Clover Yellow Mosaic Virus from Apple Trees with Leaf Pucker Disease

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ABSTRACT

A virus sap-transmitted from apple induced symptoms on herbaceous plants that differ from those induced by other viruses reported to infect apple. The virus has been isolated from cambial and/or fruit tissues, but not from petal or leaf tissues of apple trees displaying leaf pucker disease symptoms. In clarified *Chenopodium quinoa* sap, the virus has a dilution end point of 10^8 and thermal inactivation point (10 min) at 52 C, and retains infectivity at 18-20 C for 9 weeks. Particle dimensions by electron microscopy are 590 ± 10 nm × 13.5 nm. Purified virus preparations have a sedimentation coefficient of 122 S, an absorption peak at 263-265 nm, and minimum absorption at 245 nm. Antisera with titers to 1:3,200 were obtained. No serological relationship could be demonstrated with other rod-shaped viruses from tree fruits or with 14 rod-shaped viruses from other hosts. However, the apple virus proved to be serologically indistinguishable from clover yellow mosaic virus (CYMV). It has a similar host range and induces symptoms on clover that resemble those of CYMV.

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In British Columbia, the apple leaf pucker disease (10, 11, 12) has been studied extensively in its woody hosts. On cultivars McIntosh and Spartan, both foliar and fruit symptoms occur; on Spartan, symptoms include bark cankering at bases of buds, internal bark necrosis, shoot tip killing, and mild xylem pitting. Budding and grafting consistently effect a high level of transmission and symptom response within 1 year of inoculation.

Persistent efforts have been made to mechanically transmit viruses from trees displaying leaf pucker symptoms. This paper describes a virus that has been transmitted in sap to herbaceous plants from apple trees displaying leaf pucker disease symptoms, and that by electron microscopy, serological testing, and other criteria is distinct from any virus hitherto reported to infect apple.

MATERIALS AND METHODS.—*Sap transmission from apple.*—We effected all transmissions by grinding apple tissues in 2% nicotine solution and applying the resulting preparation to four young leaves of a receptor plant that had received a light application of 600-mesh Carborundum dust. Inoculated leaves were not washed.

Chenopodium quinoa Willd. and *C. amaranticolor* Coste & Reyn. were used initially as receptor hosts. *C. quinoa* was the more satisfactory receptor, and was used exclusively in later work.

Apple tissues that were sampled included petals (freshly collected or following storage at −25 C), freshly collected tip leaves, scrapings from immediately beneath the skin of fruits displaying leaf pucker symptoms, and cambial scrapings from current-season or 1-year terminal growth.

Sources of these apple tissues were trees of *Malus sylvestris* Mill. 'McIntosh' in four commercial orchards and/or young trees that had been propagated from these orchard trees by budding McIntosh to seedling rootstocks. All sampled trees displayed symptoms of the leaf pucker disease. Indexing on woody hosts had demonstrated that all the source trees were infected with chlorotic leaf spot virus (CLSV). Inoculations from most of them had also induced stem-pitting symptoms on Virginia crab and decline of Spy 227. No inoculations from any of the trees induced brown line, decline, or stem-grooving symptoms on Virginia crab; or rubbery wood, mosaic, or chat fruit symptoms on Lord Lambourne.

Symptom studies in herbaceous hosts.—Inoculum usually was derived from *C. quinoa*, either from inoculated leaves bearing local lesions or from upper leaves of systemically infected plants. When there was any evidence that transmission in apple juice had given simultaneous infection of *C. quinoa* by CLSV and the virus under study, several successive local lesion transfers from *C. quinoa* to *C. quinoa* were used for their separation. For each transfer, a lesion characteristic of those induced by the "leaf pucker" virus was cut as a 1-mm disc from an inoculated leaf and used individually for inoculation of healthy plants. Dilution series were also used for such separations.

For all transmission from *C. quinoa* to *C. quinoa* and to other herbaceous plant species, the leaf tissue was ground with 1% nicotine solution and the resulting preparation rubbed on upper surfaces of young leaves that had been dusted with Carborundum. All tests were performed in greenhouses at 21-25 C. During winter months, the natural light was supplemented with fluorescent lights over each greenhouse bench.

Gomphrena globosa L., although unsatisfactory as a receptor when apple tissues were the sources of inoculum, served as a dependable diagnostic host when infected *C. quinoa* was the source.

