Detection of Prunus Necrotic Ringspot and Prune Dwarf Viruses in *Prunus* Seed and Seedlings by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Enzyme-linked immunosorbent assay (ELISA) was used to determine the incidence of Prunus necrotic ringspot (NRSV) and prune dwarf (PDV) viruses in lots of *Prunus* seed and seedlings used by commercial nurseries in Washington and Montana between 1981 and 1983. These viruses were found rarely, if ever, in seed or seedlings of *P. armeniaca, P. besseyi, P. salicina, P. serotina,* and *P. tomentosa.* The incidence of both viruses in noncertified *P. mahaleb* seed lots (both foreign and domestic) was below the 5% tolerance limit allowed by the tree fruit virus certification programs of both states. Although NRSV was rarely detected in *P. cerasifera* seed, the incidence of PDV in noncertified seed lots ranged between 1 and 53%. PDV was detected in only two of 1,000 *P. cerasifera* seedlings, however, indicating that most PDV-infected seed did not germinate. In noncertified *P. avium* seed lots, the incidence of PDV ranged between 0 and 58% and the incidence of NRSV ranged between 0 and 28%. The incidence of either virus in *P. avium* seedlings was dependent on the incidence found in the seed lots. Only an occasional virus-infected seed was found in noncertified *P. persica* seed lots from Europe, whereas the average incidence of PDV and NRSV in domestic seed lots was 10 and 17%, respectively. Although NRSV was detected in *P. persica* seedlings of various size classes and in ungerminated seed, PDV was detected only in ungerminated or partially germinated seed. Use of ELISA to monitor virus incidence in *Prunus* seed and seedlings provides nurseries with a valuable tool for reducing the incidence of seedborne viruses in *Prunus* rootstocks.

In recent years, Canada and many U.S. states have established tree fruit virus certification programs to provide fruit tree growers with virus-tested trees (7). Prunus necrotic ringspot virus (NRSV) and prune dwarf virus (PDV) occur worldwide and are seed-transmitted in many *Prunus* species (5,8). Consequently, most stone fruit certification programs specify that *Prunus* seedling lots must be tested for these viruses (7), and to meet certification standards, they must contain fewer than 5% virus-infected plants. Although limited amounts of virus-certified seed of a few *Prunus* species are produced in Washington, Oregon, and California, the demand for certified seed exceeds the supply available in most years. Consequently, many nurseries purchase and plant large amounts of *Prunus* seed of unknown virus content with the expectation that the incidence of seed-transmitted viruses will be below 5%.

In Washinqton, *Prunus* seed is normally planted in September or October. About 1 yr later, nursery inspectors from the Department of Agriculture index 50–200 seedlings collected at random from each *Prunus* seed lot in each field to be certified. Until 1981, all seedlings were indexed by bud-inoculation onto Shirofugen flowering cherry (*P. serrulata* Lindl.), a woody indicator for both NRSV and PDV. If the incidence of seed-transmitted viruses exceeded 5% in any seedling lot, that lot was not eligible for certification. Because test results usually were not available until after the seedlings had been dug in late October or early November, nurseries sometimes invested thousands of dollars in land, labor, and time to produce a crop that could not be certified and for which there was little or no market.

In 1980, we adapted enzyme-linked immunosorbent assay (ELISA) procedures to detect NRSV and PDV in *Prunus* seed before planting and in seedlings before digging. Since 1981, these procedures have been used to test virtually all *Prunus* seed lots planted in Washington and Montana. Results of these tests are presented in this paper.

MATERIALS AND METHODS

Sample collections. Nursery inspectors in Washington and Montana collected seed from domestic seed lots in accordance with standard sampling procedures used in the respective states. For foreign seed lots, 100 seeds were usually taken from each container received, regardless of size. For seedling tests, the top two to four leaves from 50–100 commercial-grade plants were selected at random in each field, sealed in plastic bags, and delivered to the ELISA laboratory at Prosser for testing.

