Etiology

Cytopathology of Leafroll-Diseased Grapevines and the Purification and Serology of Associated Closteroviruslike Particles

F. Zee, D. Gonsalves, A. Goheen, K. S. Kim, R. Pool, and R. F. Lee

First and second authors, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456. Third author, USDA, University of California, Davis 95616. Fourth author, University of Arkansas, Fayetteville 72701. Fifth author, Department of Horticultural Sciences, New York State Agricultural Experiment Station, Cornell University, Geneva 14456. Sixth author, University of Florida, Citrus Experiment Station, Lake Alfred 33850.

We wish to thank D. K. Hummer and E. D. Williams for technical assistance.

This study was supported by grants from the American Vineyard Foundation, New York State Grape Production Research Fund, Inc., Division of Plant Industry, New York State Department of Agriculture and Markets and USDA Cooperative Agreement 58-3615-5-036.

Accepted for publication 20 April 1987 (submitted for electronic processing).

ABSTRACT

Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R., and Lee, R. F. 1987. Cytopathology of leafroll-diseased grapevines and the purification and serology of associated closteroviruslike particles. Phytopathology 77:1427-1434.

Thin-section electron microscopy of grapevines infected with two leafroll isolates from California (CA-1 and CA-2) and another from New York (NY-1) revealed the presence of flexuous rod-shaped viruslike particles in the cytoplasm of phloem parenchyma cells and in the sieve elements. Particles about 10–12 nm in diameter, of undetermined length, were associated with membranous vesicles containing fibrils and electron-dense granular material characteristic of closteroviruses. Viruslike particles purified from mature symptomatic leaves of grapevines infected with NY-1 and CA-1 leafroll isolates were between 1,800 and 1,900 nm long. In sodium dodecyl sulfate immunodiffusion tests, NY-1 antiserum reacted with CA-1, CA-2, and NY-1 isolates. With indirect enzyme-linked immunosorbent assay (ELISA), NY-1 antiserum reacted more strongly to homologous than heterologous antigens, thus indicating serological differences among the isolates. With direct ELISA, the NY-1 antiserum reacted with homologous but not with the CA-2 or CA-1 antigens. The consistent association of closteroviruslike particles with the leafroll disease supports previous suggestions that closteroviruses are the causal agents of the disease. However, final proof will require the reproduction of the disease by these viruslike particles.

Grapevine leafroll is one of the most important and widespread diseases of grapevines, occurring in all grape-producing countries in the world (29). Infected vines are abnormally sensitive to environmental stresses, and crop production can be reduced as much as 80% (29). There are generally no differences in leaf and flower development between healthy and infected vines until midsummer, when symptoms begin to appear on lower leaves near the base of infected canes. Symptoms include downward rolling of leaves and interveinal chlorosis. The symptoms spread outward from the vine trunk and become more intense as the season progresses. In late fall, leaf laminae of dark-fruited varieties turn red, while the major veins remain green (10). Although leafroll is not usually lethal, it causes erratic bearing, lowered sugar content in fruit, and delayed ripening of the crop. Brix levels of fruit from affected vines may be 25–50% lower than those from healthy vines on the same date (8–10,15). Leafroll is transmitted by grafting healthy vines with infected rootstocks or scions (10).

Although the transmissible nature of leafroll by grafting was demonstrated by Scheu in 1936 (29), the etiology of the disease has still not been determined. Various reseachers have associated viruslike particles with grapevines that are affected by leafroll. These include isometric particles (3,25), a potyviruslike particle (31,32), and closteroviruslike particles (3,7,14,21,26,33,34). In Israel, a potyvirus (790 × 13 nm) was purified from *Nicotiana glutinosa* L. that was inoculated with phenol extracts from a leafroll-diseased grapevine (31,32). In Italy, a closterovirus, designated grapevine virus A (GVA), with a modal length of 800 nm was recovered from a tobacco plant (*N. clevelandii* Gray) that had been inoculated with a leaf extract from a single vine with stem-pitting symptoms (2,5). Antiserum prepared to GVA reacted positively to numerous diseased grapevines in immunoelectron microscopy (IEM) immunosorbent electron microscopy (ISEM), including some with leafroll symptoms (21). Grape virus-A was also reported to be transmitted by mealy bugs (15,28). In Switzerland, two serologically distinct closteroviruslike particles with modal lengths of 1,800 and 2,200 nm were isolated from grapevines affected with leafroll disease (14). Antiserum prepared to the 2,200-nm particles did not react with the 1,800-nm particles by direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA). Thus, it was suggested that leafroll may be caused by two distinct closteroviruses (14). Recently, Mossop et al (24) associated closteroviruslike particles (about 1,400-nm modal length) and corresponding high molecular weight dsRNA from leafroll-affected grapevines.

Indexing on woody indicators is the accepted method for diagnosing leafroll disease of grapevines (8). This method is accurate but laborious and takes 2–3 yr to complete. The lack of a rapid diagnostic test and the failure to mechanically transmit the disease agent(s) hinder determination of the disease etiology. In this communication, we report on the cytology of leafroll-affected grapevines and on the purification and serological characterization of closteroviruslike particles isolated from grapevines affected with leafroll disease. We also show that sodium dodecyl sulfate (SDS)-immunodiffusion and ELISA can be used for detecting these particles in leafroll-diseased grapevines.

MATERIALS AND METHODS

Leafroll isolates. Three isolates, designated as CA-1, CA-2, and NY-1, were used extensively in this work. The CA-1 and CA-2 isolates were from leafroll-diseased grape cultivars Emperor and Melon obtained from the University of California, Davis. These had been indexed on woody indicators and found to be free from other known grape virus diseases. The CA-1 isolate was originally associated with the "White Emperor Disease," which was accepted to be leafroll some 27 years ago (10). The CA-1 isolate was maintained in Pinot noir and Emperor grape cultivars, whereas the CA-2 isolate was maintained in Melon. The NY-1 isolate originated from Pinot noir vines located in a commercial vineyard in upstate New York, which exhibited typical leafroll symptoms. Cuttings from four selected vines were propagated and served as sources of the NY-1 isolate. Subsequently, leaves from these vines were used for virus purification during the 1985 growing season. Cuttings of Baco noir, Cabernet Franc, LN-33, and Pinot noir, from vines with no leafroll symptoms in the California grape virus certification program, were grown as controls. All vines, except the NY-1 field samples, were maintained under greenhouse conditions in Geneva, NY.

