Disease Control and Pest Management

Location of Prunus Necrotic Ringspot Virus on Pollen Grains from Infected Almond and Cherry Trees

Anabel Cole, G. I. Mink, and Samuel Regev

Research associate, plant pathologist, and former research associate, respectively, Irrigated Agriculture Research and Extension Center, Prosser, WA 99350.

Washington State University College of Agriculture, Scientific Paper 6142, Project 1719.
Supported in part by funds provided by the Washington Tree Fruit Research Commission.
Accepted for publication 24 May 1982.

ABSTRACT

Cole, A., Mink, G. I., and Regev, S. 1982. Location of Prunus necrotic ringspot virus on pollen grains from infected almond and cherry trees. Phytopathology 72:1542-1545.

Intact pollen grains and washings from pollen grains from necrotic ringspot virus (NRSV)-infected almond and cherry trees produced strong reactions in enzyme-linked immunosorbent assay (ELISA) and intact pollen grains flocculated rapidly in latex flocculation tests. Pretreatment of pollen grains with anti-NRSV globulin completely blocked the serological reactivity of intact pollen grains when subsequently tested by ELISA or latex flocculation. No additional antigens were detected when 'blocked' pollen grains were disintegrated. Virus-specific antigens were easily removed from intact pollen by washing. In sucrose density gradients the sedimentation pattern of virus-specific antigens obtained from pollen washes indicated that both intact virus and subviral antigens were removed. The results indicate that most, if not all, NRSV associated with almond and cherry pollen is borne on the surface. Some implications of this finding are discussed.

Additional key words: cherry rugose mosaic disease.

In most stone fruit species, Prunus necrotic ringspot virus (NRSV) is spread from tree to tree via pollen (16). Evidence from cage and deflowering experiments indicate that, under field conditions, the virus invades susceptible trees primarily through flowers (2,7,8). Numerous workers have examined tree-to-tree spread through pollen (2,5,7,9,12,19), and others have demonstrated various effects of the virus on pollen morphology (1,15,18). However, virtually nothing is known about the mechanism by which NRSV is transferred from pollen to flower, or the exact site in the flower where infection occurs.

Some pollen-transmitted viruses are located inside pollen grains (3,20) whereas others can be found on the pollen surface (10). This relationship between virus and pollen may determine the actual mechanism by which the virus is transferred to flowers (10). Prior to studies on NRSV infection of stone fruit flowers, we examined the relationship between this virus and pollen from almond and cherry trees. Results of these studies and their possible significance in epidemiology of cherry rugose mosaic disease (CRM) caused by strains of NRSV (17) are presented here.

MATERIALS AND METHODS

Pollen sources. In the spring of 1980 and 1981 blossoms were collected from cherry or almond trees that had been indexed for NRSV by enzyme-linked immunosorbent assay (ELISA) during the winter (13). Anthers were removed by rubbing partially open blossoms across a wire screen or with a small vacuum device (11). The anthers were air dried at room temperature for 36 hr, and stored at 4 C. Pollen for individual experiments was prepared by swirling dried anthers in a dry glass tube by using a cyclo-mixer and collecting pollen grains that adhered to the tube walls. Microscopic observations indicated that essentially all pollen grains collected by this method were physically intact.

Antisera. Antiserum made against NRSV strain G (6) was furnished by R. W. Fulton, University of Wisconsin, Madison. Antiserum against alfalfa mosaic virus (AMV) was prepared earlier. Anti-virus globulins were prepared by the method of Clark and Adams (4).

Latex flocculation. Latex-conjugated anti-NRSV globulin was prepared by mixing one volume of latex beads (794 nm in diameter; Sigma Chemical Co., St. Louis, MO) diluted 1:15 in 0.85% NaCl with an equal volume of anti-NRSV globulin diluted to 100 μg/ml in pH 7.4 phosphate-buffered saline (PBS). After 1 hr at 25 C, the suspension was centrifuged at 6,000 *g* and the pellet was resuspended in saline containing 0.2% polyvinylpyrrolidone (PVP). The wash step was repeated twice and the final pellet was suspended in PBS containing 0.02% sodium azide.