Seed preparation. For peach (*P. persica*) and apricot (*P. armeniaca*) seed, the endocarp was removed before processing. For all other *Prunus* species, intact seeds were used. Individual seeds were placed in steel grinding cups. (The cups were prepared by machining 16-mm-diameter holes about 60 mm deep into sections [75 × 20 mm] of stock-grade bar steel.) After a seed was placed in each of 20 cups, hex-headed threadless bolts (150 × 13 mm) were inserted in the cups

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with a wooden guide frame. The bottom of each bolt was slightly tapered to facilitate grinding. Each bolt head was struck sharply with a hammer to crush the seed. Grinding buffer (2.5 ml) prepared according to Clark and Adams (1) was dispensed into each cup with an automatic syringe. The crushed seeds were triturated by rotating each bolt at about 2,000 rpm for 10 sec with a variable-speed electric drill fitted with a shaft-mounted socket wrench. The crushed endocarp acted as an abrasive during high-speed rotation and thoroughly triturated all seed parts. Triturates were poured into glass tubes and stored at <4 C for 2–4 hr. Each group of 20 samples was tested in a single ELISA plate.

Leaf preparation. Fresh leaf tissue (0.2 g) from each *Prunus* seedling was placed in a steel grinding cup and triturated in grinding buffer (2.5 ml) with a variable-speed electric drill fitted with a 3/8-in. rotary file (No. 4337, Coastal Abrasive and Tool Co., Trumbull, CT 06611). The rotary file was washed in tap water between samples of a given seedling lot and replaced with a hot water-sterilized file between lots. After trituration, the samples were poured into glass tubes and stored at <4 C for 2–4 hr.

ELISA conditions. ELISA conditions for detecting NRSV in leaf and seed triturates were the same as described earlier for leaf and dormant bud tissues (6). Antiserum against PDV was prepared in rabbits using a local virus isolate obtained from sweet cherry (*P. avium*). PDV gamma-globulin was used at 1 or 2 μg/ml and alkaline phosphatase-conjugated globulin was used at dilutions between 1:500 and 1:2,000. Incubation times for gamma-globulin, sample, conjugate, and substrate were the same as described previously for NRSV (6).

Twenty triturated samples (seed or leaf) were tested in duplicate wells in each 50-well Gilford plate. Two positive control wells in each plate contained the appropriate purified virus (1 μg/ml) or, more recently, triturates of immature fruit from infected trees. Two negative control wells in each plate contained triturates of virus-free seed or leaf tissue. The six remaining wells in each plate contained only grinding buffer to monitor background color development.

Absorbance readings at 405 nm were made using a Gilford PR-50 automatic EIA reader connected through an RS-232 interface to a Radio Shack TRS-80 Model III microcomputer. Using software developed in this laboratory, the microcomputer was programmed to do the following: 1) record individual well readings by plate position, 2) calculate positive control (PC) and negative control (NC) averages, 3) calculate a net positive control average (NPC = PC-NC), 4) calculate a sample average (SA) and net sample average (NSA = SA-NC) for each sample, and 5) calculate the percentage of NPC for each NSA. Samples having an NSA less than 10% of the NPC were considered uninfected. Samples having an NSA greater than 14% of the NPC were considered infected. Intermediate values were considered questionable reactions. In tests of seed and seedling lots for virus incidence, the occurrence of a single questionable reaction, among 100 or more samples, had essentially no effect on the results and was ignored. All plates were examined visually for spurious results caused by low-substrate meniscus or mechanical abnormalities.

RESULTS

Virus incidence in commercial seed. The incidence of PDV and NRSV in commercial lots of *Prunus* seed varied greatly with the species tested and with the seed source (Tables 1 and 2). Neither virus was detected in seed lots (200–1,000 seeds tested) of *P. armeniaca, P. besseyi* (western sand cherry), *P. maackii, P. salicina* (Japanese plum), *P. serotina* (black cherry), or *P. tomentosa* (Manchu cherry) seed imported from European sources (Table 1) or in a single domestic (noncertified) seed lot (200 seeds tested) of *P. tomentosa*.

