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Title
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This paper describes a simple procedure for purifying the Florida isolate of citrus variegation virus (F-CVV) directly from citrus tissue, the preparation of an antiserum to F-CVV, and serological tests with this serum.

Several isolates of citrus variegation virus (CVV), including F-CVV, have been purified from leaf tissue of herbaceous and citrus hosts (*1, 3, 9, 11*). Generally, citrus has not been considered a favorable increase host. Most earlier purification attempts involved clarification of extracts from infected plants with chloroform, or a mixture of chloroform and butanol.

While no antiserum to F-CVV has been prepared previously, antisera to other CVV isolates have been reported (*2*). Desjardins and Wallace (*4*) prepared the first antiserum to a California isolate of CVV, using partially purified extracts from citrus. This serum had a low titer of virus antibodies, and con-

tained antibodies-to-host antigens. Martelli *et al.* (*9*) prepared an antiserum to purified CVV from Italy, but their serum also had a low titer of virus antibodies, and reacted strongly with sap from healthy plants. Recently, Desjardins prepared a high-titer antiserum to a California isolate of CVV purified from cowpea (personal communication). A serological relationship between the California isolate of CVV and cowpea mosaic virus has been reported (*2, 5*).

I recently purified citrus leaf rugose virus (CLRV), which is related to F-CVV, directly from citrus tissue by a relatively simple procedure that included calcium phosphate gel clarification (manuscript in preparation). A specific antiserum to this virus, of moderate titer, was developed.

In the present study, procedures developed for CLRV were applied to F-CVV.

MATERIALS AND METHODS

Virus source. The isolate of F-CVV used in this study was described previously in a host-range study (*7*). It originated from the source described by Grant and Smith (*8*) and used by Corbett and Grant (*1*) in their studies on purification of CVV.

Host plants. The citrus plants used as increase hosts in this study were rooted cuttings of Eureka lemon and seedlings of Rangpur lime. Plants of *Nicotiana glutinosa* L., *N. tabacum* L. cv Turkish, and *Phaseolus vulgaris* L. cv Red Kidney were also used as increase hosts.

Cowpea plants were used as local-lesion hosts for infectivity assays.

All plants were grown in steam-sterilized potting soil and were kept in a

partially shaded greenhouse cooled by evaporative coolers. When greenhouse temperatures exceeded 27°C, plants inoculated for virus increase or assay purposes were held in a clear, plastic, air-conditioned chamber (24 ± 2°C).

Transmission. F-CVV was mechanically transmitted by conventional leaf-abrasion procedures (*7*). Citrus plants for virus increase were graft-inoculated from a single source plant.

Purification. Virus was purified by the same procedure used for CLRV (Garnsey, manuscript in preparation). This procedure included the clarification method described by Fulton (*6*), differential centrifugation, and density-gradient centrifugation. Leaf tissue was added to a cold solution containing

0.01 M sodium diethyldithiocarbamate, 0.02 M sodium-thioglycolate, and 0.02 M potassium phosphate, which had a final pH of 7.4. Thirty ml of solution were used for each 10 gm of tissue. These ingredients were homogenized in a Sorvall Omnimixer adapted for a 1-quart container. A small amount of Dow Antifoam A was included to minimize foaming. The mixer was run at low speed for one minute, to coarsely chop the tissue, then at high speed for 30 to 60 seconds. This and all other steps in the purification process were performed in an ice bath or under refrigerated conditions.

The homogenate was expressed through cheesecloth, and the debris was re-extracted with a smaller volume of buffer. Filtrates were combined and centrifuged for 15 minutes at 2,200 g. All centrifugations were performed in a Spinco model L centrifuge at 0 to 4°C. The g values are for R_{av} values of the rotor used. Calcium phosphate gel, prepared according to Fulton (6), was added to the supernatant at a rate of 6 ml per 10 gm of starting tissue, and thoroughly mixed by means of a large syringe. The gel had been concentrated by centrifugation at 2,700 g for 5 minutes, and was like paste. After standing a few minutes, this mixture was clarified by centrifugation for 15 minutes at 2,200 g, and the virus in the supernatant was pelleted by centrifugation for 90 minutes at 78,000 g. Pellets were dissolved in a small amount of resuspending buffer (RB) containing 0.005 M potassium phosphate and 0.005 M $MgCl_2$ (pH 7.2). Resuspended pellets were given an additional cycle of differential centrifugation for 10 minutes at 6,000 g, followed by 60 minutes at 150,000 g. The final high-speed pellet was resuspended in RB, and subjected to rate zonal density-gradient centrifugation for 3 hours at 63,000 g in a SW25.1 rotor. The brake was not operated during gradient centrifugation. Density gradients were prepared with a Beckman gradient former. Sucrose concentration of the gradients varied linearly from 110 to 390 mg/ml in neutral, 0.02-M potassium phosphate buffer. Following centrifugation, gradient tubes were scanned visually with top light for light-scattering zones, and then fractionated and scanned photometrically at 254 nm on an ISCO density-gradient fractionator. Virus zones from the gradient tubes were collected and dialyzed overnight against 0.02-M potassium phosphate buffer, pH 7.2. Unless otherwise noted, virus zones were collected together. The dialyzed virus solution was centrifuged for 60 minutes at 150,000 g and resuspended in a small amount of RB.

