Serological Distinction of Watermelon Mosaic Virus Isolates

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ABSTRACT

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Antisera to purified preparations of Florida isolates of WMV type 1 (WMV-1 FL) and WMV type 2 (WMV-2 FL) were used in immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated extracts from virus infected plants. Based on reciprocal tests with sera collected 2–6 mo after the first injection, WMV-1 FL and WMV-2 FL did not cross react. Among 13 other WMV isolates obtained from other areas, three types were identified: (i) isolates that were closely related to WMV-1 FL but not reactive with WMV-2 FL, viz, one isolate each from California, New York, Texas, Australia, Jordan, and Greece; (ii) isolates that were closely related to WMV-2 FL but not reactive with WMV-1 FL, viz, a single isolate each from Arizona, New York, Australia, and New Zealand and two from California; and (iii) an isolate from Morocco, which was nonreactive with either WMV-1 FL or WMV-2 FL. The SDS-immunodiffusion methods were reliable for detecting either WMV-1 or WMV-2 in extracts from symptomatic leaves from a variety of host plants. Freeze-dried leaf extracts proved convenient as reference antigens. The papaya ringspot virus was closely related serologically to WMV-1 FL but nonreactive with WMV-2 FL, whereas soybean mosaic virus was unrelated to WMV-1 FL and related to, but distinct from, WMV-2 FL.

Additional key words: cucurbit virus, legume virus, potyvirus.

Watermelon mosaic virus (WMV), a member of the potyvirus group (5,24), is a pathogen of worldwide importance in cucurbit crops (24). The biological variability of WMV strains has been documented extensively (4,5,7,8,11,16,17,22,24–27). Webb and Scott (27) divided WMV isolates from the USA into two distinct groups, which were distinguished by host range and serological properties. Those isolates that failed to infect noncucurbitaceous plants were designated WMV-1, whereas isolates that infected plants outside the Cucurbitaceae (eg, certain species of the Chenopodiaceae, Leguminosae and Malvaceae) were designated "WMV-2". An antiserum prepared to an isolate of WMV-1 reacted strongly with WMV-1 type isolates but faintly or not at all with WMV-2 isolates (27). There have been several other reports of serologic heterogeneity among WMV isolates (1,2,12,13), and there also have been reports of cytological differences among WMV isolates (3,4).

Milne and Grogan (15) found a close serologic relationship among various WMV-1 and WMV-2 isolates and concluded that they were strains of the same virus. They also reported that papaya ringspot, another potyvirus, was serologically closely related to both WMV-1 and WMV-2. Watermelon mosaic viruses also have been reported to be related to several other potyviruses (12,13,21,23,25).

This report provides evidence that the WMV complex consists of at least three serologically different groups of viruses, based on studies with antisera prepared to Florida isolates of WMV-1 and WMV-2.

MATERIALS AND METHODS

Source and culture of WMV isolates. A WMV-1 isolate obtained from W. C. Adlerz in Florida (hereafter referred to as WMV-1 FL) was propagated in pumpkin (*Cucurbita pepo* L. 'Small Sugar') and used for preparation of antiserum. An antiserum also was prepared to a Florida isolate of WMV-2 (WMV-2 FL). This isolate was obtained from naturally infected watermelon. It was mechanically inoculated to garden pea (*pisum sativum* L. 'ALASKA'), and back-inoculated to pumpkin from pea prior to routine culture in pumpkin or squash (*C. pepo* L. 'Early Prolific Straightneck'). The other isolates and their sources are listed in Table 1.

Stock cultures of each isolate were maintained in separate, screened cages (293-μm [50-mesh] screen). Pumpkin or squash plants inoculated in cotyledonary stages were used as propagative hosts and cultures were transferred every 3–6 wk by mechanical inoculation. Benomyl and dinocap sprays were used to check powdery mildew development on pumpkin and squash. In host-range trials, at least two plants of each species or cultivar were inoculated with each isolate, and the test plants were observed for 3 wk or longer after inoculation. In addition, leaf dips from inoculated plants were prepared in 2% potassium phosphotungstate, pH 6.8, and examined by electron microscopy for the presence of filamentous particles.

