Purification and Serology of Papaya Ringspot Virus

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ABSTRACT

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An isolate of papaya ringspot virus (PRV-HA) obtained from Hawaii was purified, and antiserum to the virus was produced. Papaya infected with PRV-HA had severely distorted leaves, while infected zucchini squash showed intense mosaic and some leaf distortion which was similar to that caused by watermelon mosaic virus 1 (WMV-1). Infectivity of PRV-HA in zucchini leaves was highest 21–25 days after inoculation. Virus was purified by Cs_2SO_4 density gradient centrifugation after clarification of tissue extracts by chloroform/carbon tetrachloride and concentration of virus particles by polyethylene glycol. Aggregation of virus particles was reduced by using EDTA in extraction and resuspension buffers. Antiserum produced to isolated capsid protein of PRV-HA reacted with PRV in sodium dodecyl sulfate (SDS)-immunodiffusion tests. Antiserum produced to "intact" particles of PRV-HA did not generally react with PRV in SDS-immunodiffusion tests but gave strong reactions in enzyme-linked immunosorbent assay (ELISA) tests. Sensitivity of ELISA was increased markedly if high molarity buffer (>0.2 M potassium phosphate) or EDTA was used to extract virus from tissues. With antisera to PRV-HA, no serological difference could be detected between PRV-HA, a Florida isolate of PRV, and WMV-1. Antisera to PRV-HA did not react to three other viruses in the potyvirus group.

Additional key words: papaya mosaic virus, ELISA, watermelon mosaic virus 1, SDS-serology.

Papaya ringspot virus causes one of the most destructive diseases of papaya (*Carica papaya* L.), a fruit crop which is grown throughout the tropics (5). The term "papaya ringspot virus" (PRV) was first used in the 1940s to describe a virus affecting papaya in Hawaii (12). It is transmitted by aphids in a nonpersistent manner (13) and tentatively has been placed in the potyvirus group (9,17). Another aphid-transmitted virus which often is called papaya mosaic virus has been reported in Puerto Rico (1,2), India (3), and elsewhere. This virus and PRV have similar properties, but they differ somewhat in the symptoms caused on papaya. However, the "papaya mosaic virus" described by deBokx (6) and which occurs occasionally in Florida (5), is very different from the above described viruses. The particle length of that virus is about 533 μm (6), it is not transmitted by aphids (24), and it has been placed in the potexvirus group (9, 19). The virus we describe here is of the PRV type.

Although the symptomatology and virus-vector relationships of PRV have been extensively described (13,16,21,24), little is known of its biochemical properties. Thus, we wished to purify PRV in sufficient quantities for antiserum production as a first step in the characterization of PRV. Furthermore, we felt that an antiserum to PRV would help clarify the relationship of papaya viruses from different areas of the world. During the course of the work, an abstract on the initial attempts by other workers to purify PRV was reported (23).

MATERIALS AND METHODS

Virus isolates. Two PRV isolates, designated PRV-HA and PRV-HB, were originally obtained from leaves of infected papaya trees grown in Hawaii. PRV-HA caused severe leaf distortion of papaya leaves and intense mosaic symptoms along with leaf distortion on zucchini squash (*Cucurbita pepo* L.). Papaya infected with PRV-HB also showed leaf distortion, but to a lesser degree than PRV-HA, and leaves of infected zucchini showed only a very mild mottle. Both isolates were maintained on papaya cultivar Solo.

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Virus titer in zucchini squash. Virus titers in systemically infected squash plants were measured at intervals after inoculation by assaying leaf extracts on *Chenopodium quinoa,* a local lesion host for PRV. Cotyledons of seedlings with their first leaves just developing were inoculated with infected squash leaf extracts. At about 3-day intervals following inoculation, leaf tissues from leaves of five plants were assayed as follows: samples from chronologically similar leaves (ie first leaf, second leaf, etc.) were collected and immediately pooled, weighed, ground in 0.01 M potassium phosphate, pH 7.0 (1 g/20 ml), and assayed on *C. quinoa.* Two plants (five leaves per plant) were inoculated per extract. Local lesions were counted about 10 days later and the average number of lesions per leaf was calculated. Upper surface areas of representative squash leaves were measured at each sampling date. The relative infectivity titers of squash plants, at intervals after inoculation, were estimated by considering the relationship between infectivities of sampled tissues and surface area of the plant leaves. Infectivity titer at a particular sampling date was expressed as lesions per squash plant by the following formula:

