Host Range and Serology of Prunus Necrotic Ringspot Virus Serotypes Isolated from Hops (*Humulus lupulus*) in Washington

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ABSTRACT

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Prunus necrotic ringspot virus serotypes NRSV-HP-1 (HP-1) and NRSV-HP-2 (HP-2) were isolated from hops (*Humulus lupulus*). Although both viruses produced chlorotic line pattern and ringspot on hops, they could be distinguished by their host range and by serology. Based on mini-double-immunodiffusion tests, HP-1 showed some serological similarity to Fulton's CAMV but was serologically distinct from Fulton's NRSV-G. HP-2 showed partial serological similarity to NRSV-G, HP-1, and CAMV. In enzyme-linked immunosorbent assay (ELISA), antisera prepared against HP-1 and CAMV reacted strongly with both antigens and less strongly with HP-2. Neither detected NRSV-G antigens. Antiserum against HP-2 reacted strongly with homologous antigen and less strongly with both HP-1 and CAMV antigens. Antiserum against NRSV-G reacted with homologous antigen and very slightly with HP-2. HP-2 appeared to be serologically intermediate between NRSV-G and CAMV.

In 1959, Fridlund (6) transmitted a virus from cultivated hop (*Humulus lupulus* L.) to cucumber (*Cucumis sativus* L.) that caused symptoms indistinguishable from those caused by certain isolates of the Prunus necrotic ringspot virus (NRSV). Bock (3) found that NRSV-like isolates from hop were two serologically distinct types: "A" strains, which were serologically related to isolates obtained from apple trees, and "C" strains, which were serologically related to isolates obtained from cherry. Fulton (8) reported that the A isolate of Bock was serologically identical to the apple mosaic virus (ApMV) (5), whereas the C isolate was closely related serologically to NRSV isolated from cherry. Barbara et al (2), using enzyme-linked immunosorbent assay (ELISA), found that hops in England contained either ApMV or a serotype intermediate between NRSV and ApMV. Although these and other investigations of NRSV and ApMV in hops have provided valuable information, the host ranges of the ilarviruses from hops have not been determined.

Two viruses of the Prunus ringspot group (11), NRSV-HP-1 (HP-1) and NRSV-HP-2 (HP-2), were isolated from hops in Washington State (10). Because of their similarities, HP-1 and HP-2 were compared by host range and two serological methods (mini-double-immunodiffusion [12] and ELISA). Results of these tests are reported in this paper.

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MATERIALS AND METHODS

Virus isolates and host range tests. The HP-1 isolate in this study was obtained as a single-lesion transfer from cucumbers that had been inoculated with juice from the hop cultivar Brewer's Gold. HP-2 was isolated from a "sterile" cluster-type hop cultivar. CAMV obtained from R. W. Fulton was the English ApMV isolate

from R. Cropley, described by de Sequeira (5) and serologically identical to the A isolate of Bock (8). NRSV-G, isolated from cherry and obtained from R. W. Fulton, was closely related to the C isolate of Bock. The isolates were maintained in National Pickling cucumber. Plants for the host range tests were grown in the greenhouse at 22–25 C and inoculated in the cotyledon or four-leaf stage after a preinoculation dark treatment for 16–24 hr.

Leaves dusted with Carborundum (600-mesh) were rub-inoculated with infected cucumber leaf tissue triturated in 0.03 M phosphate buffer, pH 8.0, containing 0.02 M 2-mercaptoethanol or with purified virus in the same buffer. Plants were examined regularly for symptoms. After 1 mo, both inoculated and uninoculated leaves were assayed by ELISA for virus content. Comparative host range tests were repeated at least three times on each species.

Cuttings of the cluster-type hop cultivars L_1 and E_2 (free of all known hop viruses) were inoculated in the four- to five-paired-leaf stage with partially purified preparations of HP-1 and HP-2.