Infectivity assays involved inoculating *C. quinoa* plants or half-leaves of *Gomphrena globosa* L. and counting the local lesions before systemic invasion occurred. Each dilution of the virus preparation was applied to 10 half-leaves of *G. globosa*, randomized on the test plants.

Purification.—Numerous procedures that involved combinations of various techniques for extraction, clarification, and purification were tested, and most of these yielded infective preparations. Two widely differing procedures provided the highest yields of relatively pure virus.

Citrate-chloroform procedure.—*C. quinoa* leaves were harvested 5-7 days after inoculation, when they were covered with local lesions. Fresh leaves (100 g) were chilled before being homogenized in a Waring Blendor for 2 min in a mixture of 100 ml of cold 0.5 M citrate buffer containing 0.5% thioglycolic acid, pH 6.7, and 100 ml of chloroform. The resulting emulsion was centrifuged for 20 min at 9,000 rpm, and the aqueous phase separated and centrifuged for 90 min at 28,000 rpm in a Spinco No. 30 rotor. The pellets from the high-speed centrifugation were taken up in 0.5 M phosphate buffer, pH 7.2, and the suspension was dialyzed overnight against 0.05 M phosphate buffer. Up to 3 ml of partially purified virus was layered on a 10-40% sucrose gradient, prepared in 0.05 M phosphate buffer, and centrifuged for 2 hr at 23,000 rpm in a Spinco No. SW 25 rotor. The virus was located in the gradient as a distinct light-scattering zone. The material in the zone was removed with a hypodermic syringe and dialyzed overnight against 0.01 M Tris [tris (hydroxymethyl) amino methane] -HCl, pH 7.2, buffer. The virus solution was further concentrated by high-speed centrifugation for 90 min at 38,000 rpm in a Spinco No. 40 rotor. The virus pellets readily dissolved in 0.01 Tris buffer, and were used for serology and spectrophotometric analysis.

Liquid nitrogen procedure.—All foliage of systemically infested *C. quinoa* plants was harvested 12-14 days after inoculation of the plants and ground in liquid nitrogen, using a hand-powered grinding mill. The mixture was strained through cheesecloth and combined with an equal volume of 0.004 M di-sodium phosphate, pH 8.5. After 10-min centrifugation at 10,000 rpm, the supernatant was treated with hydrated calcium phosphate (HCP) (3), then centrifuged again for 10 min at 10,000 rpm. The virus was concentrated by centrifugation for 90 min at 28,000 rpm in a Spinco No. 50 rotor. The pellet was dissolved in 0.01 M di-sodium phosphate, pH 8.5.

Procedures that involved grinding without liquid nitrogen, substitution of standard phosphate buffer for the di-sodium phosphate, and buffering with ascorbic acid and di-sodium phosphate all gave infective preparations of lower titer than those prepared by the citrate-chloroform or liquid nitrogen procedures.

Serology.—Antisera were made from preparations obtained by both the citrate-chloroform and liquid nitrogen procedures. These preparations were emulsified with Freunds' adjuvant and introduced into rabbits by intramuscular injection. Rabbits were injected 3 to 6 times with 12 mg of material/injection, and at intervals of 1 to 4 weeks. One rabbit was bled 1 week after each injection; the others were bled only after the final injection. The dilution end points of the antisera were determined by standard tube precipitin tests.

Tube precipitin tests were used to detect the virus in crude preparations from infected *C. quinoa*. Infected leaf tissue (0.2 g) was ground in 2 ml of Tris buffered saline in a glass tissue grinder, the mixture was centrifuged at 10,000 *g* for 10 min, and the clear supernatant was used to make a twofold dilution series so that the first tube had a weight/volume dilution of 1:10.

