UC Riverside
International Organization of Citrus Virologists Conference Proceedings (1957-2010)

Title

Purification, Characterization and Serology of a Mild Strain of Citrus Infectious Variegation Virus from Florida

Permalink
https://escholarship.org/uc/item/7jn7405z

Journal
International Organization of Citrus Virologists Conference Proceedings (1957-2010), 9(9)

ISSN
2313-5123

Authors
Davino, M.
Garnsey, S. M.

Publication Date
1984

DOI
10.5070/C57jn7405z

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Purification, Characterization, and Serology of a Mild Strain of Citrus Infectious Variegation Virus from Florida[1]

M. Davino and S. M. Garnsey

ABSTRACT. A newly recognized mild isolate of citrus variegation virus (CVV-2) was purified and its properties compared to a previously characterized isolate of CVV (CVV-1) and to an isolate of citrus leaf rugose virus (CLRV). The physical properties of CVV-2 were similar to those of CVV-1, except for a consistent difference in the ratio between nucleoprotein components 1 and 2. Neither CVV isolate contained the distinct NP-4 component found in CLRV. Rabbit antiserum specific to formaldehyde-fixed, purified CVV-2 was prepared. The serum reacted specifically in immunodiffusion and ELISA tests with no detectable reaction to host antigens. The serum to CVV-2 reacted homologously to CVV-1 and showed a weak cross reaction to high concentrations of CLRV. Serological procedures described will be especially useful for detecting CVV-2 which often causes only mild symptoms in diagnostic indicator plants.

Several isolates of citrus variegation virus (CVV) have been purified from leaf tissue of citrus and noncitrus hosts (4, 6, 16, 20), but few have been well characterized and strain comparisons have not been made. An isolate of CVV was recently found in Florida (13) which differed markedly in symptom expression from a previously described CVV isolate (11), but was serologically related. Symptoms in Eureka lemon caused by the mild CVV isolate also closely resembled those caused by citrus leaf rugose virus (CLRV) (10, 12).

This paper reports purification of the mild CVV isolate, production of a specific antiserum to it, and comparisons to another isolate of CVV and to CLRV.

MATERIALS AND METHODS

Virus sources. Isolates studied included the recently discovered mild isolate of CVV (CVV-2) (13), an isolate of CVV described earlier in Florida (CVV-1) which has been previously purified and characterized (9, 11), and an isolate of CLRV (CLRV-2) from Florida which also had been purified and characterized (12). Isolates of CVV-1 and of CLRV-2 were deposited previously with the American Type Culture Collection as PV 196 and PV195, respectively. All cultures were from single lesion sources passed serially from citrus through several herbaceous hosts and back to citrus by mechanical inoculation. All the isolates were increased in seedlings or cuttings of Etrog citron. Symptoms appeared 20-30 days after inoculation in new flushes of growth. Symptoms of CVV-1 were strong and distinct, whereas CVV-2 produced barely discernible mottle patterns.

Purification. Viruses were purified with a procedure very similar to that described earlier (11, 12). Young leaf tissue was added to a cold extraction buffer (10 g/30 ml) which contained 0.01 M sodium diethyldithiocarbamate, 0.02 M sodium thioglycolate, 0.02 M potassium phosphate, pH 7.6, and a small amount of Dow antifoam.

These ingredients were homogenized in a Sorvall Omnimixer® or Waring Blendor® at 2-4°C. The homogenate was filtered and centrifuged for 10 minutes at approximately 5,000 x *g*.