Virus purification. The viruses were purified by a modification of Fulton's (7) method. Infected cucumber cotyledons (1 g of tissue per 3 ml of buffer) were triturated for 2 min in a Waring Blendor in a cold solution of 0.01 M diethyldithiocarbamic acid, 0.02 M thioglycolic acid in 0.03 M sodium phosphate buffer, pH 8.0, and expressed through four single layers of cheesecloth. The homogenate was mixed with hydrated calcium phosphate (HCP) (0.8 g/g of tissue), clarified by low-speed centrifugation (3,000 × *g* for 30 min in a Beckman model J2-21 centrifuge, JA 10 rotor), and a 40% (w/v) polyethylene glycol solution containing 1 M NaCl was added at the rate of 25 ml/100 ml of supernatant. This mixture was incubated in the refrigerator for 1 hr, centrifuged at 3,000 × *g* for 50 min in the JA 10 rotor, and pellets were resuspended in 0.02 M phosphate buffer, pH 8.0, to 1/15 of their original volume. Insoluble residue was removed by centrifugation (15,000 × *g* for 20 min), and virus in the supernatant was pelleted by centrifugation (100,000 × *g* for 120 min). Pellets were suspended in 0.02 M phosphate buffer, pH 8.0, and centrifuged at 15,000 × *g* for 20 min. The supernatant was layered on 10–40% (w/v) rate-zonal sucrose density gradients and centrifuged for 3 hr at 60,000 × *g* in an SW 25.1 rotor. Visible zones were located 18–23 and 24–28 mm below the tube meniscus for HP-1 and at 17–22 and 25–28 mm for HP-2.

Serology. Antiserum against HP-1 and HP-2 was prepared by injecting an emulsion of 1:1 Freund's incomplete adjuvant containing 0.5–1 mg of purified virus intramuscularly into separate rabbits every 3–4 days for 6 wk. Four weeks after the first injection, the rabbits were bled. Titers of the antisera were 1/512 for HP-1 and 1/256 for HP-2 as determined by the ring interface test (1).

Four antisera and their respective homologus viruses were used in the serological tests. Antisera to NRSV-G and to CAMV obtained from R. W. Fulton were compared with the two Washington isolates from hop, using mini-double-immunodiffusion tests and ELISA. Mini-double-immunodiffusion tests were conducted using agarose (1.5%) cast on a polyester-based film, supported by a slide mount. The slide mount with film-supported agar was placed on a template, and wells 1 mm in diameter were punched using a vacuum system. About 2 μl of reactants was placed in each well. Raw antisera against the viruses were used undiluted. The purified virus concentration was about 100 μg/ml for each virus tested.

For ELISA, γ-globulins obtained from each antiserum were conjugated to alkaline phosphatase, Type VII (Sigma Chemical Co., St. Louis, MO), prepared according to Clark and Adams (4). Coating globulin (1 μg/ml) was incubated for 4 hr at 37 C. Purified virus (1 μg/ml) or tissue extracts were incubated overnight at 4 C. Conjugated γ-globulins diluted 1:2,000 were incubated for 4 hr at 37 C. Substrate (*p*-nitrophenyl phosphate, Sigma No. 104) was added to Polystyrene Micro ELISA Immulon plates (Dynatech Laboratories, Alexandria, VA) at a concentration of 1 mg/ml in diethanolamine, HCl buffer, pH 9.8. The plates were incubated at room temperature for 30–60 min. Sample reactions were determined by scanning plates at 405 nm in a BIO-TEK EIA Reader (BIO-TEK Instruments, Inc., Burlington, VT). Plates were standardized by adjusting the A_{405nm} values to 0.000 for buffer control wells. The A_{405nm} value recorded for healthy control wells was less than 0.05.