Electron microscopy. For negative staining of crude extracts, leaf or bark pieces were ground with a mortar and pestle in 0.01 M potassium phosphate buffer (pH 8.0) containing 2.5% nicotine (6), 0.1 M potassium phosphate buffer (pH 7.0) plus 2% polyvinyl-polypyrrolidone (PVP) (19) or 0.1 M Tris-Cl buffer (pH 8.2). Specimens were prepared by floating a Formvar-coated grid on samples for 5 min and rinsing the grid with 30 drops of distilled water before staining with five drops of 1.5% salt of phosphotungstic acid (PTA) in water (23). A carbon grating replica of 2,160 lines per milliliter served as an enlargement standard. Particle length was measured using a micro Digi-Pad (GTCO Corporation, Rockville, MD). For thin-section electron microscopy, samples (1–2 mm^2) were taken from both young and old leaves and young stems of plants showing characteristic leafroll symptoms. Similar samples were taken from healthy plants. Specimens were fixed in modified Karnovsky's fixative (16) consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 hr at room temperature under a low vacuum. After washing three times with buffer solution, the tissues were postfixed in 1% OsO_4 in the same buffer for 2 hr and bulk stained in 0.5% aqueous uranyl acetate overnight at 4 C. Tissues were then dehydrated in an ethanol series and propylene oxide before embedding in Spurr's low viscosity medium (30). Thin sections cut with a diamond knife were double stained with 2% uranyl acetate and lead citrate before being examined.

Virus purification. A modification of the method described by Gugerli et al (14) was used. Forty grams of mature symptomatic leaves were harvested fresh or stored frozen at −80 C, powdered in liquid nitrogen in a mortar and pestle, transferred to a flask containing five volumes (1 g/5 ml) of 0.5 M Tris-Cl (pH 8.2) with 4% water-insoluble PVP, 0.5% bentonite, 0.2% 2-mercaptoethanol, and 5% Triton X-100 and agitated for 30 min at 6 C. The extract was squeezed through four layers of cheesecloth, clarified by centrifugation for 30 min at 6,000 *g*, and concentrated by centrifugation (80,000 *g* for 2 hr) over 5-ml pads of 20% (w/v) sucrose dissolved in 0.1 M Tris-Cl (pH 8.2) in a Beckman type 30 rotor (Beckman Instruments, Palo Alto, CA). The resulting pellets were thoroughly dispersed in 10 ml of 0.1 M Tris-Cl (pH 8.2) with 0.01 M $MgCl_2$ followed by centrifugation (2,000 *g* for 5 min). The supernatant was then layered over a 3-ml pad of 20% sucrose and centrifuged at 30,000 rpm for 2 hr in a Beckman type 40 rotor. The pellet from the centrifugation was dispersed thoroughly in 1 ml of 0.1 M Tris-Cl, pH 8.2, with 0.01 M of $MgCl_2$. This represented our partially purified virus preparation. Final purification was achieved by Cs_2SO_4 density gradient centrifugation (12). The partially purified preparation (1 ml) was centrifuged (2,000 *g* for 5 min), 1.5 g of Cs_2SO_4 was added to the supernatant, and the final volume was made up to 10 ml with buffer (0.1 M Tris-Cl buffer, pH 8.2, with 0.01 M $MgCl_2$). The 10-ml preparation was layered over a 3-ml pad of 53% (w/w) Cs_2SO_4 in 0.1 M Tris-Cl buffer, pH 8.2, with 0.01 M $MgCl_2$ and centrifuged in a Beckman SW40 Ti rotor for 18 hr at 36,000 rpm at 6 C. The contents of the gradient were then collected in 0.4-ml fractions using an ISCO 640 gradient fractionator (Instrumentation Specialities Co., Lincoln, NB). Fractions with viruslike particles, as determined by electron microscopy, were combined and dialyzed overnight at 6 C in 0.1 M Tris-Cl, pH 8.2, with 0.01 M $MgCl_2$. This preparation is referred to as purified virus.

Antiserum production. A polyclonal antiserum was produced in a New Zealand white rabbit against purified preparations of NY-1 using tissues from field-grown Pinot noir vines. Virus preparations of NY-1 were purified from leaves collected from three different vines (R18T3, R19T6, and R19T5). All preparations were injected subcutaneously into the back of the rabbit in three consecutive weeks. The first injection consisted of 1 ml of purified preparation mixed with 1 ml of Freund's complete adjuvant. The two subsequent injections were with 1 ml of purified preparation mixed with 1 ml of Freund's incomplete adjuvant. Serum was collected weekly starting 21 days after the first injection.

SDS-immunodiffusion. Antiserum collected weekly was checked for activity by the SDS-immunodiffusion test (27). Healthy and leafroll-affected leaf extracts were prepared by grinding 1 g of leaf tissue in 1 ml of water and then 1 ml of 3% SDS in water was added to the mixture. Leaf extracts were stored at −20 C for further use. Host reactions were minimized by intragel absorption; healthy leaf extract was added to the antiserum well 45 min before loading of the antiserum and samples.

Immunoelectron microscopy. Antisera used in decoration tests included those prepared to citrus tristeza virus (CTV), papaya ringspot virus (PRV-p), GVA (21), the 2,200-nm closteroviruslike particles from leafroll-affected vines in Switzerland (14), and the NY-1 isolate. Each specimen grid was prepared by a 10-min incubation on the virus sample, rinsing with

20 drops of 0.1 M phosphate buffer (pH 7.0), and a 15-min decoration in antiserum diluted 1:50 in 0.05 M potassium phosphate buffer (pH 7.2) (22,23); the grid was then rinsed with 20 drops of buffer and 30 drops of distilled water and stained with 5 drops of 1.5% PTA.

ELISA. The NY-1 antiserum was absorbed with healthy antigens before immunoglobulin fractionation. Healthy leaf extracts were prepared by grinding 4 g of leaf tissues from healthy LN-33 or Pinot noir vines in 10 ml of 0.1 M Tris-Cl buffer (pH 8.2) with 0.01 M $MgCl_2$, 4% PVP, and 0.2% 2-ME, agitated for 30 min,

