Purification and Properties of Hibiscus Chlorotic Ringspot Virus

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ABSTRACT

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A serologically identical virus was isolated from 14 cultivars of *Hibiscus rosa-sinensis,* some of which showed mosaic or chlorotic rings, or spots of various sizes and intensity on most leaves. The virus was readily isolated when flower petals were triturated in buffer and the homogenate rubbed on *Hibiscus cannabinus* (kenaf) seedlings. The virus infected 12 of 13 species of the Malvaceae that were tested, but infected only 8 of 54 species representing 21 other families. The only nonmalvaceous genera with susceptible species were *Chenopodium, Gomphrena, Phaseolus, Vigna, Antirrhinum, Digitalis,* and *Torenia.* The best local lesion hosts were *Chenopodium quinoa* and *C. amaranticolor,* with distinct chlorotic lesions, and kenaf, which produced necrotic lesions. Yields of the single-component virus purified from kenaf averaged 0.35 mg/g of tissue. Virus particles, stained with 2% phosphotungstic acid adjusted to pH 6.8, measured 27-30 nm. An immunized rabbit produced an antiserum with a titer of 1:512. The virus was serologically unrelated to any of 43 other spherical viruses. Sap from diseased kenaf remained infectious for 30 days at 22 C, when heated to 72 C for 10 minutes, or when diluted 1×10^{-8}. Ratio of bases was A = 25.5, C = 26.4, G = 23.8, and U = 24.3. The virus probably is the same as the Hibiscus ringspot virus described in the literature.

Additional key words: electron microscopy, ribonucleic acid, RNA.

Virus-like diseases of *Hibiscus rosa-sinensis* L. include the whitefly-transmitted yellow vein mosaic (2, 15) and Hibiscus leaf curl (13) in India; line pattern of *H. rosa-sinensis* in South Africa (19); and mosaic/ringspot in Hawaii (9). None of these disease agents has been mechanically transmitted. A mosaic disease of *Abelmoschus manihot* (L.) Medic. in New Guinea (17) and a ringspot of *H. rosa-sinensis* in Nigeria (12) have been mechanically transmitted.

Chlorotic spots and mosaic were observed on the leaves of *H. rosa-sinensis* 'Flame' imported to the U.S. from El Salvador. Another introduction of *H. rosa-sinensis* from Thailand showed chlorotic ringspots. A virus was isolated from flower tissue of each source into *H. cannabinus* L. (kenaf). This report deals with the properties of a virus isolated from the cultivar Flame and the effect this virus has on certain other species, widely grown in Central America, which are troubled with virus-like disorders.

MATERIALS AND METHODS

The virus was readily isolated from source plant flower petals by triturating tissue in 10-30 volumes of 0.1 M K_2HPO_4 and rubbing the tissue extract on kenaf leaves. In addition to the virus isolates above, virus was also isolated from hibiscus growing along the roadside at Brownsville, Texas and from *H. rosa-sinensis* cultivars from nine U.S. arboretums and commercial nurseries. The cultivars were: Belle of Portugal, Betty Yellow, Double Rainbow, Golden Belle, Golden Glow, Indian Chief, Joan Lum, Overture, Rossestey, Virginia Welch, Superba Hybrid, Kainana, Waimea Pink, and unidentified types. Virus was not isolated from *H. rosa-sinensis* cultivars Double Bride, Gast-57, Jim Hendry, Royal Kahala, Rose-of-China, Vivicans, or Vulcan; nor from other *Hibiscus* species including *H. scotti* Bulf. f., *H. schzopetalus* Hook. f., *H. syriacus* L., *H. moscheutos* L., *H. coccineus* Walt., and *H. militaris* Cav.

Some of the virus antisera used to identify and determine relationships of the hibiscus virus were produced in our laboratories. They were for the viruses of: brome mosaic, broad bean mottle, bean mild mosaic *(manuscript on purification, serology, and properties in preparation),* cowpea mosaic, carnation ringspot, carnation mottle, cucumber mosaic, cherry leafroll (dogwood strain), peanut stunt, southern bean mosaic, sowbane mosaic, tobacco ringspot, tomato aspermy, and turnip yellow mosaic antisera.