In flocculation tests, approximately 20 μl of freshly prepared pollen suspended in saline were drawn into 100-μl capillary pipettes containing 20 μl of conjugated latex. The tubes were rotated for 5 min, hung vertically by using double-stick tape, and observed against a black background. Visible reactions were recorded at 10, 30, and 60 min. Absence of flocculation was confirmed by

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.

0031-949X/82/12154204/$03.00/0
©1982 The American Phytopathological Society

examination with a dissecting microscope.

ELISA conditions. Coating globulin (1 μg/ml) prepared from NRSV antiserum according to Clark and Adams (4) was incubated for 4 hr at 37 C in polystyrene cuvette pacs (Gilford Instruments, Inc., Oberlin, OH 44074). Pollen samples suspended in grinding buffer or, in some experiments, the saline washes of intact pollen grains were incubated in duplicate wells for 8–16 hr at 4 C. Conjugated globulin diluted 1:1,000 was incubated 4 hr at 37 C. Substrate (*p*-nitrophenyl phosphate, Sigma No. 104) in substrate buffer (4) was incubated 1 to 2 hr. Absorbance readings at 405 nm were made with a PR-50 EIA reader (Gilford Instruments).

Density gradient centrifugation. Linear sucrose density gradients (10–40%, w/v) were layered with 0.8-ml aliquots of pollen washes and centrifuged 90 min at 60,000 rpm in a Beckman SW 65 rotor. Gradients were fractionated into 0.6-ml aliquots by using an ISCO fractionator and tested for serological activity by ELISA.

RESULTS

Sensitivity of tests. To determine the relative sensitivity of ELISA and latex flocculation under our conditions, we compared a series of dilutions of purified NRSV in both tests (Table 1). Virus concentrations between 0.03 and 0.13 μg/ml were readily detected by ELISA but definitive latex flocculation did not occur at concentrations below 0.13 μg/ml.

Tests with intact pollen. Pollen samples from 73 trees representing 10 cherry and three almond cultivars were tested for NRSV by both ELISA and latex flocculation. No virus was detected by using either test on pollen from any of 47 trees that had previously indexed free of NRSV by ELISA. Virus was detected by ELISA associated with pollen from 26 trees, all known to be infected with NRSV. Suspensions that initially contained 0.02 g of pollen per milliliter produced $A_{405\,nm}$ values between 0.57 and 2.47 in tests in which control solutions containing purified virus at 0.1 μg/ml produced $A_{405\,nm}$ values near 1.0 and suspensions of healthy pollen produced $A_{405\,nm}$ values less than 0.1.

As expected, latex flocculation was somewhat less sensitive in detecting virus associated with pollen. Pollen samples from 20 of 26 NRSV-infected trees flocculated within 10–30 min, whereas pollen from all 47 healthy trees remained dispersed for several hours. The six pollen samples that did not produce clear flocculation reactions in these tests came from six trees (cultivar French Burlat) in one orchard. When these six samples were tested by ELISA they produced $A_{405\,nm}$ values between 0.59 and 1.0.

Infectivity test. In three experiments intact air dried pollen grains from a NRSV-infected almond tree were rubbed, without added abrasive, on leaves of three *Chenopodium quinoa* plants by using cotton swabs lightly moistened with 0.01 M neutral phosphate buffer containing 0.01 M sodium diethyldithiocarbamate and 0.01 M cysteine HCl. All nine inoculated plants developed strong symptoms of NRSV within 4 days. The presence of NRSV in each plant was confirmed by ELISA.

'Blocking' experiment. The above results strongly suggested that many virus-specific antigens including infectious virions were attached to the outer surface of pollen grains from NRSV-infected trees. We reasoned that if this were true a pretreatment of the pollen grains with virus-specific antibodies should saturate the exposed antigenic sites and block any subsequent serological reaction. This hypothesis was tested as follows: Three 0.02 g pollen samples from a healthy almond tree and three similar samples from a NRSV-infected tree were each suspended in saline (0.4 ml) and mixed with equal volumes of PBS, anti-alfalfa mosaic virus (AMV) globulin diluted to 0.5 mg/ml in PBS, or anti-NRSV globulin diluted to 0.5 mg/ml. After 30 min incubation at room temperature, the liquids were carefully drained off and each pollen treatment washed three times with saline with gentle agitation to remove unreacted antibodies. Each pollen sample was then suspended in 1 ml of fresh saline and subdivided. One subsample was tested by ELISA and latex flocculation. The remaining subsample was homogenized in a Servall Omni-Mixer for 2 min at top speed to rupture most of the pollen grains and (presumably) release internally borne virus. Homogenized treatments were then tested by ELISA and latex flocculation.

