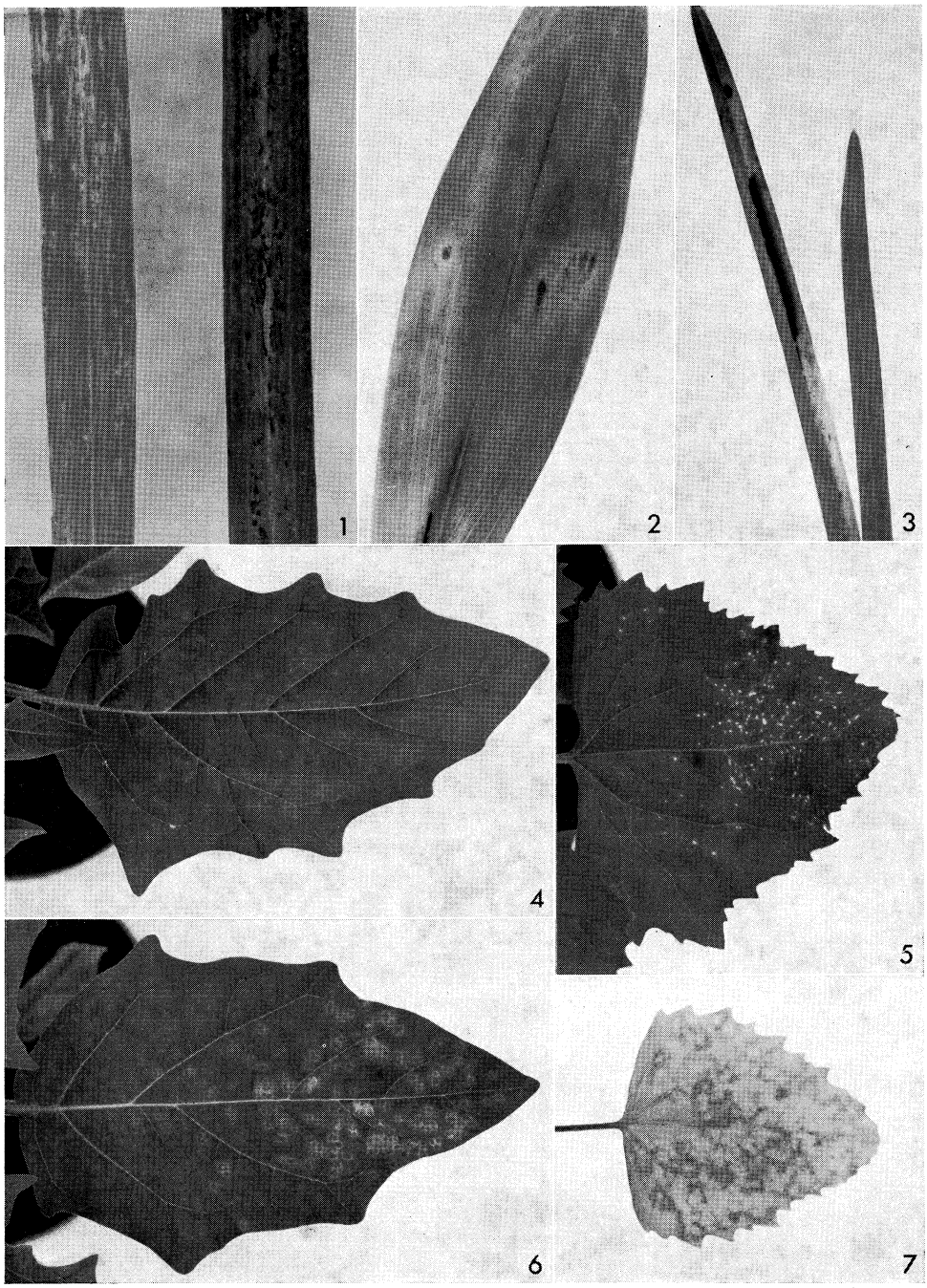
Plate 2

Fig. 1.—Electron micrograph of a purified preparation of O-TMV. Shadowing with platinum at 30°. ×40,000.
Fig. 2.—Electron micrograph of a purified preparation of CyMV. Shadowing with platinum at 30°. ×40,000.