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

UV absorption of virus solutions was monitored on a Beckman DB-G double-beam spectrophotometer.

Electron microscopy. Purified virus was stained by mixing a suspension 1:1 either with a 2 per cent solution of potassium phosphotungstate (PTA), pH 6.7, containing 0.25 per cent bovine serum albumin, or with 1.0 per cent solution of uranyl acetate. The virus mixture was placed on electron microscope grids covered with carbon-coated formvar membranes, and examined in a Phillips model 200 electron microscope. Magnification was determined by photographing a diffraction grating (52,864 lines/inch) at the same instrument magnification.

Serology. Rabbits were immunized by a combination of intramuscular and intravenous injections of purified virus in normal saline, over a several-month period. For intramuscular injections, the virus was mixed 1:1 with Freund's incomplete adjuvant. Serum was separated from blood and preserved by adding sodium azide to a final concentration of 0.02 per cent.

Agar gel double-diffusion tests were

conducted in 100×15-mm plastic petri plates loaded with 12 ml of agar solution containing either 0.75 per cent Epiagar (Colab Laboratories, Inc.) or 0.6 per cent Noble Agar (Difco Laboratories) and 0.02 per cent sodium azide. Wells were cut in the solidified agar with an Auto-Gel Punch (Grafar Corp.).

Plant extracts for gel diffusion tests were obtained by expressing sap from leaf tissue with a hand press, or by grinding tissue in a buffer containing 0.02 M sodium phosphate and 0.02 M sodium sulfite (pH 8.0).

RESULTS AND DISCUSSION

Purification from citrus. The hydrated calcium phosphate gel treatment provided good clarification of citrus extracts. The second high-speed pellet following clarification was clear to light brown in color. This pellet resuspended very rapidly, and the supernatant had to be decanted quickly to avoid loss of virus. Infectivity assays did not indicate a large loss of virus at any individual step.

Density gradients of the resuspended high-speed pellet from CVV-infected tissue normally contained four visible zones. The first zone, approximately 9 mm below the meniscus, was faintly visible with top light, and presumably consisted of fraction 1 protein (*10*). A similar zone occurred in gradients from extracts of healthy tissue. A second zone, approximately 18 to 20 mm below the meniscus, was not visible with top light, but was visible as a light-brown zone against a white background. This zone was also present in extracts from healthy plants, and probably contained phytoferritin (*1*).

Two closely spaced, light-scattering zones were readily visible just below the second zone. These lower zones were absent in extracts from healthy plants. The relative position of all zones is shown by the absorbency profiles of density gradients from extracts of F-CVV-infected and healthy Eureka lemon plants (fig. 1). The absorbency profile of the F-CVV gradient also indicated that the lower virus zone actually contained more than one component. These results are similar to others (*1*).

Small, colorless pellets were obtained from virus zones, removed from the density-gradient tubes, dialyzed, and concentrated by high-speed centrifugation. These pellets resuspended readily and were highly infectious even at a 10^{-4} dilution. Purified F-CVV caused typical symptoms in citrus and herbaceous indicator plants.

Purification results with tissue from Rangpur lime and Eureka lemon were similar. Results were consistent if young, very succulent leaves from plants growing vigorously under moderate temperatures were used. One-ml quantities of purified F-CVV with an $O.D._{260}$ of 5.0 to 8.0 were usually obtained from 100 gm of leaf tissue. F-CVV was also purified from frozen Rangpur lime tis-
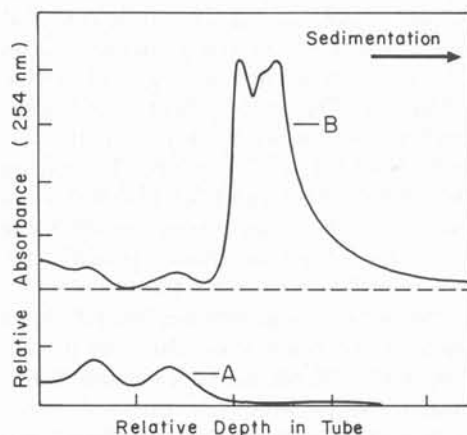