Virus-infected seeds were detected

Table 1. Incidence of prune dwarf (PDV) and necrotic ringspot (NRSV) viruses in seed lots of various *Prunus* species imported from European sources

Species	Source	Seed tested (no.)	Percent infected seed	
			PDV	NRSV
P. avium	North Carpathian Mts.	100	54.0	0.0
	West Carpathian Mts.	100	58.0	0.0
	Hungary (1)	100	28.0	0.0
	Hungary (2)	200	9.0	0.0
	Eastern Europe	600	13.2	0.2
P. armeniaca	Eastern Europe	1,000	0.0	0.0
P. besseyi	Eastern Europe	300	0.0	0.0
P. cerasifera	Bulgaria	100	11.0	0.0
	Eastern Europe (1)	1,900	39.9	0.2
	Eastern Europe (2)	100	13.0	1.0
	Rumania	100	17.0	0.0
P. cerasis	Eastern Europe (1)	100	12.0	1.0
	Eastern Europe (2)	100	7.0	6.0
	Eastern Europe (3)	100	5.0	0.0
	Rumania	100	0.0	0.0
P. maackii	Eastern Europe	500	0.0	0.0
P. mahaleb	Bulgaria	100	0.0	0.0
	Eastern Europe (1)	600	8.2	0.0
	Eastern Europe (2)	2,200	4.0	0.0
	Eastern Europe (3)	1,600	0.7	0.0
	Eastern Europe (4)	100	17.0	0.0
	Eastern Europe (5)	100	6.0	0.0
	Eastern Europe (6)	100	18.0	0.0
	Germany	200	0.5	0.0
	Rumania	100	1.0	0.0
P. padis	Hungary	100	0.0	0.0
P. persica	Hungary (1)	100	0.0	0.0
	Hungary (2)	300	1.6	0.0
P. salicina	Eastern Europe	200	0.0	0.0
P. serotina	Eastern Europe	500	0.0	0.0
P. tomentosa	Eastern Europe	200	0.0	0.0

Table 2. Incidence of prune dwarf (PDV) and necrotic ringspot (NRSV) viruses in domestic seed lots of various *Prunus* species

Species	Type of seed	Lots tested (no.)	Seed tested (no.)	Percent infected seed	
				PDV	NRSV
P. armeniaca	Noncertified	2	200	1.0	0.5
	Certified	3	300	0.3	0.0
P. avium	Noncertified	17	3,900	33.5	6.2
	Certified	13	1,300	0.3	0.0
P. besseyi	Noncertified	3	300	0.0	3.0
	Certified	2	200	0.0	0.0
P. cerasifera	Noncertified	6	600	16.6	0.0
	Certified	11	1,100	0.1	0.0
P. mahaleb	Noncertified	8	800	3.3	0.1
	Certified	11	1,100	0.0	0.0
P. persica	Noncertified	14	1,400	10.1	16.8
	Certified	6	600	0.0	0.0

most frequently in noncertified seed lots (both foreign and domestic) of *P. avium* (mazzard cherry), *P. cecerasifera* (myrobalan plum), *P. cerasus* (sour cherry), and *P. mahaleb* (mahaleb cherry) (Tables 1 and 2). In all four species, the principal virus found was PDV. The incidence of PDV in individual seed lots ranged between 9 and 58% for *P. avium*, between 11 and 40% for *P. cerasifera*, between 0 and 12% for *P. cerasus*, and between 0 and 18% for *P. mahaleb*. The incidence of NRSV in individual seed lots of *P. avium* and *P. cerasus* ranged between 0 and 6%, whereas only an occasional NRSV-infected *P. cerasifera* or *P. mahaleb* seed was detected.

Only five PDV-infected seeds were found in one of three *P. persica* seed lots from Europe, whereas the average incidence of PDV and NRSV in domestic noncertified seed lots was about 10 and 17%, respectively (Table 2).

Only trace amounts of PDV were detected in certified seed lots of *P. armeniaca, P. avium,* and *P. cerasifera*. No virus was detected in certified seed of *P. mahaleb* and *P. persica*.

Virus incidence in commercial seedlings. Even though the incidence of PDV in all noncertified seed lots of *P. cerasifera* planted in Washington and Montana was well above the 5% tolerance limit established for seedlings in both states, this virus was not detected in any of 100 commercial-grade seedlings taken at random from eight fields planted with noncertified seed and was detected in only one of 100 seedlings taken from each of two additional fields. Similar but less dramatic results were obtained with seed and seedlings of *P. avium* and *P. persica* (Table 3).

