Use of Enzyme-Linked Immunosorbent Assay Results in Efforts to Control Orchard Spread of Cherry Rugose Mosaic Disease in Washington

G. I. MINK, Irrigated Agriculture Research and Extension Center, Prosser, WA 99350, and M. D. AICHELE, Division of Plant Industry, Washington Department of Agriculture, Yakima 98901

ABSTRACT

Mink, G. I., and Aichele, M. D. 1984. Use of enzyme-linked immunosorbent assay results in efforts to control orchard spread of cherry rugose mosaic disease in Washington. Plant Disease 68:207-210.

Dormant flower buds from over 15,000 sweet cherry trees were submitted by Washington growers over a 3-yr period and tested by enzyme-linked immunosorbent assay (ELISA) for the presence of *Prunus* necrotic ringspot virus (NRSV). Approximately 28% of all trees tested were infected with NRSV, and about 20% of these were rated by ELISA as infected with strains causing cherry rugose mosaic disease (CRM). Most, but not all, CRM-infected trees examined for symptoms during the growing season showed one or more symptoms of the disease. In orchards planted with certified virus-free trees, nearly all trees rated by ELISA as CRM-infected showed severe fruit symptoms. In these orchards, diseased trees were reliably identified by ELISA during the winter months for removal before flowering. In orchards planted with noncertified trees, however, a variable percentage of the trees rated by ELISA as CRM-infected had mild leaf symptoms of no economic importance to growers, atypical symptoms, or no symptoms. Although ELISA results proved useful for detecting and mapping the distribution of NRSV in these orchards, growers were reluctant to remove trees solely on the basis of such results.

Rugose mosaic disease of sweet cherry trees (*Prunus avium* L.) is caused by strains of *Prunus* necrotic ringspot virus (NRSV) transmitted through cherry pollen and seed (6). The disease is known to occur in nearly all cherry-growing regions of the Pacific Coast states (5). Once established in an orchard, the disease is spread from tree to tree through infected pollen.

Since the implementation of the fruit tree nursery improvement program in 1962, most cherry orchards established in Washington have been planted with trees certified free from known viruses, including the cherry rugose mosaic (CRM) strains of NRSV. Nevertheless, CRM appears in many young orchards within a few years after the trees begin to flower. Initial infection sites in young orchards appear to be related to the general practice of using commercial honeybees to aid pollination (4).

Approximately 89% of the trees in most Washington cherry orchards are Bing, a self-infertile cultivar. Pollenizer cultivars, such as Black Republican, Black Tartarian, Chinook, Rainier, and Van, are planted at every third tree space in every third row. Virtually all Bing fruit are harvested for fresh market use, whereas fruit of most pollenizers are harvested for processing. Typical symptoms of CRM in most cherry cultivars include leaf enations, curling, and chlorotic blotches; reduced shoot growth; and a delay in fruit ripening that varies from a few days to several weeks, depending on the virus isolate. Delayed ripening is often accompanied by fruit malformation. Because of rapid fluctuations in fresh market prices during harvest and the extreme vulnerability of ripening fruit to cracking during June rains, a delay of even a few days in the harvest of Bing fruit can mean substantial economic losses for growers. Many growers attempt to control spread of CRM by removing visibly diseased trees, but this rarely succeeds because by the time fruit symptoms are observed, the trees have flowered and provided ample inoculum for further spread.

In 1979, methods were developed to detect NRSV-infected trees during the winter months using enzyme-linked immunosorbent assay (ELISA). In preliminary field tests (3), CRM strains of NRSV could be distinguished from the ordinary NRSV strains that are of no economic importance to growers on the basis of color intensity in ELISA. This offered growers an opportunity to identify and remove CRM-infected trees before they flowered.

In January 1980, a statewide testing program was initiated to determine the feasibility of using results from ELISA tests to control orchard spread of CRM. Although we found this approach useful under certain orchard conditions, we encountered several problems that severely limit the general application of winter testing by ELISA for control purposes.

Scientific Paper No. 6230, Project 1719, Washington State University College of Agriculture Research Center, Pullman. Supported in part by funds provided by the Washington Fruit Commission and the Washington Fruit Tree Nurserymen's Association.

Accepted for publication 29 August 1983.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

Sample collection and preparation. All samples were collected and submitted by growers between January and April of each year. Growers unfamiliar with CRM symptoms sampled their most unproductive trees first or selected a few trees at random. If CRM strains of NRSV were detected by ELISA in one or more of the first trees sampled, most growers submitted samples from an additional 20–40 surrounding trees to determine the extent of spread. Growers who had removed diseased trees from their orchards frequently sampled large numbers of trees in areas surrounding the original infection sites; some sampled entire orchards.

