Transmission and Field Spread of Raspberry Bushy Dwarf Virus

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ABSTRACT

Bulger, M. A., Stace-Smith, R., and Martin, R. R. 1990. Transmission and field spread of raspberry bushy dwarf virus. Plant Dis. 74:514-517.

The field spread of raspberry bushy dwarf virus (RBDV) in red raspberry (*Rubus idaeus*) cultivar Skeena was mapped using a double-antibody sandwich form of enzyme-linked immunosorbent assay. The incidence of infected plants was 2, 16, and 84% for the third, fifth, and seventh years, respectively. Flowers of *R. parviflorus, R. spectabilis, R. procerus, R. ursinus,* and cultivated *R. idaeus* containing thrips were sampled at two locations in the Fraser Valley of British Columbia. Total numbers of thrips were relatively high for *R. parviflorus* and *R. procerus* compared to red raspberry, *R. spectabilis,* and *R. ursinus.* Results of pollen washes and serological blocking of pollen surfaces indicated that RBDV was located on and probably in the pollen grains of *R. parviflorus.* Onion thrips (*Thrips tabaci*) did not transmit RBDV when they were allowed to feed on caged *Chenopodium quinoa* plants that had been dusted with RBDV-infested *R. parviflorus* pollen.

Raspberry bushy dwarf virus (RBDV) commonly infects many cultivars of red raspberry (*Rubus idaeus* L.). Infected plants are symptomless in some cultivars, but other cultivars show foliar yellowing and significant reductions in fruit quality, fruit size, and yield (9,15,22). Murant et al (23) showed that RBDV is transmitted through raspberry seed and pollen and that healthy plants pollinated with infected and/or infested pollen may become infected. They found that deflowered plants did not become infected with the virus. Thus, the flower is believed to be the avenue for infection. No vectors have been shown to transmit RBDV in raspberry (22), and the mechanism of RBDV transmission has not been elucidated.

Recently, Sdoodee and Teakle (24) showed that *Thrips tabaci* Lindeman transmitted tobacco streak virus (TSV) by feeding on *Chenopodium amaranticolor* Coste & Reyn. in the presence of pollen from infected tomato plants. Presumably, contaminated pollen falls into feeding wounds made by *T. tabaci,* and virus is released and infects the plant.

Because RBDV shares many of the properties of TSV and other members of the ilarvirus group, we investigated the potential for thrips transmission of RBDV. Our objectives were to determine the rate of field spread and the spatial distribution of infected plants in a susceptible red raspberry cultivar, to investigate the possibility that thrips serve as vectors, and to determine whether the virus is located in or on the pollen of infected plants.

Accepted for publication 8 January 1990 (submitted for electronic processing).

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MATERIALS AND METHODS

Mapping and spatial analysis of RBDV spread in raspberry. About 900 red raspberry plants (cultivar Skeena) in a field in Abbotsford, British Columbia, that was established from supposedly virus-free planting stock in 1981 were sampled individually in the spring of 1984, 1986, and 1988. A young leaf was removed from three canes in each plant (stool). The samples were taken before flowering in all cases except the last sampling time, when the samples were taken before and shortly after flowering.

The three leaves from each stool were pooled and tested for the presence of RBDV by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Immunoglobulin G (IgG) from polyclonal antiserum was used at 1 μg/ml for coating, and IgG from a monoclonal antibody (20) conjugated with alkaline phosphatase was used at 0.1 μg/ml as the conjugate (8).

The spatial pattern of RBDV-infected plants was assessed by calculating the variance-to-mean (VTM) ratio and ordinary runs tests for all disease assessment times (17,18). For the VTM test, the field was divided into 54 quadrats (3 × 10 plants) including 90 plants from each row (starting from the left of Fig. 1). Significant clustering was indicated by a chi-square statistic of the VTM: $\chi^2 = (n-1)V/m$, where n is the number of quadrats and V and m are the variance and mean, respectively, of the number of infected plants per quadrat.

Ordinary runs analysis was done following Madden et al (18), starting down the row represented in the upper left corner of Fig. 1 and proceeding up the next row and so on until the last row. Ninety-five plants from each row (starting from the left of Fig. 1) were used in the analysis. A clustered pattern is indicated by a standardized statistic: $Z_u < -1.64$ at $P = 0.05$ or $Z_u < -2.33$ at $P = 0.01$. Ordinary runs tests were calculated for each row (95 plants per row), and the frequency of rows suggesting nonrandomness (clustering) was calculated for each assessment time.

