Location of Prune Dwarf and Prunus Necrotic Ringspot Viruses Associated with Sweet Cherry Pollen and Seed

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We thank A. Soeldner and C. Weiss of the Electron Microscope Facility, Oregon State University, for their assistance.
Portions of a Ph.D. thesis submitted to Oregon State University by the first author.
Oregon State University Agricultural Experiment Station Technical Paper 6606.
Accepted for publication 9 October 1985 (submitted for electronic processing).

ABSTRACT

Kelley, R. D., and Cameron, H. R. 1986. Location of prune dwarf and Prunus necrotic ringspot viruses associated with sweet cherry pollen and seed. Phytopathology 76:317-322.

The location of prune dwarf virus (PDV) and Prunus necrotic ringspot virus (PNRSV) in pollen and seed from infected and healthy sweet cherry trees pollinated with pollen from infected trees was investigated by means of electron microscopy and enzyme-linked immunosorbent assay (ELISA). Transmission electron microscopy revealed viruslike particles in the cytoplasm of pollen grains from PDV-infected cherry trees. No particles were observed in pollen from PNRSV-infected or noninfected trees. Both viruses were detected by ELISA in all parts of fruit from infected trees pollinated with pollen from healthy trees. Both viruses were detected in the cotyledons, hypocotyl-radicle tissues, and testa-nucellus-endosperm tissues but not in the mesocarp of fruit from caged healthy trees bee-pollinated with pollen from infected trees or in infected fruit of healthy orchard trees hand-pollinated with pollen from infected trees. Antigen levels in the buffer from successive washings of pollen from infected trees decreased steadily. Antigen levels were high in pollen samples that had been washed, then ground in buffer, even those treated before grinding with antiserum to bind all available antigenic sites. Attempts to transmit PNRSV to cherry fruit by pollination with pollen from PNRSV-infected almond failed.

Studies have shown that prune dwarf virus (PDV) and Prunus necrotic ringspot virus (PNRSV) are transmitted by pollen in squash (5,11) and in cherry (7,8,15) and that pollen from infected plants can cause infection of pollinated plants (7). Two mechanisms of virus entry into the pollinated plant as a consequence of pollination with pollen from infected plants seem plausible: infection of the stigma from virus carried on the pollen surface (9) and direct infection of the egg during fertilization by virus carried within the pollen (1), followed by infection of the mother plant (4).

Many pollen-transmitted viruses, including PDV and PNRSV, are also seed-transmitted (7). The percentage of seed transmission of PDV and PNRSV is extremely variable, and seeds from infected trees in which antigen can be detected quite commonly produce some seedlings in which neither virus is detectable. This study was undertaken to determine the location and behavior of these viruses relative to the pollen and seed of sweet cherry. Differences in the location of a virus within cherry seed, depending on whether the virus originated from the mother plant or from the fertilizing pollen, were examined.

MATERIALS AND METHODS

Pollen collection. Flowers were collected from mature Corum sweet cherry trees infected with PDV or PNRSV and from almond trees infected with PNRSV, as determined by enzyme-linked immunosorbent assay (ELISA) (2). Control flowers were collected from trees containing neither virus. Anthers were collected by rubbing flowers over a screen. Pollen was then separated from the anthers after drying.

Transmission electron microscopy. Pollen samples gathered from trees determined by ELISA to be infected with either PDV or PNRSV, with both viruses, or with neither virus were placed in 2% potassium permanganate and centrifuged for 10 min at 3,000 *g*. Samples were stored in potassium permanganate solution at 4 C for 24 hr, resuspended in 0.2 M phosphate buffer (pH 8.0), and centrifuged. The supernatant was discarded, and the pollen was resuspended in melted 1% ion agar and poured onto glass slides to cool. Agar was cut into 1-mm squares with a razor blade and dehydrated in an acetone series that included uranyl acetate saturated in 70% acetone. Dehydrated samples were processed in propylene oxide as a transition solvent and embedded in medium-hard Spurr's resin. Sections were poststained with lead citrate and examined at 60 kV with a Philips 300 transmission electron microscope.