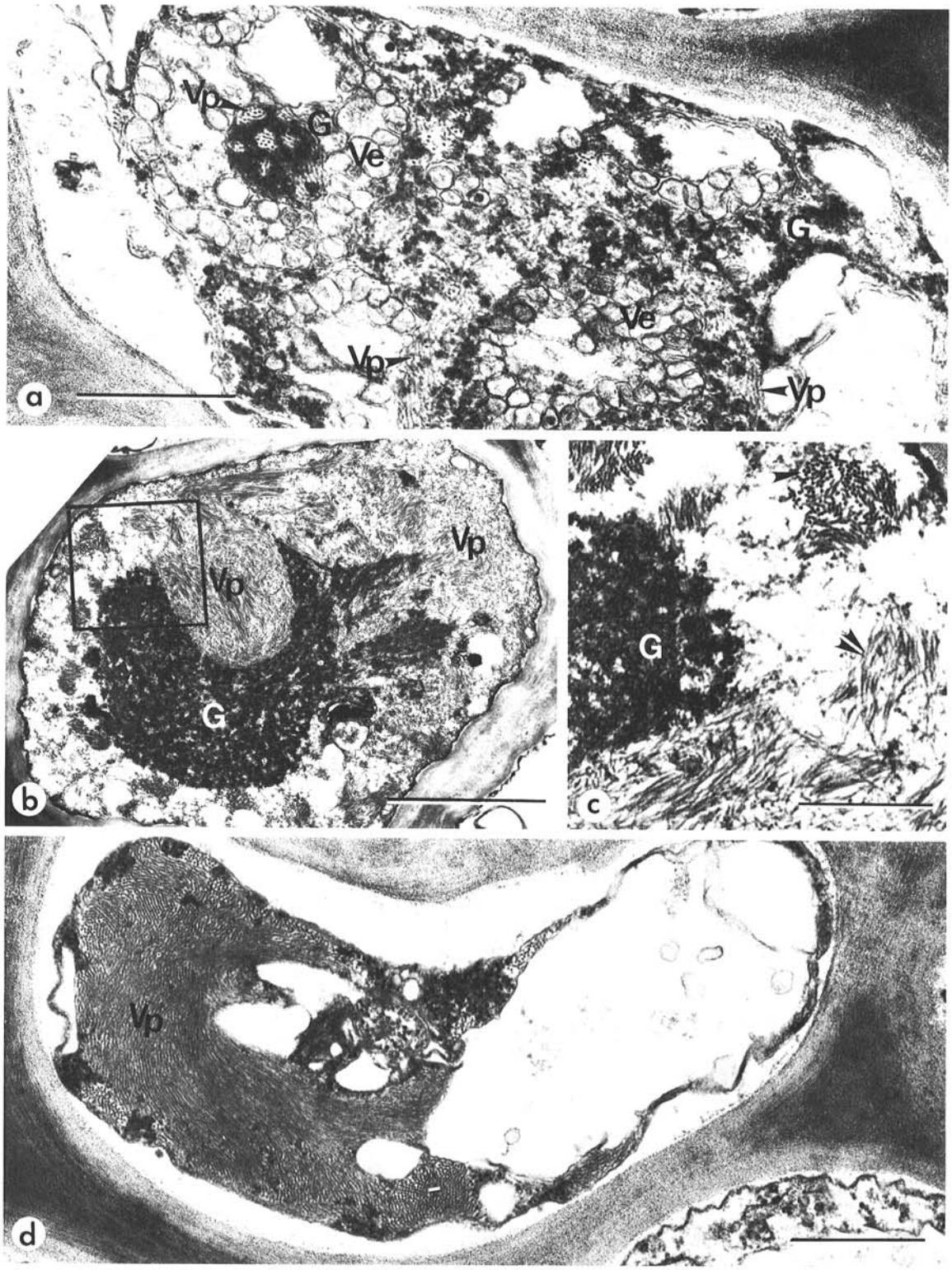

Fig. 1. Cytopathology of grapevine leaves infected with CA-1 or NY-1 isolates of leafroll. **A,** A phloem parenchyma cell containing flexuous rod-shaped viruslike particles (Vp). The particles are associated with a large number of vesicles (Ve) and patches of electron dense granular inclusion (G). A large circular body of granular inclusion (G) contains electron-lucent areas in which viruslike particles (Vp) are embedded. Emperor leaf infected with CA-1 isolate. (Bar = 0.55 μm) **B,** A sieve element containing an extremely large body of granular inclusion (G) associated with densely packed aggregates of viruslike particles (Vp). The particles in some aggregates are arranged parallel to one another. Emperor leaf infected with CA-1 isolate. (Bar = 1.66 μm) **C,** A higher magnification of the squared area of **B** showing the details of granular inclusions (G) and viruslike particles sectioned transversely (arrowhead) and longitudinally (double arrowheads). (Bar = 0.50 μm) **D,** A phloem parenchyma cell showing the cytoplasm entirely replaced by viruslike particles (Vp). Old Pinot noir leaf infected with NY-1 isolate. (Bar = 0.62 μm)

and centrifuged at 5,000 *g* for 10 min. Crude antiserum was then mixed with two volumes of healthy leaf extract and incubated for 16 hr at 6 C, followed by centrifugation (8,000 *g* for 15 min). Immunoglobulins were then isolated by ammonium sulfate precipitation and DEAE cellulose (DEAE-Sephacel, Pharmacia Fine Chemicals, Piscataway, NJ) column chromatography (4).

For indirect ELISA (17,20), plates were coated with test antigens suspended in coating buffer, blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hr, and then loaded with immunoglobulin. The immunoglobulin was preabsorbed a second time with extracts of healthy Pinot noir leaf tissue as described by Gonsalves et al (13). For direct double antibody sandwich ELISA (direct ELISA, 4), purified immunoglobulin from the NY-1 antisera was conjugated to alkaline phosphatase type VII (P4502, Sigma Chemicals Co., St. Louis, MO). Controls with buffer, healthy, and known infected samples were included in all ELISA tests. Absorption was measured at 405 nm with a Dynatech MR 580 reader (Dynatech Laboratories, Inc., Alexandria, VA).

RESULTS

Under greenhouse conditions, grapevines infected with CA-1, CA-2, or NY-1 leafroll isolates developed symptoms approximately 3 mo after budbreak. Melon vines infected with CA-2 had downward and inward rolling of leaf margins, chlorotic leaf lamina with green veins, and scorched margins. In addition to the leafrolling and green veins, Emperor vines infected with CA-1 had severely chlorotic leaves with uneven patches of reddening between veins. All Pinot noir vines infected with CA-1 or NY-1 isolates developed leafrolling and dark reddish leaves with green main veins.

Electron microscopy of infected tissues. Long flexuous rod-shaped viruslike particles measuring 10–12 nm in diameter with undetermined length were found consistently in grapevines infected with each of the three isolates of leafroll. They were similar to particles found in leafroll-diseased grapevines in studies carried out in California (33,34). The particles were only in sieve elements and parenchyma cells of the vascular bundles (Fig. 1B and D). The particles occurred in the cytoplasm but not in organelles and were usually associated with two types of structures unique to infected cells, membranous vesicles, and electron-dense granular material (Figs. 1A and B and 2).

