Characterization of a Tymo-like Virus Common in Poinsettia

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ABSTRACT

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A virus commonly present in commercially grown *Euphorbia pulcherrima* was transmitted mechanically and by grafting to *E. cyathophora* and several other euphorbiaceous species and was tentatively designated poinsettia mosaic virus (PMV). No hosts were found except *Euphorbia* spp. and these were relatively insusceptible; no techniques were found which permitted 100% infection. The thermal inactivation point of PMV was between 60 and 65 C; the dilution end point was beyond 10^{-4}. PMV withstood aging at 24 C for 8 days, but not for 10 days. It was purified by the same method as for tobacco streak virus, involving clarification with hydrated calcium phosphate, high-speed centrifugation, and acidification to precipitate host material. When centrifuged in sucrose density gradients, two zones appeared, the uppermost (50S) being empty or nearly empty particles. Only the lower zone (112S) was infectious and it consisted of well-defined particles ~25 nm in diameter which closely resembled those of turnip yellow mosaic virus. The $A_{260/280}$ ratio of unfractionated virus was 1.50, the extinction coefficient (1 mg/ml, 1 cm, 260 nm) was 8.4, and the buoyant density of the bottom component was about 1.43 g/ml. Properties and morphology resembled those of tymoviruses, but no cross-reactions were obtained with antisera to 12 tymoviruses. PMV often was detectable in crude poinsettia sap by agar gel double diffusion tests, but the ELISA technique was far more reliable. Poinsettias with no obvious symptoms often contained PMV.

From time to time distorted leaves and bracts of poinsettia (*Euphorbia pulcherrima* Wild.) have been noted. Pape (13, 14) described such symptoms in Germany in 1927 and suggested that they might be caused by a virus. Similar or somewhat milder symptoms were later observed in 1953–1954, but no evidence of transmissibility was obtained (R. W. Fulton, *unpublished*).

More recently, specimens of poinsettia with distorted leaves and bracts have appeared. Some bracts had failed to color normally and remained small and straplike or fan-shaped. On close examination, numerous leaves were found with mild mottling, which suggested virus infection. Electron microscopic examination of negatively stained leaf-dip preparations revealed viruslike particles (8).

Virus was transmitted from suspect plants by inserting slivers of poinsettia stems or petioles into slits in the upper part of stems of young euphorbiaceous species. After about 2 wk, young leaves of *Euphorbia cyathophora* J. Murr. developed prominent vein-clearing, which later became mosaic patterns. The virus also was transmitted by mechanical inoculation from poinsettia to *E. cyathophora* (8). A similar occurrence of virus-like particles in poinsettia was described by Garner et al (9).

This paper describes the transmission, host range, and some properties of this virus, designated as poinsettia mosaic virus (PMV), which indicate that it is a previously undescribed tymovirus.

MATERIALS AND METHODS

Euphorbia cyathophora was grown from seed purchased from commercial sources. According to Bailey (1), *E. cyathophora* is universally mislabeled as *E. heterophylla* in the ornamental seed trade; hence, we use the former name.

Inoculations were made by gently wiping corundum-dusted leaves with inoculum, usually prepared in 0.03 M, pH 8.0, phosphate buffer. Young leaves of *E. cyathophora* with prominent mosaic provided inoculum for host range and infectivity trials. Some euphorbiaceous species were graft-inoculated by implanting a sliver of infected stem tissue into a slit in the stem of the healthy plant and binding the wound with self-adhering latex tape.

Symptoms appeared in mechanically inoculated *E. cyathophora* in 8–14 days at 24 C. Inoculated plants were kept at least 3 wk before being recorded as not infected. In trials in which percentage of infection was recorded, total numbers of plants inoculated in several replications of a single treatment ranged from 45 to 95. *E. cyathophora* without symptoms were considered uninfected. After antiserum was prepared, such plants often were used as healthy plant controls and they never reacted with antiserum to the virus.

Experimental plants were grown in a greenhouse kept night and day at about 24 C. Seedlings were grown in a mix of composted soil, sand, and peat moss.