Washed pollen. Weighed samples (0.02 g) of pollen from noninfected cherry, cherry infected with PDV or PNRSV, and almond infected with PNRSV were each suspended in 3 ml of PBS-Tween-PVP buffer (pH 7.4) and shaken by hand for 1 min, then centrifuged at 1,100 *g* for 10 min. A 1-ml sample of the supernatant was taken for ELISA analysis, the remainder was discarded, and the pellet was resuspended in 3 ml of buffer. This procedure was repeated six times, after which the pellet was ground with a mortar and pestle. This suspension was centrifuged, a 1-ml sample of the supernatant was taken, and the pellet was resuspended and washed three more times. Light microscope examination of ground samples showed that approximately 80% of the pollen grains were disrupted. An identical set of pollen samples was processed similarly except that the samples were first incubated for 1 hr in phosphate buffer containing the γ-globulin fraction of antiserum to either PDV or PNRSV at a 1:4 dilution (1 ml globulin:4 ml sample in buffer). The entire experiment was repeated three times as described plus four times with only four washings before grinding. The results of all experiments were similar. In preliminary tests, pollen samples were given an additional ethanol wash to remove exterior lipids that might contain virus particles. No difference in results was noted, and this treatment was discontinued.

Virus in fruit. Fruit from noninfected cherry trees and trees infected with PDV or PNRSV or both were collected in 1982 and 1983. Five mature cherries from different limbs were gathered from each tree and combined as one sample. Samples were collected 5 and 17 May and 24 June. Each fruit was dissected into four parts: cotyledons, hypocotyl-radicle tissues, testa-nucellus-endosperm tissues, and a piece from the mesocarp equal in weight to the cotyledons. Each part was cut with a new razor blade to reduce the probability of surface contamination of dissected parts. Preliminary ELISA tests in which each fruit part was washed showed no differences from reactions of unwashed parts. Results

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reported here are from unwashed samples. No attempt was made to separate the remnants of the endosperm from the testa and nucellus tissues. Hypocotyl-radical and testa-nucellus-endosperm tissues were ground in 2 ml of phosphate buffer and mesocarp and cotyledon samples, in 5 ml.

Four sweet cherry trees (two Lambert and two Bing) not infected with either PDV or PNRSV were each enclosed in a cage 4.9 m (16 ft) long by 2.4 m (8 ft) wide by 3.7 m (12 ft) high. Each cage was divided in half internally, with half the tree canopy in each half-cage. At the popcorn stage of bloom development, a hive of bees was placed in each half-cage. Branches with unopened flowers from noninfected Black Republican cherry trees or from trees of that cultivar infected with PDV or PNRSV were placed in buckets of water in each half-cage as pollen sources. On 5 and 17 May and 24 June, five fruit from each half-tree were collected and tested as described.

In 1982 and 1983, orchard trees determined by ELISA to be free from PDV and PNRSV were pollinated by hand with pollen collected from trees infected with either PDV or PNRSV or both. Infected trees were also hand-pollinated with pollen collected from