[1]Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Calcium phosphate gel (8, 12) was added to the supernatant (7 ml gel per 10 g of starting tissue) and thoroughly mixed. After 5 minutes, the mixture was clarified by low speed centrifugation and centrifuged for 2 hours at 106,000 x g (R_{max}). The virus-containing pellets were dissolved in a small amount of 0.005 M potassium phosphate buffer, pH 7.8. The resuspended pellets were given an additional cycle of differential centrifugation (10 minutes at 6,000 x g, and 1 hour at 321,000 x g). The second high-speed pellet was resuspended in 0.005 M potassium phosphate and 0.005 M $MgCl_2$ (pH 7.2) (RB buffer) and subjected to rate zonal density-gradient centrifugation for 4 hours at 141,000 x g in a Beckman SW 28 Rotor®. Linear density gradients were usually prepared with a Beckman gradient former. Sucrose concentrations of 200 to 500 or 100 to 400 mg/ml in 0.05 M Tris buffer, pH 7.8 were used. After centrifugation, gradient tubes were observed visually with top light for light-scattering zones, and then fractionated and scanned at 254 nm with an ISCO® gradient fractionator and absorbance monitor. Virus zones were collected, concentrated by centrifugation for 2 hours at 106,000 x g, and resuspended in a small amount of RB buffer. Virus used as inject antigens was subjected to a second cycle of density-gradient centrifugation.

Healthy leaf tissue was also processed to evaluate the effectiveness of the purification steps and to identify host components.

An extinction coefficient of 5.2 at 260 nm was assumed for a 1 mg/ml suspension of purified virus.

Infectivity assay. Black local Cowpea and California Wonder bell pepper were used for infectivity assays. Plants were grown in steam-sterilized potting soil and were kept in a partially shaded greenhouse cooled by evaporative coolers. When greenhouse temperature exceeded 27°C, plants inoculated for virus increase or assay purpose were held in a clear, plastic-covered, air-conditioned chamber (24±2°C).

Inocula were prepared in cold 0.05 M potassium phosphate buffer, pH 7.2, and applied with sterile cotton swabs to leaves dusted with 400 mesh-carborundum. Inoculated leaves were usually rinsed with deionized water.

Formaldehyde treatment. The purified virus was treated with formaldehyde to stabilize capsid structure and enhance immunogenicity (3, 7, 15). A virus suspension in 0.05 M phosphate buffer was treated with 1% formaldehyde after recovery from the second sucrose density gradient. After 4 hours at room temperature, sucrose was added (70 mg/ml) and the virus was lyophilized and stored at -60°C until use.

Serology. A New Zealand white rabbit was immunized with a combination of intramuscular and foot pad injections of purified, formaldehyde-treated CVV-2. The purified virus was emulsified (1:1) with Freund's incomplete adjuvant prior to injection. The rabbit received about 0.65 mg of purified virus in the first injection, followed by a similar injection 14 days later. Three additional injections with approximately 1 mg quantities of purified virus were made 4, 8 and 11 months after the initial injection.

The rabbit was bled from the ear periodically and the serum was separated and stored at -20°C or lyophilized. The serum was diluted with 50% glycerol for immunodiffusion titer tests. Plant extracts for gel-diffusion tests were prepared by grinding tissue in 0.02 M sodium phosphate pH 8.0 or 0.05 M Tris pH 7.8. Purified virus was used at 100 or 200 μg/ml to titer

antisera. Agar gel double-diffusion tests were conducted as previously described (11, 12).

Enzyme-linked immunosorbent assay (ELISA) was done by the double-sandwich method essentially as described for citrus tristeza virus (1, 2) except that antigens were normally prepared in 0.05 M Tris buffer. The γ globulins were prepared by ammonium sulfate precipitation and chromatography on DEAE-cellulose (Sephacel)®. Enzyme conjugates were prepared to alkaline phosphatase (Sigma) by the single-step glutaraldehyde method.

Electron microscopy. Serologically specific electron microscopy (SSEM) of CVV was accomplished as described by Derrick and Brlansky (5). Carbon-fronted, Parolodion-coated 200 mesh copper electron microscope grids were floated for 30 minutes on a 1:500 dilution of antiserum to CVV, then washed three times with 0.05 M Tris buffer pH 8.0. Antibody-coated grids were floated for 1 hour on diluted CVV preparations, washed once with 0.05 Tris buffer pH 8.0 and three times with distilled water. Virions were positively stained by floating the grids on a 5% uranyl acetate solution in 50% ethanol for 1 minute and were washed in 95% ethanol and dried. The grids were examined with a Philips® 201 electron microscope and were photographed at instrument magnifications of 15,000 to 30,000X.

