Transmission and the Role of Honeybees in Field Spread of Blueberry Shock Ilarvirus, a Pollen-Borne Virus of Highbush Blueberry

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ABSTRACT

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The rapid and random spread of blueberry shock ilarvirus (BIShV) in commercial plantings suggested that insects played a role in transmission. Transmission from infected field plants to trap plants only occurred during bloom, indicating that flowers were the avenue for infection and flower-visiting insects including pollinators were involved. Trap plants readily became infected during bloom when a honeybee hive was included in cages with diseased field plants and trap plants. There was limited transmission in cages without a hive and no transmission when healthy field bushes were caged with trap plants either with or without honeybees. Infective

pollen survived within the hive for 1 but not 2 weeks. Western flower thrips did not transmit BIShV when they were allowed to feed on caged flowering trap plants in the presence of a source of infected pollen. Results of pollen washes, cell disruption, and electron microscopy show that particles of BIShV were on, in, and between the cells of the pollen tetrad. Pollen from infected plants germinated as readily as pollen from healthy plants. BIShV was seedborne, but at a low level. All 42 cultivars tested were susceptible to BIShV when inoculated by grafting. The primary mechanism of transmission appears to be the transfer of BIShV-contaminated pollen by honeybees from flowers on infected plants to flowers on healthy plants.

Additional keywords: *Apis mellifera*, cultivar reaction.

Necrotic shock disease of highbush blueberry (*Vaccinium corymbosum* L.), caused by blueberry shock ilarvirus (BIShV, ITCV decimal code 10.0.2.02.02), was originally observed on several cultivars in commercial fields in Washington and Oregon (21). The disease has not been detected in neighboring British Columbia or in other production areas in North America. Symptomatic bushes were initially thought to be infected with blueberry scorch carlavirus (BISV) (22) because both viruses cause blighting of both flowers and newly emerging leaves (20). Blighted flowers and leaves on bushes infected with BIShV drop by early summer, whereas wilted flowers blighted by BISV remain attached. Moreover, plants infected with BIShV produce a second flush of leaf growth, so that, by the end of the season, plants look nearly normal although they produce little fruit. In contrast, there is no second flush of growth on bushes infected with BISV. Necrotic shock symptoms usually appear for only 1 year, although they have been recorded for up to three successive seasons. Infected plants that have gone through the shock phase of the disease look normal and produce fruit, but they remain infected and serve as a source of inoculum.

A number of ilarviruses including prunus necrotic ringspot virus (PNRSV) (8,13,14) and prune dwarf virus (PDV) (17) are pollen borne, as is the blueberry leaf mottle nepovirus (BLMV) (5). Thrips have been shown to transmit tobacco streak ilarvirus (16,33) and pollen-borne viruses from other virus groups (18,28). Honeybees mediate the transmission of PNRSV (13,26), PDV (14), and BLMV (6) by moving virus-contaminated pollen from the flowers of diseased plants to flowers of healthy plants. Both PNRSV and BLMV survive in honeybee hives, which can account for long-distance spread when hives are transported from one production area to another (1,26).

Our objectives were several-fold: (i) to determine if BIShV is pollen borne, (ii) to document the natural rate of spread in commercial blueberry fields, (iii) to assess the role of honeybees and thrips in transmission, and (iv) to test the reaction of commercial cultivars to BIShV. A preliminary report covering a portion of this work has been published (2).

MATERIALS AND METHODS

Enzyme-linked immunosorbent assay (ELISA) testing. Double-antibody sandwich (DAS)-ELISA was used to detect BIShV in blueberry plants (7). All reagents were used at 100 μ l per well in flat-bottom microtiter plates (Corning Inc., Corning, NY) except the blocking step, which was at 200 μ l per well. Coating antibody was diluted in 0.01 M carbonate buffer, pH 9.6. Plates were coated for 4 to 6 h at room temperature. They were then blocked with phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 and 0.1% nonfat dried milk (PBS-Tween-Smp) and incubated at room temperature for 1 h. Leaf samples were collected into Plexiglas trays with 96 wells in an 8 $\times$ 12 format. Leaf samples were homogenized (~1:15, wt/vol) in 0.05 M borate buffer, pH 8.0, containing 0.05% Tween-20, 0.5% nicotine, 2% polyvinylpyrrolidone (molecular weight = 44,000), and 0.1% nonfat dried milk. The extracted leaf sap was added, and the plates were incubated overnight at 4°C. Plates were then washed three times with 0.5 $\times$ PBS followed by the addition of BIShV antibody conjugated to alkaline phosphatase and diluted 1:1,000 in PBS-Tween-Smp. Plates were incubated for 2 h at 28°C and washed as above. Substrate (*p*-nitrophenylphosphate) at 0.5 mg/ml in 10% diethanolamine, pH 9.8, was added, and plates were incubated at room temperature. Absorbance of each well at 405 nm (A_{405}) was read with an ELISA plate reader (MR5000; Dynatech Laboratories, Inc., Chantilly, VA, or Molecular Devices, Sunnyvale, CA). All samples were tested in duplicate wells. Absorbance values were considered positive if the A_{405} values were greater than five times the values obtained for healthy controls.

