

Incidence of Iilarviruses in Young Peach Trees in California

J. K. UYEMOTO and C. F. LUHN, USDA-ARS, Department of Plant Pathology, and W. ASAI, R. BEEDE, J. A. BEUTEL, and R. FENTON, Cooperative Extension, Department of Pomology, University of California, Davis 95616

ABSTRACT

Uyemoto, J. K., Luhn, C. F., Asai, W., Beede, R., Beutel, J. A., and Fenton, R. 1989. Incidence of ilarviruses in young peach trees in California. *Plant Disease* 73:217-220.

Indirect enzyme-linked immunosorbent assays (ELISA) of young cling peach (*Prunus persica*) trees in the Sacramento and San Joaquin valleys of California revealed that about 28% of first- to fifth-leaf trees were infected by either prune dwarf virus or Prunus necrotic ringspot virus, or by both. Peach trees infected by both viruses were severely stunted. Infection was confirmed in about one-half of the ELISA test trees by bud-chip inoculations onto Shirofugen flowering cherry trees (*P. serrulata*). These results strongly suggest that infected scion buds, rootstocks, or both were used in the propagation of nursery trees.

In mid-October 1986, a second-leaf cling peach (*Prunus persica* (L.) Batsch

Accepted for publication 30 October 1988 (submitted for electronic processing).

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1989.

'Carson') orchard (4 ha) was diagnosed to contain trees with peach stunt disease; incidence was estimated at 90%. Peach stunt disease is caused by prune dwarf virus (PDV) in certain peach cultivars (6) but more commonly by a combination of PDV and Prunus necrotic ringspot virus (PNRSV) in all cultivars (3). Serological tests of the trees in the orchard confirmed

that nine of 10 peach trees contained both PDV and PNRSV, members of the ilarvirus group (1). Although these ilarviruses are readily transmitted to healthy trees during pollination by diseased pollen (2,4), the relatively high incidence of peach stunt disease in these young trees suggested that virus-infected scions and/or rootstocks were used to produce budling trees. The incidence of ilarviruses in other young cling peach orchards in California was not known. This paper describes results for PDV and PNRSV detection by serological assays and bioassays of leaf and bud collections, respectively, derived from young cling peach trees in California.

MATERIALS AND METHODS

Orchard survey. Orchard maps and information on peach cultivars and

planting ages were obtained from regional Cling Peach Advisory Board offices in Kingsburg, Modesto, and Yuba City, CA, for orchards located in the counties of Kings and Stanislaus (southern and northern San Joaquin Valley) and Sutter-Yuba (Sacramento Valley). Selected orchards in their first- to fifth-leaf stage of growth (i.e., trees planted 1–5 yr previous to sampling) were identified and visited. For serological assays, three current-season shoots from separate scaffold branches were taken from an individual tree, bundled together, and labeled as to orchard and tree site. Three to 10 trees per orchard were sampled. These collections were made from March to late May. For graft indexing, 1-yr-old budsticks (three per tree) were removed during June to August from trees previously testing positive or negative by ELISA, and one bud from each budstick was inserted under the bark flap of a Shirofugen flowering cherry tree (*P. serrulata* Lindl.), a standard indicator host for both viruses (3,8). After 4–6 wk, each bud-chip inoculation was examined for signs of gum and internal tissue necrosis, a common host response caused by and associated only with the presence of PDV or PNRSV (8). The internal symptom was determined by cutting the bark adjacent to the inserted test bud so as to expose the phloem-xylem tissues. Because of the similarities in host response, Shirofugen assays cannot be used to distinguish between PDV and PNRSV.