RESULTS

Purification. Yields of CVV-2 purified from systemically infected, but essentially symptomless young Etrog citron leaf tissue were 1-3 mg/100 g of tissue, and were similar to those for CVV-1 by the same procedure. Good clarification of citrus extracts was obtained with HCP and the second high-speed pellet was normally clear or light brown.

When density gradients through which partially purified CVV-2 preparations had been centrifuged, were illuminated with top light, four zones were visible (fig. 1b). The first zone, which was about 0.8 cm below the meniscus, had an absorption maximum at 280 nm, was also present in gradients from extracts of healthy tissue, and was not infective. Three other prominent zones were visible between about 2.3 and 2.9 cm below the meniscus. All had an UV spectrum typical of nucleoprotein with an E_{max} at 259-261 nm and an E_{min} at 243-245 nm and a E_{260}/E_{280} ratio of 1.32 to 1.34.

Small, colorless pellets were obtained when the virus zones were removed from the density gradient tubes and concentrated by high-speed centrifugation.

When CVV-1 and CLRV-2 were purified simultaneously with CVV-2 and centrifuged on 200-500 mg/ml sucrose gradients for 4 hours at 141,000x *g*, there were four major visible components for CLRV, and three components for both isolates of CVV (fig. 1a). When visualized by top light, the two strains of CVV appeared similar; however, scanning the gradients at 254 nm revealed small but consistent differences. Profiles of CVV-2 showed a higher ratio of lower component (NP-1) to middle component (NP-2) than CVV-1 and lacked a shoulder (probably NP-4) above major upper component (NP-3). Profiles of both CVV-1 and CVV-2 also showed a shoulder below the NP-1 component. These patterns were consistent in repeated experiments.

The gradient on which the virus zones collected from the first gradient was re-centrifuged contained only the three major virus zones (fig. 1c).

The upper component (NP-4)