Fig. 1. Absorbance profile of centrifuged sucrose-density gradients of partially purified preparations from (A) healthy Eureka lemon leaves and (B) CVV-infected lemon leaves. Partially purified preparations from approximately 60 gm of leaf tissue were centrifuged for 3 hours at 25,000 rpm in a SW 25.1 rotor. Width of flow cell in scanning device was 5 mm.

sue, but yields were approximately one-half those from comparable fresh tissue.

Purification from other hosts. Several attempts were made to purify F-CVV from systemically infected plants of Red Kidney bean, *Nicotiana glutinosa* and Turkish tobacco with the same procedure described above. Purified F-CVV was obtained from each of these increase hosts, but calcium phosphate gel clarification was not as satisfactory. High-speed pellets from the second differential centrifugation were green and fairly large. Density gradients of this material were overloaded with host components, which contaminated the virus zones.

Adequate purification of F-CVV from herbaceous hosts was achieved only with additional treatment. Readsorption of the high-speed pellet with 1 to 2 ml of gel after the pellet was resuspended in 10 ml of extraction buffer removed much of the host material and allowed clean separation of the virus during subsequent density-gradient centrifugation. An alternate approach was to use two cycles of density-gradient centrifugation. After the first one, virus zones contaminated with host material were removed, dialyzed, pelleted, and given a second density-gradient centrifugation. The ratio of host constituents to virus was more favorable in the second gradient, and adequate separation occurred. Final yields of purified virus were lower than from comparable amounts of citrus tissue, possibly because of losses in extra treatments.

More experimentation might have produced a better procedure for purifying F-CVV from herbaceous hosts, but such studies were not pursued, since adequate quantities of purified virus could be readily obtained from citrus tissue. Furthermore, use of citrus as an increase host precluded contaminant viruses in herbaceous increase hosts, and allowed repeated harvest of tissue from increase plants following a single inoculation.

Properties of purified F-CVV. Samples from the lower virus zone of density gradients were about five times more infectious than were samples of the upper zone diluted to the same $O.D._{260}$ value. Infectivity in the top zone may have been due to contamination with bottom-zone particles, since separation of zones was probably not complete in these gradients. However, similar infectivity measurements have been reported previously ([1]). Further studies on separation and analysis of the various virus components would be desirable.

Sedimentation rates for the virus zones in density gradients were similar to CLRV. The lower zone sedimented slightly slower than did purified southern bean mosaic virus obtained from the Plant Virus Laboratory, University of Florida. This is consistent with the 110S value reported for F-CVV ([1]).

The absorption spectrum of purified F-CVV was typical of a nucleoprotein, with a minimum at 243 to 245 nm and a maximum at 259 to 261 nm. The $O.D._{max}/O.D._{min}$ ratio was 1.22 to 1.25 and the $O.D._{260}/O.D._{280}$ nm ratio was 1.42 to 1.45. These values are again similar to those reported earlier ([1]).

Examinations of purified F-CVV in the electron microscope revealed many disrupted and deformed particles in preparations stained with PTA. Particle structure was better preserved in preparations stained with uranyl acetate. Most particles were approximately spherical (fig. 2A) and 29 to 30 nm in diameter. The average diameter of 100 particles stained in uranyl acetate was 29.7 nm.