Table 1. Diagnostic hosts for Prunus necrotic ringspot (NRSV) serotypes NRSV-HP-1 and NRSV-HP-2 from hop

	Virus			
	NRSV-HP-1		NRSV-HP-2	
Host	Inoc. leaves	Uninoc. leaves	Inoc. leaves	Uninoc. leaves
Amaranthaceae				
Amaranthus retroflexus L.	NRL[a](4)[b]	L	L	L
Gomphrena globosa L.	L	–	–	–
Chenopodiaceae				
Chenopodium album L.	L	–	–	–
C. amaranticolor Coste. & Reyn.	L	–	CRS (6)	–
C. berlandieri Moq.	L	–	CS,NL (10,15)	CS (15)
C. capitatum (L.) Asch.	NL (10)	–	–	–
C. foliosum (Moench) Asch.	L	–	–	–
C. hybridum L.	NL (4)	M (12)	–	–
C. murale L.	NL (5)	L	NRS (18)	–
C. urbicum L.	CS (14)	–	–	–
C. vulvaria L.	L	–	–	–
Compositae				
Helianthus annuus L.	CS,NRS (5)	VC,CRS (21)	NL,CS? (5)	–
Tithonia speciosa Hook.	CS (9)	CRS,VC (14)	CS,NL (9)	CS,NRS,LP (18)
Zinnia elegans Jacq.	–	L	–	–
Z. elegans 'Thumbelina'	NL (10)	CS (20)	–	–
Cucurbitaceae				
Cucumis anguria L.	NL (5)	M (17)	NL,CS (5,15)	–
Cucurbita moschata Dcne. 'Butternut'	NL (6)	–	–	–
C. pepo (L.) Alef. 'Early Prolific Straightneck Squash'	NL (6)	–	–	–
C. pepo 'Small Sugar Pumpkin'	CS,NL (6)	–	–	–
Leguminosae				
Daubentonia tripetii (Poit) F. T. Hubb.	NRL (10)	–	0	0
Phaseolus vulgaris L. 'Prince'	L	–	–	–
Solanaceae				
Datura stramonium L.	NL (5)	CRS,NRS (10)	L	–
D. tatula L.	CS,CRS (8)	CS (25)	CS (8)	–
Nicotiana angustifolia R&B	L	–	–	–
N. clevelandii Gray	L	L	–	–
N. repanda Willd.	L	NE,D (10)	NL (20)	R (10)
N. tabacum L. 'DB 402'	NL (11)	L	–	–
N. tabacum 'Samsum'	CS (10)	–	NL (10)	–
N. tabacum 'Xanthi-NC'	NL (2)	–	L	–
Solanum melongena L.	CRS,NRS (11)	L	–	–
S. nanum L.	L	–	–	–

[a] CRS = chlorotic ringspot, CS = chlorotic spots, D = distortion, LP = line pattern, M = mottling or mosaic, NE = necrotic etch, NL = necrotic local lesions, NRL = necrotic red lesion, NRS = necrotic ringspot, R = rugose, VC = veinclearing, – = not infected, L = latent, 0 = not tested, and ? = symptom faint or questionable.
[b] Number of days for symptoms to occur.

RESULTS

Host ranges of hop viruses. The most striking differences in host ranges occurred among species of Chenopodiaceae (Table 1). The HP-1 isolate

infected *Chenopodium capitatum, C. foliosum, C. hybridum, C. urbicum,* and *C. vulvaria.* However, the HP-2 isolate did not infect any of these five *Chenopodium* species. The HP-2 isolate infected *C. berlandieri* (Fig. 1), but HP-1 was restricted to latent infection of inoculated leaves. Thus, the viruses were readily distinguished and each virus could be distinguished from the others on the basis of differential susceptibility of *Chenopodium* species. HP-1 and HP-2 had wide host ranges. HP-1 infected 60 species in 8 families, whereas HP-2 infected 45 species in 8 families among 101 species in 15 families tested. HP-1 infected three test species of the Solanaceae: *Nicotiana clevelandii, N. tabacum* 'DB 402,' and *Solanum melongena,* but HP-2 did not infect these species. In the Cucurbitaceae, *Cucurbita moschata* 'Butternut,' *C. pepo* 'Early Prolific Straightneck Squash' and *C. pepo* 'Small Sugar Pumpkin' were all infected with HP-1 but not with HP-2.