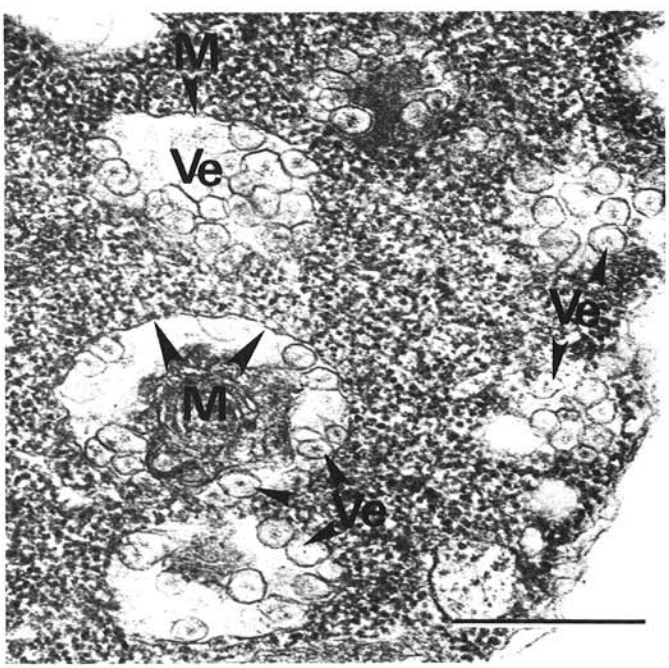

Fig. 2. A portion of phloem parenchyma cell, from a young Emperor leaf infected with CA-1 isolate, containing clusters of fibril-containing vesicles (Ve) in the cytoplasm. Each cluster is surrounded by a membrane (M). (Bar = 0.52 μm)

In young leaves, some phloem parenchyma cells contained many vesicles without viruslike particles or electron-dense granular material, suggesting that these cells were in an early stage of infection. The vesicles in these cells occurred in clusters, each of which was surrounded by a membrane, and ribosomes were packed between these clusters (Fig. 2). Individual vesicles contained fine fibrils (Fig. 2). In some other parenchyma cells, the cytoplasm was filled with fibril-containing vesicles associated with electron-dense granular material and viruslike particles (Fig. 1A). The granular material often formed large bodies of circular profile containing electron-lucent areas in which viruslike particles were embedded (Fig. 1A). Some sieve elements were filled with an extremely large granular body associated with densely packed aggregates of viruslike particles (Fig. 1B). In such cells, the fibril-containing vesicles were rare. The particles in aggregates were often parallel to one another in their long axis, but disorderly arranged particles in an aggregate were not uncommon (Fig. 1C). In older leaves, the entire cytoplasm of many parenchyma cells was replaced by aggregates of viruslike particles (Fig. 1D).

Virus purification. Flexuous rod-shaped viruslike particles were recovered consistently from symptomatic leaves of grapevines infected with the three isolates, but not from leaves of healthy Baco noir, Cabernet Franc, LN-33, or Pinot noir. The procedure of Gugerli et al (14) was improved by increasing Triton X-100 concentration in the extraction buffer from 1 to 5% to facilitate release of the particles from host debris (Fig. 3A–D) and by reducing the speed for low-speed centrifugation and eliminating organic solvent clarification. Viruslike particles were further purified by Cs_2SO_4.

Viruslike particles in Cs_2SO_4 gradients were located by electron microscopy; the highest concentrations were observed consistently in a fraction collected between 8.0 and 8.4 ml below the meniscus of the gradient. This fraction coincided with a small peak in the UV-profile of the gradient and reacted strongly against homologous antiserum in indirect ELISA (Fig. 4).

Virus yields from leafroll-diseased vines were very low, as compared with citrus tristeza virus from infected citrus (1,12), which made accurate yield measurements difficult to determine. When a 5-μl sample of a purified CA-1 or CA-2 preparation was coated onto a 400-mesh grid, the average particle count was between $<$ 1 to 50 per grid square. More particles were recovered from leaves of Pinot noir infected with CA-1 isolate than from Emperor leaves infected with the same isolate. Tissues from greenhouse and field-grown NY-1 infected Pinot noir vines gave the highest yields; purified preparations averaged 50–1,000 particles per grid square.

Viruslike particles were always recovered from mature symptomatic leaves in field and greenhouse grown vines. Asymptomatic leaves collected in fall from field-grown NY-1 infected vines also contained a high concentration of particles. Few particles were recovered from young leaves and leaves of poorly growing vines.

The type of polyvinyl-polypyrrolidone (PVP) used in the extraction buffer was critical. Few viruslike particles were recovered when water-soluble PVP-40 was used in place of water-insoluble PVP. Addition of 0.5 M urea to the extraction buffer increased the number of particles recovered, but most were in aggregates attached to host debris. Increasing the Triton X-100 concentration to 6 or 7% with urea in the extraction buffer did not improve the purification of these particles. Viruslike particles were not recovered when 0.01 M EDTA was included in the extraction buffer. Polyethlylene glycol (PEG) precipitation could not be substituted for high-speed centrifugation steps in the purification procedure.

Particles were not detected by electron microscopy of crude leaf extracts prepared by grinding diseased leaf, stem, or inner bark tissues in potassium phosphate buffer plus 2.5% nicotine or phosphate buffer plus 2% PVP or Tris-Cl buffer. Particles (approximately one particle per grid hole), however, were detected in the clarified supernatant of the initial tissue extract of the

purification procedure. Virus was not recovered by the purification procedures used for papaya ringspot virus (11), citrus tristeza (1,12), or apple chlorotic leafspot viruses (19).

Particle size. The modal length of 217 particles from different CA-1 preparations was between 1,800 and 1,900 nm. These measurements included particles isolated in preparations before and after cesium sulfate gradient centrifugation (Fig. 5). Measurements of 256 particles from NY-1 preparations purified through cesium sulfate centrifugation also have modal length between 1,800 and 1,900 nm (Fig. 5). The modal length of CA-2 particles could not be determined adequately because of low yields and excess aggregation of particles.

SDS-immunodiffusion. Two precipitation reactions were visible in SDS-immunodiffusion tests. A wider, faster moving band specific to leafroll-infected samples and a sharper, slower moving nonspecific band were observed in all cross reactions between the NY-1 antiserum and the three leafroll isolates. The nonspecific reaction was removed readily by intragel absorption with healthy antigens. No spur formation was observed between precipitin lines when NY-1 antiserum was reacted with extracts of homologous NY-1, and heterologous CA-1 and CA-2 isolates (Fig. 6). The reactivity between NY-1 antiserum and extracts of CA-2 infected leaves was slower and generally less intense than the homologous reaction. The highest specific antibody titer (1/4) was observed in serum collected 6 wk after the final injection.