RESULTS.—*Sap transmission from apple.*—Sap inoculation from apple yielded tiny etched local lesions on *C. quinoa* that differed only slightly from those induced by CLSV and occurred either alone or in association with symptoms typical of CLSV infection. Their distribution was characteristically much more sparse than that of CLSV lesions. Subsequent transmissions in sap from *C. quinoa* to *C. quinoa* and other hosts resulted in development of symptoms that could be distinguished readily from those induced by CLSV and by other apple viruses. On *C. quinoa*, the local lesions were chlorotic, gradually developed necrotic centers, and lacked the halos that characterized CLSV lesions (Fig. 1-A). The systemic symptoms differed from those of CLSV especially in severity, the chlorotic areas coalescing to give the impression of a general chlorosis. Confirmations were most reliably made on *C. globosa* which developed characteristic discrete local lesions within 36-48 hr of inoculation (Fig. 1-E). Our isolates of CLSV and apple stem-grooving virus (ASGV) do not induce symptoms on this host.

The symptoms have been induced by 16 inoculations from ground cambial tissues and by three from fruit tissues. The successful cambial tissue isolations were made in October 1969, June 1970, March 1971, and August 1971. The skin tissues that yielded the virus were from mature fruits in storage; attempts to transmit the virus from immature fruits were unsuccessful. In 1970 and 1971, petals and young leaves from the trees with infected cambial and/or fruit tissues did not yield the virus.

The sources of the virus were 10 leaf pucker-affected trees which had been propagated from four diseased trees in commercial orchards. Forty-four other trees displaying leaf pucker symptoms have not yielded the virus so far. There have been no isolations of the virus from over 200 apple trees free from leaf pucker. Inoculations from many of these trees induced symptoms on *C. quinoa* that were typical of CLSV and/or ASGV infection, whether sampled tissues were from cambium, fruit, petal, or leaf.