Blocking and washing of virus-infested pollen surfaces. Pollen was collected from known RBDV-infected and healthy plants of *R. parviflorus* Nutt. Anthers were removed from the flowers and allowed to dry in a petri plate overnight at room temperature. The dried anthers were lightly rubbed onto a 200-mesh screen, and the pollen was collected and stored in sealed tubes at 4 C. We used *R. parviflorus* pollen because of the convenient high yields and easy access to the pollen and because *R. parviflorus* is a potential source of RBDV inoculum.

Samples of 5–10 mg of pollen from infected and healthy plants were placed in 1.5-ml microfuge tubes for the blocking and washing experiments. Samples were treated with 0.5 ml of IgG (0.7 mg/ml) from RBDV polyclonal antiserum, 0.5 ml of IgG (0.7 mg/ml) from TSV (isolated from red raspberry

cultivar Willamette) polyclonal antiserum (control), or 0.5 ml of phosphate-buffered saline (PBS), pH 7.4. Samples were shaken gently on a vibrator for 1 hr at room temperature and then centrifuged in an Eppendorf centrifuge at 14,000 rpm for 5 min. The pellets were washed with 0.5 ml of PBS. The centrifugation and washing were repeated three times, and the presence of the virus in each wash supernatant was assessed by DAS-ELISA. The final pollen pellet was taken up in 0.5 ml of PBS, homogenized in a glass tissue homogenizer, and centrifuged, and the presence of RBDV in the supernatant homogenate was assessed by DAS-ELISA.

Thrips transmission experiment. A colony of *T. tabaci* was maintained on *Phaseolus vulgaris* L. and *C. quinoa* Willd. at 25 C with 16 hr of light. Adults and nymphs were collected with an aspirator and immobilized with a 20-min treatment of carbon dioxide. *C. quinoa* test plants were or were not dusted with freshly harvested pollen from infected *R. parviflorus* plants, and thrips were or were not added to plants.

To show the presence of infectious virus, pollen was washed and inoculated to *C. quinoa* for each experiment. The pollen wash supernatants were rubbed onto 2-wk-old plants that had been dusted with Carborundum. The inoculated *C. quinoa* plants were tested for the presence of RBDV by ELISA after 7, 14, and 28 days.

Eight plants in each treatment were each covered with a glass cage covered with fine mesh. Five thrips (a random mixture of adults and nymphs) were added to each cage, allowed to feed for 4–5 days, then killed with Lorsban insecticide (chlorpyrifos, 0.8 g/L) (Dow Chemical Company). The plants were tested for the presence of RBDV by DAS-ELISA 7, 14, and 28 days later. The experiment was repeated twice.

Thrips survey. Flower-inhabiting thrips were surveyed in *R. parviflorus, R. ursinus* Cham. & Schlectend., *R. procerus* P. J. Müll., *R. spectabilis* Pursh, and cultivated red raspberry. Samples were taken from five to eight sites at each of two locations (Abbotsford and Vancouver, British Columbia). At weekly intervals, 50 flowers were collected per site for each species until flowering was completed.

To extract thrips (25), flowers were immersed in 1 L of tap water containing 0.1% Triton X-100 and stirred gently for 30 sec. Floating debris was skimmed off, and the thrips were allowed to settle to the bottom. The thrips mixture was passed through a large-mesh screen (aperture 2.0 mm) placed on a funnel with a fine-mesh screen (aperture 37 μm) attached to the bottom of the funnel. The thrips that were trapped on the fine-mesh screen were collected and stored in 70% ethanol.

RESULTS

Mapping RBDV spread in raspberry. The rate and pattern of spread of RBDV in Skeena red raspberry are represented in Fig. 1. The incidence of infected plants as determined by DAS-ELISA increased from 2% in the first year surveyed to 16% in the third year and 84% in the fifth year. The map of the actual locations of infected plants for each of the surveys indicates that the virus spread more rapidly in the area of the field where the density of plants infected in the first year of sampling was higher (Fig. 1).