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Washing of pollen from BISHV-infected plants. Pollen was collected from known diseased and healthy 'Bluetta' blueberry. Pollen was collected from field bushes during bloom, but for a second experiment, branches from dormant field bushes were collected and flowers were forced in a greenhouse. In both cases, pollen was collected by tapping open flowers over a glass petri dish. Collected pollen was stored at 4°C (no more than 3 days) until tested. Pollen samples (5 mg) were placed in 1.5-ml Eppendorf microfuge tubes, washed with 0.25 ml of PBS by gentle shaking for 30 min at room temperature, and then centrifuged in an Eppendorf centrifuge at 14,000 rpm (16,000 × g) for 1 min. The pellets were resuspended in 0.25 ml of PBS, and the washing and centrifugation was repeated five times. Each supernatant was assayed for the presence of BISHV by DAS-ELISA. The final pellet was disrupted in 0.25 ml of PBS by homogenization in a glass tissue homogenizer and centrifuged. This final supernatant was likewise assayed for BISHV by DAS-ELISA.

Electron microscopy. Thin-section electron microscopy was conducted as previously described (21). Pollen was fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.0, post-stained in osmium tetroxide, destained in ethanol, and embedded in Epon 812. Sections were stained in lead acetate and uranyl acetate. Virus particles were identified using gold-labeled protein A in combination with antibodies to the virus.

Trap plants. For several experiments, potted healthy 'Berkeley' blueberry plants with numerous fruit buds were used as trap plants. Plants (45 to 75 cm tall) were purchased from a nursery and tested by DAS-ELISA prior to use. Trap plants were maintained at a site several kilometers away from other blueberry plants both before and after being used in field experiments. In early fall, these plants were sprayed with malathion insecticide and then overwintered in a screenhouse. The following spring, trap plants were monitored for disease symptoms and new emerging leaves were tested for BISHV.

Time of BISHV transmission. Trap plants were exposed to naturally infected field bushes at different times during the crop season to determine when infection takes place. Trap plants were placed between two diseased field bushes at each of 10 locations in a commercial field near La Center, WA. More than 90% of the field bushes were infected. The potted plants were fastened to steel fence posts so the canopy of the trap plant was the same height (approximately 1.4 m) as the midpoint of the fruiting region of the mature field bushes. Sets of trap plants were maintained in the field from just prior to bloom in mid-April to early leaf drop in October. From April through June, trap plants were exchanged for a fresh set of trap plants every week. Beginning in July, they were set out for 1-week periods every other week. Between mid-April and early June, trap plants were set out in pairs; flowers were removed from one plant of each pair just prior to being placed in the field. This field experiment was repeated during three cropping seasons; during the third season, trapping was discontinued in early June. Trap plants were treated with an insecticide, overwintered in a screenhouse, and tested for BISHV as described above.

Role of honeybees in transmission. Field trials were conducted on commercial farms in Whatcom and Clark counties, WA, during 1991 and 1992. Two fields per county were selected, one with naturally infected and the other with healthy bushes. Fields in each county were more than 20 km apart. Trellises were constructed and covered with greenhouse shade cloth (2.1 × 2.1-mm openings) to cage field bushes (5 to 10 bushes per cage depending on the site; four cages per farm). Approximately 20 trap plants were set on the ground beneath field bushes in each cage. At the Clark County locations, flowers were removed from three trap plants per cage immediately before they were placed in cages. Small (nuclear) honeybee (*Apis mellifera* L.) hives were placed in two cages (one hive per cage) at each location. Each hive consisted of (i) one Langstroth box, (ii) nine frames, (iii) an egg-laying queen, (iv) 2 to 3 lb of workers, and (v) 3 to 4 lb of honey. For the 1991 tests, an external feeder with sucrose solution was placed in the entrance of

hives, but for 1992, an internal feeder containing sucrose solution and pollen cake replaced one of the frames. A pan of water was maintained in all cages with hives. Hives were set on boards between field bushes with the entrance facing south. Treatments were replicated twice in each field. Hives and trap plants were kept in the cages during bloom. Hives were then moved (within 5 h) to cages that contained only fresh flowering trap plants ('Bluetta') to determine in-hive survival of infective pollen. After 7 days, the trap plants were replaced with a new set of flowering trap plants ('Northland'). The second set was also removed after 7 days. These two cultivars were selected because they flowered later than 'Berkeley' and both were susceptible to BISHV.

Role of thrips in transmission. Flowering branches were cut from healthy and diseased field bushes. The cut ends of branches were placed immediately in water. Trap plants and the branches were fumigated with sulfotep insecticide (Plant Fume 103; Plant Products Corp., Vero Beach, FL) for 15 h. Following fumigation, each trap plant was covered with a fine-mesh bag (130-µm openings) to exclude insects and contain thrips. Flowering branches were placed in some of the bags. At the same time, flower stalks of mustard plants naturally infested with western flower thrips (*Frankliniella occidentalis*) were placed in some of the bags. Controls consisted of bags containing no thrips and bags without a source of BISHV-infected pollen. Each treatment was replicated three to five times. Fresh thrips-infested mustard flower stalks were placed in the appropriate bags after 14 days. After petals had dropped from flowers of both the blueberry trap plants and branches, the mustard flower stalks and blueberry branches were removed. Trap plants remained bagged until fall, at which time the bags were removed and the plants were sprayed with an insecticide. Trap plants were then placed in the screenhouse and tested for BISHV the following spring.