Purification, polyacrylamide gel electrophoresis, and electron microscopy. The WMV-1 FL isolate was purified from systemically infected pumpkin tissue harvested from plants inoculated approximately 3 wk previously. The tissue (400 g) was homogenized with a Waring Blendor in a mixture containing 800 ml of 0.5 M potassium phosphate, pH 7.5, 2.0 g of Na_2SO_3, 200 ml of chloroform, and 200 ml of carbon tetrachloride, and the homogenate was centrifuged at 5,000 rpm for 5 min in a Sorvall GSA rotor. The aqueous phase was collected and centrifuged at 9,000 rpm for 20 min. The supernatant was removed, and carbowax (polyethylene glycol 6,000) was added at the rate of 8 g/100 ml. After it was stirred for 1 hr at 4 C, the mixture was centrifuged at 9,000 rpm for 10 min, and the pellets were resuspended in 10 ml of 0.05 M potassium phosphate, pH 7.5. The resuspended material was subjected to centrifugation in a CsCl gradient ($\bar{\rho}$= 1.28 g/ml, in 0.05 M potassium phosphate, pH 7.5 or 8.2) generated at 150,000 *g* (maximum) for 18 hr. The virus-containing zone (occurring at about three-fourths the distance from the meniscus to the bottom of the tube) was removed, diluted with an equivalent volume of buffer, and centrifuged at 10,000 rpm (12,000 *g*) in a Sorvall SS34 rotor for 10 min. The supernatant fluid was diluted further and

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subjected to high-speed centrifugation at 36,000 rpm (120,000 *g*) in a Beckman 40 rotor for 1 hr. The resulting pellet, which consisted of purified virus, was resuspended in a small volume of 0.02 M Tris buffer, pH 8.2.

A portion of the purified WMV-1 FL was stored at 4 C for about 2 mo before use as an immunogen. During storage the virion capsid protein became partially degraded as reported for other potyviruses (9,10) by presumed proteolytic activity due to a plant protease contaminant. This antigen is referred to hereafter as WMV-1 FL (deg.).

The WMV-2 FL isolate was purified from pumpkin by a similar procedure, except that the chloroform-carbon tetrachloride clarification was omitted. Instead, phosphate-buffered homogenates were clarified by treatment overnight with *n*-butanol (8 ml/100 ml homogenate) prior to clarification by slow-speed centrifugation, purification by polyethylene glycol precipitation, and equilibrium centrifugation in CsCl.

Polyacrylamide gel electrophoresis experiments were conducted in sodium dodecyl sulfate (SDS)-gels (28) as modified by Hiebert and McDonald (9).

Leaf extracts were prepared for electron microscopy in 1 or 2% potassium phosphotungstate, pH 6.8, containing 0.05 or 0.1% bovine serum albumin. Particles were photographed in a Philips 200 electron microscope and measured by comparison with a diffraction grating.

Preparation of antisera. All antisera were prepared in New Zealand white rabbits, with preparations emulsified with Freund's complete adjuvant (1:1, v/v) for the first injection and preparations emulsified with incomplete adjuvant for subsequent injections. For WMV-1 FL, the rabbit initially was given 3 mg of virus intramuscularly and 3.5 mg 1 mo later. A different rabbit was injected with the WMV-1 FL (deg.). In this case 3 mg were injected initially, followed by an injection of 4.5 mg 3 wk later. For WMV-2 FL, a rabbit was given 3.2 mg initially, followed by a second intramuscular injection of 2.2 mg 2 wk later. After the second injections, the animals were bled every 5–14 days for a period of at least 5 mo. Antisera were stored either by freezing or by lyophilization.

Serologic tests. Antisera were evaluated by immunodiffusion tests conducted in the presence of sodium dodecyl sulfate (SDS). The medium consisted of 0.8% Noble agar, 1.0% sodium azide, and 0.5% SDS (6,19). Crude antigens were usually prepared from freshly harvested leaves by grinding 1 g in 1 ml of deionized water followed by the addition of 1 ml of 3% SDS. The samples were expressed through cheesecloth and held at room temperature until use, but not longer than 1 hr. In some cases, freeze-dried antigens (20) were used.