Serological assays. Rabbit sources of antisera to PDV and PNRSV were gifts of G. I. Mink (Prosser, WA) and G. Nyland (Davis, CA), respectively. Ascites fluid prepared to a monoclonal cell line (NA70C9) (5) against PNRSV was supplied by IGEN, Inc. (Rockville, MD). The *o*-phenylenediamine substrate (12 mg/20 ml of 0.012% H₂O₂ in 0.05 M dibasic sodium phosphate and 0.025 M citric acid, pH 5.0) and peroxidase-labeled goat antirabbit (GAR) and goat antimouse (GAM) antisera were purchased from Sigma Chemical Co. (St.

Louis, MO) and United States Biochemical Corporation (Cleveland, OH), respectively. The serological reactions were done in Microtitration plates (Costar, Cambridge, MA) and read at A_{450nm} in a Titertek Multiskan MC reader (Flow Laboratories, Inc., McLean, VA).

Virus cultures of PNRSV (CH-30 and CH-61) (7) in *Chenopodium quinoa* Willd. and of PDV in cucumber (*Cucumis sativus* L. 'National Pickling') were provided by G. I. Mink. In addition, an isolate (No. 17) of PNRSV that had been transmitted from cherry to cucumber, purified, and returned to a *P. mahaleb* L. seedling was supplied by G. Nyland.

Leaf extracts were prepared by crushing (with a mortar and pestle) 0.5 g of leaf tissue in 2 ml of 0.05 M carbonate buffer, pH 9.6, containing 2% polyvinylpyrrolidone, *M_r* 40,000 (PVP-40), 0.2% egg albumin, and 0.45% sodium diethyldithiocarbamate trihydrate (DIECA) for PDV (G. I. Mink, *personal communication*) and in phosphate buffer, pH 7.0, PVP-40, and polyoxyethylene sorbitan monolaurate (0.05% Tween 20) (10) for PNRSV.

For PDV ELISA, precoating the wells with anti-PDV antibodies was not done, as virus particles bind directly to the plastic well. With PNRSV, the wells required precoating with rabbit anti-PNRSV antibodies (2 µg/ml of carbonate buffer, pH 9.6; 2 hr at 37 C) to attract and bind PNRSV particles contained in extracts placed in the wells. All tissue extracts were incubated for 1.5 hr at room temperature, and plates were rinsed thoroughly with PBS-Tween (0.01 M phosphate buffer, pH 7.0, containing 0.8% sodium chloride, 0.002% potassium chloride, and 0.05% Tween 20). Next, IgG of rabbit anti-PDV (2 µg/ml) or ascites anti-PNRSV (1:3,000 dilution in PBS-Tween, PVP-40, and DIECA) was added and incubated 1 hr at 37 C; the plates were then rinsed with PBS-Tween. This was followed by the addition of enzyme-labeled GAR (used at 0.33 µg/ml) and GAM (used at 1 µg/ml)

antibodies and incubation for 3 hr at 37 C. The plates were then rinsed, and the substrate solution was added, incubated 45 min, and read. All extracts and solutions of antibodies and substrate were used at 200 µl per well. Extracts were plated in duplicate wells.

RESULTS

Initial ELISA results with known virus cultures indicated marked differences between extracts of virus-infected and healthy host tissues. With PDV, the A_{450nm} values averaged >2.0 and 0.09, respectively, for infected and healthy cucumber sap. In tests with peach leaves, mean values were 1.35 (PDV-infected) and 0.18 (healthy). PNRSV cultures Ch-30, CH-61, and No. 17 reacted similarly to each other, with mean A_{450nm} values of 1.05, 1.17, and 0.95, respectively. Healthy tissue extracts provided mean readings of 0.23 (*C. quinoa*) and 0.17 (*P. mahaleb*). On the basis of the relatively high healthy readings with the antisera, we used A_{450nm} values of 0.25 and below as negative and 0.50 and above (twice the healthy value) as positive for both viruses. This procedure was followed even though an occasional ELISA plate yielded healthy A_{450nm} values of 0.10 or less and test samples with more than twice those healthy readings but less than 0.25; such samples were classified as ELISA-negative. When extracts consistently produced A_{450nm} values between 0.25 and 0.50, however, the tested trees were viewed with suspicion. All trees from those orchards were resampled in June or later and assayed onto Shirofugen trees to test for ilarvirus content in the peach trees.