Viability of pollen. Pollen was collected from newly opened flowers of both healthy and naturally diseased field bushes. In one test, pollen was obtained from graft-inoculated potted plants. In the latter test, pollen from self-grafted plants served as the control. Fresh pollen was seeded onto the surface of 9% sucrose agar (2.5% agar) by clasping the pedicel of each open flower with forceps, inverting the flower over the medium, and tapping the forceps with a pencil to dislodge pollen. Two to four flowers were used to seed each petri dish. In some tests, sucrose agar was amended with 100 µg of boric acid (H₃BO₃) per ml (ppm), 300 ppm of calcium nitrate (Ca[NO₃]₂·4H₂O), and 200 ppm of magnesium sulfate (MgSO₄·7H₂O). Seeded plates were incubated in darkness at 25°C for 9 to 18 h depending on the test. The surface of the agar was sprayed with phenolic rose bengal to kill and stain the pollen and pollen tubes. Blueberry pollen (microspore) is shed as tetrads composed of four pollen grains, and each grain is capable of germinating. A pollen tetrad was recorded as germinated when one grain produced a pollen tube that was longer than the diameter of the tetrad. A minimum of 50 pollen tetrads was observed microscopically on each of three to four replicate plates to determine percent tetrad and grain germination.

Detection of BISHV antigens in blueberry seedlings. Ripe berries were picked by hand from healthy and diseased field bushes. Seeds were extracted following a procedure routinely used by blueberry breeders (30). Air-dried seeds were stored in screw-capped vials at 3 to 4°C until used. For use, seeds were soaked overnight at room temperature in an aqueous solution of gibberellic acid (360 µg/ml). Treated seeds, after being rinsed briefly in tap water, were sown on the surface of a peat/sand mixture (1:1, vol/vol) in shallow plastic trays and then covered with a thin layer of sphagnum peat. Initially, trays were held under intermittent mist in a greenhouse, but were later placed under incandescent and fluorescent lights without mist. Emerging seedlings were transplanted into peat/sand mixture in plastic seedling trays with one seedling per compartment. When plants were large enough (after 1 to 3 years of growth in the greenhouse), leaves were collected and assayed for BISHV by DAS-ELISA.

Cultivar reaction to BISHV. Potted plants (2 to 3 years old) of 42 blueberry cultivars were inoculated with BISHV by grafting. Plants were obtained from commercial nurseries and tested for BISHV before use. Scion wood was cut from current year's growth of naturally infected field bushes. Two bottle grafts were made per plant, each on a separate main stem. Control plants were self-grafted. There were four plants per treatment for each cultivar. Plants were grafted during the summer and kept in the greenhouse until fall, when they were moved to the screenhouse. Plants were observed for symptoms and leaves were tested for BISHV by DAS-ELISA the following spring and for up to 3 years thereafter.

RESULTS

Rate and pattern of BISHV spread in two commercial fields. Blocks of 'Berkeley' bushes in two commercial fields were monitored for the natural spread of BISHV over a period of 5 years (Table 1). Diseased bushes were identified initially by symptoms and later by DAS-ELISA. The rate of virus spread in both fields gave a sigmoidal curve. The virus spread more rapidly at the Washington than at the Oregon site (85 versus 44% of the bushes infected after 3 years, respectively). The pattern of within-field spread was followed in the block at the Washington site. The disease first appeared in 1993 in this block of 528 bushes (Fig. 1). The initial 14 infected bushes were scattered throughout the block. In 1994, the 70 newly infected bushes were evenly distributed in the block except in the northwest quadrant, where 38 of the newly infected bushes were located. All but 12 of those 38 plants were next to another diseased bush. By 1995, 85% of the bushes in the entire block tested positive for BISHV and were evenly distributed among the quadrants. Only nine bushes in the block remained healthy in 1997, with at least two bushes in each quadrant.

TABLE 1. Rate of spread of blueberry shock ilarvirus in naturally infected blocks of 'Berkeley' blueberry bushes on two commercial farms

Year	Infected (%) ^a	
	Albany	Deming
0	0 (5) ^b	0 (4 and 5) ^b
1	0.9	2.7
2	7.0	15.9
3	44.0	84.9
4	77.9	92.9
5	... ^c	98.3

^a Total of 575 and 528 bushes at Albany, OR, and Deming, WA, respectively.
^b Number in parentheses = age of planting at year 0.
^c ... = Planting taken out.

Nearly all of the bushes in the block of 528 'Berkeley' bushes (Fig. 1) became infected before there was significant spread into the adjacent block of 'Earlblue' bushes immediately to the east. This same nonrandom pattern of movement between different cultivars was also noted for adjacent blocks of 'Bluetta' and 'Blueray' bushes on this farm. Over 70% of 'Bluetta' bushes tested positive for BISHV before infected bushes were detected in the 'Blueray' block. On a nearby farm, few 'Jersey' bushes showed symptoms or tested positive for BISHV until most 'Bluetta' bushes in the block immediately to the north were infected.

Time of transmission. Field bushes bloomed for 5 weeks each spring. In 1990, trap plants became infected during 3 of the 5 weeks of bloom (Table 2). Five of the trap plants deblossomed before going to the field also became infected. During 1 of the 2 weeks when deblossomed plants became infected, a few flowers on two trap plants were overlooked. None of the trap plants became infected in 1991, even during bloom. In 1993, transmission occurred during 2 weeks of the bloom period and all of the trap plants without flowers remained healthy.