IEM. Results of IEM are presented in Table 1. The NY-1 antiserum uniformly decorated the CA-1 and homologous NY-1 particles but not CA-2 particles. Antisera prepared to citrus tristeza or papaya ringspot viruses did not decorate any leafroll-associated particles in IEM (Table 1). Antiserum produced to the 2,200-nm particles isolated from leafroll-affected vines in Switzerland (14) did not decorate NY-1 or CA-2 particles but appeared to decorate CA-1 particles. GVA antiserum did not decorate any of the particles from all NY-1 and CA-2 preparations and particles from CA-1 preparations that were isolated from infected Emperor vines (Table 1). However, three different

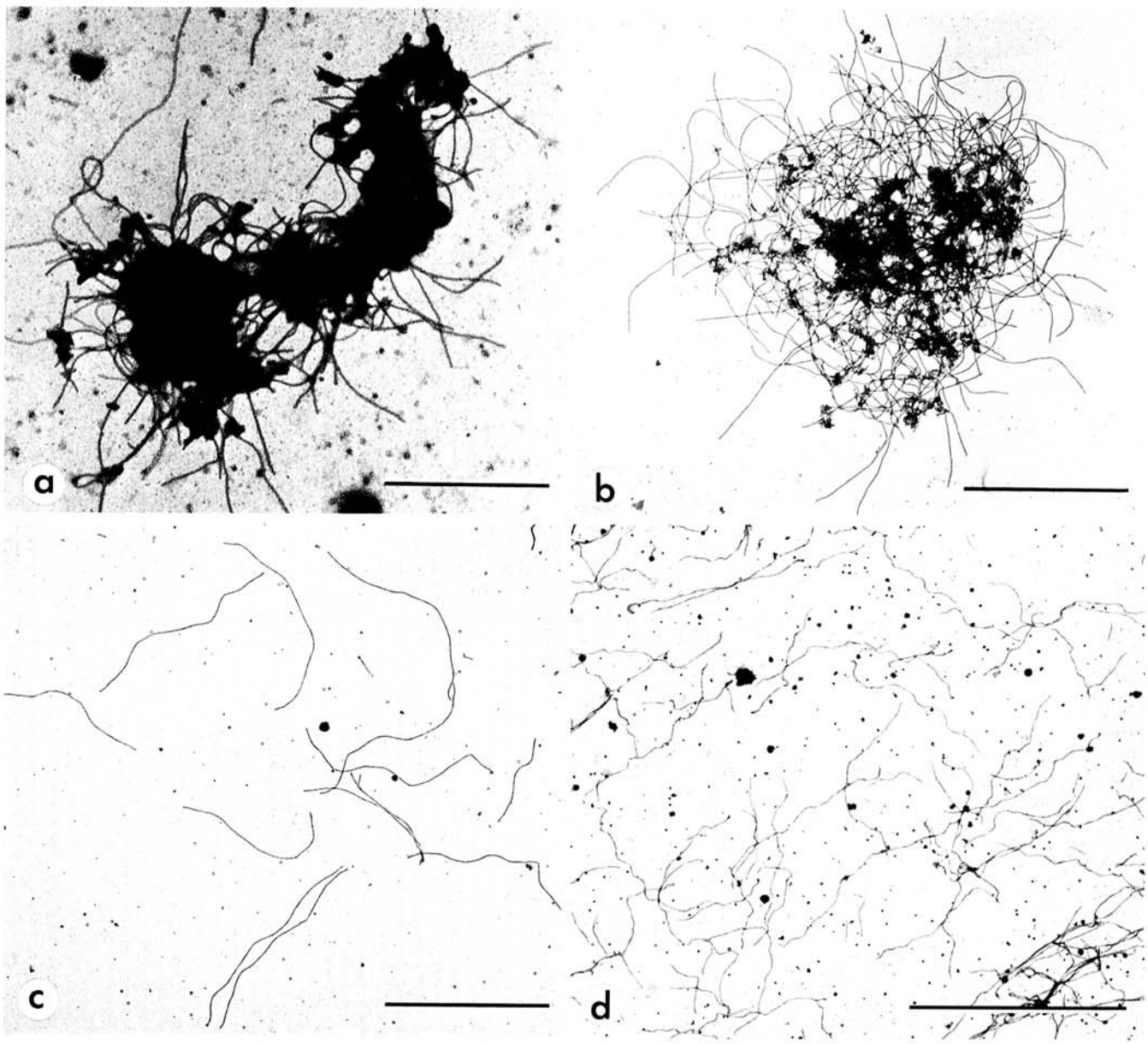

Fig. 3. Effect of Triton X-100 on purification of viruslike particles from symptomatic leaves of a grapevine infected with the NY-1 leafroll isolate. All electron micrographs of the particles were taken after cesium sulfate density gradient centrifugation. **A,** A heavily aggregated mass of viruslike particles and host debris commonly observed among individual particles when 1% Triton X-100 was used in the extraction buffer. (Bar = 1.31 μm) **B,** A more dispersed aggregate was observed when the concentration of Triton X-100 in the extraction buffer was increased to 2.5%. (Bar = 1.38 μm) **C** and **D,** Majority of the virus particles were dispersed **(C)** although some particle aggregates **(D)** could be observed when 5% Triton X-100 was used in the extraction buffer. (**(C)** Bar = 1.31 μm. **(D)** Bar = 1.78 μm)

reactions were observed when GVA antiserum was cross reacted with preparations of CA-1 infected Pinot noir vines. One, nearly all of the particles from these preparations were not decorated by GVA antiserum. Two, about 1% of shorter particles or fragments of particles were fully decorated by GVA antiserum. Three, part of some long particles were decorated by GVA antiserum in a similar way as described by Milne et al (21) for GVA.

ELISA. With indirect ELISA, immunoglobulins from NY-1 antiserum reacted more strongly to homologous antigens than to CA-1 or CA-2 antigens (Fig. 7). However, we obtained erratic results with indirect ELISA when crude leaf extracts were used as antigen sources. Preliminary data indicated that components in crude leaf extracts probably interfered with binding of viral antigens to walls of the ELISA plates. In one experiment, when crude leaf extracts, partially purified, and purified virus preparations from the same batch of leaves of a NY-1 infected plant were used as antigen sources, only partially purified and purified preparations gave positive indirect ELISA reactions (Fig. 8). However, that same crude leaf extract gave positive reactions with direct ELISA (Fig. 8). Background reactions were minimized by absorbing immunoglobulins with healthy leaf extracts just before their loading onto ELISA plates.