Herbaceous hosts.—The virus induced symptoms

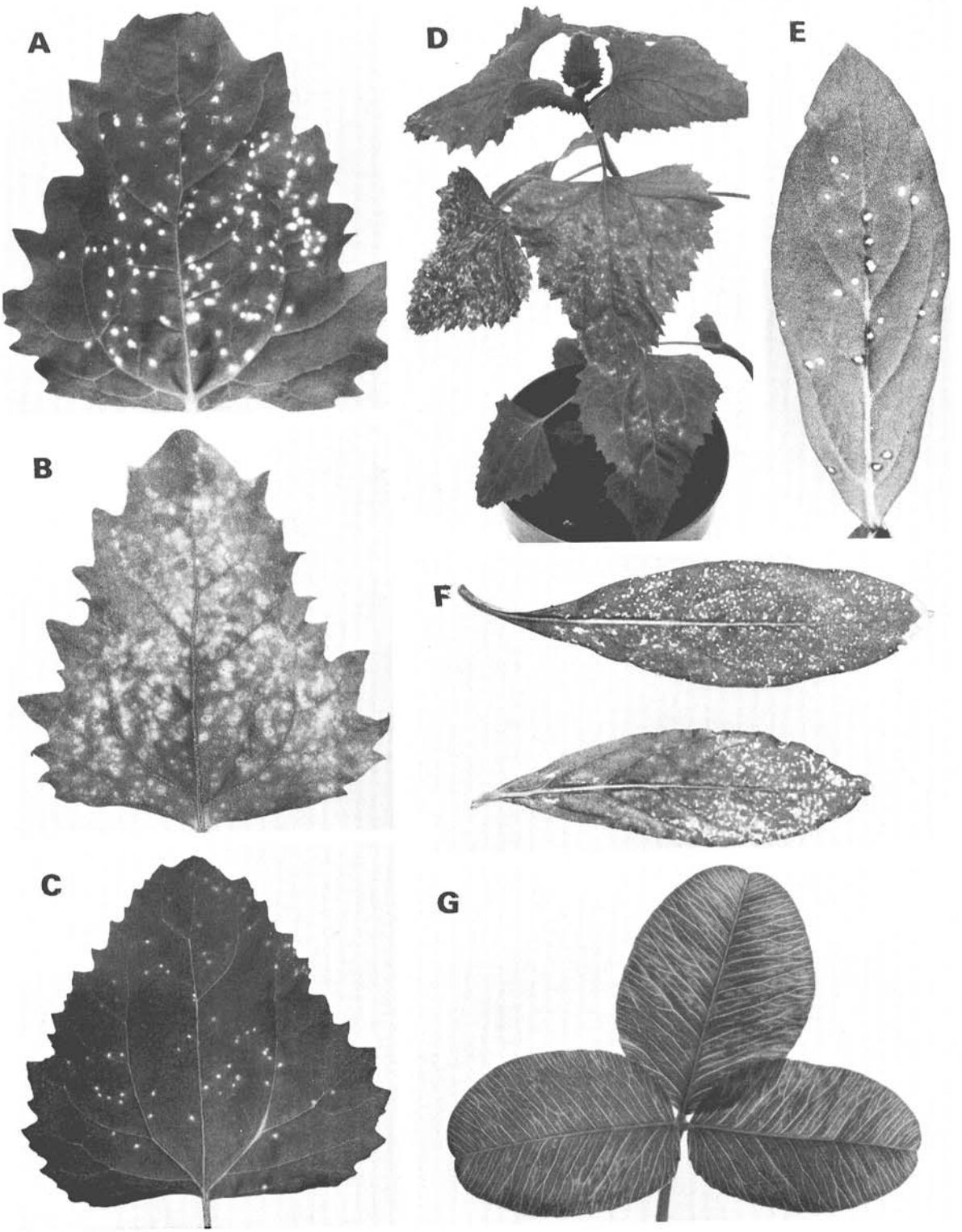

Fig. 1. Symptoms induced by apple isolate of clover yellow mosaic virus on **A)** *Chenopodium quinoa*, inoculated leaf; **B)** *C. quinoa*, systemically infected leaf; **C)** *C. amaranticolor*, inoculated leaf; **D)** *C. amaranticolor*, systemically infected plant; **E)** *Gomphrena globosa*, inoculated leaf; **F)** *G. globosa*, systemically infected leaves; **G)** alsike clover (*Trifolium hybridum*), systemically infected leaf.

on the following plants species: *C. quinoa* – large chlorotic lesions on inoculated leaves in 5-6 days (Fig. 1-A) and similar chlorotic lesions, without pattern, on systemically infected tip leaves in 12-14 days (Fig. 1-B); *C. amaranticolor* – pinpoint necrotic lesions on inoculated leaves in 5-6 days (Fig. 1-C) and chlorotic lesions without pattern on tip leaves in 12-14 days (Fig. 1-D); *C. album* L. – local and systemic chlorotic lesions at similar intervals; *Gomphrena globosa* – white, etched lesions on inoculated leaves in 36-48 hr (Fig. 1-E) and necrotic lesions with red halos on tip leaves in 14-20 days (Fig. 1-F); *Crotalaria spectabilis* Roth. – chlorotic lesions on inoculated leaves in 7 days and dark green spots on tip leaves in 14-16 days; *Beta vulgaris* L. (Swiss chard) – necrotic lesions on inoculated leaves in 6-7 days and severe stunt in 12-14 days; *Ocimum basilicum* L. – small black lesions on inoculated leaves in 6-7 days and similar symptoms on tip leaves in 14 days; *Cucumis sativus* L. 'Chicago Pickling' – pinpoint necrotic lesions on cotyledons and leaves.

Plants that developed infections without displaying symptoms included *Vigna sinensis* (L.) 'Savi' (black-eyed cowpea); *Phaseolus vulgaris* L. 'Kinghorn'; *Tropaeolum majus* L.; *Petunia hybrida* Hort. ex Vilm.; and *Cucumis maxima* Duchesne 'Buttercup'.

Plants that did not become infected included *Zea mays* L. 'Golden Bantam'; *Nicotiana glutinosa* L.; *N. clevelandii* Gray; *N. rustica* L.; and *N. tabacum* L. 'White Burley', 'F2Cl', 'X73', and 'Havana 425'.

Stability in clarified C. quinoa sap.—The thermal inactivation point in several tests was 52 C. The dilution end point in several tests reached 10^{-8}. Longevity in vitro at 18-20 C was 9 weeks, and at 4 C was 14 weeks.

Particle morphology.—Negatively stained preparations from cut leaves of infected *C. quinoa* revealed semiflexuous rods, most of which were of uniform length (Fig. 3-A). There were very few shorter particles, which may have been produced by fracturing, and there was a comparably small percentage of longer rods, which could be conceived as products of terminal aggregation. Measurements of 277 particles selected at random on electron micrographs indicated a modal length of 590 ± 10 nm (Fig. 2).

Preparations made by the citrate-chloroform procedure followed by sucrose density-gradient centrifugation were free from contaminants when examined in the electron microscope; however, considerable fracturing of particles was evident (Fig. 3-B).