RESULTS

Host range and properties of WMV-1 FL and WMV-2 FL. The WMV-1 FL isolate had filamentous particles characteristic of potyviruses. Of 183 particles measured in leaf dips from pumpkin 78% ranged from 690 to 900 nm long, with a modal class of 780 nm. The WMV-1 FL induced a systemic mosaic or mottle and leaf distortion in Small Sugar pumpkin and Early Prolific Straightneck squash, butternut squash (*C. moschata* Duch.), watermelon (*Citrullis lanatus* (Thumb.) Mansfeld), cucumber (*Cucumis sativus* L.), and a mottle or chlorotic spotting in *Luffa acutangula* Roxb. Filamentous particles were found in leaf extracts from symptomatic plants. No symptoms were induced on the following test plants nor were filamentous particles found in leaf dips from inoculated plants: *Chenopodium amaranticolor*, Coste et Reyn, *Phaseolus vulgaris* L. 'Bountiful' and 'Pinto,' *Pisum sativum* L. 'Alaska,' *Nicotiana benthamiana* Domin., and *Carica papaya* L.

The WMV-2 FL isolate also was filamentous, with 82% of 142 particles from pumpkin leaf dips ranging from 690 to 900 nm in length, with a modal class of 780 nm. This isolate induced systemic mottle or mosaic symptoms in pumpkin, squash, watermelon, and cucumber; dark green vein-banding and some leaf distortion in *N. benthamiana*; a mottle and sometimes systemic necrosis accompanied by wilting and premature death of the plants in pea; and numerous chlorotic lesions on inoculated leaves of *C. amaranticolor*. The first attempts to infect Bountiful bean resulted in a few chlorotic lesions on the inoculated primary leaves, with no systemic symptoms. When the lesions were excised, triturated in water, and inoculated to the primary leaves of Bountiful bean, local chlorotic lesions and a systemic mottle developed. When transferred back to squash from systemically infected bean, the bean isolate was serologically identical to the one maintained in pumpkin or squash (Fig. 1). Rod-shaped particles were found in

TABLE 1. Test plant and serological reactions of watermelon mosaic virus isolates

Origin of isolate	Host reactions[a]				Reactions with antisera to[b]		
	Luffa acutangula	*Nicotiana benthamiana*	*Pisum sativum* 'Alaska'	*Cucurbita pepo* 'Small Sugar'	WMV-1 FL	WMV-1 FL (deg.)	WMV-2 FL
Group I:							
Florida (W. C. Adlerz)	S	–	–	S	+	+	–
California (R. E. Webb)	S	–	–	S	+	+	–
Texas (ATCC PV-23)	S	–	–	S	+	+	–
New York (R. Provvidenti)	S	–	–	S	+	+	–
Australia (R. Greber)	S	–	–	S	+	+	–
Jordan (G. Martelli)	S	–	–	S	+	+	–
Greece (G. Martelli)	S	–	–	S	+	+	–
Group II:							
Florida (D. Purcifull)	–	S	S	S	–	–	+
California (R. E. Webb)	–	S	S	S	–	–	+
California (J. Watterson)	–	S	S	S	–	–	+
Arizona (ATCC PV-27)	–	S	S	S	–	–	+
New York (R. Provvidenti)	–	S	S	S	–	–	+
Australia (R. Greber)	–	S	S	S	–	–	+
New Zealand (W. Thomas)	–	S	S	S	–	–	+
Group III:							
Morocco (B. Lockhart)	–	–	–	S	–	–[c]	–

[a]S = Systemic infection on the basis of symptoms and presence of filamentous particles in leaf dips; – = No symptoms and no filamentous particles in leaf dips.

[b]Symbols: + = reaction of serological identity (precipitin lines fused without spurs) when compared to homologous; – = No reaction. Antisera collected from 2–5 mo after immunization were used in trials, and antigens consisted of SDS-treated extracts from pumpkin leaves.