Virus was purified by the same method as used for tobacco streak virus (7); involving homogenization of infected tissue at the rate of 1 g of tissue to 1.5 ml of 0.03 M. phosphate buffer, pH 8.0, containing 0.02 M 2-mercaptoethanol. After centrifugation at 3,000 rpm for 15–20 min, hydrated calcium phosphate equal to 0.9 of the weight of tissue was mixed with the supernatant liquid. The mixture was centrifuged at 5,000 rpm for 20 min. The clear yellowish supernatant liquid was centrifuged 4 hr in a Spinco No. 30 rotor at 30,000 rpm. Pellets were resuspended in 0.03 M EDTA, pH 6.2 and the resuspension mixture was centrifuged at 10,000 rpm for 15 min in a Spinco No. 40 rotor. The supernatant liquid was adjusted to pH 5.0 with citric acid and the precipitate was removed by centrifugation at 10,000 rpm for 15 min. The virus-containing supernatant liquid was adjusted to pH 6.2 with NaOH, diluted with 0.03 M EDTA to fill one or two centrifuge tubes and centrifuged 2 hr at 40,000 rpm. Final pellets were resuspended in 0.03 M EDTA, pH 6.2.

Before being used to produce antiserum, purified virus was centrifuged 3.5 hr in 7–25% gradients of sucrose in 0.03 M EDTA, pH 6.2. Upper and lower zones were removed with an ISCO density gradient fractionator. Separated zones were diluted with EDTA, centrifuged 2 hr at 40,000 rpm and resuspended in EDTA.

Rabbits were injected twice a week intramuscularly with 1.0–1.25 mg of bottom-zone virus emulsified in Freund's incomplete

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adjuvant. Blood was collected initially after 14 injections. Several additional bleedings were done at weekly intervals, with continued injections. Final antiserum titer was 1:2,560, as determined by microprecipitin test.

For electron microscopy, diluted virus preparations containing 1% potassium phosphotungstate, pH 7.2, were placed on carbon-coated Formvar films and observed with a JEM 7 electron microscope.

RESULTS

Virus transmission. Poinsettias from which virus was transmitted by grafting tended to become free of symptoms when grown at 24 C (8). Infected *E. cyathophora,* however, showed obvious mosaic at that temperature (Fig. 1). Mechanical transmission of virus to *E. cyathophora* from poinsettia could often be obtained, but the percentage of plants showing symptoms usually was low. Transmission from *E. cyathophora* to *E. cyathophora* regularly was obtained, but usually was less than 100%.

Electron microscope observations of dip preparations had indicated a high concentration of virus in infected tissue. The low rate of mechanical transmission suggested that the virus might rapidly become noninfectious in extracts, that an inhibitor of infection was involved, or that *E. cyathophora* was relatively unsusceptible.

To attempt to circumvent virus inactivators, various additives were included in extracts of infected *E. cyathophora* and inoculations were made with each extract to eight or more healthy seedlings of *E. cyathophora.* Additives included bentonite, polyvinyl pyrrolidone-10, poly-l-ornithine, 2-mercaptoethanol, polyethylene glycol-(PEG 6000), Celite, sodium oxalate, sodium cyanide, sodium azide, sodium diethyldithiocarbamate, nicotine, caffeine, sucrose, 0.35 M and 0.7 M mannitol, ethylene diamine tetraacetate, and several detergents. Buffers included phosphate, borate, and tris, as well as distilled water. There were variations in the number of plants infected, but no additive resulted in 100% infection. When at least six of eight plants became infected, the additive involved was retested. No additive consistently promoted a high rate of infection.

Preinoculation treatments known to increase the susceptibility of plants to mechanical inoculation also were tried. Darkening for 24 or 48 hr (2) or immersion in water at 45 C for 1 min (17) did not increase the percentage of infection.

A comparison was made of the susceptibility of young *E. cyathophora* plants having a pair of cotyledons and two small leaves with older plants having six to eight leaves. The same inoculum gave approximately the same percentage of infection of the sets of plants of different ages and different leaf areas. Inoculum applied with glass spatulas was no more effective than that applied with cheesecloth pads.