Species that were not hosts of either virus were: *Antirrhinum majus* L., *Arctotis breviscapa* Thunb., *Atriplex hymenelytra* (Torr.) Wats., *Beta vulgaris* L., *Browallia elata* L., *Capsicum annuum* L. 'Early Calwonder,' *Chenopodium botrys* L., *C. foetidum* Schrad., *Crotalaria capensis* Jacq., *C. striata* Desv., *Datura fastuosa* L., *D. meteloides* DC., *Dianthus deltoides* L. 'Auricle-eyed,' *Hibiscus palustris* L., *Ipomoea purpurea* (L.) Rooth., *I. quamoclit* L., *Lavatera arborea* L., *Lycopersicon esculentum* Mill. 'VR Moscow,' *Malva moschata* L., *Melilotus alba* Desv., *Nicotiana debneyi* Domin., *N. glutinosa* L., *N. occidentalis* Wheeler, *N. rustica* L., *N. stocktonii* Brandeg., *N. tabacum* L. 'VTI 245,' *N. velutina* Wheeler, *Phaseolus vulgaris* L. 'Kinghorn,' and 'Yakima,' *Physalis floridana* Rydb., *Pisum sativum* L. 'Alaska,' *Tetragonia expansa* Thunb., *Urtica dioica* L., *Vicia faba* L. 'Diana,' and *Vigna sinensis* (Torner) Savi. 'Blackeye.'

The serotypes isolated from hop caused similar symptoms on the following species: *Amaranthus caudatus* L., *Chenopodium polyspermum* L., *C. quinoa* Willd., *Citrullus vulgaris* Schrad. 'Jubilee,' 'Klondike,' 'Peacock,' and '#86,' *Crotalaria spectabilis* Roth., *Cucumis melo* Naud. 'Casaba,' 'Iroquois,' '#32,' and 'Hale's Best,' *C. prophetarum* L., *C. sativus* L. 'Boston Pickling' and 'National Pickling,' *Cucurbita maxima* Dcne. 'Buttercup,' *C. pepo* (L.) Alef. 'Miniature,' *Humulus lupulus* L., *Lagenaria luffa* Mol. 'Greensnake,' *L. siceraria* (Mol.) Standl. 'Caveman's Club,' *Lavetera assurgentiflora* 'Kellogg,' *Luffa acutangula* Roxenb. 'Dishrag,' *Momordica balsamina* L., *Nicandra physalodes* (L.) Gaertn., *Nicotiana megalosiphon* (Heurck & Muell.) Arg., *N. solanifolia* Walp., *N. tabacum* L. 'DB 102' and 'T. I. 787,' *Petunia hybrida* Vilm., *Trichosanthes anguina* L., *Vinca rosea* L., and *Zinnia elegans* Jacq. 'Bicolored.'

The effects of environmental factors on susceptibility and symptom expression were not investigated, although hosts were tested under various seasonal fluctuations over several years and these fluctuations did not appear to substantially affect symptoms.

The symptoms on hops caused by these NRSV serotypes appeared about 5 days after inoculation as chlorotic ringspots and line patterns. They differed only in severity, with HP-1 more severe than HP-2. Presence of virus was monitored