Other antisera were obtained for the viruses of: M. Hollings, petunia asteroid mosaic and pelargonium leaf curl; J. Fulton, elm mosaic; R. Fulton, Prunus necrotic ringspot and prune dwarf; R. Koenig, eggplant mosaic and Andean potato latent; J. Bancroft, cowpea chlorotic mottle; J. Uyemoto, peach rosette mosaic; A. Jones, elderberry latent; C. Wetter, tomato bushy stunt; R. Gamez, bean virus del mosaico rugoso; H. Moline,

Physalis mottle; C. Valenta, red clover mottle; L. Givord, okra mosaic; D. Maat, cherry leafroll and raspberry ringspot; P. Catherall, lolium mottle and cocksfoot mottle; R. Kahn, eucharis mosaic; R. Woods, Dulcamara mottle; A. Quacquarelli, chicory yellow mottle, artichoke crinkle, and almond mosaic; C. Bercks, belladonna mottle; and D. Walkey, arabis mosaic. Antisera to bean pod mottle, desmodium yellow mosaic, cucumber mosaic C strain, and peanut stunt Western strain, were obtained from the American Type Culture Collection, numbers 9, 12, 39, and 45, respectively.

Sources of pancreatic ribonuclease, anion exchange resin for base ratio studies, other specialized chemicals, and descriptions of equipment used in this study were the same as previously reported (18). Electrophoresis apparatus and procedures were the same as described by Kaper and West (11).

An experimental host range and common physical properties of the virus isolate from Flame were determined as described (18). Kenaf was used as the maintenance host for the hibiscus virus and was the source of virus for host range studies. Back indexing of inoculated symptomless experimental species was done on kenaf. Kenaf was also the source of virus that was used for bioassay in thermal inactivation, dilution end point, and aging in vitro experiments.

Purification.—Fresh kenaf leaves from plants inoculated 2-4 weeks earlier, were blended in a 0.1 M phosphate-borate extraction buffer (made up of 152 ml of 0.1 M KH_2PO_4, 8 ml of 0.1 M Na_2HPO_4, 2.16 g boric acid salt) containing 0.02 M 2-mercaptoethanol, and adjusted to pH 6.5 with 0.1 N sodium hydroxide. During blending, cold chloroform was added so that the final ratio of tissue-buffer-chloroform was 1:3:0.2 (w/v/v). The slurry was centrifuged at 12,000 *g* for 10 minutes. Virus in the clear yellow supernatant fluid was then concentrated by one of three methods: (i) centrifuged at 105,000 *g* for 75 minutes; (ii) mixed with an equal volume water-saturated ammonium sulfate, incubated 30 minutes at 4 C, and recentrifuged at 14,000 *g* to pellet the virus; or (iii) mixed to 10% with polyethylene-glycol 6000, stirred, and cooled 15 minutes at 4 C, and again centrifuged at 14,000 *g*. Pelleted virus was resuspended in 0.02 M sodium acetate buffer, pH 6.0, and after several hours these opalescent preparations were centrifuged at 10,000 *g* to remove plant materials. The virus was further purified by centrifugation for 2 hours in 10-40% sucrose gradients containing one of two ionic strengths of the above phosphate-borate buffer. They were: (i) the above 0.1 M buffer containing 40% sucrose, which was diluted with the same buffer, or (ii) 0.05 M buffer containing 40% sucrose and diluted with water.

The single opalescent band was collected by use of an ISCO system (Instrumentation Specialties Co., Lincoln, Nebraska), bioassayed, and dialyzed 24 hours in 0.02 M sodium acetate buffer; the virus was reconcentrated by centrifuging 2 hours at 105,000 *g*. The final pellets were resuspended in 0.02 M pH 6.0 acetate buffer, dialyzed again to remove traces of sucrose, and was used in phosphorus and nitrogen determinations, for RNA extraction, electron microscopy, or for immunizing a rabbit.