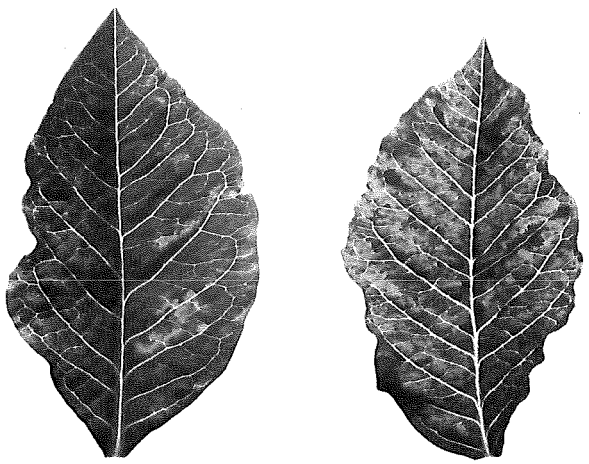

Fig. 1. Mosaic symptoms in leaves of *Euphorbia cyathophora* infected with poinsettia mosaic virus.

Inoculations made by pricking leaves with the points of 25 fascicled straight pins were effective, but somewhat less so than was leaf rubbing. In one experiment, a single set of pricks on each of two leaves resulted in mosaic symptoms in 25% of the plants, while four to 10 sets of pricks on four or more leaves per plant resulted in 12% of the plants showing symptoms.

After PMV had been purified, sets of *E. cyathophora* were inoculated with phosphate buffer containing purified virus at concentrations five to 10 times the concentration in leaf extracts. Only about half of the inoculated plants became infected.

Manipulation of inoculum composition had no significant effect on the number of plants that became infected. This suggested that the seedling population of *E. cyathophora* might be heterogenous, with some plants susceptible and some immune. This was tested in two ways. First, plants which remained healthy for 3–4 wk after an initial inoculation were reinoculated. In several trials, 50–70% of these plants became infected by the second inoculation. Thus, the group not infected by the first inoculation showed no greater resistance to infection than did the original population of seedlings.

In addition, seed was set on infected *E. cyathophora.* None of the seedlings from these plants developed symptoms before inoculation, showing that the virus was not seed-transmitted in this host. When these seedlings were inoculated, about 45% became infected. This percentage was no greater than that obtained by inoculating commercially produced seed, and gave no evidence for genetic control of susceptibility.

Most of the experiments on mechanical transmission were done in the fall and winter. In late winter and early spring the rate of transmission improved so that often 70–90% of inoculated *E. cyathophora* developed symptoms. This suggested either that plants were more susceptible or that a selection had been made in the virus for a strain that was more readily transmitted mechanically than was the original isolate from poinsettia. To test this, a series of transfers were made of the virus to *E. cyathophora* at intervals as short as possible. When the first symptom appeared on one of a group of inoculated plants, sometimes within 8 days, this leaf was used as inoculum for another group of plants. After eight successive transfers, this line of virus was compared with an isolate which had been transferred directly from poinsettia to the *E. cyathophora* supplying inoculum. The results (Table 1) showed that the line of virus transferred many times in *E. cyathophora* was no more readily transmitted than was virus recently transferred from poinsettia.

Mechanical inoculation trials with seedlings of *E. prunifolia* and *E. heterophylla* showed that no more of them developed symptoms than did those of *E. cyathophora.* PMV also was transmitted when latex exuded from cut petioles and stems was diluted with an equal amount of phosphate buffer and rubbed on *E. cyathophora.*

Because euphorbiaceous species are susceptible to certain whitefly-transmitted viruses (4), and because *Trialeurodes vaporariorum* (Westwood) is a common greenhouse pest, it was tested as a possible vector. In several trials, each involving 20–50 insects, no transmission was obtained. Similarly, no transmission was obtained with two-spotted mites (*Tetranychus telarius* L.).

Host range. Young, rapidly growing plants to be tested as hosts were inoculated with phosphate buffer extracts of young leaves of *E. cyathophora* showing distinct mosaic. Because the rate of transmission to *E. cyathophora* was only about 50%, it was thought that back-transmission to detect virus in plants which failed to develop symptoms would not be reliable; therefore, this was not done. Some species were tested serologically.

Seedlings of 105 species in 20 families were inoculated. These included most species commonly used in virus differentiation. Only the following species of the genus *Euphorbia* became infected: *E. cyathophora* J. Murr.; *E. epithymoides* L.; *E. heterophylla* L.; *E. marginata* Pursh.; *E. prunifolia* Jacq.; and *E. supina* Raf. *E. characias* L.; and *E. wulfeni* Hoppe became infected by graft inoculation and several plants of *E. fulgens* Karw. were infected when received.

In each susceptible species the sequence of symptoms was

similar, beginning as a veinclearing and becoming a mosaic. None of them developed primary symptoms on inoculated leaves. "Starch lesions" (11) could not be detected in inoculated leaves of *E. cyathophora*.