The statistics of aggregation from ordinary runs and VTM tests are summarized in Table 1. For the first assessment time, the ordinary runs and VTM tests strongly indicated a random spatial pattern of diseased plants (mapped in Fig. 1). In contrast, both tests indicated nonrandomness or clustering of plants for the second assessment time, as shown by $Z_u = -13.83$ and $P < 0.0001$ and $\chi^2 = 124.28$ and $P < 0.0001$. For the final assessment time, the ordinary runs test again indicated clustering of infected plants ($P < 0.0001$); however, the VTM test indicated randomness ($P = 0.330$). The ordinary runs test for each row showed no clumping of infected plants for 1984 ($P = 0.05$), but the frequency of rows indicating clumping was 0.67 and 0.55 for 1986 and 1988, respectively.

Blocking and washing of virus-infested pollen surfaces. The only pollen samples that resulted in positive ELISA values in the washes were those treated with TSV-specific IgG or PBS. The RBDV-globulin washes and all healthy controls gave negative ELISA results. All homogenized RBDV-infested pollen samples tested positive, including the RBDV-globulin treatment of RBDV-positive pollen (Table 2). Infectious RBDV also was found in the surface washes of Skeena raspberry pollen (*data not shown*).

Thrips transmission study. *T. tabaci* did not transmit RBDV to *C. quinoa* in any treatment tested using *R. parviflorus* infested with infectious virus, as indicated by rub-inoculations to *C. quinoa* and ELISA data. Feeding damage was easily seen in all thrips treatments, but no damage was noted in the pollen-only treatments. Thrips were also unsuccessful in transmitting RBDV when infected *C. quinoa* and *Fragaria vesca* L. pollen were used.

Thrips survey. The number of thrips collected in each *Rubus* sp. varied greatly among sites. However, samples collected at different times from the same site varied much less than samples from different sites (*data not shown*). Numbers of thrips per 50 flowers were moderate to high for *R. procerus* and *R. parviflorus* relative to raspberry, *R. spectabilis,* and *R. ursinus.* These differences

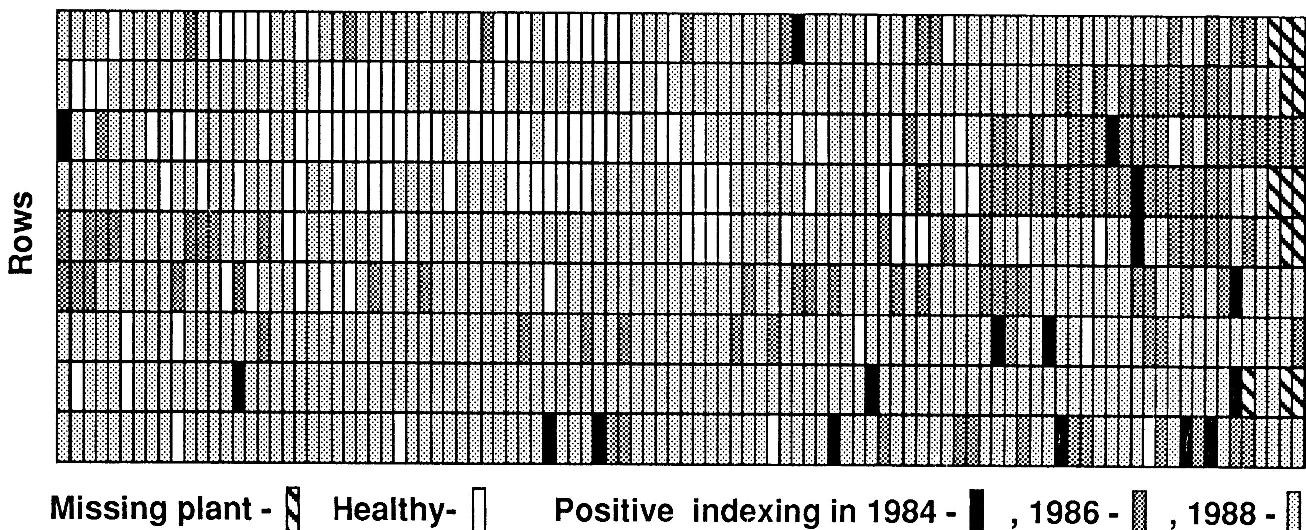

Fig. 1. The temporal and spatial distribution of red raspberry (cultivar Skeena) plants infected with raspberry bushy dwarf virus in Abbotsford, British Columbia. Each rectangle represents one group of canes spaced 0.75 m apart within rows and with 3.0 m between rows.

were consistent between the two locations (Table 3). The predominant species of thrips found in flowers were *T. madronii* Moulton and *Frankliniella occidentalis* (Pergande).