Pretreatment of pollen from the virus-infected tree with anti-NRSV globulins completely prevented any subsequent reaction with anti-NRSV globulin in ELISA or latex flocculation tests while pretreatment with PBS or anti-AMV globulin had no effect (Table 2). Similar results (not reported) were obtained with pollen from NRSV-infected cherry trees.

The results substantiate the hypothesis that NRSV is located on the outer surface of pollen grains. Furthermore, the results with homogenized pollen (Table 2) strongly suggest that no additional antigens were liberated during disruption of the pollen grains.

Removal of virus from pollen. The fact that solutions containing intact pollen reacted readily in ELISA suggested that many viral antigens were removed from the pollen surface during the sample incubation period. The ease with which these antigens can be removed was tested by washing 0.1-g pollen samples repeatedly with 1.0 ml of saline and using either gentle or vigorous agitation

TABLE 1. Relative sensitivity of ELISA and latex flocculation in detecting Prunus necrotic ringspot virus

Test	Virus concentration (μg/ml)[a]					
	0.55	0.27	0.13	0.06	0.03	0
ELISA	2.15[b]	1.72	1.24	0.65	0.27	0.02
Latex	+++[c]	++	+	±	–	–

[a] Purified virus diluted in grinding buffer (4).
[b] Absorbance values at 405 nm after 1.5 hr.
[c] Relative intensity of flocculation reaction after 30 min.

TABLE 2. Reaction of pollen[a] from healthy and Prunus necrotic ringspot virus (NRSV)-infected almond trees[b] in latex flocculation tests and enzyme-linked immunosorbent assay (ELISA) with anti-NRSV globulin after pretreatment with anti-alfalfa mosaic virus or anti-NRSV globulins

Virus content	Globulin pretreatment	Post-treatment	Test	
			Latex[c]	ELISA[d]
None	None	Wash[e]	–	0.00
	AMV	Wash	–	0.00
	NRSV	Wash	–	0.00
	None	Wash, homogenize[f]	–	0.00
	AMV	Wash, homogenize	–	0.00
	NRSV	Wash, homogenize	–	0.00
NRSV	None	Wash	++	2.10
	AMV	Wash	++	1.87
	NRSV	Wash	–	0.02
	None	Wash, homogenize	++	2.00
	AMV	Wash, homogenize	++	2.08
	NRSV	Wash, homogenize	–	0.00

[a] Suspensions containing 0.02 g of pollen in 0.4 ml of saline.
[b] Trees previously indexed by ELISA using methods described earlier (13).
[c] Degree of flocculation observed 30 min after mixing with 100 μg latex-conjugated anti-NRSV globulin per milliliter.
[d] Absorbance at 405 nm.
[e] Pollen washed three times with 1 ml of saline.
[f] Pollen disintegrated in a Servall Omni-Mixer at top speed for 2 min.

TABLE 3. ELISA reactivity[a] of liquids used to wash pollen from healthy and necrotic ringspot virus-infected almond trees

Pollen source	Wash treatment	Wash number			Pollen residue
		1	2	3	
Control	Gentle[b]	0.00	0.00	0.00	0.00
	Vigorous[c]	0.00	0.00	0.00	0.00
NRSV	Gentle	1.99	2.69	1.51	1.49
	Vigorous	1.85	1.90	1.50	0.99

[a] Absorbance at 405 nm.
[b] Mix with wood probe, then filter.
[c] Swirl 2 min in a cyclo-mixer, then filter.

during the wash. The pollen-free liquids reacted strongly in ELISA (Table 3) confirming that a portion of the viral antigens were removed by each wash. In sucrose density gradients, the serological activity associated with the first wash of almond or cherry pollen was found primarily at two locations: fractions 1 and 2, and fractions 5 and 6 (Fig. 1). In a sister tube containing purified NRSV, the virus was located in fractions 5 and 6. The results suggest that both intact and subviral antigens were removed from the pollen surface.