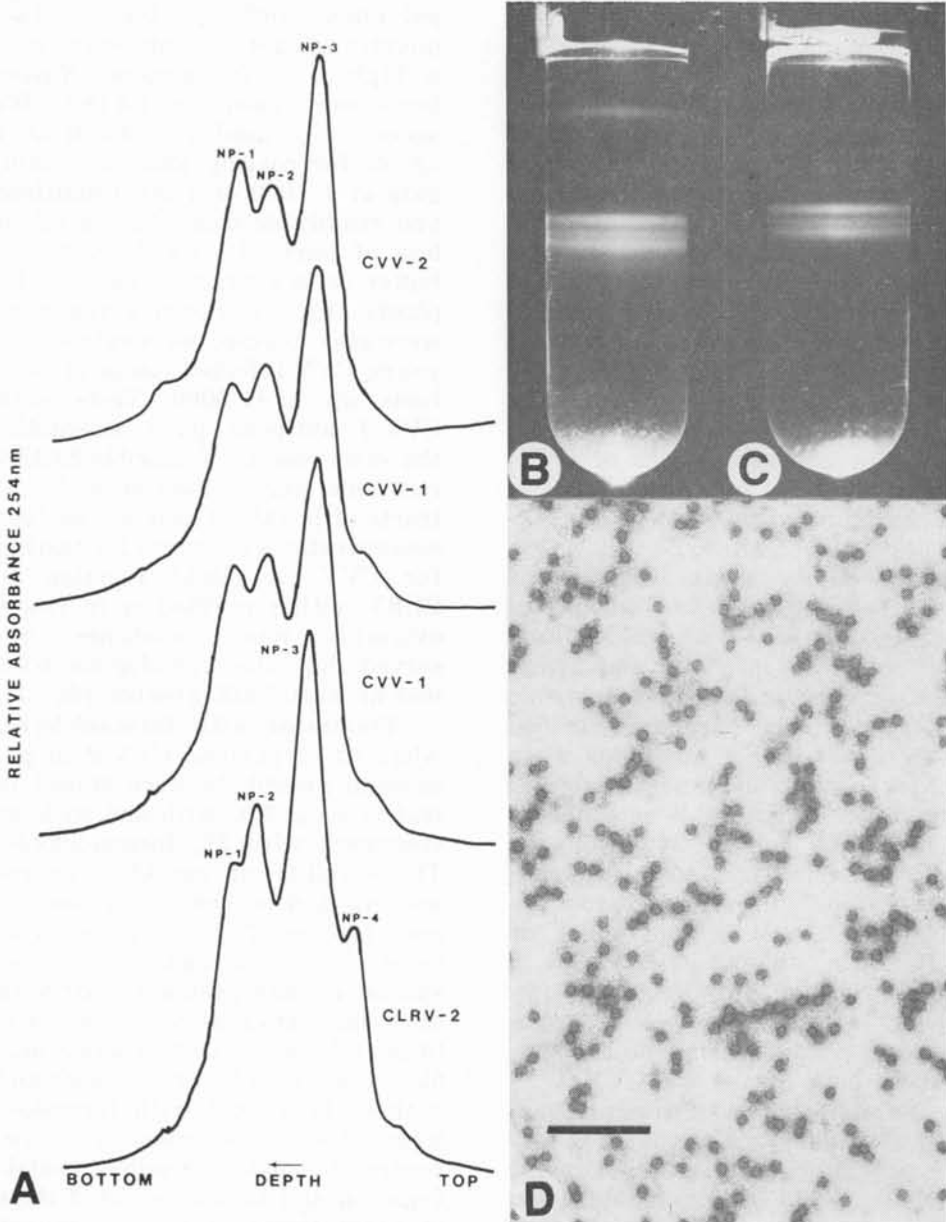

Fig. 1. A) U.V. absorption profiles of mild (CVV-2) and standard (CVV-1) isolates of citrus variegation virus and citrus leaf rugose virus (CLRV) after centrifugation in linear sucrose density gradients. Profile for CVV-2 and upper profile for CVV-1 from 100-400 mg/ml gradients centrifuged 4 hours at 28,000 rpm in SW 28 rotor. Lower CVV-1 and CLRV profiles from 200-500 mg/ml gradients centrifuged 14 hours at 24,000 rpm in SW 28 rotor. Note difference in ratio of NP-1 to NP-2 between CVV-2 and CVV-1; B) first density gradient of partially purified CVV-2 (resuspended high-speed pellet); C) second gradient of CVV-2 virus zones recovered from first gradient; D) Electron micrograph of CVV-2 particles from sucrose rate zonal gradient fixed in formaldehyde, absorbed to serologically sensitized grid, and stained with uranyl acetate. Bar = 0.3 μm.

visible in CLRV-2 gradients (fig. 1a) was not obvious in either CVV strain, especially CVV-2. This is consistent with an earlier report (14) that the NP-4 component of CLRV is much more prominent than the corresponding NP-4 component of CVV.

The slight differences in band positions noted were probably due to variation between gradient tubes. Centrifugations carried out on a 100-400 mg/ml gradients gave similar profiles (fig. 1a). Some variation in relative peak size was noted in different preparations of CVV-2, but the NP-1 peak was always in higher concentration than the NP-2 peak.

Infectivity assay. Inoculations with buffered extracts from tissues collected in the field yielded local and systemic infection and symptoms in cowpea, and mild systemic mottle in bell pepper. Purified CVV-2 was highly infectious when all three components were present. Only NP-1 and NP-2 components caused the typical symptoms of CVV in herbaceous indicator plants when inoculated singly. Direct infectivity comparison of NP-1 or NP-2 vs a combination of NP-1, 2 and 3 showed that the three component combination was more infectious than either single component for both CVV-1 and CVV-2.

Serology. The antiserum collected 7 days after the first immunization had a dilution endpoint (DEP) of 1/4 in immuno-diffusion tests. Antiserum collected 5 days after the second immunization had a DEP of 1/128. The titer gradually decreased over several months to a DEP of 1/16. A booster immunization at this point rapidly increased titer to a DEP of about 1/256. The titer subsequently decreased again but could be restored by subsequent immunizations.