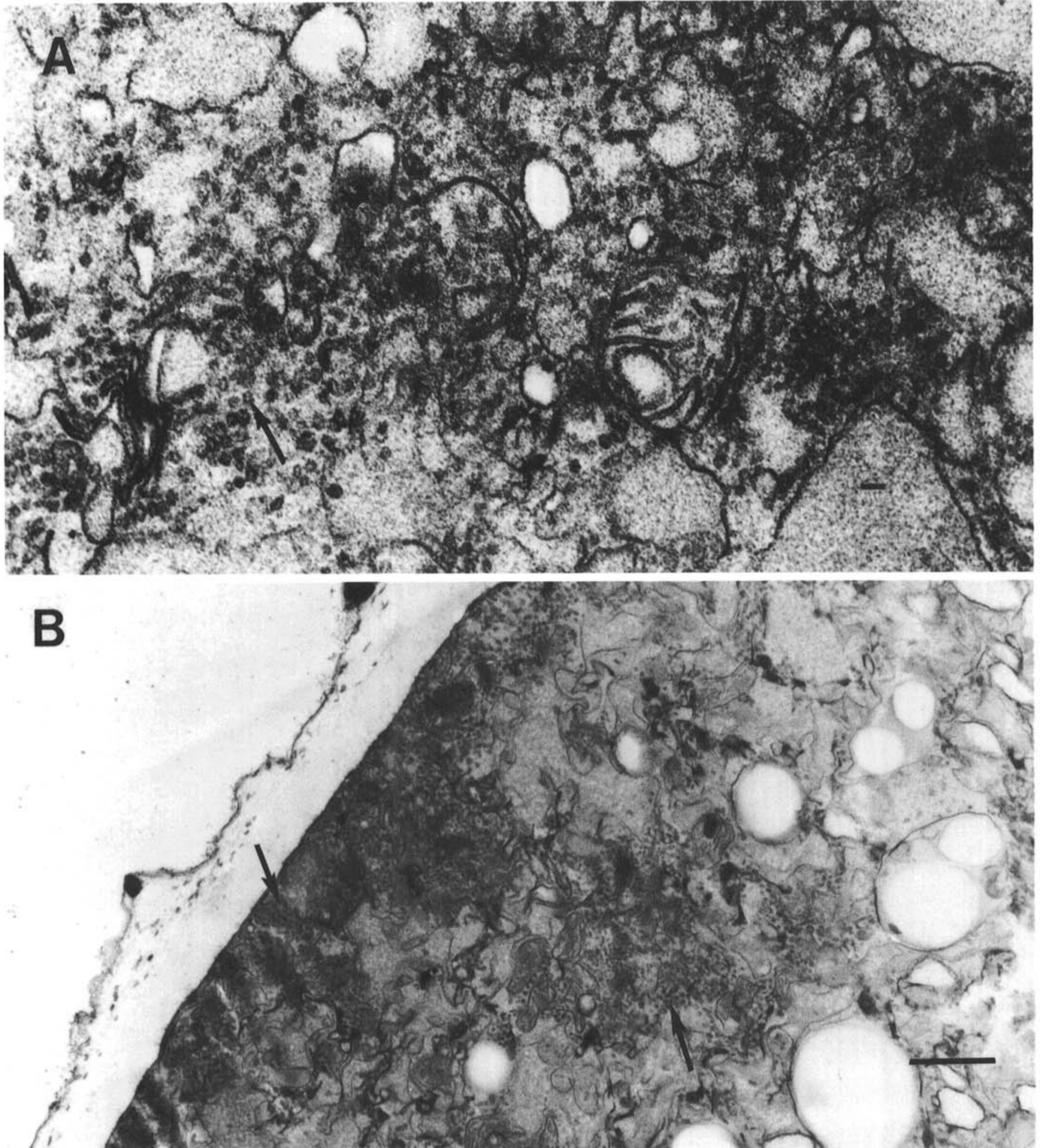

Fig. 1. A, Viruslike particles in pollen from sweet cherry tree infected with prune dwarf virus. Scale bar = 100 nm. **B,** Viruslike particles apparently concentrated at corner of pollen grain. Scale bar = 1 μm.

noninfected trees. In 1983, some noninfected trees were also pollinated with a combination of pollen from PNRSV-infected almond and noninfected cherry. Limbs with pollinated flowers were enclosed in sleeve cages until after petal-fall. Fruit were harvested at maturity, dissected, and tested as described.

ELISA procedures. The γ-globulin fractions of PNRSV antiserum against isolate G and PDV antiserum against isolate B were used in all ELISA procedures. These antisera, prepared by R. W. Fulton and purchased from ATCC, have been shown capable of detecting 31 of 34 isolates of the viruses (10). Antisera γ-globulin fractions were adjusted to an optical density of 1.4 (approximately 1 mg/ml).