Electron microscopy.—Virus from gradient fractions was analyzed with the electron microscope. Samples in 0.02 M sodium acetate buffer were placed on Formvar-coated grids and negatively stained with 2% phosphotungstic acid adjusted to pH 6.8 with KOH. Instrument magnification was calculated by use of 310- and 500-nm polystyrene latex as standards.

Immunization and serology.—Antiserum to the hibiscus virus was produced by injecting a rabbit once a week with 2-4 mg of virus in 1 ml of buffer. The first 2 weeks virus was injected intravenously. The last 4 weeks virus was emulsified with an equal volume of Freund's incomplete adjuvant and then injected intramuscularly. Bleedings of 35 ml began with the fourth injection and were repeated for 7 weeks. For serological tests, we used the gel double-diffusion method in 0.75% Ionagar No. 2 containing 0.02% sodium azide. Readings were made after 24 and 48 hours. Titer was determined by making a two-fold dilution series of samples from each bleeding and the antibodies were permitted to react with at least two concentrations of purified virus. All other antisera tested against the hibiscus virus were diluted 1:2 and 1:8.

Ribonucleic acid content determined by ratio of total nitrogen to phosphorus.—The percent RNA in a virus can be established by the determination of percent P and of the specific extinction coefficient from dry-weight-optical density data (18). For accurate data, however, large amounts of purified virus are needed. The ratio of N to P in virus provides another method for calculating percent RNA (10) and requires only about 3 mg per test. Nitrogen was determined by the micro-Kjeldahl method (10), and phosphorus determined as described (18). Multiple P and N determinations were made simultaneously on a stock solution of 1.5 - 2.5 mg/ml of virus in 0.02 M acetate buffer and on control solutions of known content of inorganic phosphorus. Trials on the N:P ratio of a tymovirus, eggplant mosaic, provided another control for these experiments. The formula of Kaper and Litjens (10) was used for calculations.

Ribonucleic acid extraction and infectivity studies.—In the extraction of RNA from purified virus, an ice bath, cold chemicals, and rotors were used throughout. Virus (20-30 mg) was mixed with final concentrations of 0.05-0.08 M sodium phosphate buffer and 2% sodium dodecyl sulfate (SDS) containing a trace of bentonite, and then was phenol-extracted as described (18). Final RNA precipitates were dissolved in water; the solution was read in the spectrophotometer to ascertain freedom of phenol and to determine concentration of RNA ($E_{260}^{0.01}$ = 250 OD), then stored at −20 C.

For determination of infectivity, extracted nucleic acid was diluted with phosphate buffer in a 10-fold series, then rubbed leaves of kenaf. We determined sensitivity of the nucleic acid to RNase by mixing the enzyme in a final concentration of 1 μg/ml with 10 μg/ml RNA and incubating at 0 C and 22 C. These preparations were bioassayed at 10-minute intervals up to 1 hour on kenaf. Base ratio of the RNA was determined by published procedures (18).

RESULTS

Symptoms and host range.—Combinations of mottling and small chlorotic leafspots, which persisted through the year (Fig. 1), were observed in most of the leaves of the virus source plant Flame, whereas small

chlorotic ringspots were the primary symptom in leaves of an unidentified *Hibiscus* sp. from Thailand (Fig. 2). The latter symptom usually faded a month or so after the leaves appeared. No symptoms were observed in the flowers of any infected hibiscus plants and flowering was not visibly affected.

