# Identification of Rugose Mosaic-Diseased Cherry Trees by Enzyme-Linked Immunosorbent Assay

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**ABSTRACT**

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Vegetative and flower buds collected during the dormant season from sweet cherry trees infected with cherry rugose mosaic (CRM) strains of *Prunus* necrotic ringspot virus (NRSV) and tested by enzyme-linked immunosorbent assay (ELISA) consistently produced absorbance readings 2.5–14 times greater than those obtained with similar tissues taken from trees infected with ordinary strains of NRSV. Procedures were developed to identify CRM-diseased trees before they flower to improve tree removal to reduce intraorchard spread of this disease. ELISA tests made during the growing season with cherry leaf and fruit tissues gave highly variable results unsuitable for diagnostic purposes.

Rugose mosaic disease (2) of sweet cherry (*Prunus avium* L.) is caused by strains of *Prunus* necrotic ringspot virus (NRSV) (6). The cherry rugose mosaic (CRM) strains of NRSV can be differentiated from ordinary strains (8) by host reaction, cross-protection in herbaceous plants, and serologic tests (9,10).

Before 1975, CRM-diseased trees were found only occasionally in commercial sweet cherry orchards in central Washington. For reasons not yet understood, the number of diseased trees has risen dramatically during the past 5 yr. In 1979, CRM was found in 40 commercial orchards. In several orchards, the disease has spread rapidly from tree to tree during the past 2–3 yr.

Grower attempts to control spread by removing obviously diseased trees have been unsuccessful, apparently for three reasons: 1) All strains of NRSV can be transmitted through pollen (8). 2) CRM strains appear to invade trees already infected with ordinary NRSV strains. 3) The symptoms that appear during the first 2–3 yr after infection with CRM strains resemble the effects of excess gibberellin, which is applied in many Washington orchards to retard ripening and thus extend the harvest season. Consequently, most growers are reluctant to pull trees until symptoms are severe and most of the fruit is unmarketable. Diseased trees with mild symptoms frequently remain in place for several years.

Accurate, early diagnosis of CRM combined with tree removal before flowering is essential to control orchard spread of this disease. However, a large percentage of sweet cherry trees in Washington orchards are infected with ordinary NRSV strains (7), which have little or no effect on fruit. To identify CRM-diseased trees under these conditions, I tested several techniques, including indexing on differential herbaceous plants and various serologic procedures. Of the methods tested, the enzyme-linked immunosorbent assay (ELISA) appeared the most useful. This report describes the ELISA procedures and their limitations.

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## MATERIALS AND METHODS

**Antisera.** Antisera prepared against the following six viruses in the *Prunus* ringspot group (8) were used to test antiserum specificity (Table 1): apple mosaic virus isolates C and P (ApMV-C, ApMV-P), *Prunus* necrotic ringspot isolate G (NRSV-G), Danish line pattern virus (DLPV), rose mosaic virus (RMV), and a hop (*Humulus lupulus* L.) isolate of NRSV. The first five antisera were prepared by R. W. Fulton (5); C. B. Skotland provided the sixth. Antivirus globulin obtained from each antiserum was conjugated to alkaline phosphatase, type VII (Sigma Chemical Co., St. Louis, MO), by the methods of Clark and Adams (3). For all subsequent tests, antiserum NRSV-G was obtained directly from R. W. Fulton, prepared for ELISA and used in polystyrene micro-ELISA plates (Dynatech Laboratories, Alexandria, VA).

**ELISA conditions.** Coating globulin (1 μg/ml) was incubated for 4 hr at 37 C. Tissue extracts triturated 1:10 (w/v) in phosphate-buffered saline containing 0.5 ml/L Tween 20, 20 g/L (w/v) polyvinylpyrrolidone (mol wt 44,000), and 2 g/L ovalbumin were incubated overnight at 4 C. Conjugated globulin (dilutions between 1:1,000 and 1:3,300, depending on the preparation) was incubated for 4 hr at 37 C. Substrate (*p*-nitrophenyl phosphate, Sigma No. 104) was added to plates at a concentration of 1 mg/ml in diethanolamine, sodium carbonate