[c]Sera collected up to 2 mo after immunization were negative; later bleedings from this rabbit gave weak reactions of undetermined specificity in tests with the Moroccan isolate.

leaf dip preparations from all species of test plants that showed symptoms after inoculation with WMV-2 FL. No symptoms were induced by WMV-2 FL on the following test plants nor were rod-shaped particles found in extracts from leaves taken above the point of inoculation: cowpea (*Vigna unguiculata* (L.) Walp. 'Knuckle Purple Hull'), *P. sativum* L. 'Little Marvel,' and *L. acutangula* Roxb.

Because the serologic specificities of different preparations of a given potyvirus can differ (10,12,19), the capsid proteins of the different virus preparations used for WMV antiserum production were analyzed by SDS polyacrylamide gel electrophoresis (Fig. 2a). These tests (Fig. 2a) revealed that freshly prepared WMV-1 FL consisted principally of native capsid protein (MW estimate 36,500), with only a trace of a lower MW component (27,500), which presumably arose by proteolytic degradation. The WMV-1 FL (deg.) preparation (stored for 2 mo), however, was almost completely degraded into the light protein form with a MW of 27,000 (Fig. 2b). The freshly prepared WMV-2 FL preparation also showed considerable degradation (Fig. 2-c). Based on the staining intensity of the respective protein zones, however, it was judged that native protein (MW estimate of 36,500) was the major species in WMV-2 FL preparations used for antiserum production.

Serologic comparison of WMV-1 FL and WMV-2 FL and their relationships to other WMV isolates. The SDS-immunodiffusion tests indicated that WMV-1 FL and WMV-2 FL were serologically distinct (Fig. 1, 3, and 4). No cross reactions were detected in reciprocal tests with antisera collected up to 6 mo after the initial injections. This was determined with antisera collected on 11 different dates from the rabbit injected with WMV-1 FL and with antisera taken on 16 different dates from the rabbit injected with WMV-2 FL. Very faint reactions of WMV-1 FL with WMV-2 FL antiserum were sometimes detected with bleedings collected later than 7 mo after the initial immunization. Neither antiserum reacted with antigens from healthy squash (Fig. 1, 3, and 4) or healthy pumpkin (Fig. 1) or with extracts from noninoculated plants of any other species used as sources of antigens in this study.

In one trial, antigens in crude leaf extracts from infected plants were prepared in SDS and were tested against antisera diluted with normal sera (19). Both the WMV-1 FL antiserum and the WMV-2 FL antiserum had titers of 1/16 and 0 against homologous and heterologous antigens, respectively.

Freeze-dried extracts from infected plants were sometimes used as antigens after it was determined that these extracts gave reactions of serological identity (no spur formation) to freshly prepared extracts. The latter usually yielded heavier precipitin bands, as reported with other viruses (20).

The immunodiffusion tests were useful for detecting WMV in extracts from several species of test plants. The WMV-1 FL isolate was detected in pumpkin, straightneck squash, butternut squash, cucumber, watermelon, and *L. acutangula*. The WMV-2 FL isolate was detected in pumpkin, straightneck squash, butternut squash, watermelon, cucumber, bean, pea, *N. benthamiana*, and *C. amaranticolor*.

When sap from *C. amaranticolor* was placed next to wells containing sap from pumpkin, nonspecific precipitation occurred that obscured immunoprecipitin lines. About 24 hr after the initial addition of antiserum and antigens to gel plates, these nonspecific precipitates were removed selectively by soaking the plates in a bath consisting of 0.05 M Tris buffer, pH 7.2 in 0.85% NaCl. The bath was stirred gently with a magnetic stirrer and the solution (about 500 ml per plate) was changed several times during a 24–48 hr washing period.

