# Three Serotypes of Prunus Necrotic Ringspot Virus Isolated from Rugose Mosaic-Diseased Sweet Cherry Trees in Washington

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**ABSTRACT**

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Three distinct serotypes of Prunus necrotic ringspot virus (NRSV) (designated CH-3, CH-9, and CH-30) were isolated from sweet cherry trees in Washington that showed similar symptoms of cherry rugose mosaic disease. Among more than 50 NRSV isolates serotyped, only one isolate of serotype CH-30 and two isolates of serotype CH-3 were found. Two of these isolates were from trees in Washington known to have been infected before 1970; the other was from Canada. Most NRSV isolates obtained from sweet cherry trees in Washington, almond and cherry trees in California, and pollen from beehives brought to Washington from California were serotype CH-9. This serotype also included isolates A, E, G, and H described earlier in Wisconsin. Serotype CH-9 isolates were transmitted from trees showing a range of symptoms.

Rugose mosaic disease of sweet cherry (*Prunus avium* L.) trees originally described by Thomas and Rawlins (16) has been known to occur in cherry-growing districts of all the Pacific Coast states and British Columbia for more than 25 yr (12). Since the mid-1970s, the incidence of rugose mosaic-diseased trees in Washington has been increasing steadily (8). Chronic symptoms include chlorotic leaf spots or blotches, leaf twisting, enations on the abaxial leaf surface, and delayed ripening of fruit that may vary from a few days to several weeks. The disease, which occurs in mild, moderate, and severe forms (10,12), is caused by strains of Prunus necrotic ringspot virus (NRSV) (11,13,14). Earlier, two reports from California indicated that NRSV strains that cause rugose mosaic disease in sweet cherry or almond calico and bud failure diseases in almond could be distinguished serologically from strains that cause ordinary necrotic ringspot diseases in these hosts (13,14). However, no specific serological data were presented in either report. Consequently, the nature of these serological relationships is unknown.

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Preliminary results obtained in Washington with enzyme-linked immunosorbent assay (ELISA) demonstrated that NRSV isolates commonly found in local cherry trees reacted strongly with an antiserum prepared against a sour cherry (*P. cerasus* L.) isolate originally described by Fulton as NRSV-G (5,6) but did not react with antisera prepared against NRSV isolates from hops or plum or with two apple mosaic virus (ApMV) antisera (8). In addition, that study indicated that dormant bud tissues from rugose mosaic-diseased trees produced absorbance readings 2.5–14 times greater than those obtained with similar tissues taken from trees infected with ordinary NRSV isolates (8). These data tended to support the earlier contention that rugose mosaic and necrotic ringspot isolates in cherry could be distinguished serologically. However, when we conducted large-scale tests by ELISA with the NRSV-G antiserum, we found that ELISA results often failed to distinguish rugose mosaic-diseased trees from NRSV-infected trees that showed any of the following: no symptoms, symptoms typical of ordinary NRSV isolates, or symptoms atypical of rugose mosaic (10). In one orchard, we consistently failed to detect virus in one 40+-yr-old tree even though the tree showed severe rugose mosaic symptoms (*unpublished*). These results precipitated the current study in which we found that at least three distinct serotypes of NRSV can be isolated from rugose mosaic-diseased trees. A brief report was presented earlier (11).

## MATERIALS AND METHODS

**Field inoculations.** Cherry trees identified during visual and serological surveys as infected with biological variants of NRSV were bud-inoculated to 2-yr-old Bing trees in the field. The first author collected field isolates of NRSV from almond orchards in California in cooperation with G. Nyland, Davis, CA, that were subsequently indexed on young Bing trees. All cherry and almond isolates used in these tests produced symptoms typical of NRSV when indexed on *P. serrulata* Lindl. cv. Shirofugen (13).

**Virus isolates.** Isolates of NRSV were transmitted from cherry or almond trees by mechanical inoculation (8) to greenhouse-grown *Chenopodium quinoa* Willd. seedlings. One rugose mosaic isolate (Dobi) was obtained from tissue provided by A. J. Hansen, Summerland, BC. Isolates were purified from *C. quinoa* leaf tissue as described earlier (8). Purified antigens of NRSV-hop and ApMV-hop were provided by C. B. Skotland, Prosser, WA (15).