Regardless of how trees were selected for testing, the sampling procedures were the same. Eight dormant flower spurs were taken from various locations around each tree. At least one spur was taken from each major scaffold limb. All spurs from a given tree were sealed in a plastic bag, labeled, and submitted in groups of 11–22 trees. Nearly all samples were processed within 5–7 days after collection.

Four subsamples (a total of 16 buds) were prepared from each tree tested. Each subsample consisted of four buds, two buds from each of two spurs. Each four-bud sample was triturated with a variable speed ¼-in. drill fitted with a ⅜-in. rotary file in a metal grinding cup containing grinding buffer (2.5 ml) prepared according to Clark and Adams (1). After trituration, samples were stored 2–4 hr at 4 C to allow large particulate matter to settle.

ELISA test. Coating globulin (1 μg/ml) and conjugated globulin (diluted between 1:1,000 and 1:3,000) were prepared as described earlier (3) from NRSV-G antiserum provided by R. W. Fulton. All tests were performed in 50-well polystyrene plates. Coating globulin was incubated 4 hr at 37 C. After three 3-min washes, each of the four subsamples from a given tree was pipetted into a single well (four wells per tree, 11 trees per plate). The remaining wells contained

buffer only (one well) or triturates of buds from healthy (one well), ordinary NRSV-infected (one well), or CRM-infected (three wells) trees. All tissue samples were incubated 10 hr at 4 C. Conjugated globulin and substrate (*p*-nitrophenyl-phosphate) were incubated at 21 C for 3 hr and 1.5 hr, respectively. Absorbance readings at 405 nm (A_{405}) were made with a Gilford PR-50 EIA reader (Gilford Instruments, Inc., Oberlin, OH 44074).

In preliminary tests, purified preparations of the ordinary and CRM strains of NRSV, when tested by ELISA at the same nucleoprotein concentrations, produced A_{405} values that were virtually identical. Dormant flower buds from CRM-diseased trees, however, consistently produced A_{405} values 2.5–14 times higher than similar buds from trees infected with ordinary NRSV strains (3), suggesting that strains inducing CRM symptoms occurred in these tissues at much higher concentrations than strains inducing no visible symptoms.

To establish useful limits for evaluating ELISA results, we made 130 separate comparisons of the following treatments (each treatment was triturated in 2.5 ml of grinding buffer): 1) four buds from a healthy tree, 2) four buds from a tree infected with an ordinary NRSV strain, 3) one bud from a CRM-diseased tree, and 4) four buds from a CRM-diseased tree. Although the test conditions were the same for each comparison, substrate incubation times were adjusted to give A_{405} values for treatment 4 that ranged between 0.45 and 2.74 (highest reading obtainable with our PR-50 reader). In every test, the PR-50 reader was adjusted to zero on well position No. 1, containing grinding buffer alone.

Visual observations. Although some growers removed a few infected trees before bloom each year, most of the tested trees in 85 orchards were observed at least once during this study, and selected trees in some orchards were observed repeatedly. Observations for both leaf and fruit symptoms were made between mid-May and harvest (usually mid- to late June). Because the final decision for tree removal was made by the grower, his appraisal of the possible economic importance of any fruit symptoms was considered in evaluating the correlations between ELISA results and visual observations.

RESULTS

Preliminary evaluation of ELISA data. In the 130 evaluation tests reported in Table 1, none of the A_{405} values for healthy buds (treatment 1) exceeded 0.05, and in over 90% of the tests, the A_{405} values for treatment 1 were below zero. Absorbance values for treatments 2 and 3 averaged 23 and 90%, respectively, of treatment 4 values (Table 1).

In 76% of the 130 comparisons, the A_{405} values obtained with four buds infected with an ordinary NRSV strain (treatment 2) were less than 30% of the values obtained with four buds infected with the CRM strain (Table 2). Similarly, in 75% of the comparisons, absorbance values obtained with a single CRM-infected bud (treatment 3) were 70% or more of the values obtained with four infected buds. In only 25% of the comparisons were values between 30 and 70% of treatment 4 obtained with either treatment 2 or 3.

Based on the above data, all tests with orchard trees were evaluated as follows: 1) If one or more subsamples from a given tree produced A_{405} values that exceeded 75% of the positive control (four CRM-infected buds), the tree was rated as CRM-infected. 2) If the highest subsample value was less than 30% of the positive control, the tree was considered infected with an ordinary NRSV strain. 3) If the highest subsample value was between 30 and 75% of the positive control, the tree was considered NRSV-infected but no strain evaluation was made.