Role of honeybees in the transmission of BISHV. In Whatcom County, 67% of the trap plants in cages with a honeybee hive and diseased field bushes became infected (Table 3). There was only a low level of transmission (17%) to trap plants in cages with diseased field bushes but no hive. Trap plants caged with virus-free field bushes remained healthy, regardless of whether or not a hive was in the cage. There was no transmission to any trap plant in Clark County fields in 1991, regardless of treatment (Table 3). The lack of transmission was consistent with the results of the time of infection study conducted in the same field that year. When the honeybee study was repeated for the second year, transmission occurred in fields with diseased bushes in both counties (Table 4). As before, the level of infection was highest when trap plants were

TABLE 2. Transmission of blueberry shock ilarvirus to healthy trap plants placed next to diseased field bushes during bloom

Year	Week during bloom	Transmission, no. trap plants infected/no. exposed	
		With flowers	Flowers removed
1990	17 April – 24 April	0/10	0/10
	24 April – 1 May	0/10	0/10
	1 May – 8 May	5/10	3/10
	8 May – 15 May	2/10	2/10
	15 May – 22 May	5/10	0/10
1993	22 April – 28 Apr	0/10	0/10
	28 April – 5 May	1/10	0/10
	5 May – 12 May	3/10	0/10
	12 May – 19 May	0/10	0/10
	19 May – 26 May	0/10	0/10

Fig. 1. The spatial and temporal distribution of 'Berkeley' blueberry bushes infected with blueberry shock ilarvirus in a commercial planting in Whatcom County, WA. Each rectangle represents a single bush. Bushes are set 1.5 m apart in rows with 3.0 m between rows.

placed in cages containing both diseased field bushes and a honeybee hive. There was no noticeable reduction in fruit set or berry size on field bushes in cages without hives. Only one trap plant that had its flowers removed became infected and that plant was in a cage with diseased field bushes in Clark County. Only one 'Bluetta' trap plant became infected, when exposed for 1 week to bees from hives that came directly from cages containing diseased field plants. None of the 'Northland' trap plants, exposed to bees during the second week, became infected, suggesting that BISHV survives in the hive for up to 1 week. In both years of the honeybee study, weather during the 2 weeks following exposure of hives to field bushes was favorable for honeybee activity, and bees were observed foraging on the 'Bluetta' and 'Northland' trap plants. Inexplicably, two trap plants (one 'Bluetta' and one 'Northland') caged with hives that originated in healthy control fields tested DAS-ELISA-positive for BISHV.

Transmission by thrips. Only one of the trap plants in this experiment tested positive for BISHV, and it came from a mesh bag containing BISHV-infected donor pollen but no thrips. None of the trap plants in cages with thrips became infected. Moreover, thrips were not detected in blueberry flowers at two field sites where BISHV was actively spreading and a majority of the bushes tested positive for BISHV.

Association of BISHV particles and pollen. Virus antigen was detected in the first buffer washing, and absorbance readings decreased in subsequent washings (Table 5), suggesting that virus particles were present on the surface of pollen tetrads or that some of the pollen grains ruptured during the first several washes. Homogenizing the pollen tetrads after the sixth washing released additional virus antigen. Virus particles were found between and within individual pollen grains (Fig. 2).

Viability of pollen. BISHV had no impact on either tetrad or grain germination for pollen from 14 of the cultivars inoculated by grafting (Bluecrop, Bluejay, Bluegray, Bluetta, Collins, Darrow, Duke,

Earliblue, Elliott, Jersey, Patriot, Spartan, Toro, and 1613-A; data not shown). There was a slight increase (32 to 45%; $P < 5\%$) in pollen grain germination for diseased 'Bluejay' plants and a decrease (93 to 62%; $P < 1\%$) in pollen tetrad germination for 'Patriot' plants when compared with pollen from healthy plants of the two cultivars. Pollen from naturally infected 'Berkeley' field bushes germinated as well as that from healthy field bushes. Pollen tubes arising from infected pollen were as long as those produced by healthy pollen.

Detection of BISHV antigen in seedlings. None of the seedlings flowered or exhibited any symptom of the shock disease before being tested for BISHV. BISHV was detected in only 3 of 250 (1.2%) plants grown from seed originating from diseased plants. All of the seedlings arising from seed from healthy bushes tested negative for the virus.

Cultivar reaction to BISHV. None of the self-grafted plants showed shock symptoms or tested positive for BISHV, regardless of cultivar. Conversely, plants of all 42 cultivars were susceptible to shock

TABLE 5. Reaction of pollen from 'Bluetta' bushes infected with blueberry shock ilarvirus (BISHV) in double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) following successive washes with phosphate-buffered saline and homogenization of cells

Pollen source	ELISA absorbance values ^a				
	Wash 1 ^b	Wash 2	Wash 3	Wash 6	Homogenized pollen
BISHV-infected 1 ^c	2.120	2.030	1.131	0.127	2.434
BISHV-infected 2 ^d	2.346	1.985	1.247	0.056	2.158
Healthy	0.048	0.036	0.043	0.042	0.039

^a A₄₀₅; average of duplicate samples each tested in duplicate wells.

^b Each wash was with 0.25 ml of phosphate-buffered saline.