All samples analyzed by ELISA were treated as described by Clark and Adams (2). Wells were coated with purified γ-globulin (250 μl/well) diluted (1:500) in coating buffer (pH 9.6) for 4 hr at 37 C. Wells were washed three times with PBS-Tween buffer (pH 7.4), then 250 μl of the sample was placed in each well and incubated overnight at 4 C. Wells were washed again as described, and 250 μl of conjugated antiserum (1:500, conjugate:PBS-Tween-PVP buffer, pH 7.4) was placed in each well and incubated at 37 C for 4 hr. Plates were washed again as described and 300 μl/well of substrate was added. Absorbance values for 405 nm wavelength light (A_{405}) generated by *p*-nitrophenol in ELISA reactions were recorded 30 min after addition of substrate, using a Gilford PR-50 EIA automatic analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074).

RESULTS

Transmission electron microscopy. Viruslike particles about 45 nm in diameter were observed in thin sections of pollen from trees infected with PDV (Fig. 1A) or with PDV and PNRSV. Although seen throughout the cytoplasm of the pollen, particles appeared to concentrate in the areas beneath the pores at each corner of the grain (Fig. 1B). Particles were not observed in pollen from healthy or PNRSV-infected trees. Subsequent examination of pollen from PDV-infected trees prepared in the same manner except for fixation in 4% glutaraldehyde and postfixation in osmium tetroxide showed particles about 25 nm in diameter with similar distribution. Resolution of specimens with this fixation procedure was extremely poor, however. Attempts to specifically stain particles in potassium permanganate-fixed sections with ferritin-tagged antibodies to PDV and PNRSV (12) were unsuccessful.

Washed pollen. ELISA reactions (A_{405}) were compared among all treatments (Tables 1 and 2). Supernatant fluids from buffer washings of pollen from infected trees not pretreated with antiserum (Figs. 2A, 2C, and 3A) contained progressively less antigen after each successive washing. A_{405} values increased significantly when washed pollen grains were ground. Values for washings after grinding decreased with each wash, and the value for the last wash was not higher than that for pollen from noninfected trees. A_{405} values were not higher than those of noninfected controls for the first six washings of pollen pretreated with antiserum (Figs. 2B, 2D, and 3B), but values increased significantly when these samples were ground.

Virus in fruit. Both PDV and PNRSV were readily detectable in ground suspensions of immature cherry fruit and seed collected 5 and 17 May from infected trees. No antigen was detected, however, in fruit collected on these dates from caged healthy trees bee-pollinated with pollen from infected trees. In mature fruit collected 24 June and dissected, neither virus was detected in mesocarp tissue of fruit from healthy trees produced by either hand- or bee-pollination with pollen from infected trees. However, PDV and PNRSV were detected in the cotyledons, hypocotyl-radicle tissues, and testa-nucellus-endosperm tissues of the seeds from these fruit (Fig. 4). Both PDV and PNRSV were detected in all tissues examined, including mesocarp, of fruit from infected trees, whether pollen used for pollination was from infected trees or from healthy trees (Fig. 5). No virus was detected in fruit resulting from hand-pollination of healthy trees with a combination of pollen from PNRSV-infected almond trees and healthy cherry trees.

DISCUSSION

Transmission electron microscope observations of viruslike particles within pollen grains suggest that PDV is carried internally by cherry pollen. Diameter of the observed particles was larger than that reported for PDV in purified extract (6), but several factors indicate that these particles are indeed PDV: (i) The particles were observed only in pollen from PDV-infected trees; (ii) when used in long fixation times, potassium permanganate is known to cause disruption of viral proteins (14) and could have caused particles to swell (13); and (iii) glutaraldehyde/osmium fixation resulted in smaller particles that were also observed only in pollen from PDV-infected trees. Potassium permanganate was chosen as a fixative

TABLE 1. Effect of washing pollen on detection of Prunus necrotic ringspot virus (PNRSV) by ELISA[a]