$$\left[\left(\begin{array}{c}\text{lesions per}\\ \textit{C. quinoa}\text{ leaf}\end{array}\right)\times\frac{\text{total area of squash leaf (cm}^2\text{)}}{\text{sampled area of squash leaf (cm}^2\text{)}}\right]_{\text{1st leaf}}+\left[\quad\right]_{\text{2nd leaf}}+\ldots\ldots=\text{lesions per plants.}$$

Virus purification. The method that was finally adopted for purification of PRV was a modification of the procedure described by Purcifull and Hiebert (20) for purifying WMV-1. The PRV-HA isolate was used in all purification studies. Leaves of systemically infected zucchini which had been inoculated with leaf extracts of PRV-HA-infected papaya or zucchini were harvested 21–25 days after inoculation and homogenized with a Waring blender in 0.5 M potassium phosphate, pH 7.5 (2 ml/g tissue) containing 0.01 M EDTA and 0.1% sodium sulfite. Chloroform and carbon tetrachloride, each at 0.5 ml/g tissue, were added as the tissue was being ground. After centrifugation of the homogenate at 8–10,000 *g* for 10 min, polyethylene glycol MW 6,000 (PEG) was added to the supernatant at the rate of 8 g/100 ml and the mixture was stirred for

1–4 hr at 6 C. Precipitated virus, along with host debris, was recovered by centrifugation (10,000 *g* for 10 min), and resuspended by stirring for 1 hr or more in PE buffer (0.1 M potassium phosphate + 0.01 M EDTA, pH 7.0) equal to one-fifth the original volume of clarified supernatant. After centrifugation to remove more host debris, virus was precipitated from the supernatant by adding PEG (5%) and NaCl (0.3 M). Precipitated virus was collected by centrifugation (10,000 *g* for 10 min) and resuspended in PE buffer equal to one-twentieth the volume of the original clarified supernatant.

Papaya ringspot virus was further purified by centrifugation in Cs_2SO_4 gradients in a Beckman SW36 rotor (8). Cs_2SO_4 (0.15 g/ml) was dissolved in the virus suspension which was then layered (10 ml per tube) over a 3.0-ml cushion of 53% (wt/wt) Cs_2SO_4 which was buffered with PE. The tube contents were centrifuged at 30,000 rpm for 18–22 hr at 6 C. The viral zone, located about midway down the tube, was carefully collected with a Pasteur pipet, diluted with two volumes of PE buffer, and host material was removed by centrifugation (10,000 *g* for 10 min). The virus suspension was usually dialyzed in PE buffer overnight and given another cycle of Cs_2SO_4 density gradient centrifugation followed by dialysis to remove Cs salts. On occasion, virus was detected and collected from the gradients by using an ISCO (Instrumentation Specialties Co., Lincoln, NB 68504) UA5 monitor and an ISCO 640 fractionator.

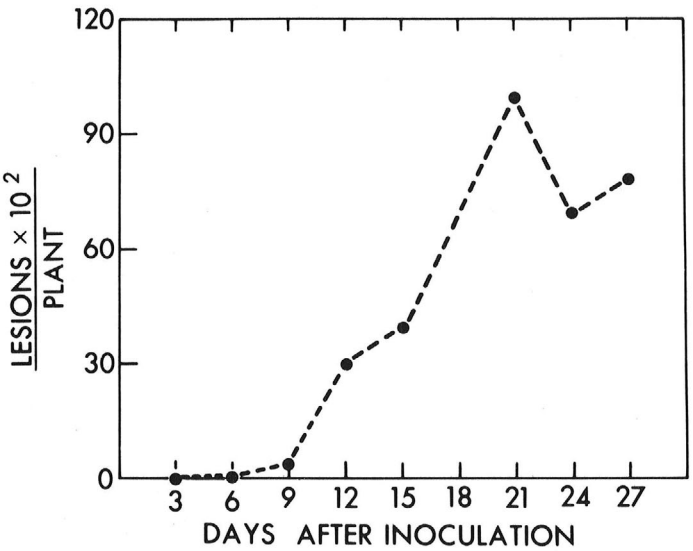

Fig. 1. Infectivity titer of papaya ringspot virus in leaves of zucchini squash plants in relation to time after inoculation. Lesions per plant refers to the calculated sum of lesions (see Materials and Methods for formula) as measured by sample inoculations of zucchini leaf extracts to *Chenopodium quinoa*.