The antiserum to CVV-2 did not react to crude or concentrated extracts of healthy tissue in agar gel immunodiffusion tests. Subsequently aliquots of antiserum with a high titer in immunodiffusion tests were used for ELISA. We successfully used γ globulin at 1 μg/ml for coating and our conjugate at 1/1000 or 1/2000 dilutions and readily detected 0.5 ng/ml or less of purified CVV-2 diluted in buffer or in extracts from healthy plants (fig. 2). Positive reactions were also obtained with extracts of young CVV-infected tissue at dilutions up to 1/5000. Tests with CVV-1 antigens gave essentially the same results. Negligible ELISA reactions were observed with extracts of healthy tissue at reactant concentrations optimum for testing for CVV. A slight reaction to CLRV, either purified or in tissue extracts, was sometimes observed, but the homologous titer was at least 100X greater (fig. 2).

Treatment with formaldehyde. Aliquots of purified CVV-2 in potassium phosphate were stored in test tubes at 5°C with and without treatment with 2% formaldehyde. The stability of capsid structure was checked by comparing absorbancy profiles of the virus preparations after centrifugation in linear sucrose density gradients. After 10 days' incubation at 5°C, there was little difference in absorbance profiles between CVV-2 stored with and without treatment with formaldehyde. The virus zones were recovered by cushion gradient centrifugation and incubated for 3 days at room temperature. Subsequent centrifugation in a linear sucrose gradient showed that degradation had occurred, but that there was little difference associated with the formaldehyde treatment.

Other experiments are in progress to determine the best concentration of formaldehyde and other chemicals to preserve the virion structure. A weaker ELISA reaction was obtained to formaldehyde-treated virus than to the same

concentration of untreated virus (fig. 2).

Electron microscopy. The diameters of particles in purified preparations ranged from about 26 to 35 nm for CVV-2, and 24 to 35 nm for CLRV. The mean was 29.8 and 28.3, respectively. These results are in accordance with earlier reports (14) and since the minimum and maximum size did not differ from those reported earlier, we did not measure particles from single gradient components. The purified virus particles appeared largely intact, but were somewhat pleomorphic (fig. 1c).

DISCUSSION

Our results show that the mild strain of CVV recently discovered in Florida (CVV-2) has physical properties generally similar to the more severe strain (CVV-1) described earlier, but that it contains a somewhat different ratio of the major nucleoprotein components. Differences in ratio percentage be-