Wash no.	Pollen from healthy cherry trees		Pollen from PNRSV-infected almond trees		Pollen from PNRSV-infected cherry trees	
	Treated with antiserum	Not treated with antiserum	Treated with antiserum	Not treated with antiserum	Treated with antiserum	Not treated with antiserum
1	0.032	0.036	0.008	0.421	0.039	2.700[b]
2	0.032	0.029	0.020	0.050	0.046	0.387
3	0.033	0.030	0.025	0.041	0.043	0.074
4	0.024	0.018	0.029	0.036	0.044	0.053
5	0.028	0.017	0.013	0.032	0.047	0.051
6	0.016	0.018	0.013	0.019	0.042	0.053
Grind	0.015	0.035	2.438	2.244	2.700[b]	2.700[b]
7	0.012	0.028	0.097	0.080	0.816	0.297
8	0.009	0.026	0.050	0.029	0.150	0.044
9	0.011	0.009	0.028	0.020	0.076	0.043
Pollen ground without washing	···	0.036	···	2.700[b]	···	1.845

[a]Values are average of three replicate wells made from each treatment sample. γ-Globulin fraction (1.4 OD) used in 1:500 dilution in PBS-Tween buffer; readings taken on Gilford PR-50 EIA automatic analyzer 30 min after addition of substrate.

[b]Maximum possible absorbance reading by machine used.

TABLE 2. Effect of washing pollen on detection of prune dwarf virus (PDV) by ELISA[a]

Wash no.	Pollen from healthy cherry trees		Pollen from PDV-infected cherry trees	
	Treated with antiserum	Not treated with antiserum	Treated with antiserum	Not treated with antiserum
1	0.037	0.238	0.043	2.292
2	0.045	0.044	0.053	1.236
3	0.025	0.028	0.043	0.280
4	0.033	0.010	0.041	0.174
5	0.033	0.007	0.022	0.124
6	0.009	0.023	0.029	0.090
Grind	0.047	0.061	1.892	1.316
7	0.031	0.033	0.747	0.299
8	0.020	0.013	0.355	0.133
9	0.022	0.014	0.333	0.120
Pollen ground without washing	···	0.349	···	2.700[b]

[a]Values are average of three replicate wells made from each treatment sample. γ-Globulin fraction (1.4 OD) used in 1:500 dilution in PBS-Tween buffer; readings taken on Gilford PR-50 EIA automatic analyzer 30 min after addition of substrate.

[b]Maximum possible absorbance reading by machine used.

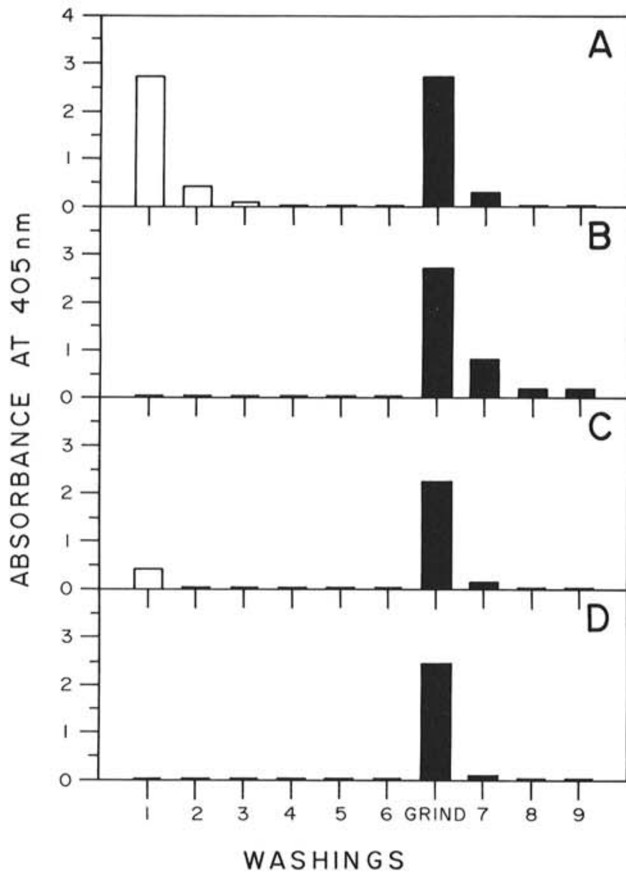

Fig. 2. Absorbance values for 405 nm wavelength light of *p*-nitrophenol generated in ELISA reactions of supernatant from successive washings, grinding, and further washings of sweet cherry and almond pollen samples from trees infected with Prunus necrotic ringspot virus (PNRSV) when tested for PNRSV antigen. Cherry pollen **A**, not pretreated and **B**, pretreated with PNRSV antiserum; almond pollen **C**, not pretreated and **D**, pretreated with PNRSV antiserum. Values for pollen from noninfected control trees were between 0.009 and 0.036.