DISCUSSION

The epidemiological significance of pollenborne viruses stems from their potential for being transmitted horizontally from an infected host to a healthy host (12). Many viruses are pollenborne; however, most infect only the embryo of the developing seed, and the parent plant does not become systemically infected. Mandahar (19) stated that epidemiologically important pollenborne viruses are those that spread horizontally in the field and back-infect pollinated plants. These viruses fall into the ilarvirus and nepovirus groups. Four economically important diseases in which pollen and the flower have been linked with horizontal spread in the field are tobacco streak and raspberry bushy dwarf of *Rubus* spp. (2,6,7,23) and prunus necrotic ringspot and prune dwarf of *Prunus* spp. (3,10).

RBDV can spread rapidly in raspberry, as shown in the present study (Fig. 1) and in previous studies by Murant et al (23). The spatial pattern of spread we found shows that the area of greatest density of infected plants in 1986 and 1988 was in the area that had the greatest initial inoculum density in 1984. The ordinary runs and VTM test statistics indicated a transition from a random distribution of infected plants in 1984 to a clustered distribution (on average) in 1986. For the 1988 data, the ordinary runs test suggested a clumping ($P < 0.001$) of infected plants, whereas the VTM test suggested a random pattern ($P = 0.330$). This discrepancy may be explained by the fact that one of the requirements for the VTM test of aggregation ($\chi^2 = [n-1]V/m$) to be applicable is that the number of infected plants must be low in relation to what could be obtained (17). A random pattern of infected plants indicates that at the time of assessment the virus was not spreading from plant to plant within the field, whereas clusters of infected plants suggest that the virus is spreading from plant to plant (18).

To determine the mechanism of pollen transmission of RBDV, the location and infectivity of the pollenborne virus should be known. The results of our polyclonal blocking study show that the virus is infectious when pollen washes and homogenates are inoculated to *C. quinoa*. The virus is found on the surface of the pollen and is easily washed off. The evidence also indicates that RBDV systemically infects the pollen, because the homogenate of infected pollen treated with anti-RBDV globulin was strongly positive by ELISA and positive in bioassays. Massalski and Cooper (21) found that cherry leaf roll virus was located in and on infected pollen of cherry, birch, and walnut. Cole et al (5) found that prunus necrotic ringspot virus was located exclusively on the surface of the pollen of almond and cherry. They speculated that foraging bees carrying pollen with infectious virus could abrade flower parts and infect plants. They did not determine how long the virus remained infectious on dry pollen.

Our results indicate that *T. tabaci* does not transmit RBDV to *C. quinoa* while feeding in the presence of pollen infected and infested with virus. The additional evidence that RBDV spreads rapidly in the field (no known vector), that thrips are not common in raspberry flowers (including the mapped field), and that the virus is likely to systemically infect pollen of *Rubus* spp. lends support to the hypothesis that RBDV infects raspberry by germ tube growth into the style and/or the embryo sac and subsequent spread in the vascular system. This mechanism of spread, which some investigators consider unlikely (13), is based on observations that the zygote is isolated from maternal (nucellar) tissue, for example, in barley infected with barley stripe mosaic virus (4) and in soybeans infected with tobacco ringspot virus (26). However, it is not known whether there are vascular connections between the zygote and nucellar or other vascular tissue in *Rubus* spp.; therefore,

Table 1. Statistics of aggregation from ordinary runs tests (U) and variance-to-mean ratio estimates (VTM) for the infected plants mapped in Fig. 1

Year Statistic	Observed	Expected	Standard deviation	Mean	Z_u[a]	χ^2[b]	P[c]
1984							
U	35.00	34.32	0.98		1.20		0.547
VTM	1.02	1.00	0.70	0.48		26.54	0.434
1986							
U	134.00	221.47	6.29		−13.83		<0.0001
VTM	4.78	1.00	4.29	3.85		124.28	<0.0001
1988							
U	133.00	227.00	9.63		−9.71		<0.0001
VTM	1.12	1.00	5.30	25.2		28.60	0.330

[a] Standardized variable; large negative values indicate clustering.
[b] Chi-square statistic; significant clustering is indicated when $\chi^2 > 71.0$ ($P = 0.05$; 53 df).
[c] Significance level.