A proof of pathogenicity of the virus by return inoculation to poinsettia (*E. pulcherrima*) was complicated by a high rate of occurrence of the virus in this species and by the uncertainty of its detection by transmission to *E. cyathophora*. Seedlings of *E. pulcherrima* were apparently virus-free and for this reason they were inoculated. In numerous trials, no virus could be transmitted from uninoculated seedlings to *E. cyathophora* either by grafting or by mechanical inoculation. PMV could be transmitted to poinsettia seedlings, however, by grafting with pieces of infected *E. cyathophora* stems, or in relatively low percentages, by mechanical inoculation. The infected plants developed a slight mottle on young leaves, which became a mild mosaic (Fig. 2).

Virus properties. In property studies, sets of plants, usually eight, were inoculated with sap of infected *E. cyathophora* that had been treated in various ways. The thermal inactivation point of the virus (10 min) lay between 60 and 65 C. In aging trials at 24 C, *E. cyathophora* sap was slightly infectious after 8 days, but not after 10 days or more.

The infectivity-dilution curve of the virus (Fig. 3) was unusually flat, the number of plants infected decreased less than expected with dilution, if it is assumed that a chance infection by a single particle is sufficient to result in a plant with mosaic. Apparently, *E. cyathophora* plants are relatively unsusceptible, although the dilution end point of the virus was beyond 10^{-4}.

PMV remained active in infected *E. cyathophora* tissue kept at −14 C for at least several weeks. The virus also retained infectivity when tissue was finely diced and dried cold over $CaCl_2$.

Purification of PMV. Property trials showed that PMV withstood freezing, so infected tissue of *E. cyathophora* was frozen as a first step in purification. PMV was not removed from leaf extracts by hydrated calcium phosphate (6), which did remove green material and cellular debris upon low-speed centrifugation of the mixture with leaf sap. Therefore, the purification method used for tobacco streak virus (7) was used. Virus pellets after high-speed centrifugation were resuspended in 0.03 M EDTA, pH 6.2.

Yields of virus from *E. cyathophora* systemically infected 3 to 6 wk were around 0.33 mg per gram fresh weight of infected tissue. PMV was also purified from infected poinsettia (*E. pulcherrima*), but yields were much lower and preparations were of a brownish gray color.

The extinction coefficient for purified virus was 8.4 (1 mg/ml, 260 nm, 1 cm), based on dried samples of about 10 mg. Upon analytical centrifugation, two zones were apparent, with S values of 50S and 112S. The $A_{260/280}$ ratio of unfractionated virus was about 1.50.

Electron microscopy. Purified PMV was layered on gradients of 7–25% sucrose made up in 0.03 M EDTA, pH 6.2, and centrifuged 3.5 hr at 24,000 rpm in a Spinco SW 25.1 rotor. The zones were collected with an ISCO density gradient fractionator. Sucrose was removed and top and bottom particles were separately concentrated by high-speed centrifugation. Particle resuspensions in 0.03 M EDTA were diluted in water and negatively stained with potassium phosphotungstate.

Top particles appeared to be empty proteinaceous shells (Fig. 4), but seemed to be surrounded by poorly defined debris. That this debris might be nucleic acid attached to the exterior of the empty shells was suggested by an $A_{260/280}$ ratio slightly greater than one. The top particles measured about 26 nm in diameter, slightly larger than the bottom paritcles. Top particles were not infectious, but reacted strongly with antiserum prepared against bottom particles.

Bottom particles had an average diameter of about 25 nm and were constructed of rather clearly-defined morphological subunits (Fig. 4). Their close resemblance to particles of turnip yellow mosaic virus was apparent (Fig. 4).

Serology of PMV. Antiserum was prepared by injecting rabbits intramuscularly twice a week with 0.7–1.0 mg of virus emulsified in Freund's incomplete adjuvant. After nine injections, the serum had a microprecipitin titer of 1:640; after 16 injections, the titer was 1:2,560.

PMV was readily detectable in saline agar gel double diffusion tests with antiserum at 1:20 or 1:40 and undiluted sap of infected *E. cyathophora*. It often was detected in poinsettia sap by gel diffusion, but repeated testing showed that false negatives were not uncommon. Latex agglutination was an unsatisfactory technique because unsensitized latex often agglutinated when mixed with poinsettia sap.