During the initial extractions, each sample was processed twice, i.e., tissues were crushed in phosphate buffer for ELISA of PNRSV and in carbonate buffer for ELISA of PDV. We soon discovered, however, that extracts in carbonate buffer also worked well for PNRSV, provided the wells were precoated with anti-PNRSV antibodies. Thereafter, all tissue extractions were done in carbonate buffer.

For leaf extracts made during the collection period of 23 March to 27 April 1987, the incidence of PNRSV and PDV among first-leaf trees averaged 24%. Overall, 28% of the trees of all ages were infected by one or both ilarviruses (Table 1). Eighteen of the 41 test orchards (March–April collection) were resampled and indexed onto Shirofugen trees. When only 158 directly compared orchard trees were considered, ELISA detected 81 virus infections and Shirofugen detected 82. Thus, all trees with leaf extracts that yielded mean ELISA values above 0.50 (virus-positive), except for one tree below 0.25 (virus-negative), were confirmed by the bioassays (Table 2). The presence of serologically different strains of PNRSV,

Table 1. Incidence of Prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) in cling peach trees in California orchards, 1987^a

Year planted	Leaf stage ^b	No. of orchards sampled	No. of orchards diseased	No. of trees infected with:			No. of trees infected/total (%)
				PNRSV	PDV	PNRSV + PDV	
1987	First	12	4	22	0	5	27/111 (24)
1986	Second	4	0	0	0	0	0/39 (0)
1985	Third	15	7	25	5	17	47/129 (37)
1984	Fourth	8	5	10	3	5	18/63 (29)
1983	Fifth	2	2	8	1	0	9/20 (45)
Total		41	18	65	9	27	101/362 (28)

^aData are based on enzyme-linked immunosorbent assay (ELISA) of collections during 23 March to 27 April. ELISA values (A_{450nm}) ≥ 0.50 (twice the mean of healthy controls) were classified as positives.

^bNumber of sets of leaves produced in the orchard trees, e.g., first-leaf stage = tree sampled same year as planted.

but not of PDV, was evident also. This occurred in only two orchards; several extracts gave values of 0.27–0.34 in orchard S and of 0.41–0.49 in orchard N (Table 2). Healthy peach extract values in the respective test plates were 0.10 and 0.20. With strict adherence to A_{450nm} of 0.50 for virus-positive, these extracts were classified as ELISA-negatives. However, as they also produced readings above the stated figure for healthy (A_{450nm} of 0.25), we were alerted to the possibility that the trees, especially in orchard S, were indeed infected. All ELISA-tested trees were bioassayed on Shirofugen, which showed six virus-positive trees in orchard S and nine in orchard N. These results confirmed that extracts yielding A_{450nm} values between 0.25 and 0.50 were virus-positive and that a heterologous virus serotype was present. Such virus strains would be barely detected using antibodies of a monoclonal cell line produced to the type strain of PNRSV (5).

During 5–22 May 1987, 21 additional orchards, consisting mostly of second-leaf trees (nine orchards) and third-leaf trees (six orchards) were sampled. ELISA results were erratic. For example, three orchards with ELISA-positives of 0/12 (number trees positive/number trees tested), 1 PDV/11, and 0/10 showed one, nine, and three virus-positives, respectively, on Shirofugen; the single Shirofugen-positive in the first orchard was ELISA-positive for PNRSV for tissue sampled on 21 April. Results in two other orchards sampled on 22 May were quite different from the ones just noted. In one orchard, all 10 trees sampled were PNRSV-positive by ELISA, with A_{450nm} values averaging 0.88. In the other orchard, 16 of 17 trees sampled were PNRSV-positive (ELISA A_{450nm} values averaging 0.92), and five were also infected by PDV (ELISA A_{450nm} values averaging 0.71). All 27 trees from both orchards indexed virus-positive on Shirofugen, i.e., one more than indicated by ELISA. These findings, in contrast to the earlier May results, agree favorably with ELISA tests performed on trees sampled during April. Among the entire May collection of 151 samples, the ELISA procedure detected 27 virus-infected trees: 10 PNRSV, 14 PDV, and 3 PNRSV + PDV.