DISCUSSION

Results of the experiments reported here indicate that most, perhaps all, of the NRSV antigens associated with pollen from infected almond and cherry trees are located on the pollen surface. As might be expected, a portion of these antigens can be easily removed by gentle washing. Similar results were obtained by R. I. Hamilton (*personal communication*) who used bee-collected pollen. However, some NRSV antigens remain on pollen even after three successive vigorous washes indicating great variability in either the location or strength with which the antigens are attached.

At least a portion of the surface-bound antigens appear to be intact virus which, under our conditions, remained infectious for more than the 36 hr the pollen was being air dried. We have made no attempt to determine how long NRSV can remain infectious on dried pollen, but in earlier studies (14) the virus was transmitted from a pollen sample that had been stored in a beehive for 10 days. Although numerous environmental factors can adversely affect virus exposed to the pollen surface, circumstantial evidence suggests that infectivity may be retained for several days under certain conditions.

Field experiments have provided strong circumstantial evidence that NRSV infects cherry trees only through flowers (2,7). Infected trees produce pollen which is characterized by various morphological and physiological abnormalities (1,12,15,18). The degree to which these abnormalities are expressed depend upon the tree cultivar and the virus isolate used. Implied, but not stated, in these reports seems to be an assumption that pollen-borne NRSV occurs internally and, for transmission to occur, it is necessary for pollen to germinate, penetrate the flower style and perhaps initiate fertilization. Our finding that NRSV occurs primarily on the pollen surface suggests that viable pollen may not be necessary for pollen transmission of this virus. In view of the ease with which we could transmit NRSV from pollen to *C. quinoa* plants by simple abrasion of the leaves it is conceivable that cherry flowers might be infected by bees abrading flower parts with NRSV-contaminated pollen. Studies are now underway to determine which, if any, flower parts are susceptible to mechanical inoculation.

Although the potential for mechanical transmission of NRSV remains, for the moment, speculative, it has practical implications in current studies on the epidemiology of cherry rugose mosaic disease in some western states. Each year, approximately 50,000 beehives are moved from Washington to California to pollinate various stone fruit crops and then returned to Washington in time to pollinate sweet cherry (*Prunus avium*) orchards (14). Large amounts of NRSV-contaminated pollen are collected and stored while the hives are in California. Many bees that reenter Washington still carry contaminated pollen on their bodies (14). If NRSV can infect cherry trees through abrasions on flower parts caused by foraging bees it would not be necessary that they carry viable pollen or even pollen from a compatible species.