To increase the sensitivity of serological detection in poinsettia, the ELISA technique (3) was applied, with the alkaline phosphatase:*p*-nitrophenyl phosphate system. The virus was readily detectable in infected poinsettia sap, even when this was diluted 1:1,000. In one comparative trial of 20 samples (including some that had consistently tested negative by infectivity and agar gel double diffusion tests) 15 gave positive ELISA reactions. Concurrently, the same saps were tested by agar gel double diffusion and four positive reactions were obtained. Virus was detected in most of about 150 poinsettias obtained from commercial greenhouses, although many were not obviously diseased. Mechanical transmission of virus from randomly selected plants confirmed the serological tests. Seedlings of *E. pulcherrima* consistently tested negative by ELISA.

Relationships of PMV. The presence of an empty top component in preparations of purified virus, a restricted host range, the high concentration of virus in infected tissue, and a morphology very similar to that of turnip yellow mosaic virus suggested that PMV should be grouped with the tymoviruses (10).

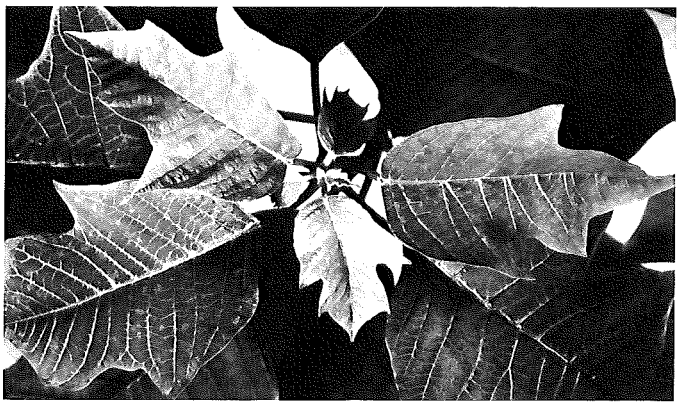

Fig. 2. Mottling in *Euphorbia pulcherrima* resulting from mechanical inoculation with poinsettia mosaic virus.

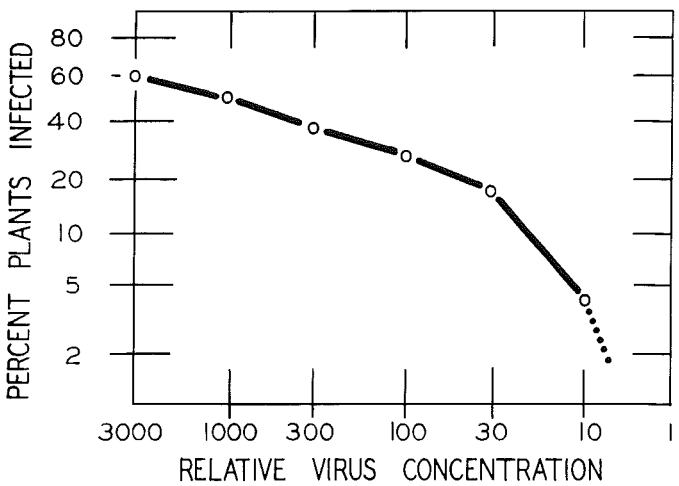

Fig. 3. Dilution infectivity curve of poinsettia mosaic virus inoculated to *Euphorbia cyathophora*. The highest virus concentration (3,000) represents crude sap diluted 1:30.

Comparisons were made of the sedimentation rates of bottom particles of PMV and bottom particles of turnip yellow mosaic virus and of desmodium yellow mottle virus (15). In sucrose density gradients, particles of each virus sedimented to the same depth in tubes run at the same time. When bottom particles of PMV and each of the other viruses were mixed before layering on gradients, only one zone was formed during 3.5 hr of centrifuging.

In attempts to determine the buoyant density of bottom particles of PMV, the virus evidently was disrupted by CsCl because no sharp band was evident. When preparations of bottom particles of PMV were treated with 9% formaldehyde and then centrifuged in CsCl, a single band was formed just slightly below the band formed by TYMV bottom particles. In comparison with the buoyant density of TYMV (1.42 g/ml), the buoyant density of PMV was estimated as about 1.43 g/ml. Exposure to formaldehyde did not alter the buoyant density of TYMV.