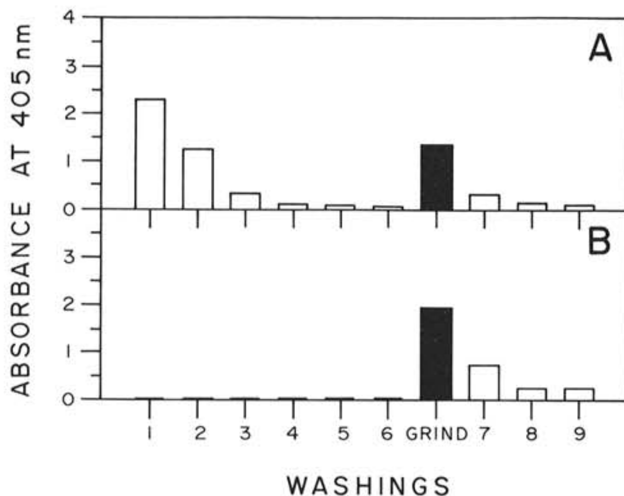

Fig. 3. Absorbance values for 405 nm wavelength light of *p*-nitrophenol generated in ELISA reactions of supernatant from successive washings, grinding, and further washings of sweet cherry pollen samples from trees infected with prune dwarf virus (PDV) when tested for PDV antigen. **A**, Pollen not pretreated and **B**, pollen pretreated with PDV antiserum. Values for pollen from noninfected control trees were between 0.009 and 0.047.

because ribosomes, which are difficult to distinguish from PDV and PNRSV particles, do not stain well after use of this fixative. The apparent concentration of particles beneath the pollen grain pores is believed to be an artifact. Because the fixative enters through the pores, particles cluster near these openings, whereas internal virus particles do not become fixed and are thus lost during subsequent processing. The possibility exists that the observed particles are some virus for which the trees were not tested. Considering the number of trees from which pollen was examined (over 50), however, the occurrence of such particles only in samples from PDV-infected trees seems unlikely.

PNRSV was not observed in electron microscope examinations of pollen from infected trees. This may have been due to disruption of the virus during the preparation process.