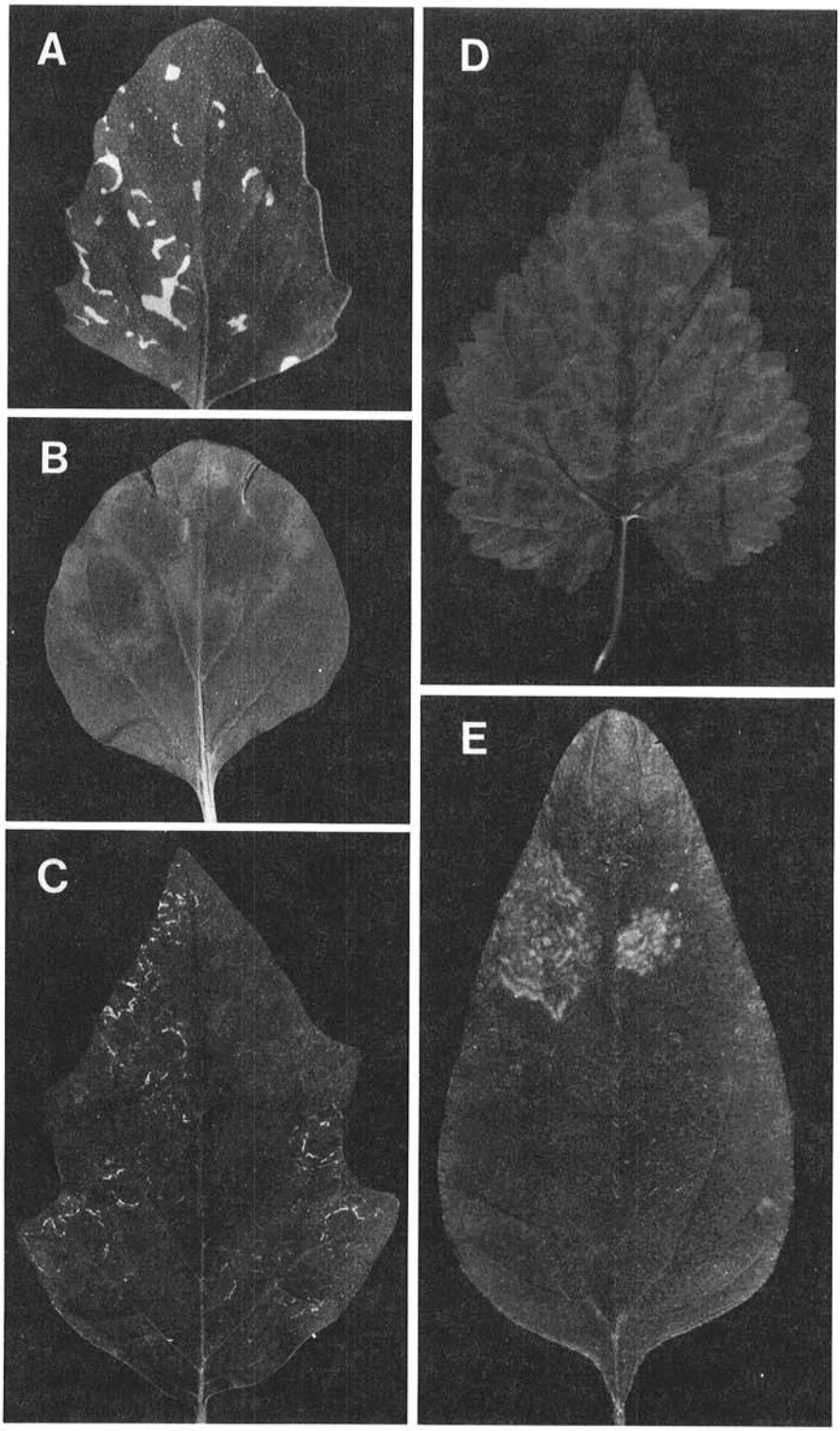

Fig. 1. Chlorotic and necrotic ringspots incited by **(A)** Prunus necrotic ringspot virus (NRSV-HP-2) on *Chenopodium berlandieri* on inoculated leaf, **(B)** Prunus necrotic ringspot virus (NRSV-HP-1) on *Solanum melongena* on inoculated leaf, **(C)** NRSV-HP-1 on *Datura stramonium* on uninoculated leaf, **(D)** NRSV-HP-2 on *Humulus lupulus* on inoculated leaf, and **(E)** NRSV-HP-1 on *Helianthus anuus* on uninoculated leaf.

periodically with ELISA.