We mechanically inoculated (with the isolate from Flame) 60 species of plants representing 22 families

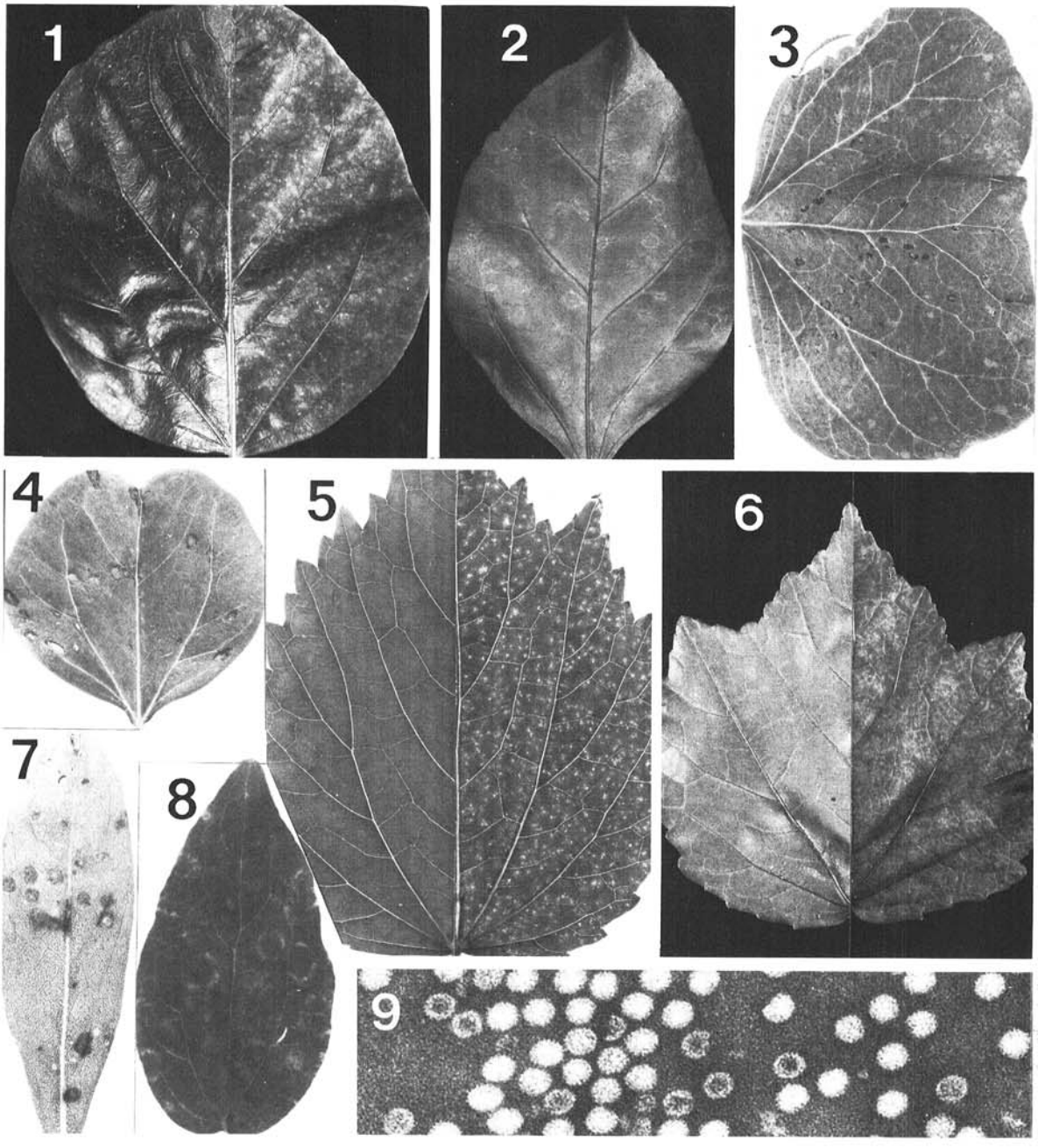

Fig. 1-9. (1-2) Leaves of *Hibiscus* introductions showing spots and rings from which plants the virus isolates were obtained, **1)** *H. rosa-sinensis* from El Salvador and **2)** *Hibiscus* sp. from Thailand. (3-4) Local lesions incited by hibiscus chlorotic ringspot virus (HCRSV) in **3)** *Gossypium hirsutum* and **4)** *H. cannabinus*. (5-6) Systemically infected leaves from **5)** *H. cannabinus* and **6)** *H. acetocella*. The left half of each leaf in Figures 1, 5, and 6 are healthy controls. (7-8) Leaves from seedlings mechanically inoculated with HCRSV, **7)** red lesions in *Gomphrena globosa* and **8)** Systemic chlorotic ringspots in *H. rosa-sinensis*. **9)** Particles of HCRSV × 181,000. About 20% of the particles have disintegrated or have been penetrated by the phosphotungstic acid stain.