Preparations made by the liquid nitrogen-HCP procedure exhibited concentrated mats of particles terminably aggregated to such a degree that recognition of single entities was difficult (Fig. 3-C). Dilutions of such preparations were compared with particles of tobacco mosaic virus (TMV) -type strain on the same grid. The mean particle width of the "leaf pucker virus" was almost precisely three-quarters that for TMV (Fig. 3-D), which suggested a mean particle diameter of 13.5 nm, and a diameter distribution from 13 to 15 nm.

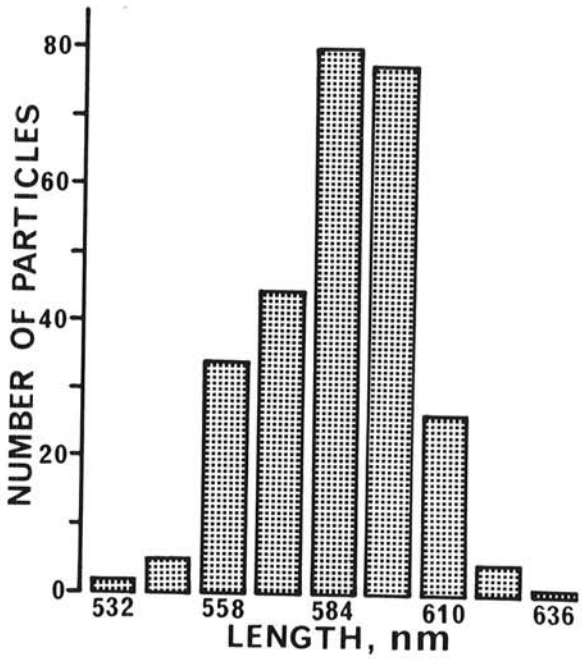

Fig. 2. Histogram showing the length distribution of 277 particles of the apple isolate of clover yellow mosaic virus. Measurements are for leaf dip preparations from infected *Chenopodium quinoa*, negatively stained with uranyl acetate.

One such preparation, diluted with distilled water (0.3 ml:1 ml) for electron microscope examination, remained in the laboratory at approximately 21 C for 3 days before application to the grids. Most of the particles were fractured to very short lengths. This sequence was repeated experimentally with the same result. On the other hand, a comparable virus preparation that was removed from refrigeration immediately before dilution and examination exhibited the spaghettilike mats described above.

Ultraviolet absorption.—Purified preparations removed from a sucrose density-gradient and dialysed overnight against 0.01 M Tris buffer had an absorption spectrum characteristic of a nucleoprotein with a low nucleic acid content. Each virus preparation had an absorption peak at 263-265 nm and absorbed minimally at 245 nm. Solutions, after correction was made for light scattering at 320 nm, had a 260:280 absorption ratio of 1.18 (mean for four preparations) and a max:min ratio of 1.29.

Analytical ultracentrifugation.—The virus was detected in the analytical ultracentrifuge when crude sap extracted from infected *C. quinoa* leaves was examined. The virus sedimented in the crude sap at an uncorrected value of 90-110 S. Sap that was clarified by low-speed centrifugation and dialysed overnight against 0.01 M neutral phosphate buffer contained material that sedimented at 115-118 S. After extrapolation to infinite concentration, the $S_{20,w}$ value for this material was 122 S.