**Table 1.** ELISA absorbance readings[a] with necrotic ringspot virus-infected cherry tissues[b] and antisera prepared against various *Prunus* ringspot viruses

| Antiserum[c] | Original virus source | Leaf tissue[d] | | | Bud tissue[e] | | |
|---|---|---|---|---|---|---|---|
| | | Rugose | Nonrugose | Control | Rugose | Nonrugose | Control |
| ApMV-C | Apple | 0 | 0 | 0 | 0 | 0 | 0 |
| ApMV-P | Apple | 0 | 0 | 0 | 0 | 0 | 0 |
| RMV | Rose | 0 | 0 | 0 | 0 | 0 | 0 |
| DLPV | Plum | 0 | 0 | 0 | 0 | 0 | 0 |
| NRSV | Hops | 0 | 0 | 0 | 0 | 0 | 0 |
| NRSV-G | Cherry | 0.9 | 0.1 | 0 | 1.2 | 0.12 | 0 |

[a] Absorbance at 405 nm.
[b] 0.2 g tissue per 2 ml of buffer.
[c] α-globulin = 1 μg/ml; enzyme conjugated globulin = 1:2,000 dilution. Substrate incubation time = 45 min.
[d] Collected and tested in June 1978.
[e] Collected and tested in January 1979.

buffer, pH 9.8. Plates were incubated at room temperature until the control wells produced absorbance readings at 405 nm ($A_{405}$) near 1.0 (usually 1–2 hr).

**Virus isolates.** In August 1977, four pairs of 18-mo-old, virus-free *P. avium* 'Bing' trees were bud-inoculated with NRSV isolates collected either from symptomless trees or from trees exhibiting a range of CRM symptoms. During 1978, each inoculated tree exhibited symptoms typical of those observed on the original inoculum source (Table 2). During the winter of 1978–1979, dormant vegetative and flower buds were collected from each tree and tested by ELISA.

Several NRSV isolates were transmitted from newly emerging cherry leaf tissues to *Chenopodium quinoa* Willd. seedlings by rub-inoculating Carborundum-dusted leaves with triturates in 0.01 M neutral phosphate buffer containing 2% nicotine sulfate. All isolates transmitted from trees exhibiting CRM symptoms produced systemic necrosis on *C. quinoa* and systemic mosaics on *Gomphrena globosa* L. Isolates transmitted from symptomless trees (including isolates transmitted from *P. domestica* L. 'Italian Prune' infected with Fulton's isolates A, E, H, and G (4) and maintained by P. R. Fridlund) produced only chlorotic mottle and rings on *C. quinoa* and failed to infect *G. globosa* systemically.

**Virus purification.** Isolates CRM-3 and O-27 (Table 3) were purified from *C. quinoa* leaf tissue (20 g) triturated in a Waring Blendor with 50 ml of 0.01 M neutral phosphate buffer and strained through cheesecloth. The extract was adjusted to pH 4.5 with acetic acid, stored at room temperature for 30 min, and centrifuged 15 min at 10,000 rpm in a Beckman number 30 rotor and then 90 min at 37,000 rpm in a number 40 rotor. The pellets were suspended overnight in 0.01 M neutral phosphate buffer at 4 C. After a second cycle of differential ultracentrifugation, the preparations were layered on 10–40% rate zonal sucrose density gradients and centrifuged 2 hr at 24,000 rpm in an SW-25.1 rotor. The virus zones were removed and were concentrated into neutral phosphate buffer by ultracentrifugation.

**Dormant samples.** Between January and April 1979, dormant flower buds were collected from trees in commercial cherry orchards. One spur with 6–8 buds was taken from each quadrant of a given tree at a point about 2 m above ground. Two buds from each cluster were triturated in 2 ml of grinding buffer and tested in a single well (four wells per tree). Samples producing absorbance values ($A_{405}$) 80% or more of that obtained with a standard CRM control were rated as positive for CRM strains. Samples producing absorbance values less than 75% of the CRM control were rated as infected with ordinary NRSV strains.