including six genera of the family Malvaceae. The limited experimental host range included 12 of 13 species tested in the Malvaceae (Fig. 3-6). Other hosts were: *Antirrhinum majus* L., *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Digitalis purpurea* L., *Gomphrena globosa* L. (Fig. 7), *Phaseolus vulgaris* L. cv. Pinto, *Torenia fournieri* Lind., and *Vigna unguiculata* subsp. *cylindrica* (L.) van Eseltine. Hibiscus virus did not incite a symptom in *Antirrhinum, Digitalis, Torenia,* or *Vigna* and was restricted to the inoculated leaves. The virus incited symptoms in other species (Table 1).

The hibiscus virus did not infect one or more species in any of the following genera: *Amaranthus, Atropa, Beta, Brassica, Calendula, Capsicum, Catharanthus, Celosia, Convolvulus, Coreopsis, Crotalaria, Cucumis, Cucurbita, Cynoglossum, Datura, Dianthus, Dolichos, Euphorbia, Helianthus, Ixora, Lycopersicon, Matricaria, Nepeta, Nicandra, Nicotiana, Pelargonium, Petunia, Physalis, Pisum, Platycodon, Raphanus, Salpiglossis, Salvia, Solanum, Tagetes, Tetragonia, Zea,* and *Zinnia*.

The most useful herbaceous hosts for studying hibiscus virus isolates were *C. quinoa, C. amaranticolor,* and kenaf. All three were especially sensitive to the isolates tested and developed distinct local lesions within 2 weeks. Responses among the eight mechanically inoculated hibiscus species ranged from no detectable infection in *H. mutabilis* to necrotic local lesions within 6 days (Fig. 4) followed with systemic mosaic or chlorotic spots in kenaf (Fig. 5). The symptoms appeared in fully expanded leaves of kenaf and progressed up the stem to a point three-to-five leaves below the growing point. Infected plants were 30-50% shorter than control plants 1 month after inoculation. Okra [*Abelmoschus esculentus* (L.) Moench] reacted only with necrotic local lesions while red-leafed *H. acetocello* showed only systemic veinal chlorosis which later disappeared (Fig. 6). Some *H. rosa-sinensis* seedlings responded, with systemic vein clearing followed by tiny chlorotic spots like those in the source plant (Fig. 1) while others developed chlorotic rings and leaf distortion (Fig. 8). Cotton (Fig. 3), okra and gomphrena (Fig. 7) reacted with local lesions in some trials.

Stability in vitro and aphid transmission.—Sap extracted from kenaf remained infectious when heated to 72 C, incubated at room temperature for 30 days, or diluted up to 1×10^{-8}. Time required for kenaf to react with systemic symptoms correlated inversely with the concentration of infectious virus (Table 2). The virus was

TABLE 1. Symptoms caused by Hibiscus chlorotic leafspot virus in mechanically inoculated seedlings

Family and species	Primary reaction or symptoms
Amaranthaceae	
Gomphrena globosa	Red local lesions
Chenopodiaceae	
Chenopodium amaranticolor	Chlorotic local lesions
C. quinoa	Chlorotic local lesions
Fabaceae (Leguminosae)	
Phaseolus vulgaris 'Pinto'	Pin point necrotic local lesions
Vigna unguiculata subsp. *cylindrica*	Latent
Malvaceae	
Abelmoschus esculentus (okra)	Necrotic lesions - not systemic
A. manihot	Latent infection
Abutilon buchii	Mild systemic chlorosis of veins
Althaea spp.	Mild systemic mosaic
Gossypium hirsutum	Necrotic or chlorotic lesions - no systemic infection
G. sturtianum	Latent infection
Hibiscus acetocella	Systemic chlorosis of veins
H. californicas	Latent infection
H. cannabinus (kenaf)	Necrotic lesions and systemic mosaic
H. lasiocarpos	Latent local infection
H. mutabilis	No infection
H. rosa-sinensis	Local and systemic chlorotic spots and rings
Malva alcea	Mild systemic mosaic
Malvastrum spp.	Mild systemic mosaic
Scrophulariaceae	
Antirrhinum majus	Latent
Digitalis	Latent
Torenia fournieri	Latent