Serological studies. Antisera from HP-1, CAMV, HP-2, and NRSV-G were tested against purified antigens of HP-1, NRSV-G, HP-2, CAMV, and healthy plant extract in mini-double-immunodiffusion tests (Fig. 2). HP-1 antiserum in the center well (Fig. 2A) reacted with all viruses except NRSV-G, forming coalesced precipitin lines to HP-2, CAMV and HP-1. Antiserum to CAMV in the center well (Fig. 2B) was similar to HP-1 but reacted to NRSV-G, which was strongly spurred by HP-1 and HP-2. Also, HP-1 formed a spur over HP-2. HP-2 antiserum in the center well (Fig. 2C) reacted with all viruses tested. The similarity between HP-2 and NRSV-G was seen by a formation of a strong coalesced precipitin line. CAMV and HP-1 produced a weak precipitin line that coalesced when reacted with antiserum to HP-2, forming a strong HP-2 spur over that of CAMV and HP-1. When reacted to HP-2 antiserum, there were three precipitin lines formed to CAMV virus, one that coalesced with HP-1 and one that appeared to spur over HP-1 and NRSV-G. NRSV-G produced a strong reaction with antiserum to HP-2 with a strong spur over HP-1. Antiserum to NRSV-G (Fig. 2D) reacted with NRSV-G, forming a spur over HP-2. This shows NRSV-G and HP-2 share partial serological identity, but the spur formed by NRSV-G over HP-2 indicates HP-2 does not contain some components present in NRSV-G. No preciptin lines were formed between NRSV-G antiserum and HP-1 or CAMV. This clearly shows that NRSV-G does not share antigenic sites in common with HP-1.

As shown in Table 2, NRSV-G was not detected by CAMV or HP-1 in ELISA. The relationship between ELISA absorbance values of HP-2 and NRSV-G, though very low (0.084–0.087), gave indication of slight agreement; however, because of low absorbance, ELISA could not be used to readily distinguish between the two viruses. In mini-double-immunodiffusion tests (Fig. 2C,D), HP-2 and NRSV-G showed partial serological relatedness. CAMV and HP-1 were closely related in ELISA.