ELISA results. Between 1980 and 1982, over 15,000 trees from 104 orchards were tested by ELISA, with 12,322 trees tested in 1980 alone (Table 3). Approximately 28% of all trees tested were infected with NRSV, and about 20% of these were rated as infected with CRM strains. The incidence of CRM strains appeared to vary greatly with the manner in which test trees were selected (Table 4). As might be expected, the incidence of CRM-infected trees was lowest in trees selected at random and highest in trees selected from problem areas. No virus was detected in a limited number of samples submitted from six orchards.

All trees were tested in 16 orchards. Bing was the principal variety in 11 of these, Lambert in three, and Rainier in two. In the Bing orchards, the incidence of NRSV infection ranged from 0 to 63.5% (Table 5). The incidence of virus infection was highest in older orchards and in those planted with noncertified trees. Orchards established initially with virus-free certified nursery trees (orchards 1–3) contained a few scattered trees rated by ELISA as CRM-infected but no trees rated by ELISA as infected with ordinary NRSV strains (Table 5). By contrast, orchards established before the availability of virus-free certified trees (orchards over 20 yr old) contained both CRM and ordinary NRSV strains at incidences above 10% (Table 5).

Correlations between ELISA results and symptom expression on Bing trees. In 78 of 85 orchards, we observed typical CRM leaf and fruit symptoms on one or more of the Bing trees rated by ELISA as CRM-infected. The extent to which we could reliably identify diseased trees with ELISA varied greatly among orchards, however. The 78 orchards could be divided into two general types: group 1, orchards (34) in which a majority of the trees rated by ELISA as CRM-infected showed severe leaf and fruit symptoms, and group 2, orchards (44) in which only a small percentage of the trees rated as CRM-infected showed severe leaf and fruit symptoms.

Group 1 orchards were 20 yr old or less, had been planted originally with virus-free certified trees, and contained only a few CRM-infected trees. Most of the infected trees occurred singly or in small clusters (Table 5, orchards 2 and 3). Comments from the growers regarding the first recognized fruit symptoms suggested that the causal virus had been introduced naturally into these orchards

Table 1. Summary of absorbance (A_{405}) values obtained in enzyme-linked immunosorbent assay with dormant flower buds[a] from trees infected with an ordinary or a cherry rugose mosaic (CRM) strain of necrotic ringspot virus (NRSV)

No. of tests	Treatment 4		Treatment 3			Treatment 2		
	Range	Avg.	Range	Avg.	%[b]	Range	Avg.	%[b]
16	0.45–0.99	0.73	0.31–1.31	0.64	88	0.01–0.45	0.14	19
38	1.00–1.99	1.59	0.81–2.51	1.47	92	0.14–0.72	0.35	22
38	2.00–2.71	2.37	0.92–2.74	1.90	80	0.21–0.90	0.50	21
38	2.74[c]	2.74	1.42–2.74	2.69	98	0.16–1.45	0.80	29
Avg.		1.86		1.69	90		0.45	23

[a] Treatment 1 = four buds from a healthy tree (all A_{405} values were less than 0.05); treatment 2 = four buds infected with an ordinary NRSV strain; treatment 3 = one bud infected with a CRM strain; treatment 4 = four buds infected with a CRM strain.
[b] Percent of treatment 4 average.
[c] Maximum value obtainable with PR-50 reader = 2.74.

Table 2. Distribution of A_{405} values obtained with dormant flower buds from healthy trees and trees infected with necrotic ringspot virus

Treatment[a]	Treatment A_{405} values expressed as percent of treatment 4 values			
	0–1%	2–30%	31–69%	70–>100%
1	130[b]	0	0	0
2	0	99	31	0
3	0	0	33	97
4	0	0	0	130

[a] Treatment 1 = four buds from a healthy tree (all A_{405} values were less than 0.05); treatment 2 = four buds infected with an ordinary strain of necrotic ringspot virus (NRSV); treatment 3 = one bud infected with a cherry rugose mosaic (CRM) strain of NRSV; treatment 4 = four buds infected with a CRM strain.
[b] Number of tests in which A_{405} values were in the percent range indicated.

within the past 10–12 yr. For these orchards, diseased trees were reliably identified in the winter ELISA tests.