TABLE 2. Physical properties of Hibiscus chlorotic ringspot virus in vitro: correlation of relative concentration of virus with time for systemic symptoms to appear in kenaf

Treatment	Number of days before symptoms appeared
Thermal inactivation point (C, 10 minutes)	
Control	14
60	14
65	15
70	20
75	no symptoms
Aging in vitro (days, 22 C)	
4	14
10	20
15	35
30	49
60	no symptoms
Dilution end point	
Of virus in crude kenaf juice	
Dilution	
10^{-1}	14
10^{-5}	14
10^{-6}	21
10^{-7}	27
10^{-8}	35
10^{-9}	no symptoms
Of purified virus (μg/ml)	
10^{-1}	14
10^{-2}	15
10^{-3}	21
10^{-4}	33
10^{-5}	40

not transmitted by aphids, *Myzus persicae* (Sulzer), from kenaf to kenaf during four trials.

Purification.—Each method of purification produced highly infectious single-component virus preparations. Using an approximated specific extinction coefficient of $E_{260}^{0.01} = 46$ OD, yields ranged from 0.25 mg/g to 0.5 mg/g of leaf tissue among the replicates when the virus was concentrated by ultracentrifugation or by precipitation with ammonium sulfate, but were 50% less when virus was concentrated with polyethylene glycol (PEG 6000). When virus was purified through gradients containing borate buffer, yields averaged 10% more with 0.1 M than with 0.05 M borate.

Rate zonal centrifugation of concentrated virus resulted in a single heavy opalescent band. When the gradients were fractionated, a single sharp ultraviolet (UV)-light-absorbing peak was observed. The fractions that produced this peak were infectious at 1:50,000 dilution to kenaf. The hibiscus virus sedimented in gradients more rapidly than turnip yellow mosaic virus ($=S_{20,w}=117S$) but less rapidly than arabis mosaic virus ($=S_{20,w}=126S$) which served as markers. The average uncorrected $A_{260:240}$ and $A_{260:280}$ ratios were 1.19 and 1.45 respectively.

Electron microscopy.—Negatively stained virus from gradient fractions had intact particles, 27-30 nm in diameter, and particles that were partially filled with stain (Fig. 9). Some of the particles with densely stained cores probably had been degraded during staining rather than previously existing intact as empty shells. This assumption was supported by the presence of partially fragmented or irregular margins on densely stained particles.

Serology.—Hibiscus virus antiserum collected 3 weeks after the first injection had an antibody titer of 1:256 and rose to a maximum of 1:512 with no healthy plant antibody titer. Gel diffusion serology was a rapid and reliable technique for detecting the virus in crude sap of most experimental species; but no reactions were obtained with leaf sap from infected *H. rosa-sinensis,* probably because of low virus titers.

The virus isolates from each of the 14 cultivars of *H. rosa-sinensis* from the USA and from the *H. rosa-sinensis* from Thailand were serologically indistinguishable from the original isolate obtained from Flame.

None of the antisera to other viruses reacted with the hibiscus virus in crude juice or in concentrated purified form during any of three-to-five trials. Likewise, hibiscus virus antisera did not react with any of 12 spherical viruses which were available for tests.