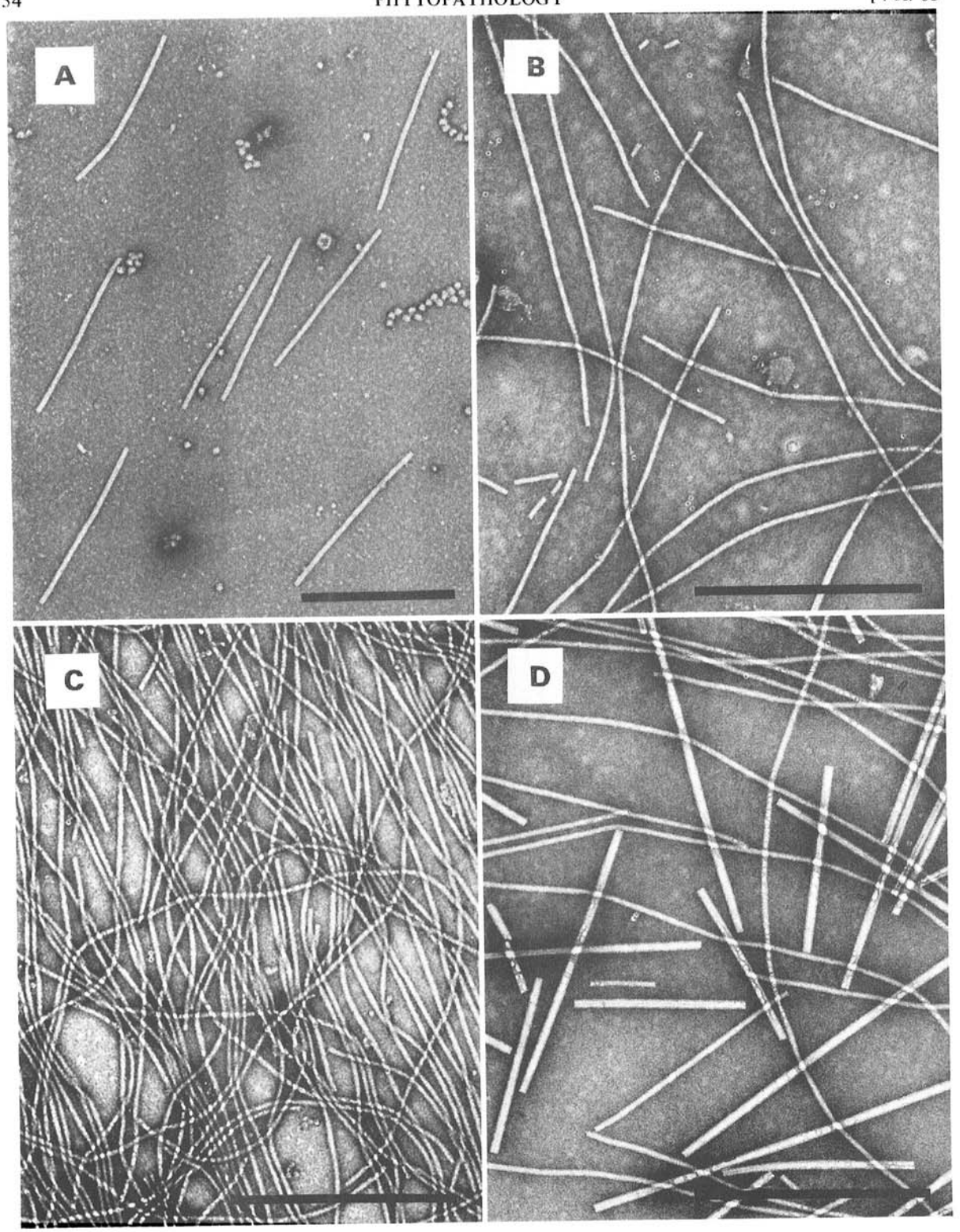

Fig. 3. Negatively stained electron micrographs of the apple isolate of clover yellow mosaic virus (CYMV). Bar represents 500 nm. **A)** Particles in a leaf dip preparation from infected *Chenopodium quinoa*. **B)** Preparation after purification by the citrate-chloroform procedure, showing end-to-end aggregation and fracturing of particles. **C)** Extensive end-to-end aggregation in a partially purified virus preparation. **D)** Mixture of CYMV and tobacco mosaic virus. The narrower particles are CYMV.

Sap that was clarified and concentrated by ultracentrifugation yielded a pellet that, when dissolved in a buffer and examined in the analytical ultracentrifuge, exhibited a pattern that showed a single peak for the virus and a broad hump for additional material that sedimented faster than the virus. The relative proportion of the virus and the additional material was dependent upon the molarity of the suspending buffer. Pellets suspended in a buffer of high molarity (e.g., 0.5 M, pH 7.2 phosphate) yielded a pattern with a higher peak and a lower hump than did comparable pellets suspended in a low molar buffer. This observation suggested that the peak corresponded with primarily unaggregated virus particles, whereas the hump corresponded with aggregated virus particles. When partially purified virus was layered on a 10-40% sucrose gradient and centrifuged for 90 min at 23,000 rpm, the material corresponding to the virus peak sedimented as a single opalescent zone, whereas the material corresponding to the hump sedimented to the bottom of the sucrose gradient. This more dense material, when tested serologically and when examined in the electron microscope, had a high virus concentration which was taken as further evidence that the "hump" material was, in fact, aggregated virus.