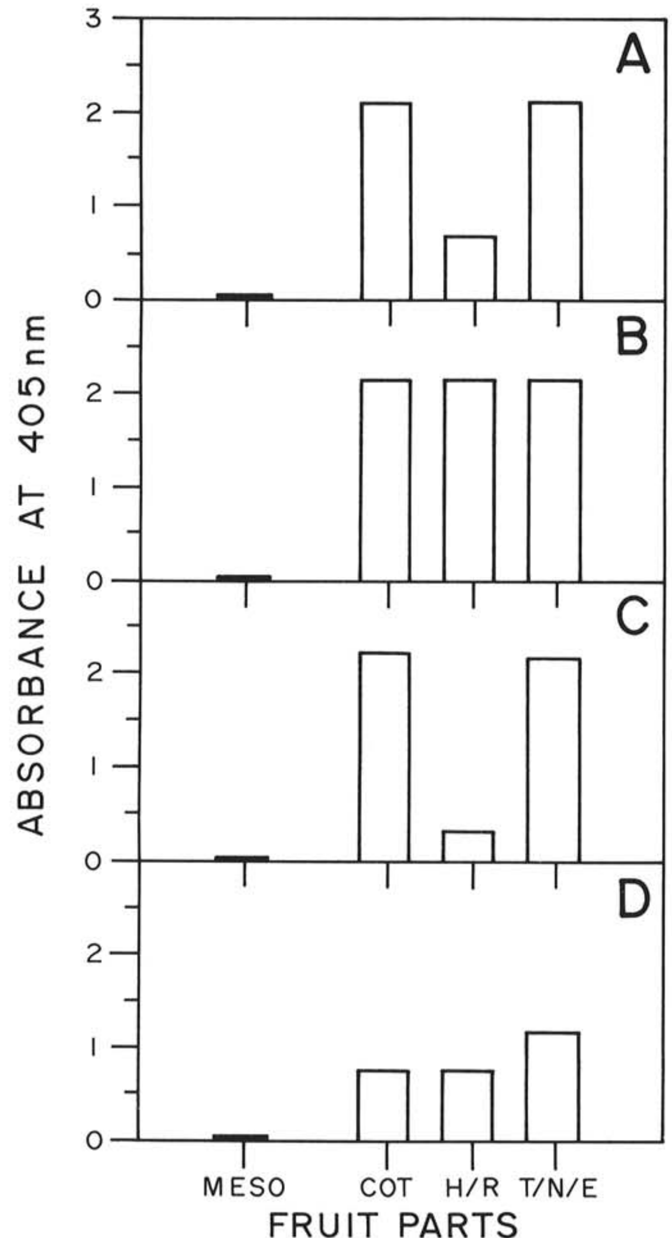

Fig. 4. Absorbance values for 405 nm wavelength light of *p*-nitrophenol generated in ELISA reactions to fruit gathered on 24 June from healthy trees pollinated with pollen from infected trees. Fruit from caged trees bee-pollinated with pollen from **A**, trees infected with Prunus necrotic ringspot virus (PNRSV) when tested for PNRSV antigen and **B**, trees infected with prune dwarf virus (PDV) when tested for PDV antigen. Fruit from trees hand-pollinated with pollen from **C**, PNRSV-infected trees when tested for PNRSV antigen and **D**, PDV-infected trees when tested for PDV antigen. MESO = mesocarp, COT = cotyledons, H/R = hypocotyl-radicle tissues, T/N/E = testa-nucellus-endosperm tissues.

The detection of antigen in the first, and sometimes also the second, wash of pollen samples from infected trees indicates that these viruses are external and can be washed off or that the pollen grains were disrupted during preparation and an internal virus is represented. There appear to be marked differences between external PNRSV from almond and from cherry pollen. Initial washings of almond pollen were consistently lower than those of cherry pollen, possibly indicating that external PNRSV is much less common in almond. This suggests that virus location may vary for different cultivars of cherry as well, and future research should not ignore this possibility. The subsequent increase in A_{405} values after disruption by grinding indicates that virus was released from inside the pollen. The A_{405} values for the first six washes of pollen pretreated with antiserum were not higher than those for pollen from healthy trees, indicating effective blockage of all accessible antigenic sites. In every test, however, values increased dramatically after grinding, from at or near the level of healthy controls to the maximum level the machine was capable of detecting (somewhat less for pollen from PDV-infected trees). We believe these results support a model of an internal location for these viruses.