Serology. Antisera were produced to intact PRV-HA and to SDS-degraded capsid protein of PRV-HA (7). Each antigen type was mixed 1:1 with Freund's incomplete adjuvant and injected into the hip muscles of white New Zealand rabbits. A rabbit was sensitized to intact PRV-HA by two injections of intact virus (about 1 mg per injection) given 1 wk apart. Booster injections were given 5, 12, 16, and 22 wk after the initial injection. Blood was collected at about weekly intervals starting 3 wk after the initial injection. A second rabbit was sensitized to PRV capsid protein in the following way. Initially, the rabbit was injected with an SDS-degraded preparation of PRV. Subsequent injections were with isolated PRV capsid protein that had been denatured by heating in phosphate-buffered SDS containing 2-mercaptoethanol, electrophoresed in SDS-polyacrylamide gels, and recovered as previously described (7). In some instances, protein was located in unstained gels by using a stained sister gel as a marker. The rabbit was sensitized by two initial injections 1 wk apart followed by booster injections 4, 9, and 14 wk after the initial injection. The amount of capsid protein injected was not measured, but was estimated to be less than 0.5 mg per injection. Blood was collected at weekly intervals as described above.

Antisera were tested by SDS-immunodiffusion (18) and ELISA (4). Media for SDS-immunodiffusion tests contained 0.8% Noble agar, 0.5% SDS, and 1.0% sodium azide dissolved in distilled water. Leaf tissues were homogenized (1 g/ml) in 1% SDS and applied to wells 7 mm in diameter and 5 mm apart at the nearest point. Color development of substrate solutions in ELISA tests was measured in a microcell attached to a sipper system of a Beckman 25 UV-spectrophotometer.

Electron microscopy. Virus preparations were negatively stained with 2% phosphotungstic acid, pH 7.2, containing 0.05% bovine serum albumin. Samples were spread on Formvar-coated grids and examined with a JEOL JEM100B electron microscope.

RESULTS

Virus titer in zucchini squash. The relationship between infectivities of leaf tissue extracts and the leaf surface area of a squash plant were used to estimate virus titer at intervals after inoculation. By this method, we could estimate the time when the combination of infectivity and the amount of tissue available for virus purification was highest. Significant infectivity was first detected in uninoculated leaves 9 days after inoculation of