Group 2 orchards ranged in age from 20 to more than 40 yr. So far as we could determine, all had been planted with noncertified trees. In these orchards, more than 10% of the trees tested were rated by ELISA as CRM-infected and an additional 10–25% were rated as infected by ordinary NRSV strains. In five such orchards, the incidence of CRM-infected trees ranged from 12 to 33% (Table 5, orchards 7–11).

Nearly all of the CRM-infected Bing trees in group 2 orchards showed mild leaf symptoms (enations or leaf curling, or both), but fruit ripening was either not affected or delayed only slightly. Because these diseased trees consistently expressed only mild fruit symptoms year after year, growers were unwilling to remove them for economic reasons.

In group 2 orchards, the distribution pattern of CRM-infected trees, as determined by ELISA, suggested to us that portions of these orchards had been planted with infected trees. In some cases, infected pollenizers appeared to have been planted, and many of the adjacent Bing trees had subsequently become infected. In other orchards, the distribution pattern suggested to us that some, but not all, Bing trees were infected when planted. Although a majority of the CRM-infected trees in these orchards showed only mild symptoms, nearly every group 2 orchard contained a few trees with severe leaf and fruit symptoms. These severely diseased trees occurred singly or in small groups scattered in much the same pattern as observed in group 1 orchards. However, we were unable to distinguish by ELISA the trees that perennially expressed mild fruit symptoms from those that showed severe symptoms. Many trees rated by ELISA as CRM-infected showed mild to moderate symptoms year after year, whereas some trees suddenly showed severe symptoms and continued to express severe symptoms annually, suggesting that several biological variants (mild, moderate, and severe) of the CRM strains may occur in Washington orchards.

Occurrence of atypical CRM symptoms. In seven orchards, the appearance of CRM-diseased Bing trees was in some way atypical. In one orchard, all trees rated CRM-infected by ELISA showed no leaf symptoms even though the fruit ripened 7–14 days later than adjacent healthy trees. In this orchard, all diseased trees were tested for and found to be free from the causal agent of little cherry disease, which can also delay ripening. In six orchards, we observed no leaf or fruit symptoms over a 3-yr period on any Bing tree that had been rated by ELISA as CRM-infected.

Symptom expression on varieties other than Bing. Over 1,500 Lambert trees from three orchards 30–40 yr old were tested and over 50% were rated by ELISA as CRM-infected. Only five adjacent trees in one orchard expressed typical CRM symptoms, however. All other infected trees either were symptomless or showed only mild leaf enations.

Several hundred CRM-infected trees of the major pollenizer varieties were observed during this study. Most infected Black Republican, Black Tartarian, Chinook, Rainier, or Van trees showed mild leaf enations and occasionally mild leaf curling. A few Van and Black Republican trees also showed mild fruit symptoms (2–4 days delay in fruit ripening). Among all of the pollenizer trees rated by ELISA as CRM-infected, only one Chinook, two Black Tartarian, two Van, and four Rainier trees had leaf and fruit symptoms severe enough to justify tree removal.

Table 3. Incidence[a] of necrotic ringspot virus (NRSV) in cherry tree samples submitted by Washington growers over a 3-yr period

Year	Trees tested (no.)	NRSV-infected (%)	Percent rated[b] as: CRM	?	NRS
1980	12,322	29.4	19.8	2.0	7.6
1981	2,120	31.2	22.7	2.5	6.0
1982	571	23.7	17.7	0.0	6.0
		Mean 28.1	20.1	1.5	6.5

[a] As determined by ELISA.
[b] Based on sample A_{405} values: CRM = at least one subsample value exceeded 75% of cherry rugose mosaic (CRM) control value; ? = highest subsample value between 30 and 75% of CRM control value; NRS = highest subsample value between 10 and 30% of CRM control value.

Table 4. Incidence[a] of necrotic ringspot virus (NRSV) in cherry tree samples submitted in 1980 for various reasons

Trees selected	Trees tested (no.)	NRSV-infected (%)	Percent rated[b] as: CRM	?	NRS
At random	1,462	10.8	9.4	1.2	0.1
From problem areas	2,619	49.0	40.7	2.5	5.8
From entire orchards	8,241	26.3	15.1	2.0	9.2

[a] As determined by ELISA.
[b] Based on sample A_{405} values: CRM = at least one subsample value exceeded 75% of cherry rugose mosaic (CRM) control value; ? = highest subsample value between 30 and 75% of CRM control value; NRS = highest subsample value between 10 and 30% of CRM control value.