**Antisera.** Antisera to local NRSV isolates were prepared in rabbits by either of two standard methods: 1) four weekly intravenous injections made with 2-ml volumes of purified antigen (0.5 mg/ml) suspended in 0.01 M neutral phosphate buffer or 2) four weekly intramuscular injections made with 1-ml volumes of purified antigen (0.1 mg/ml) emulsified with equal volumes of Freund's complete (first injection) or incomplete (remaining injections) adjuvant. Sera were collected weekly beginning 1 wk after the final injection. One antiserum prepared against isolate CH-3 produced strong reactions in both agar gel tests and ELISA and was used for all tests. Two antisera against isolate CH-9 produced strong reactions in agar gel tests but reacted weakly or not at all in ELISA. One antiserum prepared against isolate CH-30 produced strong reactions in both agar gel tests and ELISA.

**Test conditions.** Agar gel double-diffusion tests were performed in 90-mm-diameter plates containing 0.7% Noble agar in 0.1 M neutral phosphate buffer and 0.7% sodium azide. Wells were 6 mm in diameter with 5 mm between the edges of the antisera and antigen wells. Antisera were diluted 1:2 in 0.1 M neutral phosphate buffer. All antigen preparations contained 70–140 g/ml nucleoprotein

suspended in 0.1 M phosphate buffer. Conditions for double-sandwich ELISA were the same as previously described (8).

## RESULTS

**Agar gel double-diffusion tests with purified antigens.** More than 50 NRSV isolates were transmitted from cherry and almond tissue to *C. quinoa* and serotyped using antisera against isolates CH-3, CH-9, and CH-30. Among these, we found three isolates (CH-3, CH-9, and CH-30) that could be distinguished from each other by spur formation in agar gel tests (Table 1). All three isolates were transmitted from rugose mosaic-diseased trees with similar symptoms. Identical symptoms were produced on young Bing trees bud-inoculated with tissue from the original source trees (Table 2).

Antisera prepared against isolates CH-3 and CH-30 distinguished all heterologous combinations of the three antigens by the formation of obvious spurs (Table 1). Antiserum CH-9 did not distinguish between isolates CH-3 and CH-9 but did distinguish between either isolate and CH-30.

In tests with CH-30 antiserum, all three antigen types produced visible reactions within 24–48 hr. However, when antisera to either CH-3 or CH-9 were used, antigens of CH-3 and CH-9 produced visible reactions within 24–48 hr, whereas reactions associated with CH-30 antigens were not observed until after 72–120 hr. Purified antigens of CH-9 and isolates serologically identical to CH-9 produced two visible reaction lines when tested against CH-30 antiserum.

Although isolates Ch-3, CH-9, and CH-30 have many epitopes in common, each appears to possess epitopes unique to that isolate. These isolates represent three distinct serotypes of NRSV.

Only a single isolate of the CH-30 serotype has been found thus far (Table 2). This isolate was obtained from the 40+-year-old tree mentioned earlier in which we failed to detect NSRV using ELISA with Fulton's (5,6) NRSV-G antiserum. Two isolates of the CH-3 serotype were found: one from a 40+-yr-old tree in Washington that showed severe rugose mosaic symptoms, the other, from a rugose mosaic-diseased tree in British Columbia. All of the remaining isolates tested, including the four isolates initially described on the basis of symptomatology by Fulton (5,6) as A, E, G, and H, appeared to be serologically identical to CH-9 in agar gel tests (Table 2). Included among these CH-9 serotypes were isolates from rugose mosaic-diseased trees in Washington and almond calico-diseased trees in California. However, also included among the CH-9 serotypes were isolates from both cherry and almond trees showing symptoms of ordinary necrotic ringspot.

In tests not reported, we found that antisera prepared against Fulton's isolate A and an additional Washington isolate CH-61 each produced results similar to those reported here for antiserum CH-9. In our tests, Fulton's antiserum NRSV-G reacted weakly in agar gel plates with isolates CH-3 and CH-9 and not at all with isolate CH-30.