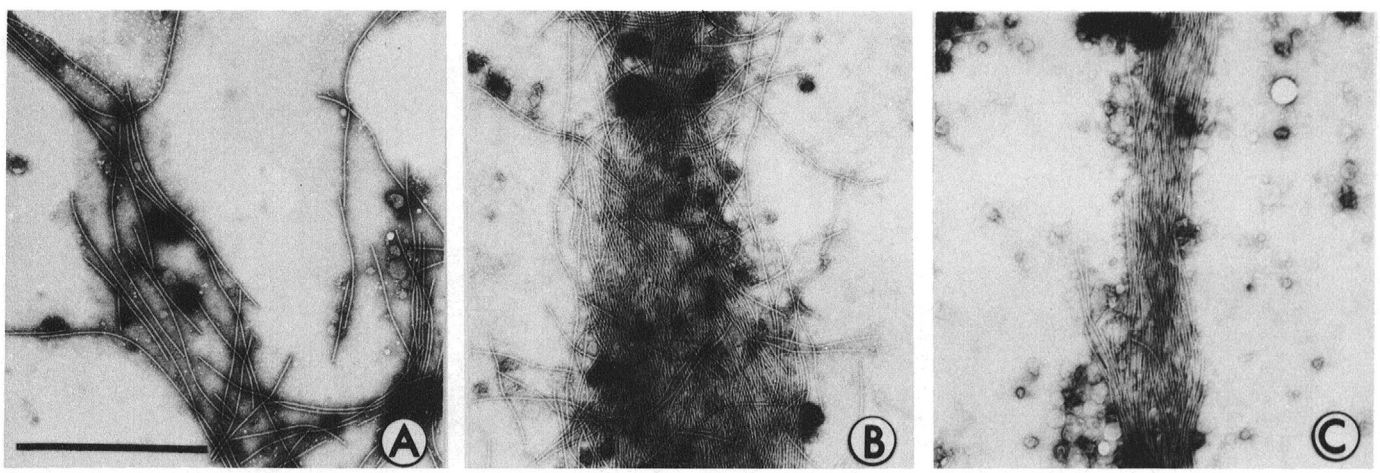

Fig. 2. Electron micrographs depicting the effects of buffers on reducing aggregation in suspensions of papaya ringspot virus (PRV). **A,** 0.09 M boric acid + 0.02 M EDTA, pH 8.35; **B,** 0.02 M potassium phosphate + 0.15 M NaCl + 0.02% NaN_3, pH 7.4 (PBS); and **C,** 0.05 M potassium phosphate, pH 7.5. Aggregated PRV preparations (as in C) suspended in 0.05 M potassium phosphate, pH 7.5, were diluted 1/5 with the respective buffers and then dialyzed against the same buffer. Aggregation was not reduced in 0.05 M Na acetate, pH 5.0, or in PBS + 0.005% Tween-20. Bar = 1 μM.

cotyledons (Fig. 1). The relative infectivity per plant was highest 21–27 days after inoculation. A zucchini plant had 5–6 leaves 21–25 days after inoculation, the time when leaves were harvested for virus purification.

Factors affecting virus purification. In our initial trials, we followed exactly the purification procedure used for WMV-1 by Purcifull and Hiebert (20). However, suspensions of PRV did not form a virus zone after centrifugation in CsCl. A viral band was observed after centrifugation in Cs_2SO_4. Furthermore, many PRV particles aggregated and settled out of solutions during dialysis in 0.05 M potassium phosphate, pH 7.5, to remove Cs salts.

To try to dissociate aggregated virus suspensions, we dialyzed dilute aggregated virus preparations against various buffers. Aggregation appeared to be less in buffers with EDTA (Fig. 2), which indicated that EDTA had reduced aggregation. Moreover, we got higher yields of virus and less aggregation when EDTA was added to the purification buffers of Purcifull and Hiebert (20).

The viral zone in the first-cycle Cs_2SO_4 gradients was usually slightly greenish and had a matted appearance. A zone of translucent, viscous, UV-absorbing material located just below the virus zone (Fig. 3) often obscured the viral zone when an ISCO fractionator was used to collect the virus. Papaya ringspot virus was conveniently collected from Cs_2SO_4 gradients with a Pasteur pipet. It was critical to remove green material from the collected virus suspension by centrifugation before dialysis. If the virus suspension was dialyzed and then centrifuged, much of the virus often sedimented with the green host material. Purified virus preparations showed little side-to-side aggregation, but significant end-to-end aggregation was observed (Fig. 4). Virus yields were about 5 mg per 100g of tissue assuming an $E^{0.1\%}_{260nm} = 2.4$ which is similar to that used for potyviruses such as tobacco etch (22).

Serology. Protein obtained by SDS-degradation of purified PRV showed one-to-three zones when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Initial observations indicated that the largest protein was predominant in freshly purified preparations (Fig. 5c), while the smaller species were predominant in older PRV preparations (Figs. 5a,b). Presumably, the smaller protein species were proteolytic products of the largest protein species. Similar observations have been reported for viruses in the potyvirus group (10,20). Preliminary MW estimates of the proteins were 36,000, 31,000, and 26,000. Antiserum was produced to material eluted from gel slices corresponding to the regions which contained all protein species.