^c Pollen collected from 'Bluetta' field bushes infected with BISHV.

^d Pollen collected from flowers on branches taken from 'Bluetta' field bushes infected with BISHV that were forced in the greenhouse.

TABLE 3. Transmission of blueberry shock ilarvirus to healthy trap plants in cages with healthy or diseased field bushes by foraging honeybees, 1991

Location ^a	Treatment	No. trap plants infected/no. tested			
		Trap plants from cages		Longevity in hive ^b	
		Replication 1	Replication 2	Week 1	Week 2
Clark Co.	Virus-free source bushes without hive in cage	0/16 (0/3) ^c	0/14 (0/3)		
	Virus-free source bushes with hive in cage	0/15 (0/3)	0/16 (0/3)	0/15	0/15
	Diseased source bushes without hive in cage	0/17 (0/3)	0/17 (0/3)		
	Diseased source bushes with hive in cage	0/16 (0/3)	0/15 (0/3)	1/15	0/15
Whatcom Co.	Virus-free source bushes without hive in cage	0/21	0/19		
	Virus-free source bushes with hive in cage	0/18	0/20	NT ^d	NT
	Diseased source bushes without hive in cage	4/21	3/20		
	Diseased source bushes with hive in cage	13/21	15/21	0/30	0/30

^a Trap plants in cages with source bushes for 21 days (23 April to 14 May) in Clark County and for 18 days (30 April to 18 May) in Whatcom County.

^b Flowering 'Bluetta' and 'Northland' trap plants were used during weeks 1 and 2, respectively.

^c Number in parentheses = trap plants with flowers removed at the time they were placed in cages with source bushes.

^d NT = not tested.

TABLE 4. Transmission of blueberry shock ilarvirus to healthy trap plants in cages with healthy or diseased field plants by foraging honeybees, 1992

Location ^a	Treatment	No. trap plants infected/no. tested			
		Trap plants from cages		Longevity in hive ^b	
		Replication 1	Replication 2	Week 1	Week 2
Clark Co.	Virus-free source bushes without hive in cage	0/17 (0/3) ^c	0/17 (0/3)		
	Virus-free source bushes with hive in cage	0/17 (0/3)	0/16 (0/3)	0/13	0/15
	Diseased source bushes without hive in cage	0/17 (0/3)	0/17 (0/3)		
	Diseased source bushes with hive in cage	7/17 (1/3)	9/17 (0/3)	1/15	0/15
Whatcom Co.	Virus-free source bushes without hive in cage	0/18	0/16		
	Virus-free source bushes with hive in cage	0/19	0/19	0/15	0/13
	Diseased source bushes without hive in cage	2/22	1/23		
	Diseased source bushes with hive in cage	17/22	13/23	0/18	0/11

^a Trap plants in cages with source bushes for 28 days (2 to 30 April in Clark County and 7 April to 5 May in Whatcom County).

^b Flowering 'Bluetta' and 'Northland' trap plants were used during weeks 1 and 2, respectively.

^c Numbers in parentheses = trap plants with flowers removed at the time they were placed in cages with source bushes.

disease when grafted with scion wood from naturally diseased field bushes (Table 6). The overall transmission rate was >90%. When transmission did not occur, the failure was usually associated with poor graft unions. With few exceptions, shock symptoms were limited to the year after inoculation. Plants exhibited only minor blighting or were asymptomatic in subsequent years, although they still tested positive for BISHV. The exceptions were ‘Nelson’, ‘O’Neal’, and ‘Blueray’. For ‘Nelson’ and ‘O’Neal’, only one and three plants, respectively, were positive for BISHV 1 year after grafting. By the second season, all four plants of both cultivars were positive. For ‘Blueray’, none of the plants grafted with diseased scion wood tested positive for BISHV or exhibited shock symptoms the year following inoculation. During the second year, three plants tested positive and were symptomatic that year. The fourth plant exhibited shock symptoms and was BISHV-positive in the third year; it was the only plant expressing symptoms that year.

DISCUSSION

The results presented in this report demonstrate that (i) BISHV is pollen borne, (ii) the virus spreads rapidly in commercial plantings, (iii) infection takes place only during bloom, and (iv) honeybees (not thrips) are involved in transmission.

BISHV particles were found on, in, and between the cells of blueberry pollen. Particles of raspberry bushy dwarf virus (RBDV) (3) and BLMV (5) were also on and in the cells of pollen of their respective hosts. All three pollen-borne viruses are transmitted horizontally from infected host to healthy host; this form of transmission is epidemiologically important (12). Once BISHV was introduced into a block of bushes of a single cultivar, the number of diseased bushes progressed geometrically from year to year, and then the rate slowed after a majority of the bushes were infected. This same pattern of rapid spread was found for PNRSV in a block of ‘Montmorency’ sour cherry (9), for BLMV in a block of ‘Jersey’ highbush blueberry (6), and for RBDV in a block of ‘Skeena’ red raspberry (3). The authors of sour cherry and blueberry reports attributed the decline in the transmission rates to the diseases reaching a stationary phase with only a few remaining sites for new infections. Both PNRSV (31) and BLMV (5) markedly reduced pollen germination, and this may have accounted for the change in infection rate if viable pollen is required for transmission. The decline in the infection rate for BISHV apparently is not linked to pollen viability, because the virus does not reduce pollen germination.