**ELISA results with purified antigens.** Because Fulton's NRSV-G isolate appeared serologically identical to our CH-9 isolate and our CH-9 antiserum was not useful for ELISA (produced uniformly low readings), we used the NRSV-G antiserum as the representative antiserum for the CH-9 serotype. Typical ELISA results with various antigens and antisera combinations are illustrated in Table 3.

Antisera against ApMV-P and the hop isolate of NRSV did not react with the NRSV isolates from sweet cherry. Of the

**Table 1.** Homologous and heterologous relationships[a] among purified antigens of three Prunus necrotic ringspot virus isolates originally transmitted from rugose mosaic-diseased trees

| Antiserum[b] in center well | Antigen[c] in left well | Precipitin line patterns with antigen in right well | | |
|---|---|---|---|---|
| | | CH-3 | CH-9 | CH-30 |
| CH-3 | CH-3 | F[d] | Sr[e] | Sr |
| | CH-9 | Sl | F | Sr |
| | CH-30 | Sl | Sl | F |
| CH-9 | CH-3 | F | F | Sr |
| | CH-9 | F | F | Sr |
| | CH-30 | Sl | Sl | F |
| CH-30 | CH-3 | F | Sr | Sl |
| | CH-9 | Sl | F | Sl |
| | CH-30 | Sr | Sr | F |

[a] Agar gel double-diffusion tests.
[b] Each antiserum was diluted 1:2 in 0.1 M neutral phosphate buffer.
[c] Antigen concentrations ranged between 70 and 140 μg/ml.
[d] Reaction lines appeared to fuse.
[e] Obvious spur formed in the direction indicated; Sr = spur to the right, Sl = spur to the left.

**Table 2.** Range of symptoms associated with isolates of each of three Prunus necrotic ringspot serotypes

| Serotype | Isolate | Source[a] | Original disease[b] | Symptons on *Chenopodium quinoa*[c] | Symptons on Bing |
|---|---|---|---|---|---|
| CH-3 | CH-3 | WA cherry | sCRM | CL, Mo, N | CRM |
| | Dobi | CAN cherry | CRM | CL, Mo, N | CRM |
| CH-9 | CH-9 | WA cherry | CRM | NL, DB | CRM |
| | CH-38 | WA cherry | sCRM | NL, DB | CRM |
| | Al-7 | CA almond | AlC/bf | NL, DB | CRM |
| | Al-5 | CA almond | Albf | NL, DB | CRM, Calico |
| | Al-2 | CA almond | AlC[d] | CNL, CR, NS | Calico |
| | Al-8 | CA almond | NRS | CNL, CR, Mo | NRS[e] |
| | Al-9 | CA almond | NRS | CNL, CR, R | NRS[e] |
| | BH-1 | CA pollen[f] | ? | CNL, CR, R | ··· |
| | BH-2/1 | CA pollen[f] | ? | CNL, CR, R | ··· |
| | CH-27 | WA cherry | NRS | CNL, CR, R | Mo |
| | Fulton A | PRF prune | NRS | CNL, CR, R | Mo |
| | Fulton E | PRF prune | NRS | CNL, CR, Mo | Mo |
| | Fulton G | PRF prune | NRS | CNL, CR, Mo | Mo |
| | Fulton H | PRF prune | NRS | CNL, CR, R | Mo |
| CH-30 | CA-30 | WA cherry | CRM | CS, CR, Mo | CRM |

[a] State or country abbreviations: CA = California, CAN = Canada, and WA = Washington; PRF = isolates originally found in Wisconsin by Fulton (7) but maintained in Italian prune trees at Prosser, WA, by P. R. Fridlund.
[b] Albf = virus-induced almond bud failure, ARC = almond calico, AlC/bf = both almond calico and bud failure symptoms, CRM = moderate cherry rugose mosaic, sCRM = severe cherry rugose mosaic, NRS = ordinary necrotic ringspot symptoms, and ? = original tree not observed.
[c] CL = chlorotic lesions on inoculated leaves, NL = necrotic lesions on inoculated leaves, CNL = chlorotic lesions with necrotic centers on inoculated leaves, CR = chlorotic rings on top leaves, DB = necrosis and die back of plant, Mo = mottle on tip leaves, N = necrosis of tip leaves, NS = necrotic spots on tip leaves, and R = recovery.
[d] Isolate transmitted to herbaceous plants and back to almond (13).
[e] Symptoms described by Nyland et al (13).
[f] Pollen taken from beehive entering Washington from California (8).

three cherry antisera, only NRSV-G reacted weakly with the NRSV-hop isolate.