Antiserum produced to isolated PRV capsid protein reacted with PRV in SDS-immunodiffusion tests (Fig. 6). A distinct immuno-

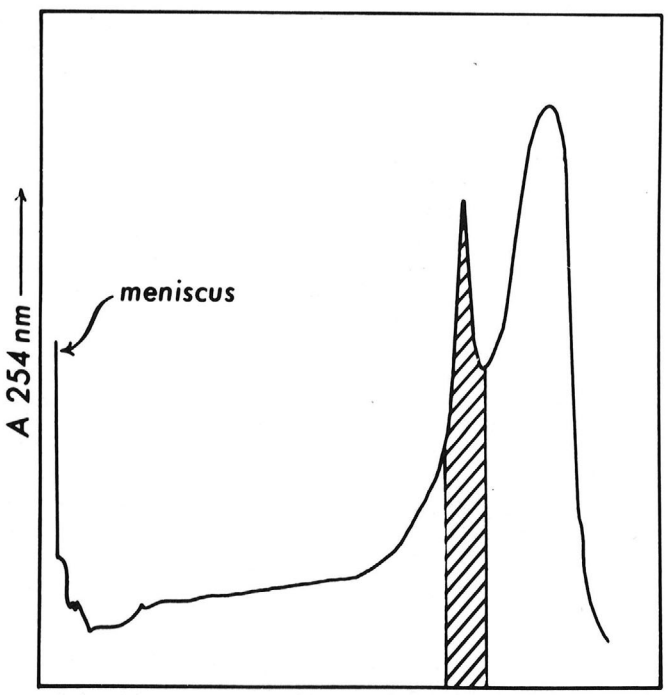

Fig. 3. Ultraviolet-absorbance profile of partially purified papaya ringspot virus after centrifugation in a Cs_2SO_4 density gradient. The shaded peak contains virus while the large UV-absorbing zone below the virus contains a translucent, viscous material. Material from the shaded area was collected and further purified by centrifugation in Cs_2SO_4.

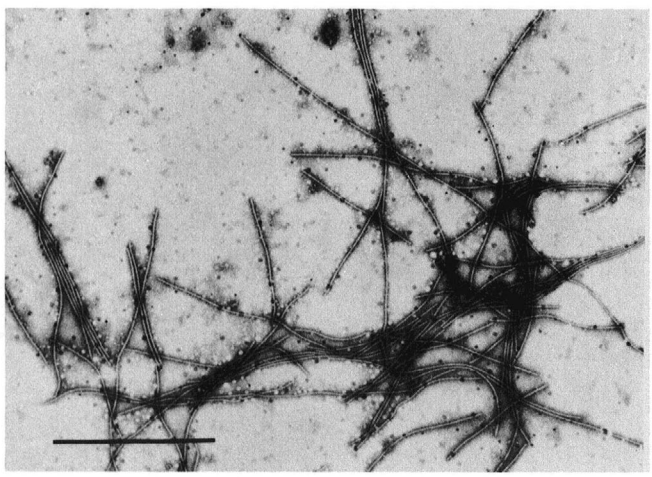

Fig. 4. Purified papaya ringspot virus preparation showing some side-to-side aggregation, but significant end-to-end aggregation. Virus was suspended in 0.1 M potassium phosphate + 0.01 M EDTA, pH 7.0. Bar = 1 μM.