The random pattern of spread for BISHV within a block of a single cultivar did not fit the reported foraging pattern for the honeybee (34). Honeybees tend to work a particular area and return to the same area if floral rewards of either nectar or pollen are adequate. Individual bees return to the same part of the field, group of

bushes, or even the same bush on subsequent trips, often for several days. The area worked by an individual bee may only be several square meters (15). If an infected bush were in the area, one would expect BISHV to spread to the neighboring bushes. This pattern of localized spread from an established infection foci was only noticed in the northwest quadrant of the test block in 1993. This pattern was similar to that reported for other pollen-borne viruses. Mink (26) found cherry trees newly infected with the cherry rugose strain of PNRSV around sites of initially infected trees. Similarly, Childress and Ramsdell (6) reported blueberry bushes with new infections of BLMV around established infection centers and new infection foci. They concluded that a single foraging honeybee on an infected bush had the potential to spread virus-contaminated pollen to several bushes in a single season.

Later, Boylen-Pett et al. (1) reported that (i) blueberry pollen was readily transferred from bee to bee within the hive, (ii) BLMV-contaminated pollen remained infectious for several days within the hive, and (iii) some bees from one colony visit other colonies or apiaries (“drifting”) as far away as 600 m. Sandoval et al. (32) recovered BLMV-infected pollen from pollen traps placed on hives up to 2.3 km from a source field. Based on this information and our own data showing that BISHV survives in the hive for up to 1 week, the likelihood of a bee foraging on healthy bushes and returning to those same plants with virus-contaminated pollen is increased. As the proportion of diseased plants in a field increases, so does the probability that infected pollen is carried to flowers on healthy bushes; either by the honeybee collecting the contaminated pollen or another foraging worker who picks up virus-contaminated pollen in the hive (in either its own or another hive). Because of in-hive transfer of pollen and drifting, every foraging honeybee has the potential to transmit BISHV, even if it never visits an infected bush. While honeybees forage in a nonrandom fashion, factors such as in-hive survival and transfer of pollen mask the recognized pattern of honeybee foraging and result in random distribution of the virus.

While the pattern of spread through a block planted to a single cultivar appeared to be random, our observations found that spread between adjacent blocks planted to different cultivars was not random. In the latter case, the pattern may be linked to the time of flowering and the relative attractiveness of flowers of different cultivars. ‘Bluetta’ and ‘Earliblue’ begin to flower before ‘Berkeley’, ‘Blueray’, and ‘Jersey’, but under climatic conditions in the Pacific Northwest there is considerable overlap in the bloom periods of all five cultivars. McGregor (25) suggested that, for cross-pollination between cultivars, the flowers of each need to be equally attrac-

Fig. 2. Electron micrograph showing blueberry shock ilarvirus (BISHV) particles within membrane-bound vesicles of ‘Bluetta’ blueberry pollen. A, Normal antiserum and B, BISHV-specific antiserum combined with gold-labeled protein A (bar represents 300 nm).

TABLE 6. Blueberry cultivars susceptible to blueberry shock ilarvirus (BISHV) by graft transmission^a

Plant	Cultivar	Plant	Cultivar
Northern highbush	Berkeley*	N. highbush (cont.)	Nui
	Bluechip		Olympia
	Bluecrop*		Patriot*
	Bluegold		Pemberton*
	Bluejay*		Puru
	Bluehaven*		Rancocas*
	Blueray*		Reka
	Bluetta*		Rubel*
	Burlington		Sierra
	Collins*		Spartan*
	Coville		Sunrise
	Darrow*		Toro*
	Dixi*		Washington
	Duke*		Weymouth*
	Earliblue*		1613-A (Hardiblue)*
	Elliott*		N51G (Eberhardt)
	Friendship	Southern highbush	O’Neal
	Herbert*		Northblue
	Jersey*		Northcountry
	Meador*		Northland*
	Nelson		Northsky

^a * = Cultivars in commercial plantings found to be naturally infected with BISHV.

tive so bees will forage indiscriminately between the two. The pattern of spread between 'Berkeley' and 'Earliblue' may be due in part to the documented unattractiveness of 'Earliblue' flowers (10,24). Honeybees may start working the slightly earlier-flowering 'Earliblue', switch to the more attractive 'Berkeley' flowers when its flowers begin to open, and not return to 'Earliblue'. If BISHV had been first introduced into 'Earliblue', the pattern of spread into 'Berkeley' may have been quite different. It is also possible that the pattern would have been the same, because BISHV first moved through the early flowering 'Bluetta' blocks before moving into the adjacent blocks of 'Blueray' and later-flowering 'Jersey' bushes.

Transmission of BISHV to trap plants from hives, after the hives were removed from a source of the virus, was limited to 1 week. In-hive survival may be responsible for long-distance spread of BLMV (1) and PNRSV (26). For PNRSV, pollen remained infective in hives during the time they were transported from almond fields in California to cherry orchards in Washington. To date, BISHV has been detected only in commercial highbush blueberry fields in Washington and Oregon. The number of commercial fields in these two states where the virus has been detected has increased since 1987 (19,23). Some fields in Washington are located just a few kilometers south of the Canadian border, yet BISHV has not been detected in commercial fields immediately north of the border in the Fraser River Valley of British Columbia (19,23). A Canadian quarantine prevents U.S. honeybee hives from moving across the international boundary, and this may explain why blueberry fields in southwest British Columbia remain free of the disease.