Production of antiserum. About 3 ml of purified F-CVV ($O.D._{260} = 3$ to 6) were injected into a rabbit over a six-week period by intraveinal and intramuscular routes. Test bleedings during, and for two weeks after these injections, indicated a low titer of F-CVV antibodies. A subsequent injection of about 1.5 ml of purified virus ($O.D._{260} = 5$) resulted in a surge of antibody produc-
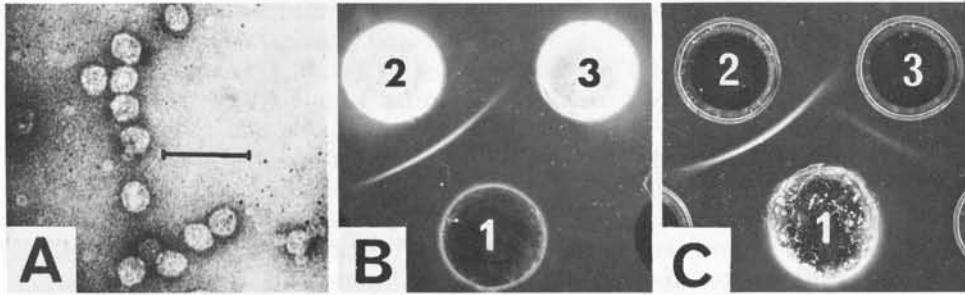
Fig. 2. *A*, electron micrograph of purified F-CVV particles stained with uranyl acetate. Bar = 100 nm. *B*, serological reaction in an agar gel plate between undiluted F-CVV antiserum (well 1), an extract from young, F-CVV-infected lemon leaves concentrated 5 times by differential centrifugation (well 2), and a 5-times concentrate of young, healthy lemon leaves (well 3). *C*, serological reaction between F-CVV antiserum (well 1), purified F-CVV (well 2), and purified citrus leaf rugose virus (well 3). A healthy extract of lemon leaves was added to well no. 1 about 3 hours before addition of the other reactants to cause intragel adsorption of antibodies to host antigens which might be present in the serum. Note formation of spur at junction of precipitation lines, indicating heterologous relationship between the two viruses.

tion, and after 10 days, a serum was obtained with a dilution end-point of $\frac{1}{64}$ to $\frac{1}{128}$ in agar gel double-diffusion plates. Titer slowly decreased after about a month, and an additional booster injection of about 1.5 ml of virus of similar concentration was given. Titer increased to the previous level and then slowly decreased.

Injection of greater quantities of virus, fixation of the virus, use of a different rabbit, or a different injection schedule might have resulted in greater antibody production. It is also possible that F-CVV, like some spherical viruses, is not strongly antigenic (*10*).

Serological tests. The antiserum obtained reacted to purified F-CVV (fig. 2C), and to buffered extracts of F-CVV-infected citrus, cowpea, Red Kidney bean and *Nicotiana glutinosa* plants in gel diffusion tests. It did not react visibly with similar extracts from healthy plants. No reaction was obtained with extracts of healthy Eureka lemon concentrated 5 times (fresh weight) by differential centrifugation, while a similar concentrate of infected tissue produced a strong reaction (fig. 2B). Buffered extracts of young, infected citrus leaves (1 gm of tissue triturated in 4 ml of

buffer) produced visible lines when tested against diluted antiserum. One zone of precipitation usually formed between the antigen and antibody wells. A second line sometimes formed, depending on the test conditions, the preparation of the antigen, and the concentration of the reactants. Proper concentration of the reactants was essential for production of clear, sharp precipitation lines (*10*). Purified F-CCV at a concentration of $O.D._{260} = 2$ gave an optimum reaction with undiluted serum.

Antiserum to F-CVV reacted in a heterologous manner to purified CLRV (fig. 3C) and to extracts from CLRV-infected plants. Spurs formed at the junction of the precipitation lines, and the heterologous titer was lower. This heterologous reaction was similar to that observed between CLRV antiserum and purified F-CVV (Garnsey, manuscript in preparation).

Antiserum prepared to F-CVV reacted to a California isolate of CVV in extracts from infected bean, cucumber, and lemon tissue and to a preparation of California CVV purified from *Gomphrena globosa* L. It also reacted to a California isolate of crinkly leaf virus in extracts from infected lemon

tissue (P. R. Desjardins, personal communication).

The F-CVV antiserum did not react with purified cowpea mosaic virus (CP MV), provided by Dr. C. L. Niblett. Antiserum to this source of CPMV also failed to react with purified F-CVV. Virus dilutions from $O.D._{260}$ of 2.0 to 0.25 were tested against sera diluted $\frac{1}{2}$ to $\frac{1}{1024}$. Antisera to cowpea mosaic (Arkansas strain), broad bean true mosaic, and squash mosaic viruses provided by Dr. R. J. Shepherd also failed to react with F-CVV. While these results do not rule out a serological relationship between F-CVV and CPMV, they at least indicate that F-CVV is not closely related to these serotypes of CP MV. A higher titer serum to F-CVV would be desirable.

SUMMARY

A Florida isolate of citrus variegation virus (F-CVV) was purified from infected citrus and herbaceous plant tissue by combination of calcium phosphate gel clarification, differential centrifugation, and density-gradient centrifugation. Antiserum prepared by injecting purified F-CVV into rabbits reacted specifically to purified virus and to extracts from F-CVV-infected citrus and herbaceous hosts. A serological relationship between citrus leaf rugose virus and F-CVV was confirmed, but no evidence was found for a serological relationship between F-CVV and cowpea mosaic virus.

ACKNOWLEDGMENTS

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