Serology.—Antisera obtained from the three rabbits which were bled only after the final injection was made had dilution end points of 1:3,200, 1:3,200, and 1:1,600. For the animal that was bled 1 week after each injection, the titer after the first injection was 1:100; after the second, 1:1,600; and after the third, 1:3,200.

By means of tube precipitin tests, the virus was detected readily in crude *C. quinoa* preparations by a strong precipitation visible at 1:80 dilution and a slight precipitation visible at 1:320 dilution. Controls for which leaf tissue from healthy plants was used had no visible precipitate in any of the tubes. Crude extracts that were from *C. quinoa* infected with isolates from six test trees, and that were tested against antiserum derived from a seventh isolate, all produced precipitation comparable to that in the homologous tests.

When partially purified preparations (by the liquid nitrogen procedure) of the virus were tested against antisera of other rod-shaped viruses also isolated from tree fruits, and against antisera of rod-shaped viruses with comparable particle dimensions from various hosts, all reacted negatively. The other viruses tested included: carnation latent virus, chrysanthemum B virus, Zygocactus virus, potato S virus, potato aucuba mosaic virus, and red clover vein mosaic virus. In precipitin tests performed by R. M. Lister, Purdue University, Lafayette, Ind., a purified preparation of our apple virus did not react with three antisera of ASGV isolate C-431, and one antiserum each of ASGV isolate E-36, CLSV from apple, CLSV from peach, and tobacco mosaic virus. Purified preparations of CLSV and ASGV provided by Lister did not react with our apple virus antiserum.

Relationship to clover yellow mosaic virus.—After the virus had been characterized by all procedures described above, H. E. Waterworth, USDA, Glenn Dale, Md., obtained our apple virus antiserum for tests against frozen preparations of isolates of bean common mosaic virus, bean yellow mosaic virus, clover yellow mosaic virus, henbane mosaic virus, pea streak virus, and potato X and Y viruses. Tests were negative with all these viruses except clover yellow mosaic virus (CYMV), which yielded moderate to weak precipitations at antiserum dilutions to 1:128.

The isolate of CYMV from Waterworth recovered from frozen pea tissue by inoculations made on *C. quinoa*, and new CYMV isolates obtained from clovers at Vancouver and Summerland, were used as antigens in further tests. CYMV antiserum from Waterworth was used at Summerland; frozen antiserum of Pratt (6) was used at Vancouver. At Summerland, purified preparations of five isolates from apple gave reactions with CYMV antiserum comparable to those obtained using homologous antiserum; the three CYMV isolates in clarified juice gave reactions with the apple virus antiserum at all dilutions comparable to those for apple virus homologous tests. The results of reciprocal tests at Vancouver, in which the virus from apple, the Vancouver isolate of CYMV, and an isolate of white clover mosaic virus (WCMV) were tested with antisera prepared against all of these, indicated that the apple virus and CYMV were identical serologically but provided no evidence of the relationship of either of these to WCMV.

Transmission in juice of the three CYMV isolates and an isolate of the virus from apple to herbaceous plants gave responses that indicated differences among all four isolates. Whereas the incubation period for local lesions on *G. globosa* was 44 hr for the apple isolate and two of the CYMV isolates, it was consistently 5 days for the Vancouver CYMV isolate. Whereas the Vancouver CYMV isolate and the apple isolate induced similar systemic symptoms on *C. quinoa*, the Waterworth and Summerland CYMV isolates did not produce systemic symptoms on this host. The Waterworth CYMV isolate failed to give systemic symptoms on *C. amaranticolor*. On garden pea (*Pisum sativum* L. 'Homesteader'), all isolates induced yellowing and dropping of inoculated leaves and chlorotic spots as systemic symptoms. Transfer in juice of an isolate from apple to young plants of alsike clover (*Trifolium hybridum* L.) and to crimson clover (*T. pratense* L.) resulted in the appearance within 2 weeks of symptoms that were typical of those induced by CYMV (Fig. 1-G).