With direct ELISA, the NY-1 antiserum gave strong specific reactions to the NY-1 isolate (Fig. 8), but not to CA-1 and CA-2 isolates. Unlike indirect ELISA, consistent specific reactions were obtained with crude leaf extracts from NY-1 infected vines. The best reactivity was obtained at 2 μg/ml of purified immunoglobulin for coating, 1/50 antigen dilution and 1/1500 enzyme conjugate

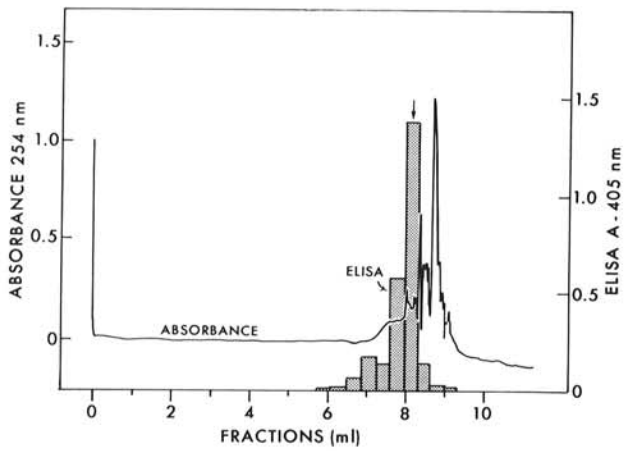

Fig. 4. Distribution of viruslike particles, NY-1 isolate, in cesium sulfate density gradient after centrifugation for 18 hr at 36,000 rpm. Samples from each of the 0.4-ml fractions were diluted 1:20 in ELISA coating buffer and tested by indirect ELISA using NY-1 antiserum. Electron microscopy of fractions showed that virus particles were in fractions showing high ELISA readings. The arrow shows where the largest number of particles was observed.

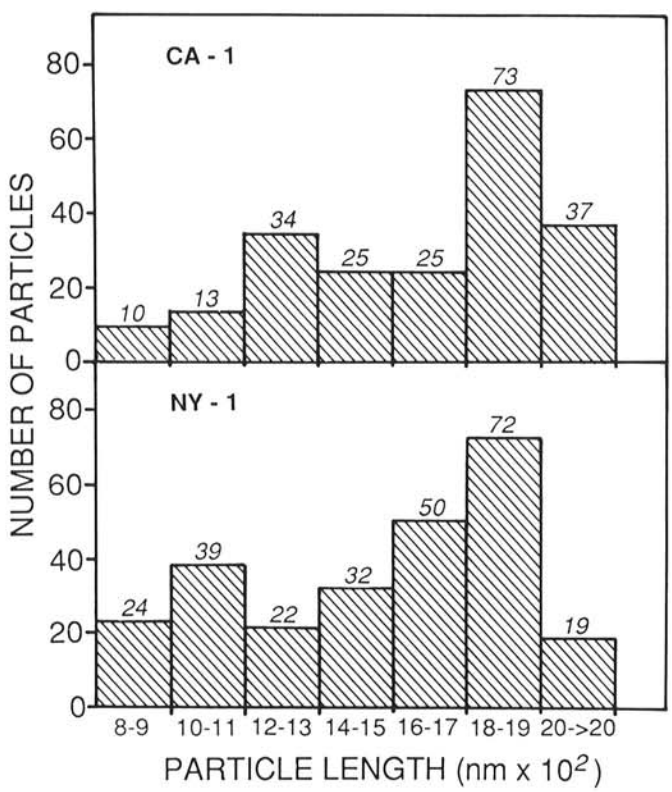

Fig. 5. Particle measurements of CA-1 (top) and NY-1 (bottom) leafroll isolates.

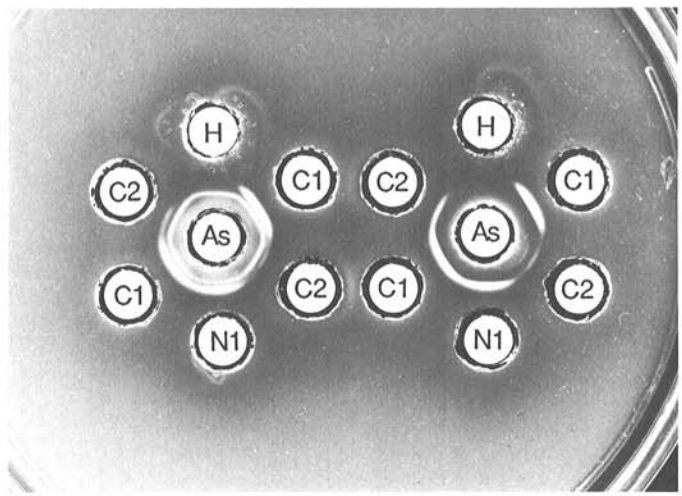

Fig. 6. Reactions of antiserum prepared to NY-1 leafroll isolate against leaf extracts of healthy LN-33 (H), and diseased leaf extracts from vines infected with NY-1 (N1), CA-1 (C1), and CA-2 (C2) leafroll isolates in SDS-immunodiffusion tests. Antiserum well (As) located on the right was preabsorbed with healthy leaf extract before the loading of antiserum and samples and the antiserum well (As) on the left was not. Note how preabsorption eliminated the nonspecific reaction.

TABLE 1. Reactivity of leafroll isolates to various antisera in immunoelectron microscopy

Isolates	Normal serum	Antisera[a] NY-1	GVA	2200	PRV	CTV
CA-1/Pinot noir						
Leaf	–[b]	–	–,p[c]	–	–	–
Stem	–	–	–, 1%+[d], p	–	–	NT[e]
CA-1/Emperor	–	–	–	Unsure[f]	–	–
CA-2	–	+[g]	–	–	–	–
NY-1						
R18T3	–	+	–	–	–	–
R19T5	–	+	–	–	–	–
R19T7	–	+	–	–	–	–
Papaya ringspot	–	–	–	–	+	–
Tristeza	–	–	–	–	–	+

[a] Antisera: NY-1 = NY-1 leafroll isolate, GVA = grapevine virus A, 2200 = leafroll isolate with 2200 nm particles, PRV = papaya ringspot virus p, CTV = citrus tristeza virus.
[b] – = No decoration.
[c] p = Half-naked and half-decorated particles, less than 1% of the particle population.
[d] %+ = Percentage of particles in the population that showed positive decoration.
[e] NT = Not tested.
[f] Unsure = Some particles appear to be decorated but the decoration was not clearly positive.
[g] + = Positively decorated particles.

dilution. Background color was not observed with healthy controls.

DISCUSSION

Our electron microscopic evidence shows that grapevines affected with leafroll disease have cytopathic structures and viruslike particles that are typically induced in plants by closterovirus infections (e.g., 18). Furthermore, closteroviruslike particles were consistently isolated from these leafroll-affected grapevines. Serological data from SDS-immunodiffusion, ELISA (direct and indirect), and ISEM tests suggest that the closteroviruslike particles from the leafroll isolates we tested are serologically related but not necessarily identical. Our results add to the body of evidence that associates closteroviruslike particles with leafroll disease (e.g., 7,14,24,26,33,34) and thus further suggests that the disease is caused by one or more closteroviruses.