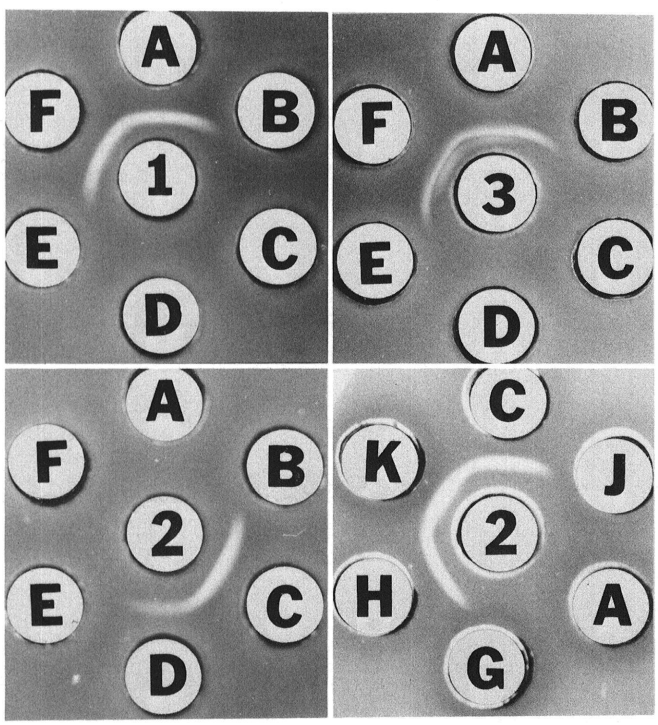

Fig. 1. Serologic distinction of WMV-1 FL and WMV-2 FL and relationships of watermelon mosaic virus isolates. The center wells contain: 1 = antiserum to WMV-1 FL; 2 = antiserum to WMV-2 FL; 3 = antiserum to WMV-1 FL (deg.) which was degraded by storage for several weeks at 4 C prior to injection. The peripheral wells contain SDS-treated extracts from squash or pumpkin leaves infected with: A = WMV-1 FL; B = WMV-Moroccan isolate; C = WMV-2 FL; D = WMV-2, Webb's California isolate; F-WMV-1, Webb's California isolate; G = WMV-1, American Type Culture Collection isolate PV-23; H = WMV-2 FL in squash after subculture in bean; K = WMV-2, American Type Culture Collection isolate PV-27. Wells E and J contained extracts from healthy pumpkin and healthy squash, respectively.

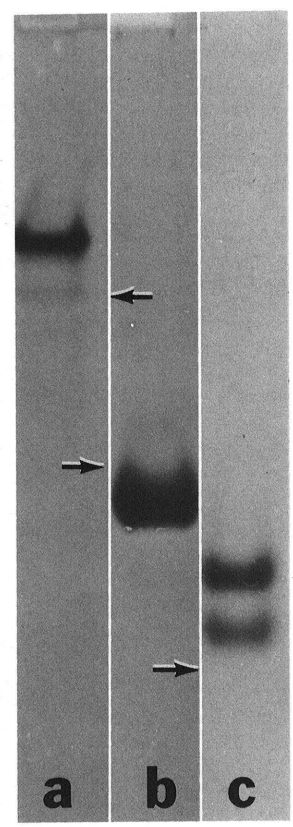

Fig. 2. Polyacrylamide gel electrophoresis (in the presence of 0.1% SDS) of aliquots of purified preparations used for preparation of antisera to WMV-1 FL (a), WMV-1 FL (deg.) (b), and WMV-2 FL (c). Arrows mark the positions of the marker protein carbonic anhydrase (MW 29,000 d) for each respective gel. Electrophoresis was from top to bottom in a 10% gel for 4 hr in (a), in a 10% gel for 6 hr in (b), and in a 6% gel for 3 hr in (c). Molecular weight estimates are ~36,500, 34,000, and 27,500 d for components in (a) and are ~36,500 and 31,400 d for the components in (c). The fast component in (b) is about 27,000 d.

Squash was inoculated on the cotyledons with a mixture of WMV-1 FL and WMV-2 FL inocula, and leaves that showed systemic symptoms apparently contained antigens of both viruses, as shown by immunodiffusion tests (Fig. 3). In such cases, the precipitation lines formed by WMV-2 often were quite weak.

The other WMV isolates studied fell into one of three types (Table 1 and Fig. 1 and 4): (i) those that reacted identically with WMV-1 FL but did not react with WMV-2 FL antiserum; (ii) those that reacted identically with WMV-2 FL but not with WMV-1 FL antiserum; and (iii) the Moroccan isolate, which was nonreactive with the WMV-1 FL, WMV-2 FL, and WMV-1 FL (deg.) antisera. Some collections (taken more than 2 mo after initial immunization) of the WMV-1 FL (deg.) antiserum reacted weakly with sap from Moroccan WMV-infected plants, but the specific nature of this reaction was not determined. In the absence of a homologous antiserum to the Moroccan isolate, our negative results with that virus and our WMV antisera are regarded as tentative.