Table 2. Reaction of pollen from *Rubus parviflorus* infected with raspberry bushy dwarf virus (RBDV) in enzyme-linked immunosorbent assays (ELISA) following pretreatment with anti-RBDV globulin or anti-tobacco streak virus (TSV) globulin

		ELISA absorbance values[a]					
Virus	Globulin treatment	Wash 1[b]	Wash 2	Wash 3	Wash 4	Homogenate[c]	Bioassay[d]
None	None	0.000	0.000	0.000	0.000	0.000	−
	TSV	0.000	0.000	0.000	0.000	0.000	−
	RBDV	0.000	0.000	0.000	0.000	0.000	−
RBDV	None	2.305	1.750	0.536	0.156	2.233	+
	TSV	2.744	2.476	1.304	0.588	1.995	+
	RBDV	0.000	0.000	0.000	0.000	2.062	+

[a] Absorbance at 405 nm; average of two replications; threshold value was 0.1 unit.
[b] Each wash was with 0.5 ml of phosphate-buffered saline (PBS).
[c] Homogenized with glass homogenizer in 0.5 ml of PBS and centrifuged at 10,000 *g*.
[d] Inoculation of *Chenopodium quinoa* with pollen homogenate.

Table 3. Survey of thrips inhabiting flowers of *Rubus* spp. in two locations in British Columbia

	Vancouver		Abbotsford	
Species	Total thrips (no.)	Average[a] (no.)	Total thrips (no.)	Average[a] (no.)
R. parviflorus	6,465	274.6 (280.7)	1,071	53.5 (48.7)
R. procerus	971	64.7 (52.4)	467	46.7 (21.1)
R. ursinus	94	4.1 (5.5)	256	12.8 (13.7)
R. spectabilis	20	2.8 (3.0)	...[b]	...
R. idaeus	...	...	58	2.2 (2.2)

[a] Average number of thrips per 50 flowers from each of five to eight sites at each location (standard deviations in parentheses).
[b] Not tested.

the suggested mechanism of RBDV transmission cannot be ruled out.

Murant et al (23) showed that RBDV could be transmitted to Lloyd George raspberry plants when unopened flowers were emasculated, brushed with infected pollen, and enclosed in cellophane bags until fruit formed. In this case, the wounds for infection could have been caused by flower-inhabiting thrips, the emasculation process, or the method of brushing pollen onto the flowers.

Our thrips survey indicated relatively few thrips (2.2 per 50 flowers) in cultivated raspberry flowers. However, many thrips were found in *R. parviflorus* flowers. We observed that honeybees and bumblebees were very common in *Rubus* spp. in the field during flowering. These insects carry large quantities of pollen and are known to damage flower parts while foraging in the flower (11). Thrips have been shown to carry comparatively little pollen (16). Considering the feeding habits of bees and thrips, we speculate that bees are more likely to play a role in transmitting RBDV than are thrips.

In British Columbia *R. parviflorus* is found wherever red raspberries are grown. Credi et al (8) found that *R. parviflorus* was commonly infected by RBDV even in areas where red raspberries were not grown. *R. parviflorus* is commonly found growing at the edges of cultivated red raspberry fields (8). Because RBDV is pollen-transmitted, and because *R. parviflorus* pollen is cross-compatible with red raspberry (14) and is commonly found in proximity to raspberry, and because the flowering periods of red raspberry and *R. parviflorus* overlap, it is possible that *R. parviflorus* could be a source of inoculum. Converse (6) found that 14% of boysenberry plants tested positive for RBDV in an area in Oregon 3 miles from the nearest cultivated *Rubus* spp. This result may be explained by the presence of wild *Rubus* spp. that are infected with RBDV.

We have not elucidated the mechanism of RBDV transmission. However, the ability of RBDV to cause significant yield losses (9,15,22) and our data showing rapid field spread indicate RBDV's potential as a serious pathogen in North America. Introduction of the resistance-breaking strain of RBDV (1) to North America would make the virus an even greater threat to red raspberry production. Our data also point to some interesting areas for further research, such as the role of bees in the spread of RBDV in raspberry and the role *R. parviflorus* plays as a source of inoculum. Pollen transmission of RBDV could be investigated in detail using gold-labeled antibodies and electron microscopy to follow the movement of the virus from pollination to postfertilization of healthy flowers.

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