Ribonucleic acid.—The N:P ratio averaged 11.15 from three determinations. With this value in the formula of Kaper and Litjens (10), the RNA of the hibiscus virus was 14.1% of the virus particle. Yields of RNA extracted from known amounts of virus ranged from 70-80% of the total calculated nucleic acid in the sources. The RNA produced a typical UV light absorption curve with an $A_{max/min}$ ratio of 2.05. The RNA molecular weight was about 1.55×10^6 Daltons and was determined by comparison with the RNA's from turnip yellow mosaic virus and cucumber mosaic virus during electrophoresis in 2.4% polyacrylamide gels. Hibiscus virus RNA was infectious in concentrations as low as 10^{-3} μg/ml. All infectivity was abolished within 10 minutes when 10 μg/ml of nucleic acid was incubated at 22 C in the presence of 1.0 μg/ml of RNase. The average ratio of bases of digested RNA was: A = 25.5, C = 26.4, G = 23.8, and U = 24.3.

DISCUSSION

Our hibiscus virus does not appear to be any of the following agents which cause diseases in other malvaceous species: the Malva virus (8), Malva yellows (5), infectious chlorosis of Malvaceae (6), Abutilon mosaic (4), cotton mosaic (3, 16), or yellow vein mosaic of Malvastrum (14) or okra (15). Based on our studies, our hibiscus virus appears not to be any of the common viruses of cotton (11). It probably is not the same as either hibiscus line pattern virus (19) or the mechanically transmissible virus of *Abelmoschus manihot* (17); and is not serologically related to the virus from *A. esculentus* recently described by Givord and Hirth (7). Although our virus infected all three member species of the Scrophulariaceae, it is not scrophularia mottle virus.

Our virus may be the same as that reported to cause mosaic and ringspot symptoms in *H. rosa-sinensis* in Hawaii (9). We isolated virus, which was serologically identical to the hibiscus virus from eight of the nine cultivars which originated in Hawaii. Based on host range and symptoms, our virus could be the same as the hibiscus ringspot virus described by Lana in Nigeria (12).

There are many reports of virus or virus-like diseases of *Abutilon* (4, 6), *Euphorbia, Phaseolus, Dioscorea, Ipomoea, Sida, Rynchosia,* and malvaceous crops (3, 6) in South and Central America where our infected hibiscus plant originated. Our host range data indicate that the hibiscus virus probably is not the same as any of the causal agents of diseases in these crops.

We observed distinct local lesions in cotton, okra, and gomphrena plants rubbed with the hibiscus virus and virus was recovered from these species back into kenaf. These species, however, were not reliable local lesion hosts because in some trials or weather conditions, they became infected but were without lesions.

We observed a single opalescent band and a single sharp ultraviolet light-absorbing peak in sucrose gradient profiles which suggested that it is a single-component virus. Yet several kinds of particles were observed in electron photomicrographs of samples from these fractions (Fig. 9). Most particles were intact, but some were partially RNA-devoid, and others appeared ruptured. These latter particle types may be due to the instability of the virus in the buffer or in PTA stain.

It seems significant that our hibiscus virus contained only 14.1% RNA since single-component spherical viruses with less than 18% RNA are rare. Only Prunus necrotic ringspot and Tulare apple mosaic viruses have been reported to contain comparable levels of RNA, 16% and 12%, respectively (1). Those values, however, were obtained on preparations of mixed particles from more than one density-gradient band; some particles probably contained little or no RNA and would explain the relatively low final values. The tymovirus, used as the control in our N:P experiments, contained 39% RNA.

Our data suggest that this hibiscus virus may be widespread in warm regions of the U. S., especially in Texas, Florida, and Hawaii, where 12 of the 14 virus-infected cultivars originated. The virus was isolated from

14 of 22 cultivars of *H. rosa-sinensis,* but not from any of six other species. Likewise, it either did not infect or did not incite symptoms in five other mechanically inoculated species. Our results are consistent with those of Hendrix and Murakishi (9) who reported that five of six other species that were graft-inoculated with the virus failed to develop symptoms of infection. Consequently, the virus may occur in certain other species, and in a latent state, in some cultivars of *H. rosa-sinensis*.

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