Although viruslike particles could be consistently isolated from leafroll-affected vines, low yields made it difficult to determine their biochemical and physical properties in more detail. Unlike citrus tristeza virus (12), which is concentrated in young leaves and bark tissue, highest virus yields were from mature symptomatic tissue. This is similar to what Gugerli et al (14) reported for their strains of leafroll. Because mature symptomatic leaves are difficult to obtain throughout the year, it was important to harvest and store as much of these leaves when they were available. We kept our leaf samples at −80 C and could still recover viruslike particles after 6 mo of storage. Actively growing diseased vines were maintained in our greenhouse throughout the year. We rotated our vines with 2 mo of cold storage for every 6–8 mo of growth to ensure vigor and maximize virus yield.

The purification procedure that we developed is based on the method described by Gugerli et al (14). Several modifications and additions to their method helped us to obtain more consistent and purer preparations. Increasing the concentration of Triton X-100 and using a cesium sulfate density gradient centrifugation step cut down on particle aggregation and host contaminants. The two high-speed centrifugation steps were essential, but they also contributed to increased particle aggregation. It was therefore important to thoroughly resuspend the high-speed pellets to minimize particle loss.

Viruslike particles in preparations of NY-1 and CA-1 had modal lengths of 1,800–1,900 nm (Fig. 5). This modal length corresponds to the length of the 1,800-nm particles that Gugerli et al (14) reported for one of their strains of leafroll, but is different from that reported by Mossop et al (24), which was 1,400 nm for their leafroll isolate. Taking our results together with those of Gugerli et al (14) and Mossop et al (24), it is conceivable that closteroviruslike particles of various lengths may cause the leafroll disease. In fact, Gurgerli et al (14) have suggested that leafroll may be caused by two distinct strains (1,800 and 2,200 nm) of closteroviruses. However, our measurements should be taken with caution since they were from purified or partially purified preparations. It is conceivable that particles in these preparations were fragmented or aggregated during the purification process. Furthermore, Mossop et al (24) observed that the closteroviruslike particles that they associated with leafroll-affected vines appeared to fragment readily in negative stained preparations. It would have been preferable to also have measurements of particles from crude 'leaf' dips, but we failed in numerous attempts to detect particles by this method.

Based on IEM and direct ELISA tests, Gurgerli et al (14) concluded that their 1,800- and 2,200-nm particles were serologically unrelated. Our IEM tests using Gugerli's antiserum to the 2,200-nm particles did not show reaction with our isolates except possibly with CA-1 particles (Table 1). There was a slight decoration on some of the CA-1 particles by Gugerli's antiserum. Our data also suggest the existence of serological differences between the CA-1, CA-2, and NY-1 isolates. Further tests using homologous antisera and virus preparations should be done to clarify these relationships.

Data from IEM tests and particle measurements lead us to conclude that our leafroll isolates are not related to GVA. The modal length of GVA is 800 nm (5), whereas those of CA-1 and NY-1 are 1,800–1,900 nm. Antiserum to GVA did not decorate any of the NY-1, CA-2 particles, or CA-1 particles isolated from infected Emperor vines. A few particles from CA-1 preparations of infected Pinot noir vines were decorated by GVA antiserum. However, these GVA-decorated particles were probably due to contamination of GVA in the Pinot noir stock. Milne et al (21) showed that GVA is widespread in grapevines, including those

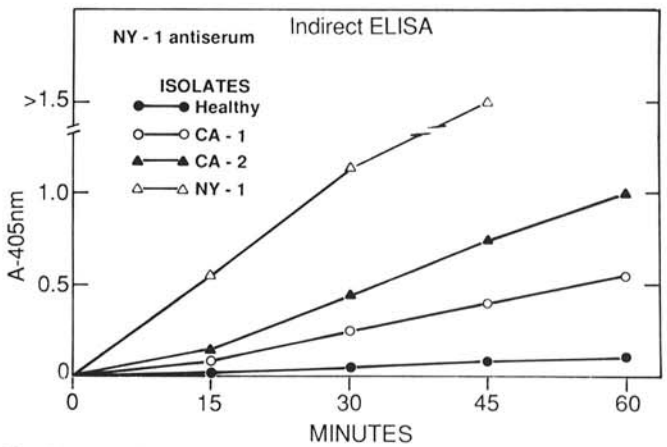

Fig. 7. Reactivity of partially purified preparations of NY-1, CA-1, and CA-2 isolates against purified NY-1 immunoglobulin in indirect ELISA.

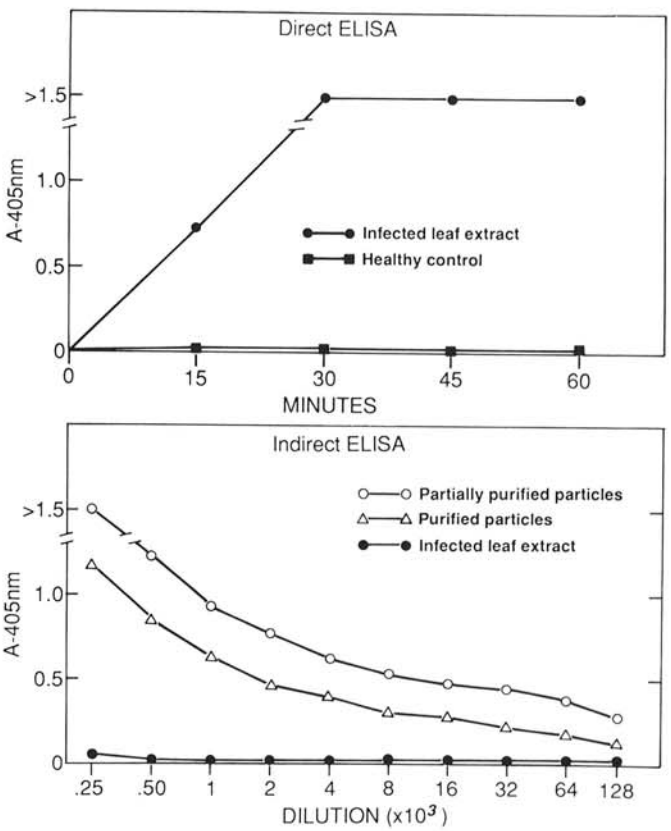

Fig. 8. Reactions of various preparations of NY-1 infected Pinot noir vines in direct (top) and indirect (bottom) ELISA using NY-1 antiserum. Same tissue source was used for both direct and indirect ELISA. Note that crude leaf extracts from infected vines show positive reaction in direct but not in indirect ELISA. However, partially purified and purified preparations from these crude leaf extracts gave positive reaction by indirect (bottom) and direct (data not shown) ELISA. Crude tissue extract for direct test is at 1/50 dilultion.

affected with leafroll, stem pitting, and also healthy grapevines. We also observed few half-decorated and half-naked particles in preparations containing particles which reacted to GVA antiserum. Milne et al (21) suggested that these are end-to-end aggregates of two serologically distinct particles. No such particles were observed in Emperor infected with the CA-1 isolate.