The virus isolates that reacted identically with WMV-1 were those isolates that infected *L. acutangula* but not pea and *N. benthamiana* (Table 1). Those isolates that reacted identically with WMV-2 FL infected the same test plants as the latter: ie, pea and *N. benthamiana* but not *L. acutangula* (Table 1). The Moroccan isolate failed to infect any of these hosts (Table 1).

Relationship of WMV-1 FL and WMV-2 FL to other potyviruses. Two isolates of papaya ringspot virus obtained from R. A. Conover were propagated in squash. The papaya ringspot viruses gave reactions of serologic identity when compared with WMV-1 FL, but they failed to react with the WMV-2 FL antiserum (Fig. 4).

In reciprocal immunodiffusion tests, soybean mosaic virus was shown to be closely related to but distinct from WMV-2 FL (eg, see spur formation in Fig. 4). No cross-reactions were observed in reciprocal tests between WMV-1 FL and soybean mosaic virus and their antisera.

Both the WMV-1 FL and the WMV-2 FL isolates were serologically distinct from three other potyviruses that have been reported to infect cucurbits, viz, lettuce mosaic (Florida isolate) in Alaska pea, turnip mosaic (a Florida isolate) in *Nicotiana clevelandii* Gray × *N. glutinosa* L. hybrid, and the severe strain of bean yellow mosaic virus from R. Provvidenti (18) in *N. benthamiana*.

DISCUSSION

This study has shown that there are at least two and possibly three serologically distinct types of WMV. Our serology findings support the observations of Webb and Scott (27), Bakker (2), and Arteaga et al (1), who reported evidence that WMV-1 and WMV-2 are serologically different, but contrast the results of Milne and Grogan (15), who reported that a very close serological relationship exists between WMV-1 and WMV-2. Unfortunately, their isolates were not available for direct comparison in our test system (K. S. Milne, *personal communication*). Webb and Scott (27) reported that antiserum to a South African isolate of WMV (WMVX) (25) was nonreactive with WMV-1 and WMV-2, and they suggested that there might be a third virus in the WMV complex. Horvath, et al (11) also suggested that there are three types of WMV isolates. Therefore, the terms "WMV-1" and "WMV-2," which were originally used to describe North America WMV isolates, may not adequately define WMV strains on a global basis.

Host ranges have been used widely in the past to classify WMV strains. The WMV isolates from Greece and Jordan were placed in the WMV-2 group on the basis of their ability to infect *C. amaranticolor* and/or *C. quinoa* (14). Susceptibility of *C. amaranticolor* to the isolate from Jordan also was determined in our laboratory by R. Baum (*personal communication*). The serologic (Table 1) and cytologic (14) properties of the isolates from Greece and Jordan, however, show that these viruses more closely resemble WMV-1 FL than WMV-2 FL. Hence, the reliability of *C. amaranticolor* as a host for typing WMV strains needs reevaluation, along with the entire matter of WMV strain classification.

The WMV-1 FL and WMV-2 FL isolates were distinguishable also by differences in their cross reactivities with certain other potyviruses. The papaya ringspot virus was found to be closely related to WMV-1 FL but was nonreactive with WMV-2 FL. These results contrast those of Milne and Grogan (15), who reported a close relationship of papaya ringspot virus to both WMV-1 and WMV-2. Soybean mosaic virus (this report) and blackeye cowpea mosaic virus (12,13,23) are both closely related to but distinct from WMV-2. Neither of these two "legume viruses" reacted with WMV-1 in reciprocal tests, however.