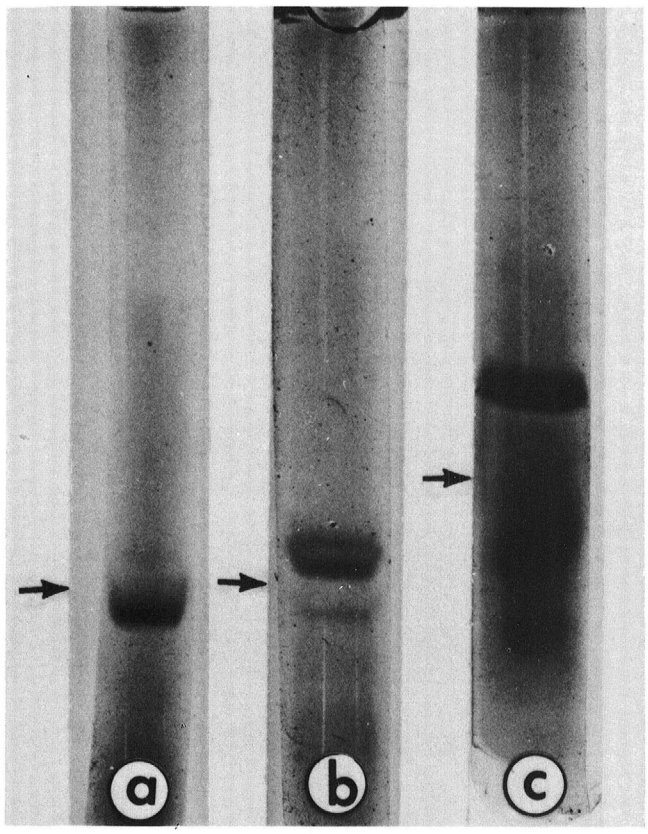

Fig. 5. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of purified papaya ringspot virus-HA preparations. Preparations were degraded with SDS **a,** 40 days, **b,** 27 days, and **c,** immediately after purification. Arrows denote the location of carbonic anhydrase (29,000 MW), the marker protein. Electrophoresis was from top to bottom in 7.5% gel. Gels (a) and (b) were run simultaneously while gel (c) was from another experiment. Different purified preparations were used in each gel. Preliminary MW estimates were 36,000 and 26,000 in (a); 36,000, 31,000, and 26,000 in (b); and 36,000 in (c).

precipitin zone plus a much weaker and slower diffusing zone were usually observed. On the other hand, antiserum to "intact" PRV did not react with PRV in SDS-immunodiffusion tests except that antisera from some bleedings gave weak reactions (Fig. 6).

Initial ELISA tests conducted by using the procedure of Clark and Adams (4) gave very poor results with antiserum to intact PRV-HA. Since we had observed that EDTA reduced aggregation, various buffers, with and without EDTA, were compared with the extraction buffer used by Clark and Adams. Sensitivity of ELISA for detecting PRV in leaves of papaya and zucchini squash was greatly affected by the type and strength of extraction buffers (Table 1). The presence of 0.1 M EDTA in the standard ELISA extraction buffer increased the sensitivity of the ELISA test tenfold. High-molarity phosphate buffers (0.4 M and 0.25 M) also gave much better results than the standard ELISA extraction buffer, even without EDTA. However, the efficiency of 0.1 M potassium phosphate was increased by the addition of EDTA. Addition of Tween-20 or polyvinyl pyrrolidone-40 to the extraction buffers did not have significant effects. In all subsequent ELISA tests, 0.25 M potassium phosphate + 0.1 M EDTA, pH 7.5, was used as the extraction buffer. Antiserum to isolated PRV capsid protein was not used in ELISA tests.

ELISA and SDS-immunodiffusion tests were used to check the relationship of PRV-HA to several potyviruses and isolates of PRV. Watermelon mosaic virus 1, PRV-HA, and a Florida isolate of PRV (PRV-340) gave reactions of identity in SDS-immunodiffusion tests in which antisera to PRV-HA and WMV-1 were used, while WMV-2 did not react with either antiserum (Fig. 7). Antisera to PRV-HA and WMV-1 also gave strong reactions to PRV-HB, PRV-340, and WMV-1 in ELISA tests. Isolates of bean yellow mosaic virus, cowpea aphid-borne mosaic virus, and watermelon mosaic virus 2 did not react with antiserum to PRV-HA in ELISA or SDS-immunodiffusion tests.

DISCUSSION

The nomenclature of papaya viruses has been a source of confusion (5), primarily because symptomatology has been used extensively for describing papaya viruses. In particular, the terms papaya mosaic and papaya ringspot have been often used to describe viruses which are transmitted nonpersistently by aphids but show somewhat different symptoms (1–3,12,13). Serology can now be used, as an additional tool, for determining relationships among aphid-borne papaya viruses.

In Hawaii, two aphid-transmitted papaya viruses have been reported and these are referred to as papaya ringspot and papaya mosaic viruses (11,12). Papaya ringspot was first reported in Hawaii in the 1940s (12) and papaya mosaic in 1963 (11). According to Ishii and Holtzmann (11), Hawaiian isolates of PRV and papaya mosaic virus are distinct viruses based on symptomatology, host range, and the absence of cross protection between viruses. The virus isolate reported here has been previously referred to as papaya mosaic virus in Hawaii (11). We have shown that PRV from Florida is serologically related to papaya mosaic virus from Hawaii. Both viruses also cause similar symptoms on papaya (cf 17). Thus, we conclude that the PRV isolate from Florida is similar to papaya mosaic virus (11) in Hawaii.