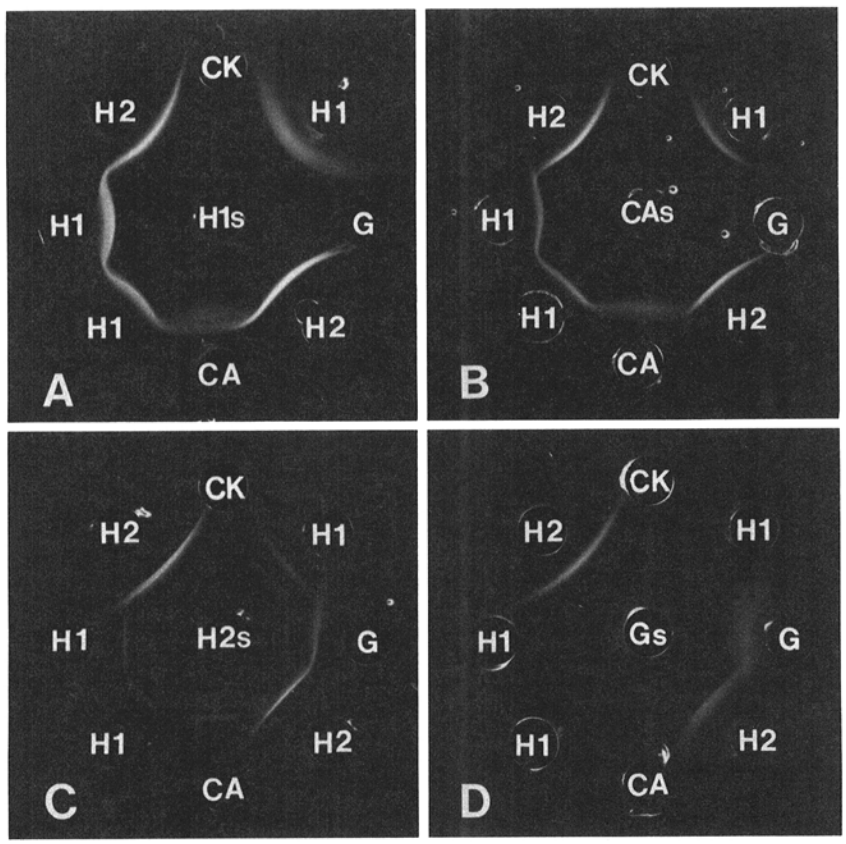

Fig. 2. Serological relationships among isolates of Prunus ringspot virus (NRSV) and apple mosaic virus (ApMV) determined by mini-double-diffusion tests in 1.5% agarose. Central wells contained antiserum to **(A)** H1s = NRSV isolate from hop (NRSV-HP-1), **(B)** CAs = Fulton's ApMV isolate (CAMV), **(C)** H2s = NRSV isolate from hop (NRSV-HP-2), and **(D)** Gs = Fulton's PNRV isolate from cherry (NRSV-G). Peripheral well CK contained clarified healthy cucumber sap. The remaining wells contained purified antigens (100 μg/ml) of virus: H1 = NRSV-HP-1, G = NRSV-G, H2 = NRSV-HP2, and CA = CAMV.

Table 2. Reactions of Prunus necrotic ringspot virus (NRSV) serotypes and apple mosaic virus (ApMV) antisera to strains of NRSV and ApMV in enzyme-linked immunosorbent assay[a]

	Antiserum[c]			
Virus[b]	CAMV	NRSV-HP-1	NRSV-G	NRSV-HP-2
CAMV	1.999	0.901	0.013	0.462
NRSV-HP-1	1.870	1.766	0.018	0.554
NRSV-G	0.036	0.007	0.904	0.087
NRSV-HP-2	0.783	0.193	0.084	1.755

[a] Absorbance at 405 nm; healthy control value less than 0.05.
[b] Virus concentration = 1 μg/ml.
[c] γ-Globulin = 1 μg/ml, enzyme-conjugated globulin = 1:2,000 dilution, and substrate incubation time = 1 hr.

DISCUSSION

This is the first extensive host range determination for NRSV isolates from hops. Although they had many common hosts, the isolates were readily detected and distinguished from one another by differential symptoms on several hosts, including *C. hybridum, C. urbicum, D. stramonium,* and *S. melongena.* HP-1 could be separated from HP-2 by differential susceptibility of *C. hybridum.* Conversely, HP-2 could be isolated on *C. berlandieri.* HP-1 had a wider host range than the HP-2 and usually produced the more severe symptoms.

Based on mini-double-immunodiffusion tests, HP-1 was serologically different from HP-2 and closely related to CAMV. HP-2 showed partial serological similarity to NRSV-G and HP-1 showed partial serological similarity to CAMV. NRSV-G was distinct from HP-1 and CAMV. Data obtained with ELISA confirm these results, except only a very slight positive reaction between HP-2 and NRSV-G was obtained. This may be an indication of an intermediate relationship between HP-2 and NRSV-G.

Although the initial objective was to distinguish between the two viruses isolated from hops in Washington, the primary importance of this study lies in its application for epidemiology and certification work. Host range, mini-double-immunodiffusion, and ELISA can be used for certain purposes, although each test has limitations. Particularly in ELISA, one individual antiserum cannot detect which specific virus is present. Halk et al (9) found HP-1 and HP-2 gave reactions with monoclonal antibodies characteristic of ApMV. This comparison helps define the antigenic relationships among these viruses.

Chlorotic line patterns and ringspots are common symptoms seen in commercial hop yards in Washington State. These symptoms are particularly prevalent in late fall when temperature becomes cooler and during cool periods following warmer periods during the summer. Both hop viruses are capable of producing the

symptoms observed in commercial hop yards. Whether they are the sole cause of such symptoms remains to be determined. These two viruses reduce both hop cone yield and brewing value.

ACKNOWLEDGMENT

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