Previous tests for serodiagnosis of WMV in crude plant extracts have not been considered reliable due to inconsistencies in obtaining positive reactions (15,24). The SDS-immunodiffusion

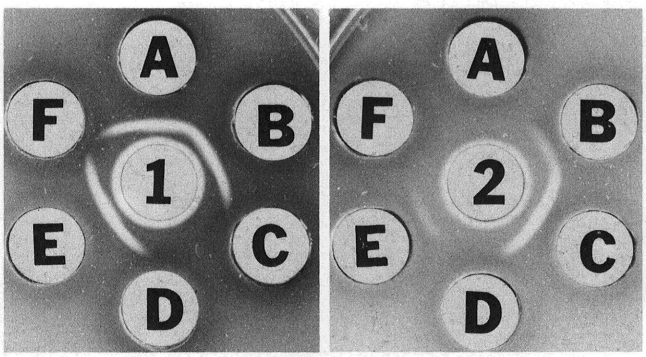

Fig. 3. Serologic detection of WMV-1 and WMV-2 antigens in plants inoculated with both viruses. The center wells contain 1 = antiserum to WMV-1 FL; 2 = antiserum to WMV-2 FL. The outer wells contain SDS-treated extracts from healthy squash (wells D and F) or from squash plants inoculated with WMV-1 FL alone (well A) WMV-2 FL alone (well C), or with both WMV-1 and WMV-2 (wells B and E).

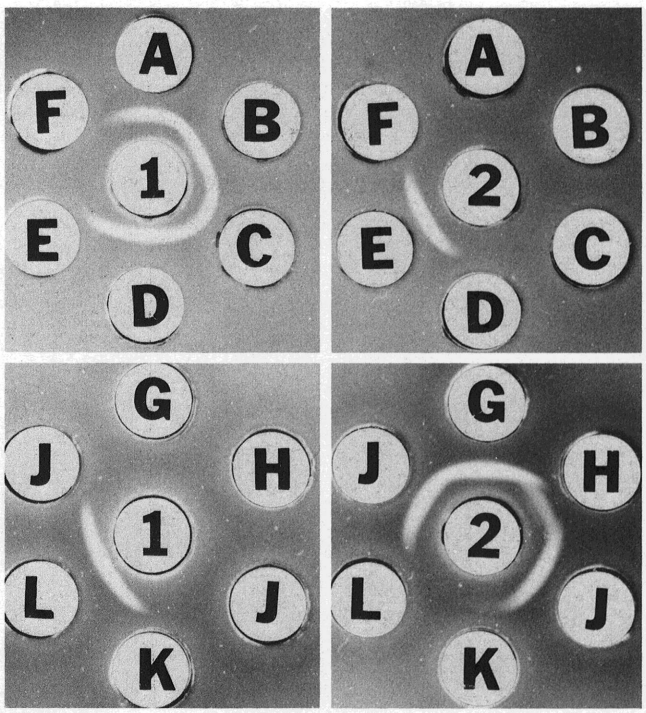

Fig. 4. Serologic reactions of WMV-1 and WMV-2 antisera with papaya ringspot (PRSV) and soybean mosaic (SoyMV) viruses. Wells 1 = WMV-1 FL antiserum and wells 2 = WMV-2 FL antiserum. The outer wells contain SDS-treated plant extracts as follows: Wells A and C = two isolates of PRSV in squash (both isolated from papaya in Florida by R. A. Conover); well B = WMV-1 FL in squash; well D = WMV-1 from the ATCC in squash; E = WMV-2 FL in squash; F = healthy squash; G = WMV-2 FL in *Nicotiana benthamiana*; H = SoyMV in *N. benthamiana*; J = WMV-2 FL in pumpkin; K = noninfected *N. benthamiana*; L = WMV-1 FL in pumpkin.

techniques employed in this study, however, consistently have given good results for detecting and distinguishing WMV-1 FL and WMV-2 FL isolates in crude leaf extracts from a variety of species in greenhouse-grown plants or in field samples (Purcifull, *unpublished*; T. A. Zitter, *personal communication*). Furthermore, we have used these methods to distinguish cucumber mosaic virus, squash mosaic virus, WMV-1 FL, and WMV-2 FL in cucurbit leaf extracts (Purcifull, Christie and Hiebert, *unpublished*). As with numerous other viruses (19,20), freeze-dried leaf extracts have proved convenient as sources of reference antigens for the cucurbit viruses.

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