Although the literature is confusing, it appears that papaya virus diseases are generally grouped as ringspot or mosaics and are associated with poty and potex viruses (5). We agree with deBokx's (6) suggestion that the name papaya ringspot virus be used for viruses about 780 nm long with potyvirus characteristics (9,17) and papaya mosaic virus be used for viruses about 530 nm long with potexvirus characteristics (9,19).

Purcifull and Hiebert (20) showed that PRV and WMV-1 reacted with WMV-1 antiserum in SDS-immunodiffusion tests. We have confirmed their observations and found that these viruses also showed reactions of identity when tested with PRV antiserum. Furthermore, WMV-1 and the PRV-HA isolate produced similar symptoms on squash. Thus far, the only differences between PRV-HA and the WMV-1 isolate that we tested are that PRV-HA caused local lesions on *C. quinoa* and *C. amaranticolor,* and WMV-1 did not infect papaya. However, WMV isolates from Greece and Jordan, which were designated as WMV-1 based on cytology (14) and serology (20), produced lesions on *C. quinoa* and/or *C. amaranticolor* (14,20). It would be interesting to further explore the relationship between WMV-1 and PRV.

The dramatic effects of high-molarity buffer and of EDTA in improving the sensitivity of ELISA may have been because these buffers minimized virus aggregation in the tissue extract. Or, it may have simply released more virus particles from the cells. Wey et al (23) also showed that EDTA prevents PRV aggregation in tissue extracts. Nevertheless, the effects of the extraction buffer should be taken into account when ELISA is used to determine serological relationships among viruses.

A rogueing program has been used in recent years to suppress the spread of PRV in certain papaya growing areas in Hawaii (15). Trees are rogued after visual symptoms appear. Since PRV is transmitted by aphids, the detection and rogueing of infected trees

TABLE 1. Effect of extraction buffers on the sensitivity of ELISA[a] for detecting papaya ringspot virus-HA in extracts of papaya leaves

Tissue source[b]	Buffers								
	0.4P[c]	0.4 P 0.1 E	0.25 P	0.25 P 0.1 E	0.1 P	0.1 P 0.1 E	Std[d]	Std 0.1 E	0.1 E
Healthy papaya	0.05	0.04	0.05	0.05	0.04	0.05	0.07	0.06	0.05
PRV-HA infected papaya	2.17	1.46	2.34	1.85	0.57	1.55	0.12	0.97	1.15

[a] Enzyme-linked immunosorbent assay. The reacted substrate was diluted 1/4 before the optical density was measured at 405 nm. Coating γ-globulin at 5 μg/ml. Enzyme-γ-globulin at 1/400 dilution.

[b] Tissues were homogenized (1/20) in the respective buffers.

[c] Numbers refer to molarity (M). P = potassium phosphate, E = EDTA, and all buffers were at pH 7.4–7.5.

[d] Std = "standard ELISA buffer" (0.02 M potassium phosphate + 0.15 M NaCl + 2% PVP + 0.005% Tween-20 + 0.02% NaN_3, pH 7.4). (See Reference 4).