All three cherry isolate antisera reacted strongly with isolates of the CH-9 serotype. However, CH-3 antiserum reacted weakly with isolate CH-30 and the CH-30 antiserum reacted weakly with isolate CH-3. In ELISA tests not reported, results with our antiserum CH-61 were essentially the same as those obtained with Fulton's NRSV-G antiserum.

**Table 3.** Specific reactivity of purified antigens of Prunus necrotic ringspot virus (NRSV) when tested by double-sandwich enzyme-linked immunosorbent assay against five antiserum-conjugate systems

| Antigen (1 μg/ml) | Cherry serotype | Mean $A_{405nm}$ values (antiserum/conjugate) | | | | |
|---|---|---|---|---|---|---|
| | | CH-3 | NRSV-G | CH-30 | NRSV-Hop | ApMV-P |
| CH-3 | CH-3 | 2.73 | 2.73 | 0.36 | 0.00 | 0.00 |
| Al-2 | CH-9 | 2.30 | 2.28 | 2.75 | 0.00 | 0.00 |
| CH-27 | CH-9 | 2.42 | 2.33 | 1.22 | 0.00 | 0.00 |
| CH-30 | CH-30 | 0.20 | 0.20 | 2.31 | 0.00 | 0.00 |
| NRSV-Hop | ··· | 0.05 | 0.30 | 0.09 | 1.05 | 0.87 |
| ApMV-Hop | ··· | 0.05 | 0.09 | 0.05 | 0.00 | 2.74 |
| Control | ··· | 0.05 | 0.06 | 0.05 | 0.00 | 0.00 |

## DISCUSSION

Serological relationships among isolates identified as NRSV or ApMV have become increasingly complex as more isolates are examined. The first two isolates of each virus to be purified and characterized serologically were found to be either closely related (NRSV-G and Danish line pattern virus) or identical (ApMV and rose mosaic virus) serologically (7). However, the NRSV isolates from *Prunus* spp. produced distinct spur reactions with the ApMV isolates from *Malus* or rose in agar gel diffusion tests and thus were recognized as serotypes (4,7). Yet because each isolate was associated with a different disease, the disease name designations were retained (7). This distinction seemed appropriate in view of the fact that NRSV-G and ApMV antigens did not react with heterologous antisera in ELISA (1). However, the discovery of "intermediate" isolates in hops (1,15) and roses (2), which react in ELISA with both antisera, blurs the clear distinction between viruses.

In this study, all isolates from cherry and almond appear to be of the NRSV type; in ELISA, they reacted with NRSV-G antiserum but not ApMV-P antiserum. However, in agar gel diffusion tests, three of the isolates appeared different enough to be considered distinct serotypes. As far as we are aware, this is the first report of three serologically distinct virus isolates recovered from plants of the same crop that showed similar, if not identical, diseases.

It is of interest to note that the single source trees of isolates CH-3 and CH-30 were known to have been infected before 1970, when local beekeepers began transporting beehives annually to California to pollinate almond and cherry trees (9). Although the original infection sources of these two isolates are unknown, it seems likely from the age of the trees and history of the two orchards involved that these two serotypes may be representative of the NRSV isolates that were common in Washington many years ago. The single source tree of the CH-3 type isolate from Canada was located in a region where use of California bees is uncommon. In contrast, the source trees of isolates CH-9, CH-38, and numerous other CH-9 type isolates found recently are all known to have been infected since 1970. All these source trees are located in orchards where beehives from California are used each spring to aid pollination. The fact that only isolates of the CH-9 serotype were detected in California almond (Table 2) and cherry (*unpublished*) orchards and in pollen from beehives entering Washington from California (Table 2) is consistent with the hypothesis that NRSV isolates are frequently introduced into Washington orchards by honeybees in hives brought from California (9).

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