Koenig and Givord (12) described a continuous range of serological relationships among 11 tymoviruses. PMV was tested in agar double diffusion plates against antisera (at 1:10) to Kennedya yellow mosaic, okra mosaic, Dulcamara mottle, eggplant mosaic, Andean potato latent, Belladonna mottle, Physalis mottle, turnip yellow mosaic, Clitoria yellow vein, Scrophularia mottle, Plantago mottle, and gumbo mosaic viruses. No cross reactions were observed. Tests with antiserum to turnip yellow mosaic and desmodium yellow mottle viruses at 1:4 also were negative.

TABLE 1. Percentages of *Euphorbia cyathophora* becoming infected when inoculated with a line of virus mechanically transmitted many times (old isolate) or with a virus newly transferred from poinsettia to *E. cyathophora*.

Isolate	Dilution in phosphate buffer[a]					
	1×10^{-2}	3×10^{-2}	1×10^{-3}	3×10^{-3}	1×10^{-4}	3×10^{-4}
Old	62	47	19	19	8	0
New	43	54	39	25	0	0

[a]0.3 M, pH 8.0.

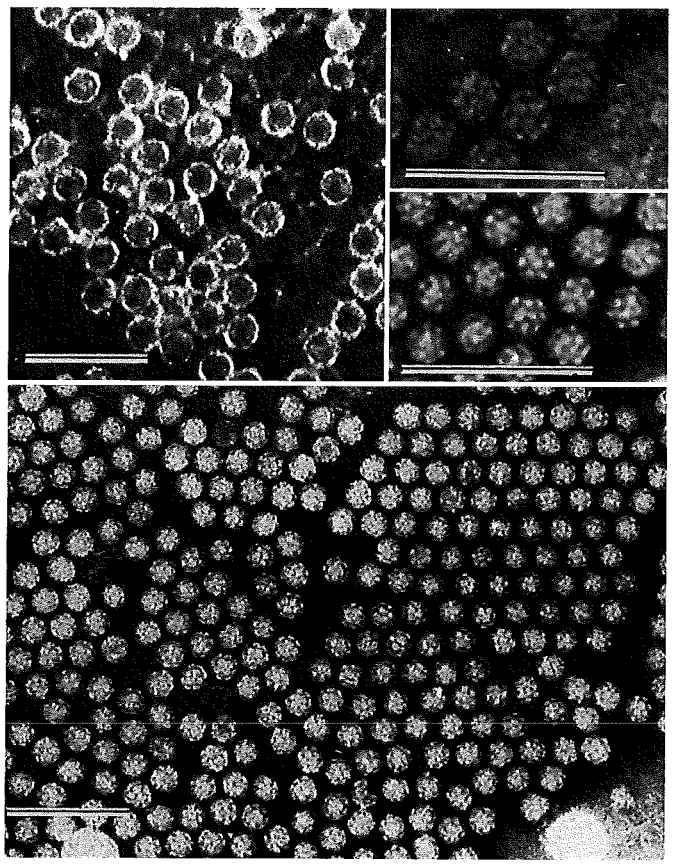

Fig. 4. Negatively stained electron micrographs of poinsettia mosaic virus. Upper left, top particles; below, bottom particles. Upper right micrograph is turnip yellow mosaic virus; beneath it, poinsettia mosaic virus. Bar represents 100 nm.

DISCUSSION

The association of various leaf and bract distortions with other diseases of poinsettia has been noted (5,16), without definite evidence that they were the result of a specific pathogen. PMV has not been shown to cause the leaf and bract malformations which have prompted growers' inquiries. This will require mechanical inoculation of plants known to be virus-free and the development of symptoms on these plants and their absence on uninoculated controls.

Cuttings from poinsettia carrying PMV do develop leaf distortions, while known PMV-free plants do not. This evidence, however, does not eliminate the possibility of other agents being associated with the infected cuttings.

The properties and morphology of PMV fit those of the tymovirus group reasonably well. The known serological cross reactions among members of this group suggest that similar cross reactions might be found with PMV. The fact that they were not may be related to the use of most antisera diluted 1:10, which was necessitated by the small amounts available.

The low percentage of infection following mechanical inoculation of what appeared to be a suitable host is not characteristic of most tymoviruses. No reason is apparent why poor infection was obtained, but it may be incorrect to assume that all infected plants inevitably developed systemic symptoms.

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