Table 5. Incidence[a] of necrotic ringspot virus (NRSV) in 11 Bing cherry orchards

Orchard number	Tree status[b]	Approx. age (yr)	Trees tested (no.)	NRSV-infected (%)	Percent rated[c] as: CRM	?	NRS	Probable inoculum source[d]
1	VF	20	448	0.0	0.0	0.0	0.0	None
2	VF	20	1,078	1.9	1.6	0.3	0.0	Bees
3	VF	20	374	5.6	4.8	0.8	0.0	Bees
4	?	20	532	3.8	1.7	0.6	1.5	Bees
5	?	20	286	13.3	3.2	1.0	9.1	Bees
6	?	20	449	9.1	1.6	2.4	5.1	Bees
7	NC	25	863	28.3	12.2	1.5	14.6	Infected Bing trees
8	NC	25	1,332	32.5	19.7	1.2	11.6	Infected pollenizer variety
9	NC	25	517	40.0	14.1	7.9	18.0	Infected nursery trees
10	NC	30	335	51.9	24.8	5.3	21.8	Infected nursery trees
11	NC	30	446	63.5	33.2	12.8	17.5	Infected nursery trees

[a] As determined by ELISA.
[b] VF = tree propagated from sources free from known viruses; ? = new owners unsure of source of original trees; NC = noncertified trees from commercial nurseries.
[c] Based on sample A_{405} values: CRM = at least one sample value exceeded 75% of cherry rugose mosaic (CRM) control value; ? = sample value between 30 and 75% of CRM control value; NRS = highest sample value between 10 and 30% of CRM control value.
[d] Based on distribution pattern of infected trees.

DISCUSSION

For many years, researchers have characterized the NRSV strains found in fruit trees by comparing their symptomatology in 1) herbaceous plants with viruses A, E, G, and H, originally described by Fulton (2), or 2) woody plant indicators with four broadly defined strains (5): ordinary necrotic ringspot (NRS), recurrent ringspot (RRS), cherry rugose mosaic (CRM), and almond calico (AlC). Although either procedure can be useful for certain purposes, neither is practical to evaluate large numbers of diseased trees. Consequently, in a large-scale testing program such as the one reported here, certain assumptions must be made. Our assumption at the beginning of this study was that the NRSV isolates occurring in dormant flower buds at high concentrations were the isolates (or strains) that induced disease (CRM) of economic importance to cherry growers.

In young orchards where the incidence of NRSV was low and where the virus appeared to have been introduced in recent years, presumably by bees, there was a good correlation between ELISA results and symptom severity. Because relatively few trees were involved, growers were willing to accept the test results and remove trees rather than risk continued spread. In orchards where the incidence of NRSV was relatively high, however, ELISA results failed to distinguish among biological variants, many of which caused little or no economic damage. Consequently, winter test results were of little practical value to those growers. The sharp decline in the numbers of trees submitted for testing over the 3-yr period (Table 3) reflects this situation.

Although the initial objective of using ELISA results in efforts to control orchard spread of CRM was only partially successful, the test procedures have proved useful for other purposes. We are currently mapping the distribution and spread of NRSV variants in selected orchards under defined conditions. In addition, nearly 10,000 registered *Prunus* seed and scion source trees maintained by growers of certified fruit tree nursery stock are tested annually by ELISA for all forms of NRSV and for prune dwarf virus, another pollen-borne virus.

ACKNOWLEDGMENTS

We wish to thank Rosalie Ripley, Richard Ray, and Leola Lewis of the ELISA Laboratory, Prosser, WA, for technical assistance.

LITERATURE CITED

1. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
2. Fulton, R. W. 1957. Comparative host ranges of certain mechanically transmitted viruses of *Prunus*. Phytopathology 47:215-220.
3. Mink, G. I. 1980. Identification of rugose mosaic-diseased cherry trees by enzyme-linked immunosorbent assay. Plant Dis. 64:691-694.
4. Mink, G. I. 1983. The possible role of honeybees in long distance spread of Prunus necrotic ringspot virus from California into Washington sweet cherry orchards. Pages 85-91 in: Plant Virus Epidemiology. R. T. Plumb and J. M. Thresh, eds. Blackwell Scientific Publications, Oxford.
5. Nyland, G. 1961. Sweet cherry rugose mosaic virus in California. Tidsskr. Planteavl. 65:106-110.
6. Nyland, G., Gilmer, R. M., and Moore, J. D. 1974. "Prunus" ringspot group. Pages 104-132 in: Virus Diseases and Noninfectious Disorders of Stone Fruits in North America. U.S. Dep. Agric. Agric. Handb. 347. 433 pp.