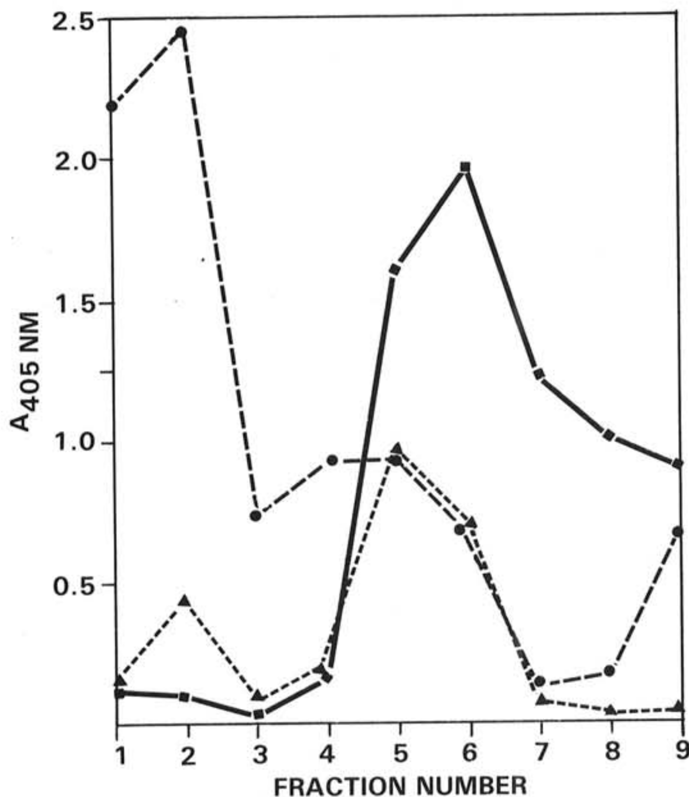

Fig. 1. Sedimentation profiles of purified Prunus necrotic ringspot virus (NRSV) (40 μg) (■——■) and antigens associated with washings of pollen from NRSV-infected cherry (●——●) and almond (▲——▲) trees after 90 min of centrifugation on sucrose density gradients (10–40%, w/v) at 60,000 rpm in a Beckman SW 65 rotor. Serial 0.6-ml fractions were removed from each gradient (top to bottom) with an ISCO fractionator and tested for NRSV antigens by ELISA.

LITERATURE CITED

1. Basak, W. 1966. Influence of prunus necrotic ringspot virus on pollen of sour cherry. Polska Akad. Nauk. Bull. Ser. Sci. Biol. 14:797-800.
2. Cameron, H. R., Milbrath, J. A., and Tate, L. A. 1973. Pollen transmission of prunus ringspot virus in prune and sour cherry orchards. Plant Dis. Rep. 57:241-243.
3. Carroll, T. W. 1974. Barley stripe mosaic virus in sperm and vegetative cells of barley pollen. Virology 60:21-28.
4. 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. 34:475-484.
5. Demski, J. W., and Boyle, J. S. 1968. Spread of necrotic ringspot virus in a sour cherry orchard. Plant Dis. Rep. 52:972-974.
6. Fulton, R. W. 1968. Serology of viruses causing cherry necrotic ringspot, plum line pattern, rose mosaic, and apple mosaic. Phytopathology 58:635-638.
7. 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.
8. George, J. A., and Davidson, T. R. 1964. Further evidence of pollen transmission of necrotic ringspot and sour cherry yellows viruses in sour cherry. Can. J. Plant Sci. 44:383-384.
9. Gilmer, R. M., and Way, R. D. 1960. Pollen transmission of necrotic ringspot and prune dwarf viruses in sour cherry. Phytopathology 50:624-625.
10. Hamilton, R. I., Leung, E., and Nichols, C. 1977. Surface contamination of pollen by plant viruses. Phytopathology 67:395-399.
11. King, J. R. 1955. The rapid collection of pollen. Proc. Am. Soc. Hortic. Sci. 66:155-157.
12. Marenaud, C., and Llager, G. 1976. Étude de la diffusion de virus de type ILAR (taches annulaires nécrotiques) dans un verger de cerisier (*Prunus avium*). Ann. Amelior. Plantes 26:357-363.
13. Mink, G. I. 1980. Identification of rugose mosaic-diseased cherry trees by enzyme-linked immunosorbent assay. Plant Dis. 64:691-694.
14. Mink, G. I. 1982. The possible role of honeybees in long distance spread of *Prunus* necrotic ringspot virus from California into Washington sweet cherry orchards. In: Plant Virus Disease Epidemiology. Federation British Plant Pathologists Blackwell Series. (In press).
15. Nyeki, J., and Vertesy, J. 1974. Effect of different ringspot viruses on the physiological and morphological properties of Montmorency sour cherry pollen. Acta Phytopathol. 9:23-29.
16. 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. 437. U.S. Government Printing Office, Washington, DC. 433 pp.

17. Nyland, G., and Lowe, S. K. 1964. The relation of cherry rugose mosaic and almond calico virus to Prunus ringspot virus. (Abstr.) Phytopathology 54:1435-1436.
18. Schimanski, H., and Schade, C. 1974. Nachweis des Nekrotischen Ringflecken-Virus der Kirsche im Pollen der Vogelkirsche (*Prunus avium* L.) und des Chlorotischen Ringflecken-Virus der Kirche im Pollen der Steinweichsel (*Prunus mahaleb* L.). Arch. Phytopathol. Pflanzenschutz 10:3-6.
19. Way, R. D., and Gilmer, R. M. 1958. Pollen transmission of necrotic ringspot virus in cherry. Plant Dis. Rep. 42:1222-1224.
20. Yang, A. F., and Hamilton, R. I. 1974. The mechanism of seed transmission of tobacco ringspot virus in soybean. Virology 62:26-37.