ELISA results of five trees infected by both PNRSV and PDV in leaf collections of 21 April and 15 May are noteworthy. In April, all extracts were highly reactive and produced mean A_{450nm} values of 1.08 (PDV) and 1.11 (PNRSV). In May, tissue extracts gave a mean A_{450nm} value of 0.80 for PDV, whereas values of the five trees for PNRSV were 0.46, 0.43, 0.22, 0.22, and 0.14. Controls of virus-infected and healthy trees produced mean A_{450nm} values of, respectively, 1.04 and 0.15 for PDV ELISA plates and 0.71 and 0.21 for PNRSV ELISA plates.

All trees infected by both PNRSV and

Table 2. Efficacy of serological assays and bioassays for detection of Prunus necrotic ringspot virus (PNRSV) in four California cling peach orchards

Tree sites	Orchard Z		Orchard H		Orchard N		Orchard S	
	ELISA ^a	Shiro ^b	ELISA	Shiro	ELISA	Shiro	ELISA	Shiro
1	1.26	+	—	—	—	—	—	—
2	1.01	+	—	—	0.43	+	0.32	+
3	1.25	+	—	—	0.41	+	0.34	+
4	1.27	+	—	—	0.61	+	—	—
5	1.17	+	0.76	+	0.63	+	—	—
6	1.18	+	—	—	0.46	+	0.27	+
7	1.10	+	—	—	0.45	+	—	—
8	—	—	—	—	0.47	+	—	+
9	1.02	+	—	—	0.72	+	0.28	+
10	1.76	+	—	—	0.49	+	—	+
PNRSV ^c	1.03		0.79		1.61		1.06	
Healthy ^c	0.23		0.24		0.20		0.10	

^a Enzyme-linked immunosorbent assay negatives (—) produced A_{450nm} values (mean of two wells) approximating those of healthy controls.

^b Shirofugen flowering cherry indicator tree; + = positive, — = negative.

^c Plant standards; values represent means from duplicate wells.

PDV were stunted. Trees infected by either PNRSV or PDV appeared normal in size compared with healthy trees (i.e., negative serological assays or bioassays) located in the same orchard.

DISCUSSION

The apparent healthy status of second-leaf trees (Table 1) is misleading. All four orchards sampled in the second-leaf stage showing no infected trees were planted with the cultivar Dr. Davis. Among seven additional Dr. Davis orchards in their first-, third-, or fourth-leaf stage, only a single PNRSV infection (a third-leaf tree) was identified, indicating that the cultivar is usually free from PNRSV and PDV. The one infection probably resulted from use of an infected cling peach seedling rootstock (*P. persica* 'Nemaguard'). Overall, our orchard surveys indicated that approximately one in four peach trees was infected by PNRSV or PDV, or both. Trees infected by both viruses showed symptoms of peach stunt disease (3). Although PNRSV spreads rapidly in peach trees via the pollination process (9), the high virus incidence among first-leaf trees, where flowering is absent, suggests that infected scion buds and/or rootstocks were used to produce nursery trees. In an effort to correct this problem at the nursery level, legislation was recently enabled (effective 1 January 1988) for a mill tax of 0.25% on all stone, pome, and nut trees and grapevines sold in California (11). Some of the revenue produced by the tax will be used to support the California Department of Food and Agriculture's nursery certification and ELISA laboratory programs. Such support should prove immediately beneficial to the California peach nursery and orchard industries in reducing the incidence of PNRSV and PDV.