We found that transmission of BISHV only occurs during bloom. Transmission appeared to be limited to those weeks during bloom when the weather was most favorable for foraging by honeybees (34). The lack of transmission at the Clark County site in 1992 was most likely due to a combination of poor synchronization of bloom between field bushes and trap plants and unfavorable weather conditions for honeybee foraging. Prunus necrotic ringspot virus transmission to cherry trees was also limited to the bloom period (13). Honeybees are highly efficient vectors of BISHV, and they have ample opportunity to transfer pollen from flowers on infected plants to those on healthy plants. In contrast, the efficiency of other flower-visiting insects may be poor, accounting for the low level of transmission to trap plants in cages without a hive. 'Montmorency' cherry trees remained healthy as long as flowers were removed before they opened (4). Removal of flowers from red raspberry prevented transmission of RBDV (3). Flowers must be present for high levels of BISHV transmission. Removing flowers from trap plants prevented transmission of BISHV in time of infection trials conducted in 1993. In the 1991 trials, however, 5 of the 50 trap plants without flowers became infected. A few flowers developed on two of the plants during their 1-week exposure because they were missed when blossoms were removed. Transmission to the remaining three plants and to one trap plant without flowers from a cage containing infected field plants suggests that a low level of transmission might occur in the absence of flowers.

Mink (27) hypothesized that honeybees move ilarvirus-infected pollen, and possibly thrips covered with contaminated pollen, from flowers of infected plants to flowers of healthy plants and that feeding wounds made by thrips permitted entry of the virus. Thrips do not appear to be involved in the transmission of RBDV, because very few thrips were found on red raspberry flowers and attempts to transmit RBDV to cucumber with the onion thrips (*Thrips tabaci*) were unsuccessful (3). However, higher numbers of thrips were found in flowers of *Rubus parviflorus* and *R. procerus*, which serve as wild reservoirs for RBDV. If thrips were involved in the transmission of BISHV, thrips in our experiment would have had to visit flowers with infected pollen, pick up contaminated pollen, and then migrate to the healthy trap plant before inoculation could take place.

We also found that BISHV is seed borne, pollen viability is not impacted, and all cultivars tested were susceptible to BISHV. We did not test the seed directly for the presence of BISHV, but in-

stead tested seedlings grown from seed collected from berries harvested from diseased and healthy plants. The low rate of BISHV transmission was nearly identical to that of BLMV (5). This method of transmission is of little consequence to the highbush blueberry industry, because plants for commercial plantings are propagated vegetatively by rooted cuttings or tissue culture. If seeds disseminated by birds germinate and the resulting bushes flower, then those plants might serve as a reservoir for the virus. BLMV was found in wild *Vaccinium* spp. and in *V. corymbosum* plants that most likely originated from seed carried from the nearby commercial field with infected plants (32). Highbush blueberry is not native to the Pacific Northwest, and volunteer plants are not found near commercial plantings.

Red raspberry pollen from cultivars infected with RBDV did not germinate as well as pollen from healthy plants (11). The viability of blueberry pollen infected with the BLMV was markedly reduced (5). Reduced pollen viability, including abortion, is also reported for prunus necrotic ringspot virus and prune dwarf virus in sour cherry (31). This does not appear to be the case for BISHV, because pollen viability was not reduced and berries from diseased bushes appeared to be just as large as berries from healthy bushes. If fewer ovules had been fertilized due to aborted pollen, it might have been reflected in berry size, because seed number is positively correlated with berry size (29). BISHV may spread more rapidly than other pollen-borne viruses that interfere with pollen germination.

Although all of the cultivars tested were susceptible to BISHV, there may be mechanisms of resistance associated with pollination and fertilization that were bypassed because plants were inoculated by grafting. Our data suggest that the buildup of BISHV in 'Blueray' plants takes longer to reach detectable levels than in other cultivars. Recovery from the shock phase of the disease, if coupled with a return to productivity, suggests that significant yield reductions may not be permanent. Results from a field study monitoring the productivity of bushes as they become infected, pass through the shock phase, and appear to have recovered will be the subject of a future report.

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LITERATURE CITED

1. Boylan-Pett, W., Ramsdell, D. C., Hoopingarner, R. A., and Hancock, J. F. 1991. Honeybee foraging behavior, in-hive survival of infectious, pollen-borne blueberry leaf mottle virus and transmission of the virus in highbush blueberry. *Phytopathology* 81:1407-1412.
2. Bristow, P. R., Windom, G. E., and Martin, R. R. 1992. Period of transmission of blueberry scorch carlavirus and blueberry shock ilarvirus in the field. (Abstr.) *Phytopathology* 82:1085.
3. 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.
4. 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.
5. Childress, A. M., and Ramsdell, D. C. 1986. Detection of blueberry leaf mottle virus in highbush blueberry pollen and seed. *Phytopathology* 76:1333-1337.
6. Childress, A. M., and Ramsdell, D. C. 1987. Bee-mediated transmission of blueberry leaf mottle virus via infected pollen in highbush blueberry. *Phytopathology* 77:167-172.
7. 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.* 34:475-483.
8. Cole, A., Mink, G. I., and Regev, S. 1982. Location of prunus necrotic