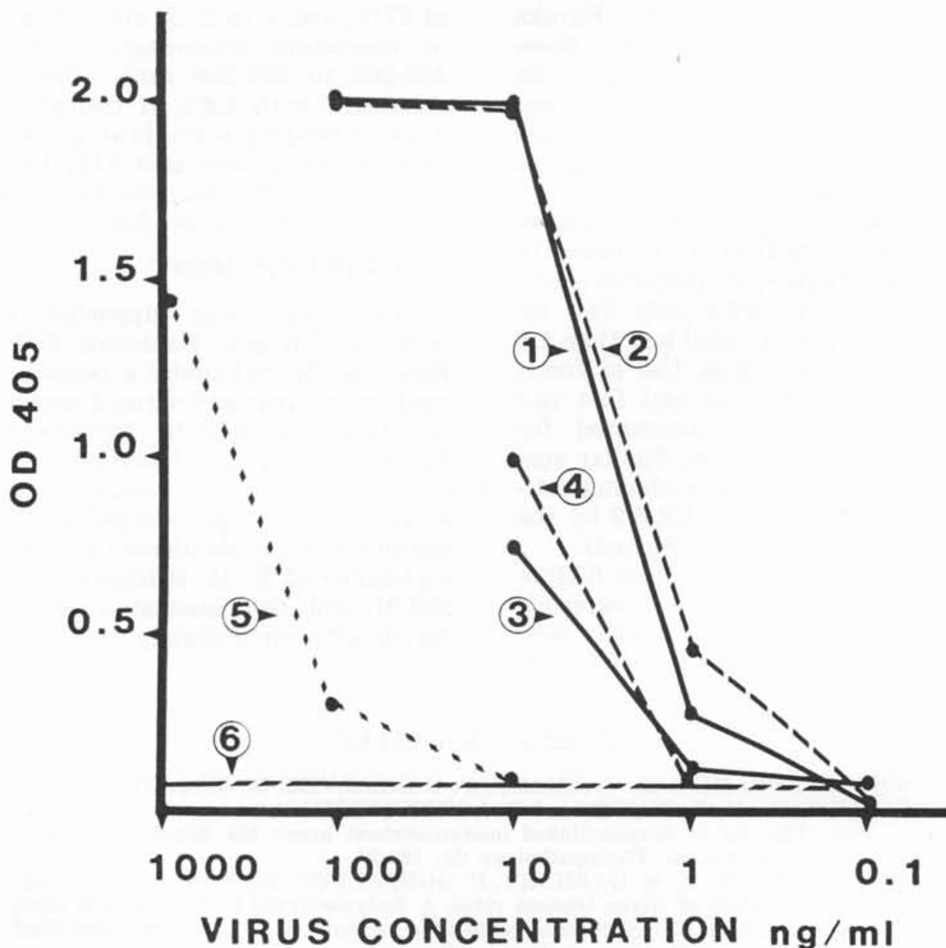

Fig. 2. Reaction of antiserum to citrus variegation virus isolate 2 in double sandwich enzyme-linked immunosorbent assay (ELISA) tests 1) purified CVV-2 diluted in buffer, 2) CVV-2 diluted in a healthy sap extract, 3) formaldehyde-treated CVV-2 diluted in buffer, 4) formaldehyde-treated CVV-2 diluted in sap extract, 5) citrus leaf rugose virus (CLRV) diluted in buffer, and 6) buffer only. γG concentration for coating was 1 μg/ml and γG-alkaline phosphatase conjugate was used at a 1/1000 dilution. (OD_{405} values shown were obtained with optical plate reader and are not true values for 1-cm path length).

tween CV components have been reported in earlier work (18) as an effect of seasonal climatic changes. Since our CVV-1 and CVV-2 isolates were inoculated at the same time and were increased under the same constant conditions of light, humidity and temperature, the different ratios we observed among the NP-1, 2 and 3 components are attributable to the strain difference. Both immunodiffusion and ELISA tests indicate that CVV-1 and CVV-2 are very closely related serologically, if not identical. Although symptoms produced in Eureka lemon by CVV-2 are similar to those produced by CLRV-2 (12), the gradient profiles for these viruses are clearly different and they are clearly different serologically in ELISA tests.

While it has been difficult to produce high-titered antisera to CVV in the past, we prepared a useful antiserum with only two injections which totaled less than 1.5 mg of purified virus. Use of formaldehyde-fixed virus and foot pad injection probably accounted for the improved success. Similar success was obtained in producing antisera to CVV-1 and CLRV-2 by the same methods (unpublished).

Preliminary results from ELISA tests of field-collected materials suggest that ELISA can be a reliable indexing method for CVV (13). Since symptoms of CVV-2 are often undetectable in normally symptomatic hosts such as Etrog citron, serological procedures will be valuable for indexing work. Sensitivity of ELISA for CVV compares favorably with other plant virus systems and is easily achieved under standard test conditions.

The effects of formaldehyde on stability of purified virus particles *in vitro* were less than expected and further tests are warranted. Rana *et al.* (18), obtained a 25% increase in O.D. after a 24-hour incubation at 27°C, and a shift of about 8 nm in maximum wavelength (from 258-260 to 267-268 nm) after a treatment with 1.8% of formaldehyde, indicating a single strandedness of the nucleic acid (17, 19), however, our shorter treatment did not produce the same effect.

ACKNOWLEDGMENT

This paper was supported in part by Consiglio Nazionale delle Ricerche (Rome) under a research project on virus and virus diseases of citrus developed by Istituto di Patologia vegetale, Universita di Catania. We also gratefully acknowledge the expert technical assistance of C. T. Henderson and the assistance of R. H. Brlansky with SSEM and the assistance of R. Smith with photography.