**Table 2.** ELISA absorbance readings obtained with dormant buds[a] from 3-yr-old Bing cherry trees[b] infected with known isolates of necrotic ringspot virus

| Tree no. | Inoculum source | Symptoms on: Source trees[c] | Symptoms on: Inoculated trees[d] | $A_{405}$ Vegetative buds[e] | $A_{405}$ Flower buds[e] |
|---|---|---|---|---|---|
| 1 | None | None | None | 0.00 | 0.05 |
| 2 | None | None | None | 0.02 | 0.01 |
| 3 | C-116/31 | None | Chlorotic spots[f] | 0.14 | 0.20 |
| 4 | C-116/31 | None | Chlorotic spots[f] | 0.24 | 0.24 |
| 5 | C-100/33 | Typical CRM[g] | Typical CRM | 1.20 | 1.25 |
| 6 | C-100/33 | Typical CRM | Typical CRM | 1.20 | 1.15 |
| 7 | C-124/48 | Severe CRM[h] | Severe CRM; dieback | 0.03 | 0.02 |
| 8 | C-124/48 | Severe CRM | Severe CRM | 0.90 | 0.85 |
| 9 | B-1 | Green fruit[i] | Typical CRM | 1.05 | 1.05 |
| 10 | B-1 | Green fruit[i] | Typical CRM | 1.10 | 1.10 |

[a] Collected in January 1979.
[b] Graft-inoculated in August 1977.
[c] Observations made in June, July, and August 1977.
[d] Observations made in June and July 1978.
[e] Four buds triturated in 4 ml of buffer.
[f] A few transient chlorotic spots appeared during early June 1978.
[g] Leaf twisting, leaf curl, delayed ripening, and fruit distortion.
[h] Severe leaf distortion and chlorotic mottle; typical CRM fruit symptoms.
[i] Fruit still green 3–4 wk after adjacent trees harvested. No apparent leaf symptoms.

**Table 3.** ELISA absorbance readings obtained with leaves[a] of *Chenopodium quinoa* infected with cherry rugose mosaic (CRM) isolates and ordinary (O) isolates of necrotic ringspot virus (NRSV)

| NRSV isolate | Isolate source | Systemic symptoms on *C. quinoa* | $A_{405}$ Experiment 1 | $A_{405}$ Experiment 2 |
|---|---|---|---|---|
| CRM-2 | Tree 8[b] | Necrosis | 1.15 | ... |
| CRM-3 | Tree 9[b] | Necrosis | 0.86 | ... |
| CRM-8 | Tree 5[b] | Necrosis | 0.94 | ... |
| CRM-9 | Tree 6[b] | Necrosis | 1.20 | 1.05 |
| CRM-11 | Tree 12[b] | Necrosis | 1.10 | ... |
| O-22 | Fulton A[c] | Chlorotic rings | 0.36 | 0.37 |
| O-23 | Fulton E[c] | Chlorotic rings | 0.34 | ... |
| O-24 | Fulton H[c] | Chlorotic rings | 0.35 | 0.36 |
| O-25 | Fulton G[c] | Chlorotic rings | 0.46 | ... |
| O-27 | Cherry[d] | Chlorotic rings | 0.35 | 0.32 |
| O-29 | Cherry[d] | Chlorotic rings | 0.33 | ... |
| Healthy control | None | None | 0.02 | 0.01 |

[a] Leaves were selected for uniformity of intensity of symptoms.
[b] Same trees used in Table 2.
[c] Maintained in Italian prune trees by P. R. Fridlund.
[d] Symptomless cherry trees that indexed positive for NRSV on *P. serrulata* Lindl. cv. Shiro-fugen (8).

**Table 4.** Variation in ELISA absorbance readings[a] obtained with individual flower buds from trees infected with various isolates of necrotic ringspot virus

| Tree[b] no. | Virus content | Bud no. 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | None | 0.03 | 0.03 | 0.02 | 0.02 | 0.04 | 0.02 | 0.02 | 0.02 | 0.02 | 0.03 | 0.04 | 0.04 |
| 3 | NRSV | 0.11 | 0.10 | 0.12 | 0.13 | 0.13 | 0.09 | 0.15 | 0.17 | 0.11 | 0.16 | 0.15 | 0.21 |
| 5 | CRM | 0.90 | 1.10 | 1.25 | 1.25 | 1.00 | 1.20 | 1.20 | 1.20 | 1.40 | 1.10 | 1.10 | 1.20 |
| 7 | CRM | 0.80 | 0.08 | 0.98 | 0.09 | 0.87 | 0.14 | 0.02 | 0.02 | 0.03 | 0.03 | 0.03 | 1.00 |
| 9 | CRM | 1.25 | 1.25 | 1.40 | 1.40 | 1.20 | 0.90 | 0.72 | 1.30 | 0.95 | 1.20 | 1.10 | 1.10 |

[a] $A_{405}$ values.
[b] Same trees used in Table 2.