Based on ELISA and Shirofugen indexings of the same test trees,

serological assays of leaf collections in March and April, but not May, compared favorably with bioassays. The erratic early May ELISA results may be attributed to the high day temperatures (at or above 38 C for 12 days between 1 and 15 May 1987), which likely affected virus multiplication in diseased trees. Nonetheless, all of the bioassays of the trees tested by ELISA during March–April (the figure of 94 positives includes, for discussion purposes, the so-called ELISA-negatives listed in Table 2 for orchards N and S) detected three more virus infections (for a total of 97) than indicated by serological assays. One explanation for this discrepancy is that since budsticks were collected 3–5 mo after bloom, the additional Shirofugen-positives represented the detection of current-season, pollen-borne virus transmissions. Alternatively, on the basis of the relatively low A_{450nm} values for four other trees in the same orchard, the two ELISA-negative, Shirofugen-positive trees found in orchard S (Table 2) probably contained a heterologous virus serotype that is presumed to be represented there. If that were the case, differences in antigenicity and/or low virus titers of collected leaves would react weakly at best and therefore be difficult to detect with the single source of PNRSV antiserum used in our tests. In an earlier study, four serotypes of PNRSV and one of apple mosaic virus were tested by ELISA, and some antiserum sources recognized only homologous or closely related virus strains (7). It is likely that use of an antiserum specific to our heterologous virus isolate would enhance our ability to detect it. An isolate from orchard N is currently being increased for virus purification and antiserum production.

ACKNOWLEDGMENT

We wish to thank the Cling Peach Advisory Board for providing financial assistance for this study.

LITERATURE CITED

1. Fulton, R. W. 1983. Ilarvirus group. No. 275 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.
2. George, J. A., and Davidson, T. R. 1963. Pollen transmission of necrotic ringspot and sour cherry yellows viruses from tree to tree. *Can. J. Plant Sci.* 43:276-288.
3. Gilmer, R. M., Nyland, G., and Moore, J. D. 1976. Prune dwarf. Pages 179-190. in: *Virus Diseases and Noninfectious Disorders of Stone Fruits in North America*. U.S. Dep. Agric. Agric. Handb. 437. U.S. Government Printing Office, Washington, DC.
4. Gilmer, R. M., and Way, R. D. 1963. Evidence for tree-to-tree transmission of sour cherry yellows virus by pollen. *Plant Dis. Rep.* 47:1051-1053.
5. Halk, E. L., Hsu, H. T., Aebig, J., and Franke, J. 1984. Production of monoclonal antibodies against three ilarviruses and alfalfa mosaic virus and their use in serotyping. *Phytopathology* 74:367-372.
6. Milbrath, J. A. 1961. The relationship of stone fruit ringspot virus to sour cherry yellows, prune dwarf and peach stunt. *Tidsskr. Planteavl.* 65:125-133.
7. Mink, G. I., Howell, W. E., Cole, A., and Regev, S. 1987. Three serotypes of *Prunus* necrotic ringspot virus isolated from rugose mosaic-diseased sweet cherry trees in Washington. *Plant Dis.* 71:91-93.
8. Nyland, G., Gilmer, R. M., and Moore, J. D. 1976. *Prunus* ringspot group. Pages 104-132 in: *Virus Diseases and Noninfectious Disorders of Stone Fruits in North America*. U.S. Dep. Agric. Agric. Handb. 437. U.S. Government Printing Office, Washington, DC.
9. Schmitt, R. A., Williams, H., and Nyland, G. 1977. Virus diseases can decrease peach yields. *Cling Peach Q.* 13:17-19.
10. Uyemoto, J. K., Bockelman, D. L., and Claflin, L. E. 1980. Severe outbreak of corn lethal necrosis disease in Kansas. *Plant Dis.* 64:99-100.
11. Woolley, R. 1988. New tax on nursery stock supports certification programs. *Cling Peach Rev.* 24:17.