LITERATURE CITED

1. BAR-JOSEPH, M., S. M. GARNSEY, D. GONSALVES, M. MOSCOVITZ, D. E. PURCIFULL, M. F. CLARK, and G. LOEBENSTEIN
1979. The use of enzyme-linked immunosorbent assay for detection of citrus tristeza virus. Phytopathology 69: 190-94.
2. BAR-JOSEPH, M., S. M. GARNSEY, D. GONSALVES, and D. E. PURCIFULL
1980. Detection of citrus tristeza virus. I. Enzyme-linked immunosorbent assay (ELISA) and SDS-Immunodiffusion methods, p. 1-8. *In* Proc. 8th Conf. IOCV, IOCV, Univ. California, Riverside.
3. BEKKER, M. F.
1966. M.Sc. Dissertation, University of Stellenbosh.
4. CORBETT, M. K., and T. J. GRANT
1967. Purification of citrus variegation virus. Phytopathology 57: 137-43.
5. DERRICK, K. S., and R. H. BRLANSKY
1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66: 815-20.
6. DESJARDINS, P. R., and J. V. FRENCH

1970. Purification of the citrus infectious-variegation virus by density gradient electrophoresis. Virology 40: 746-51.

7. FRANCKI, R. I. B., and N. HABILI
   1972. Stabilization of capsid structure and enhancement of immunogenicity of cucumber mosaic virus (Q strain) by formaldehyde. Virology 48: 309-15.
8. FULTON, R. W.
   1959. Purification of sour cherry necrotic ringspot and prune dwarf viruses. Virology 9: 522-35.
9. GARNSEY, S. M.
   1968. Additional non-citrus hosts for the Florida isolate of citrus variegation virus. Phytopathology 58: 1433-34.
10. GARNSEY, S. M.
    1968. A citrus crinkly-leaf-type virus recently discovered in Florida. Proc. Fla. State Hort. Soc. 81: 79-84.
11. GARNSEY, S. M.
    1974. Purification and serology of a Florida isolate of citrus variegation virus, p. 169-75. *In* Proc. 6th Conf. IOCV, Univ. California, Div. Agric. Sci., Richmond.
12. GARNSEY, S. M.
    1975. Purification and properties of citrus-leaf rugose virus. Phytopathology 65: 50-7.
13. GARNSEY, S. M., N. BAKSH, M. DAVINO, and J. P. AGOSTINI
    1983. A mild isolate of citrus variegation virus found in Florida citrus. p. 188-195 this volume.
14. GONSALVES, D., and S. M. GARNSEY
    1976. Association of particle size with sedimentation velocity of the nucleoprotein components of citrus variegation and citrus leaf rugose viruses, p. 109-15. *In* Proc. 7th Conf. IOCV, IOCV, Univ. California, Riverside.
15. HOLLINGS, M., and O. M. STONE
    1962. Use of formalin-treated antigen in the production of antiserum to a plant virus. Nature (London) 194: 607.
16. MARTELLI, G. P., G. MAJORANA, and M. RUSSO
    1968. Investigations on the purification of citrus variegation virus, p. 267-73. *In* Proc. 4th Conf. IOCV, Univ. Florida Press, Gainesville.
17. MAYOR. H. D., L. JORDAN, and M. ITO
    1969. Deoxyribonucleic acid of adeno-associated satellite virus. J. Virology 4: 191-94.
18. RANA, G. L., A. QUACQUARELLI, and G. P. MARTELLI
    1974. Some properties of citrus variegation virus, p. 165-68. *In* Proc. 6th Conf. IOCV, Univ. California, Div. Agric. Sci., Richmond.
19. SINSHEIMER, R. L.
    1959. A single stranded deoxyribonucleic acid from bacteriophage X 174. J. Molec. Biol. 1: 43-53.
20. YOT-DAUTHY, DANIELLE, and J. M. BOVE
    1968. Purification of citrus crinkly-leaf virus, p. 255-63. *In* Proc. 4th Conf. IOCV, Univ. Florida Press, Gainesville.