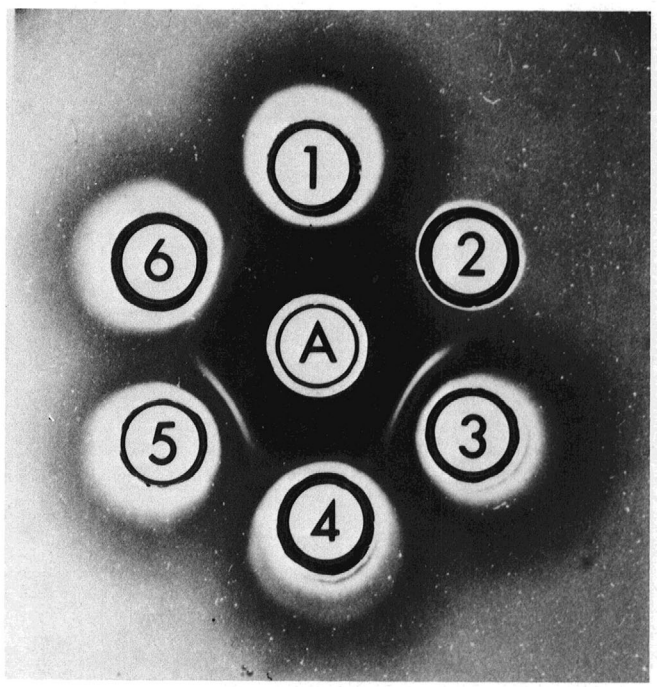

Fig. 6. Reaction in sodium dodecyl sulfate (SDS)-immunodiffusion tests of antisera prepared to "intact" papaya ringspot virus (PRV)-HA and to isolated PRV-HA capsid protein against extracts of PRV-HA-infected zucchini squash leaves. Wells contain: A = PRV-HA antigen: 1 = normal serum; 2 = 1% SDS; 3,5 = antiserum to isolated PRV-HA protein; and 4,6 = antiserum to "intact" PRV-HA.

before symptoms appear may possibly minimize the inoculum source for aphids. ELISA tests with PRV antisera may be particularly beneficial in detecting infected papaya trees before symptom expression.

Measures used to control PRV will depend on the prevalence of the virus within and adjacent to papaya orchards. Some potential control measures are cross-protection, breeding for tolerance or resistance, and eradication. The successful purification of PRV and the production of antisera to PRV should help in the development of each of these measures.

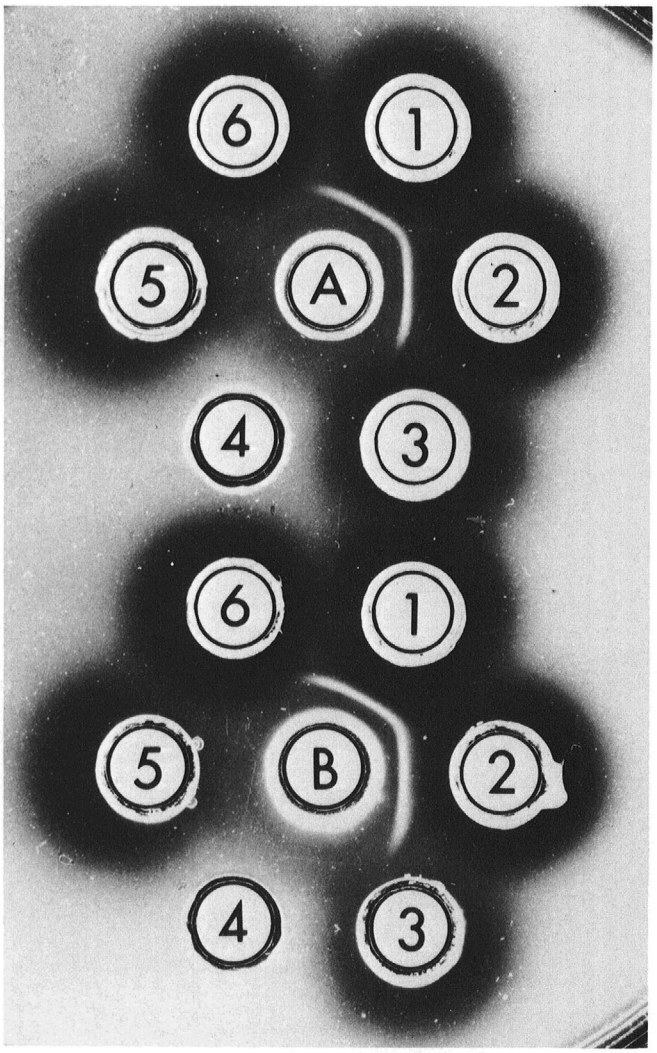

Fig. 7. Reactions in sodium dodecyl sulfate (SDS)-immunodiffusion tests of antisera prepared to isolated papaya ringspot virus (PRV)-HA capsid protein and to watermelon mosaic virus (WMV)-1, against PRV-HA, WMV-1, and WMV-2. Wells contain: A = WMV-1 antiserum; B = PRV-HA antiserum; 1 = PRV-HA; 2 = WMV-1; 3,5 = extract from healthy zucchini squash leaves; 4 = 1% SDS; and 6 = WMV-2. Extracts of PRV-HA, WMV-1, and WMV-2 were from infected zucchini squash leaves.

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