- ringspot virus on pollen grains of infected almond and cherry trees. *Phytopathology* 72:1542-1545.
9. Davidson, T. R., and George, J. A. 1964. Spread of necrotic ringspot virus and sour cherry yellows viruses in Niagara Peninsula orchards. *Can. J. Plant Sci.* 44:471-484.
 10. Filmer, R. S., and Marucci, P. E. 1963. Blueberry pollination studies, 1962. *Hortic. News* 44:16-18.
 11. Freeman, J. A., Daubeney, H. A., and Stace-Smith, R. 1969. Increased pollen abortion caused by viruses in four red raspberry cultivars. *Can. J. Plant Sci.* 49:373-374.
 12. Garrett, R. G., Cooper, J. A., and Smith, P. R. 1985. Virus epidemiology and control. Pages 269-297 in: *The Plant Viruses*, Vol. I. R. I. B. Francki, ed. Plenum Press, New York.
 13. George, J. A., and Davidson, T. R. 1963. Pollen transmission of necrotic ring spot and sour cherry yellows viruses from tree to tree. *Can. J. Plant Sci.* 43:276-288.
 14. Hamilton, R. I., Nichols, C., and Valentina, B. 1984. Survey of prunus necrotic ringspot virus and other viruses contaminating the exine of pollen collected by bees. *Can. J. Plant Pathol.* 6:196-199.
 15. Haydak, H. M. 1973. Activities of honeybees. Pages 36-90 in: *The Hive and the Honeybee*. R. A. Grout, ed. Dadant and Sons, Hamilton, IL.
 16. Kaiser, W. J., Wyatt, S. D., and Pesho, G. R. 1982. Natural hosts and vectors of tobacco streak virus in eastern Washington. *Phytopathology* 72:1508-1512.
 17. Kelly, R. D., and Cameron, H. R. 1986. Location of prune dwarf virus and prunus necrotic ringspot virus in sweet cherry pollen and fruit. *Phytopathology* 76:317-322.
 18. Krczal, G., Albouy, J., Damy, I., Kusial, C., Deogratias, J. M., Moreau, J. P., Berkemann, B., and Wohanka, A. 1995. Transmission of Pelargonium flower break virus (PFBV) in irrigation systems and by thrips. *Plant Dis.* 79:163-166.
 19. MacDonald, S. G., and Martin, R. R. 1990. Survey of highbush blueberries for scorch viruses. *Can. Plant Dis. Surv.* 70:96.
 20. MacDonald, S. G., Martin, R. R., and Bristow, P. R. 1989. Viruses capable of causing a scorch disease of highbush blueberry. *Acta Hort.* 241: 295-300.
 21. MacDonald, S. G., Martin, R. R., and Bristow, P. R. 1991. Characterization of an ilarvirus associated with a necrotic shock reaction in blueberry. *Phytopathology* 81:210-214.
 22. Martin, R. R., and Bristow, P. R. 1988. A carlavirus associated with blueberry scorch disease. *Phytopathology* 78:1636-1640.
 23. Martin, R. R., and MacDonald, S. G. 1988. Survey for blueberry scorch virus and tomato ringspot virus in highbush blueberry in B.C., Washington, and Oregon. *Can. Plant Dis. Surv.* 68:98.
 24. Marucci, P. E. 1966. Blueberry pollination. *Am. Bee J.* 106:250-257.
 25. McGregor, S. E. 1976. Insect pollination of cultivated crops. *USDA-ARS Agric. Handb. No. 496*, Washington, D.C.
 26. Mink, G. I. 1983. The possible role of honeybees in long-distance spread of prunus necrotic ringspot virus from California into Washington sweet cherry orchards. Pages 85-91 in: *Plant Virus Epidemiology*. R. T. Plumb and J. M. Tresh, eds. Blackwell Scientific Publications, Oxford, England.
 27. Mink, G. I. 1992. Ilarvirus vectors. *Adv. Dis. Vector Res.* 9:261-281.
 28. Mink, G. I. 1993. Pollen- and seed-transmitted viruses and viroids. *Annu. Rev. Phytopathol.* 31:375-402.
 29. Moore, J. N., Reynolds, B. D., and Brown, G. R. 1972. Effect of seed number, size and development on fruit size of cultivated blueberries. *HortScience* 7:268-269.
 30. Morrow, E. B., Darrow, G. M., and Scott, D. H. 1954. A quick method of cleaning berry seed for breeders. *Proc. Am. Soc. Hortic. Sci.* 63:265.
 31. Nyeki, J., and Vertesy, J. 1974. Effects of different ringspot viruses on the physiological and morphological properties of cv. Montmorency sweet cherry pollen II. *Acta Phytopathol. Acad. Sci. Hung.* 9:23-29.
 32. Sandoval, C. R., Ramsdell, D. C., and Hancock, J. F. 1995. Infection of wild and cultivated *Vaccinium* spp. with blueberry leaf mottle nepovirus. *Ann. Appl. Biol.* 126:457-464.
 33. Sdoodee, R., and Teakle, D. S. 1987. Transmission of tobacco streak virus by *Thrips tabaci*: A new method of plant virus transmission. *Plant Pathol.* 36:377-380.
 34. Winston, M. L. 1987. *The Biology of the Honeybee*. Harvard University Press, Cambridge, MA.