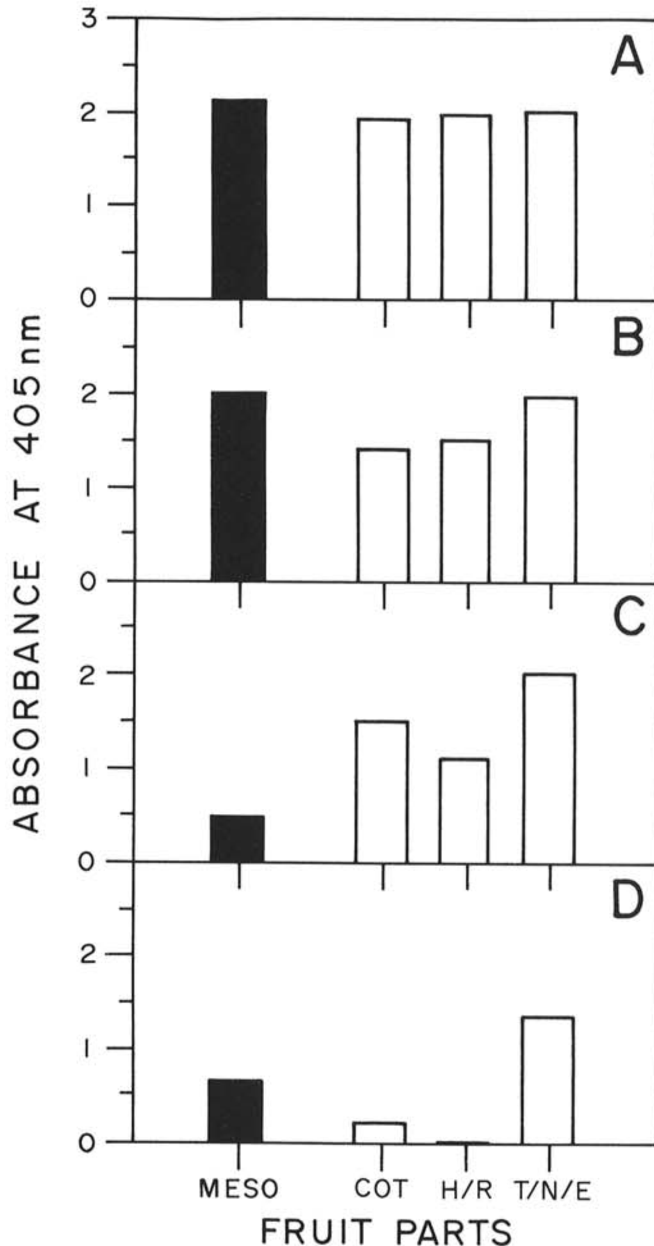

Fig. 5. Absorbance values for 405 nm wavelength light of *p*-nitrophenol generated in ELISA reactions to fruit gathered from infected orchard trees on 24 June. Reaction of fruit from **A,** trees infected with prune dwarf virus (PDV) to PDV antiserum, **B,** trees infected with PDV and Prunus necrotic ringspot virus (PNRSV) to PDV antiserum, **C,** PNRSV-infected trees to PNRSV antiserum, and **D,** trees infected with both viruses to PNRSV antiserum. MESO = mesocarp, COT = cotyledons, H/R = hypocotyl-radicle tissues, T/N/E = testa-nucellus-endosperm tissues.

Our results do not agree with those of Cole et al (3), but in repeated tests we were unable to duplicate their results. It may be that their method of pollen disruption by mixing was not as efficient as our grinding procedure; in preliminary trials, we were unable to achieve satisfactory levels of disruption with their method. It may also be that their pollen contained no internal virus, owing to differences in the cultivars used. The possibility that grinding releases more externally located but previously undetected virus particles seems remote, since ethanol treatment to remove all exterior coating from the pollen was determined effective by both light and electron microscopy.

The confinement of virus to the seeds of fruit produced by pollination of healthy trees with pollen from infected trees, while fruit from infected trees contained virus throughout, could have a number of explanations. Separation of the remnants of the endosperm from the testa and nucellus tissues was not attempted. Therefore, this portion of each fruit sample contained tissues (testa and nucellus) deriving from the megasporogenic parent and a tissue (endosperm remnants) that is a product of fertilization. If pollen transmission of these viruses requires fertilization, with infection beginning in the fertilized egg, then fruit whose virus source was the fertilizing pollen should contain virus in tissues that result from fertilization but none in tissues arising solely from the megasporogenic parent. Likewise, in fruit whose virus source was the megasporogenic parent, one would expect to detect virus in tissues deriving solely from that parent and perhaps also in tissues resulting from fertilization. Although we do not observe this distribution in as clear-cut a pattern as might be observed if the remnants of the endosperm could be reliably separated from the testa and nucellus tissues, what we do find is a pattern of distribution that strongly suggests fertilization is indeed a method of virus transmission to cherry seed. This is substantiated by the complete lack of transmission to cherry fruit of PNRSV by almond pollen, which is incapable of fertilizing a cherry. It is still quite possible, however, that tree-to-tree transmission of these viruses involves exterior particles.

Continued studies of these viruses within germinating pollen and developing embryos are under way and should yield a more complete picture.

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