In the case of *P. avium*, if the incidence of either PDV or NRSV in the seed lot was below 15%, no infected seedlings were found. Levels of PDV sufficient to prevent certification were found in seedlings from seed lots that contained 17% or more infected seed. For *P. persica* 'Halford' seedlings, the incidence of both viruses was less than 5% regardless of the incidence in seed. For *P. persica* 'Elberta,' however, the incidence of NRSV in seedlings was only slightly less than that found in the seed.

These results indicated that many virus-infected seeds either did not germinate or did not produce commercially acceptable seedlings. In May 1983, we collected and tested every peach seedling, regardless of size, and each ungerminated seed from about 2 m of row in each of nine commercial fields that had been planted with noncertified seed in 1982. The seedlings were graded into four size classes: 1) vigorous growth, 2) moderate stunting, 3) pronounced stunting, and 4) germinated but not emerged. A fifth class included all ungerminated seed. Results (Table 4) demonstrated that PDV was found only in ungerminated seed or in seedlings that did not emerge. Although NRSV was detected in a few seedlings of each size class, the incidence was greatest in ungerminated or barely germinated seed.

DISCUSSION

Between 1981 and 1983, we detected trace amounts of NRSV and PDV in noncertified seed lots of *P. armeniaca, P. besseyi, P. maackii, P. salicina, P. serotina*, and *P. tomentosa*. Most commercially available seed of these six species appeared to be harvested from virus-free trees even though the source trees were not part of any organized virus certification program. Because these seed lots were obtained from several locations in Europe and the United States, our inability to detect either virus in the seed indicates that field spread of NRSV and PDV in these species occurs slowly, if at all, at most locations. Until a high incidence of either virus can be demonstrated in noncertified seed of these species, it would appear sufficient, for certification purposes, to monitor virus incidence only in seed. Using ELISA techniques, the seed could be monitored before importation, before purchase, or before planting. For states producing large numbers of seedlings of these six species for certification, elimination of seedling testing would substantially reduce indexing costs.

Although every noncertified lot of *P. cerasifera* seed planted in Washington and Montana for the past 3 yr contained more than 5% PDV-infected seed, the virus was rarely detected in seedlings, presumably because PDV-infected seeds do not germinate. This supposition was supported by comments from nursery workers who reported poor seedling emergence for seed lots with more than 20% PDV-infected seed. Some nurseries now submit samples of *P. cerasifera* seed for ELISA indexing before purchasing the entire lot.

The incidence of seedling infection in *P. avium, P. mahaleb,* and *P. persica* found in these studies was similar to that found by other workers (2–4) using biological indexing techniques. In all cases, the incidences of PDV and NRSV in seedlings were considerably lower than those found in seed.

Our results demonstrate that ELISA can be used effectively to determine the incidence of NRSV and PDV in both *Prunus* seed and seedlings. Use of these techniques can greatly reduce the risks faced by nursery workers who must use noncertified *Prunus* seed.

Table 3. Incidence of prune dwarf (PDV) and necrotic ringspot (NRSV) viruses in seedlings grown from virus-infected seed lots of *Prunus avium* and *P. persica*

		P. avium		*P. persica*[a]	
Lot no.	Sample	PDV	NRSV	PDV	NRSV
1	Seed	40	0	20	18
	Seedling	7	0	1	1
2	Seed	22	0	15	28
	Seedling	6	0	0	2
3	Seed	17	12	12	35
	Seedling	6	3	0	2
4	Seed	15	0	15	11
	Seedling	0	0	0	4
5	Seed	8	0	1	10
	Seedling	0	0	0	7

[a] Lots 1–4 were Halford; lot 5 was Elberta.

Table 4. Incidence of prune dwarf (PDV) and necrotic ringspot (NRSV) viruses in peach seedlings of different size classes grown in fields planted with noncertified seed

Size class[a]	No. of samples[b]	PDV	NRSV	Total
1	228	0.0	2.6	2.6
2	172	0.0	2.3	2.3
3	97	0.0	4.1	4.1
4	110	0.9	11.8	12.7
5	247	12.6	7.3	19.9

[a] 1 = Vigorous growth, 2 = moderate stunting, 3 = pronounced stunting, 4 = germinated but not emerged, and 5 = all ungerminated seed.
[b] Combined samples from nine fields.

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