Although our data and those of others (e.g., 7,14,24,26,33,34) suggest a consistent association of closteroviruses with vines affected with leafroll disease, the etiological significance of these particles remains to be determined. Several major factors make it difficult to determine this conclusively. For example, the expression of leafroll symptoms in the standard grapevine virus indicator plants takes at least 2 yr to show up after inoculation under New York conditions. This makes it difficult to do follow up experiments because of the long incubation period. Also, lack of a local lesion host and herbaceous host adds to the difficulty in identifying and characterizing the causal agent(s) of leafroll. We feel the development of a routine method for isolating closteroviruslike particles from grapevines and the development of antisera to these viruslike particles will help us in our attempts to determine whether these closteroviruses are indeed the causal agent(s) of the leafroll disease. For example, the detection of these particles in crude extracts of affected grapevines by ELISA and SDS-immunodiffusion tests will enable us to determine whether infection has occurred in inoculated grapevines even before symptoms develop.

LITERATURE CITED

1. Bar-Joseph, M., Gumpf, D. J., Dodds, J. A., Rosner, A., and Ginzberg, I. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75:195-198.
2. Boccardo, G., and d'Aquilio, M. 1981. The protein and nucleic acid of a closterovirus isolated from a grape vine with stem pitting symptoms. J. Gen. Virol. 53:179-182.
3. Castellano, M. A., Martelli, G. P., and Savino, V. 1983. Virus like particles and ultra structural modifications in the phloem of leafroll-affected grape vine. Vitis 23-39.
4. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. J. Gen. Virol. 34:275-483.
5. Conti, M., Milne, R. G., Luisoni, E., and Boccardo, G. 1980. A closterovirus from stem-pitting-diseased grapevine. Phytopathology 70:394-399.
6. Corbett, M. K., Kasdorf, G. G., Engelbrecht, D. J., and Wiid, J. 1984. Detection of viral-like particles by electron microscopy of negatively stained extracts from grapevines. S. Afr. J. Enol. Vitic. 5:46-49.
7. Faoro, F., Tornachi, R., Fortusini, A., and Belli, G. 1981. Association of a possible closterovirus with grapevine leafroll in northern Italy. Riv. Pathel. Veg. Ser. IV 17:183-189.
8. Goheen, A. C. 1970. Virus and viruslike diseases of the grapevine. Pages 209-212 in: Virus Diseases of Small Fruits and Grapevines. N. W. Frazier, ed. University of California, Berkeley.
9. Goheen, A. C., and Cook, A. J. 1959. Leafroll (Red-leaf or Rougeau) and its effects on vine growth, fruit quality, and yields. Am. J. Enol. Vitic. 10:173-181.
10. Goheen, A. C., Harmon, F. N., and Weinberger, J. H. 1958. Leafroll (white Emperor disease) of grapes in California. Phytopathology 48:51-54.
11. Gonsalves, D., and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70:1020-1032.
12. Gonsalves, D., Purcifull, D. E., and Garnsey, S. M. 1978. Purification and serology of citrus tristeza virus. Phytopathology 68:553-559.
13. Gonsalves, D., Trujillo, E., and Hoch, H. C. 1986. Purification and some properties of a virus associated with cardamom mosaic, a new member of the potyvirus group. Plant Dis. 70:65-69.
14. Gugerli, P., Brugger, J.-J., and Bovey, R. 1984. L'enroulement de la vigne: Mise en evidence de particules virales et developpement d'une methode immuno-enzymatique pour le diagnostic rapide. Rev. Suisse Vitic. Arboric. Hortic. 16:299-304.
15. Hewitt, W. B. 1968. Viruses and virus diseases of the grapevine. Rev. Appl. Mycol. 47:433-455.
16. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137A.
17. Koenig, R. 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40:309-318.
18. Lister, R. M., and Bar-Joseph, M. 1981. Closteroviruses. Pages 809-844 in: Handbook of Plant Virus Infections, E. Kurstak, ed. Elsevier/North-Holland Biomedical Press, New York.
19. Lister, R. M., and Hadidi, A. F. 1971. Some properties of apple chlorotic leaf spot virus and their relation to purification problems. Virology 45:240-251.
20. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. Phytopathology 72:1018-1022.
21. Milne, R. G., Conti, M., Lesemann, D. E., Stellmach, G., Tanne, E., and Cohen, J. 1984. Closterovirus-like particles of two types associated with diseased grapevines. Phytopathol. Z. 110:360-368.
22. Milne, R. G., and Lesemann, D. E. 1984. Immunosorbent electron microscopy in plant virus studies. Methods Virol. 8:85-101.
23. Milne, R. G., and Luisoni, E. 1977. Rapid immuno electron microscopy of virus preparations. Methods Virol. 6:265-281.
24. Mossop, D. W., Elliott, D. R., and Richards, K. D. 1985. Association of closterovirus-like particles and high molecular weight double-stranded RNA with grapevines affected by leafroll disease. N. Z. J. Agric. Res. 28:419-425.
25. Namba, S., Yamashita, S., Doi, Y., and Yoda, K. 1979. A small spherical virus associated with the Ajinashika disease of Koshu grapevine. Ann. Phytopathol. Soc. Jpn. 45:70-73.
26. Namba, S., Yamashita, S., Doi, Y., Yoda, K., Terai, Y., and Yano, R. 1979. Grapevine leafroll virus, a possible member of closterovirus. Ann. Phytopathol. Soc. Jpn. 45:497-502.
27. Purcifull, D. E., and Batchelor, D. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant virus inclusions. Fla. Agric. Exp. Stn. Tech. Bull. 88.
28. Rosciglione, B., Castellano, M. A., Martelli, G. P., Savino, V., and Cannizzaro, G. 1983. Mealybug transmission of grapevine virus A. Vitis 22:331-347.
29. Scheu, G. 1936. Mein Winzerbuch. Reichsnahrstands-Verlags, G. M. B. H., Berlin. 274 pp.
30. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-34.
31. Tanne, E., Sela, I., and Harpaz, I. 1974. Transmission of grapevine leafroll to herbaceous plants. Phytopathol. Z. 80:176-180.
32. Tanne, E., Sela, I., Klein, M., and Harpaz, I. 1977. Purification and characterization of a virus associated with the grapevine leafroll disease. Phytopathology 67:442-447.
33. Tzeng, H. L. C. 1984. Anatomical and tissue culture studies of corky-bark-, rupestris-stem-pitting-, and leafroll-affected grapevines. M. Sc. thesis. University of California, Davis. 65 pp.
34. Tzeng, H. L., and Goheen, A. C. 1984. Electron microscopic studies on the corky bark and leafroll virus disease of grapevines. (Abstr.) Phytopathology 74:1142.