## RESULTS

**Indexing conditions.** Fresh cherry leaf tissue and dormant buds infected with NRSV strains reacted with only one of six antisera tested (Table 1). However, the results suggested that antiserum NRSV-G might be useful for differentiating ordinary NRSV strains in cherry tissue from strains that induce CRM. To establish the reliability of NRSV-G antiserum for selectively identifying CRM-diseased trees, dormant vegetative and flower buds from 3-yr-old Bing trees previously inoculated with known isolates of NRSV were collected and tested (Table 2). Tissue from five of six

CRM-diseased trees produced strong ELISA reactions with absorbance readings of 0.85–1.25, compared with 0.14–0.24 for tissues from two trees infected with ordinary NRSV and 0–0.05 for tissues from healthy cherry trees.

Although no virus was detected with buds taken from tree 7 (Table 2), this tree had exhibited severe rugose mosaic symptoms on some limbs the previous summer. The possibility of irregular virus distribution was examined by testing individually 12 buds taken from different limbs on each of three CRM-diseased trees (including tree 7) and from one tree infected with the ordinary NRSV strain. The results (Table 4) indicate that although three of the four young trees were systemically infected with the various NRSV isolates, tree 7 was not—only 5 of the 12 buds gave absorbance readings above 0.1. Four of the five values were between 0.8 and 1.0. In the same plate, all buds from the other CRM-diseased trees gave values of 0.72–1.4, while buds from the NRSV-diseased tree gave values of 0.09–0.21.

Table 4 clearly shows that single infected flower buds provide ample virus for detection by ELISA. Furthermore, absorbance values obtained with buds from CRM-diseased trees were consistently 2.5–14 times greater than those obtained with buds containing ordinary NRSV. Absorbance values increased only slightly when up to four buds were included in the 2-ml triturate (Table 5).

**Specific reactivity.** The differences in absorbance values obtained with dormant buds from CRM-diseased trees and with similar tissues from trees infected with ordinary NRSV were independent of the gamma globulin concentration, the enzyme-conjugated globulin concentration, and substrate incubation time. Similar differences in absorbance values were obtained during the winter and early spring months using leaves of greenhouse-grown *C. quinoa* plants displaying differential symptoms of CRM and ordinary NRSV isolates (Table 3). During the summer months, however, when greenhouse temperatures often approached 30 C, *C. quinoa* plants infected with CRM isolates usually exhibited systemic chlorotic mottling with little or no necrosis, and similar absorbance values were obtained with all infected leaves regardless of isolate.

**Table 5.** ELISA absorbance readings[a] obtained with triturates containing different numbers of dormant flower buds

| Tree[b] no. | Virus content | No. buds/2 ml 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 1 | None | 0.02 | 0.02 | 0.03 | 0.03 |
| 2 | NRSV | 0.10 | 0.14 | 0.15 | 0.16 |
| 5 | CRM | 1.15 | 1.15 | 1.05 | 1.30 |
| 9 | CRM | 1.00 | 1.25 | 1.50 | 1.60 |

[a] $A_{405}$ values.
[b] Same trees used in Table 2.

To determine whether the high absorbance values obtained with CRM-diseased tissues during the winter were due to a greater specific reactivity between CRM isolates and NRSV-G antiserum, purified isolates CRM-3 and O-27 were adjusted to the same nucleoprotein concentration ($A_{260}$=0.050) and diluted in a series of twofold dilutions. The dilutions were tested in the same ELISA plate. Virtually identical absorbance values were obtained with both isolates at comparable dilutions, suggesting similar specific reactivity of the different NRSV isolates and antiserum NRSV-G.

**Cherry leaf and fruit tissue.** Tests made with leaf and fruit tissues collected during the growing season gave highly varying results. Composite leaf samples collected from CRM-diseased trees and tested early in the growing season (late April–early June) produced either no reactions or readings only slightly above the noninfected controls. Absorbance readings with CRM-diseased tissues increased somewhat as leaf and fruit symptoms became more apparent. However, the 2.5- to 14-fold difference in absorbance values noted with dormant buds from CRM-diseased and ordinary NRSV-infected trees was not found consistently with cherry leaf tissues. Table 6 illustrates the variable reactions obtained with samples collected just before harvest in mid-June from a single tree with distinct rugose mosaic symptoms on all branches. Obviously, virus distribution in leaves and fruit varied widely.