DISCUSSION.—There appears to be adequate evidence that the described virus infects apple tree tissues. It has been transmitted to herbaceous hosts 19 times, at various seasons of the year over a 2-year period, from cambial and/or fruit tissues of ten trees. Isolates have been obtained from one of these trees on five occasions, from another on three occasions, and from three others on two occasions. Nine of these trees have yielded the virus each time isolation has been attempted. Repeated attempts to isolate the virus from numerous other trees have been unsuccessful. The transmissions were made in

glasshouse compartments from which all infected herbaceous plants had been removed. Symptoms have never appeared on noninoculated plants in these compartments. Legume viruses have never been studied in, or introduced to, this greenhouse complex.

Proof that the virus is the cause of apple leaf pucker disease is less conclusive. Such an association is suggested, because the virus has been isolated from ten trees that were propagated from affected trees in four widely separated British Columbia orchards, and has never been isolated from trees that were free from the leaf pucker disease. Our lack of success in isolating the virus from 44 additional trees displaying leaf pucker symptoms may indicate a lack of correlation in occurrence of the virus and the disease, or may merely manifest difficulty in transferring this virus from apple to its herbaceous hosts. Adequate proof that this is the causal agent of leaf pucker disease will necessitate return of the virus to its woody hosts and production of symptoms. Such trials are in progress.

An unusual experience has been the failure to transmit this virus from ground tissue of petals and leaves of trees that yielded the virus in ground cambial and fruit tissues. This differs from the experience of other workers (1, 5, 8, 9) and ourselves, for whom petals and/or leaves have proved the most satisfactory source for isolation of CLSV and ASGV. It is possible that this reflects merely an added difficulty in detecting infections of the "leaf pucker" virus in receptor plants that display the more profuse and obvious local lesions characteristically induced by CLSV.

Our experience in isolating this virus suggests that undue reliance on petals and leaves as virus sources can be a factor rationing success in isolation of viruses infecting pome fruits.

The propensity of the virus for almost infinite terminal aggregation under most of the purification regimes we have tried, and its tendency to fragmentation under other such regimes, have made some phases of its characterization difficult. Most of the exploration of purification procedures has been directed toward obtaining a yield of particles with truly characteristic dimensions, and preparations that would provide meaningful S values. The reasonable uniformity of particle dimensions obtained for leaf dip preparations suggests that these are reliable. The wide range of S values obtained for partially purified preparations provided an indication of the degrees of particle aggregation that could occur, but did not represent the true sedimentation constant. The value of 122 S obtained for the virus in clarified sap that was dialysed but not centrifuged agrees well with the value of 121 S obtained for CYMV by Pratt & Reichmann (7).

Before the virus had been established as a serotype of CYMV, ample evidence had been adduced that it differed from CLSV, ASGV, and TMV, three rod viruses previously reported to infect apple tissues. It was distinguished from CLSV and ASGV by the characteristically high titers attained in *C. quinoa* and by its stability in vitro. It differed from all three viruses in particle dimensions, herbaceous host range, and behavior in serological tests. That it is a strain of CYMV is supported by the serological evidence, by general similarity of the host ranges, by the comparable sedimentation coefficients, by the high titers reached in *C. quinoa*, and by the ability of the apple isolate to induce symptoms on alsike and crimson clover characteristic of those caused by CYMV. There are minor discrepencies in host reactions, in heat inactivation thresholds, and in virus particle lengths. The differences in host reactions can be attributed fairly confidently to pathogenic differences among isolates of a virus for which such variability has already been reported (2, 6). The difference in heat inactivation points, between 52 C for the apple isolate and 58 C reported (6) for CYMV, may be within a range of variation governed by differing test regimes. The difference in particle lengths [590 ± 10 nm for the virus from apple, 530 nm (7) for CYMV] is a fairly wide one. However, we suspect that it merely reflects differences in purification and electron microscopy procedures. Electron micrographs of the apple virus portray particles that are less flexuous than those in most published electron micrographs of viruses in the potato X virus group. Presumably, the more rigid the particles remain the more likely they are to attain a maximum measurable length. Thus, despite the discrepancy in particle length, and although the range of particle lengths for the potexvirus group (4) is thus slightly broadened, the serological evidence and other similarities leave no doubt that the virus from apple is a strain of CYMV.

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