Plate 3

Fig. 1.—Selected fields of an electron micrograph of a mixture of CyMV and U_1-TMV preparations negatively stained with phosphotungstic acid. Reversed print at magnification of 215,000.
Fig. 2.—Selected fields of an electron micrograph of a CyMV preparation stained with uranyl acetate to show the helical structure of the rods. Printed at magnification of 215,000.

ORCHID VIRUSES



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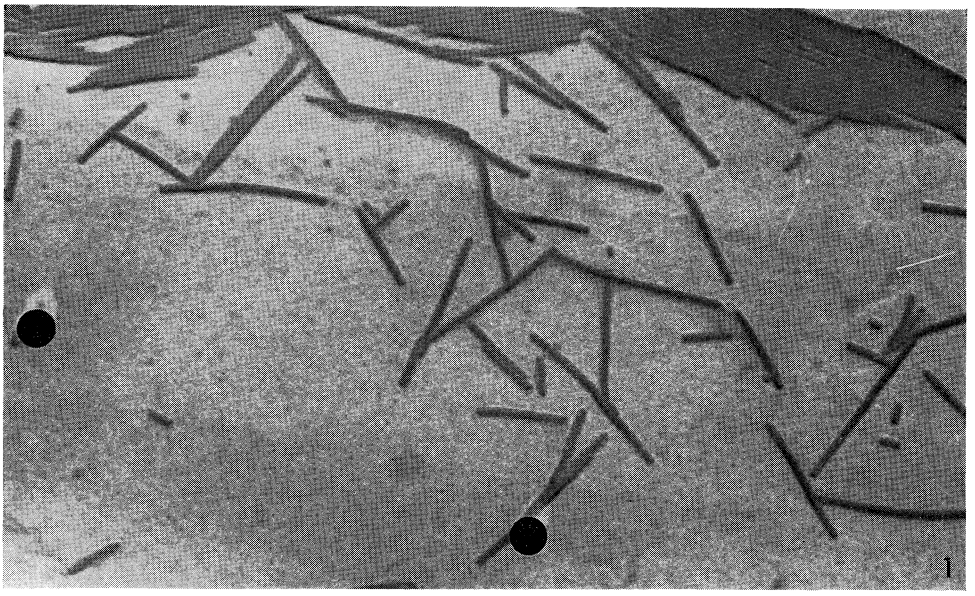

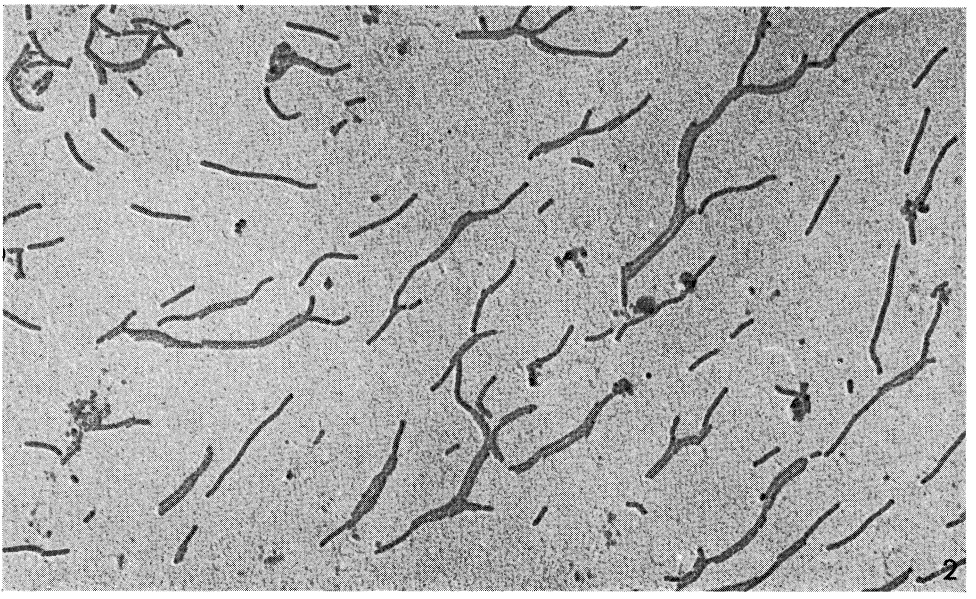

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