**Use of ELISA to index cherry orchards.** Four commercial cherry growers were contacted by letter in March 1979 and asked to provide coded samples from trees they suspected to have CRM disease and from one or more trees they considered healthy that were located at least 10 tree spaces from any possibly diseased trees. Four samples were collected from each tree, tested, and rated by ELISA. In June, I visually inspected each coded tree for CRM symptoms. Of the 50 trees tested (Table 7), 30 exhibited definite CRM symptoms before harvest. By ELISA, 29 of these 30 CRM-diseased trees were rated as infected in March. The single diseased tree not detected apparently was mislabeled, because symptoms were uniform throughout the tree in June, and repeated tests produced positive ELISA results.

In a second test, 65 trees were preselected from a commercial orchard where rapid disease spread had been mapped for 2 yr. Each tree had exhibited no symptoms in either 1977 or 1978 but was located within three tree spaces of a CRM-diseased tree. In 1979, 11 of the 65 trees exhibited definite CRM symptoms. Of these, 10 had been rated as diseased by ELISA. The one CRM-diseased tree not detected exhibited symptoms only on one small branch approximately 3 m above ground. Samples taken from the symptomless portion of this tree repeatedly tested negative for CRM.

An additional eight trees rated as CRM-diseased by ELISA exhibited no visible symptoms in 1979. Each tree was adjacent to or separated by one tree from an obviously diseased tree. All eight trees were probably recently infected by a CRM strain and are expected to develop CRM symptoms within 2 yr.

## DISCUSSION

Cherry trees infected with CRM strains of NRSV can be identified by ELISA

**Table 6.** ELISA absorbance readings of leaf and fruit samples taken from one tree with rugose mosaic symptoms

| Sample[a] | Symptoms | Leaf or fruit number 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Expanded leaf | Shot hole | 1.70 | 0.90 | 1.00 | 0.85 | 1.20 | 1.00 |
| Expanded leaf | Curl | 0.00 | 1.20 | 0.54 | 0.56 | 0.00 | 0.00 |
| Expanded leaf | Twisting | 0.00 | 0.60 | 0.64 | 0.52 | 0.46 | 0.58 |
| Expanded leaf | None | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Tip leaf | None | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Fruit | None | 0.00 | 0.49 | 0.00 | 0.00 | 0.00 | 0.00 |
| Fruit | Green | 0.60 | 0.57 | 0.85 | 0.60 | 0.00 | 0.00 |

[a] 0.2 g tissue triturated in 2 ml of buffer.

**Table 7.** Correspondence between ELISA indexing results obtained with dormant flower buds supplied by growers in four commercial orchards and the expression of rugose mosaic symptoms

| Orchard | No. of trees tested | No. of trees: With symptoms ELISA+ | With symptoms ELISA− | Without symptoms ELISA+ | Without symptoms ELISA− |
|---|---|---|---|---|---|
| 1 | 18 | 14 | 0 | 0 | 4 |
| 2 | 5 | 4 | 0 | 0 | 1 |
| 3 | 10 | 2 | 0 | 0 | 8 |
| 4 | 17 | 9 | 1 | 0 | 7 |
| Total | 50 | 29 | 1 | 0 | 20 |

during the dormant season with a high degree of accuracy. Identification was possible even in orchards more than 15 yr old, where virtually every tree was infected with ordinary NRSV strains (7). Equally important, CRM infections can be detected before symptoms are visible. Although the irregular distribution of virus in some trees can result in misdiagnosis, the use of ELISA combined with an aggressive tree removal program should give growers an opportunity they do not now have to control intraorchard spread of CRM disease.

Using ELISA to differentiate among NRSV strains or serotypes is not new. Serotyping of NRSV isolates in plum and hop cultivars has been reported (1); however, the consistent differences in absorbance values obtained with various virus-antisera combinations were assumed to result from differences in specific reactivity among reactants. Preliminary results given here indicate that at least one CRM strain and one ordinary NRSV strain possess the same specific reactivity toward the one antiserum used. Ongoing studies of this phenomenon among the NRSV strains that infect cherry suggest that CRM strains are identifiable in cherry buds because they occur at concentrations considerably greater than those of ordinary NRSV strains.

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