Acquisition and Transmission of Blueberry Shoestring Virus by Its Aphid Vector *Illinoia pepperi*

K. M. Morimoto, D. C. Ramsdell, J. M. Gillett, and W. G. Chaney

Graduate research assistant, professor, research technician, and postdoctoral research fellow, respectively; first three authors, Department of Botany and Plant Pathology, and last author, Department of Microbiology and Public Health, Michigan State University, East Lansing 48824.

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ABSTRACT

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Blueberry shoestring virus (BBSSV) causes a serious disease of highbush blueberry in Michigan, several other states, and Canada. Radioimmunosorbent assay was used to detect BBSSV in individual aphid vectors (*Illinoia pepperi*) allowed acquisition access feeding periods of 1, 6, 14, 24, 48, and 72 hr on infected blueberry leaves or on Parafilm® sachets containing purified virus in 20% (w/v) sucrose in phosphate buffer. The first significant detectable amount of virus acquisition was observed between 12 and 24 hr from sachets and between 6 and 12 hr from infected tissue. Both acquisition curves became asymptotic between 24 and 48 hr. The curve was much flatter for diseased tissue compared to the curve from the relatively higher concentration of virus in sachets. Individual aphids varied considerably in amount of virus taken up over a 24-hr acquisition access feeding period (0.15 to 11.3 ng per aphid). Uptake of BBSSV by aphids from symptomatic versus symptomless infected blueberry leaves was similar. A 24-hr acquisition access feeding period on BBSSV-diseased plants followed by inoculation access feeding periods of 1, 6, 14, 24, 48, 96, and 192 hr on healthy 1-yr-old blueberry plants resulted in 5–28% BBSSV infection for all inoculation access feeding periods.

Additional key words: *Vaccinium corymbosum*, virus-vector relations.

Blueberry shoestring disease is an economically important virus disease caused in highbush blueberry, *Vaccinium corymbosum* L., by the blueberry shoestring virus (BBSSV). This is the most widespread virus-caused disease of cultivated highbush blueberries in Michigan, the nation's leading producer of this crop. It is estimated that annual losses caused by this disease are $3 million in Michigan (13). In addition to its occurrence in Michigan, the disease has been reported in New Jersey, Washington State, North Carolina, and Nova Scotia (13,14). The only known vector of BBSSV is the blueberry aphid, *Illinoia pepperi* MacGillivray (12).

The most common symptom on shoestring-diseased plants is elongated reddish streaking on current and 1-yr-old stems. Severely affected leaves are crescent or strap-shaped. Other common symptoms include red veinbanding or red oak leaf patterns on leaves and a red-to-purple cast on immature berries. Latent periods as long as 4 yr between infection and symptom expression are common under field conditions (13). Infected bushes have decreased vigor and may eventually die due to winter injury.

In recent years, growers have begun to use insecticides to control the aphid vector and to rogue symptomatic bushes in an effort to remove inoculum sources. However, symptomless infected bushes still may serve as sources of inoculum for the disease. Virus-vector relationships need to be more fully investigated so effective control tactics can be developed.

Because of the low virus titer in shoestring-affected high bush blueberry, the long latent period, and the lack of an herbaceous host (12), standard acquisition and transmission experiments with BBSSV are difficult and time-consuming.

Several researchers have detected virus pathogens of herbaceous host plants in individual aphid vectors by using enzyme-linked immunosorbent assay (ELISA) (2,3,15). Gillett et al (6) showed that radioimmunosorbent assay (RISA) is more reliable than ELISA or immunosorbent electron microscopy (ISEM) for detecting BBSSV, a low-titered woody plant virus, in individual *I. pepperi*. Virus-vector relationships and epidemiology can be studied with the use of ultra-sensitive serological techniques such as ELISA, RISA, and ISEM; virus-vector relationship and epidemiological studies are practical possibilities.

The purpose of this research was to use RISA and ELISA to study the acquisition of BBSSV and its transmission to blueberry by its aphid vector and to gain further information concerning the virus-vector relationship, which is essential for determining more effective blueberry shoestring disease control strategies.

MATERIALS AND METHODS

Aphids. Healthy 1-yr-old rooted cuttings of *Vaccinium corymbosum* L. 'Jersey' were used as host plants for the nonviruliferous aphid colony. The virus-free colony was started from nymphs deposited by a gravid adult female onto moist filter paper in a petri dish. The culture was maintained with an 18-hr photoperiod supplemented by cool-white fluorescent light, and day and night temperatures were 23 and 18 C, respectively.

Virus and antiserum. Blueberry shoestring virus was purified from frozen infected blossoms of *V. corymbosum* cultivar Jersey as previously described (11,12). Antiserum was produced in a New Zealand white rabbit after three weekly intramuscular injections of BBSSV (1.2 mg per injection, based upon $E_{260\,nm}^{0.1\%} = 5.2$). The virus preparation was emulsified with an equal volume of Freund's complete adjuvant for the first injection; subsequent injections consisted of virus emulsified with equal volumes of Freund's incomplete adjuvant. Bleedings were taken at weekly intervals beginning 1 wk after the last injection. The antiserum reacted with purified preparations of BBSSV to a dilution of 1:1024 (v/v) in 0.85% sodium chloride in agar gel double diffusion tests. There was no antiserum reaction to purified healthy blueberry blossom tissue undiluted or diluted twofold to 1/125.

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Enzyme-linked immunosorbent assay. Prior to the use of blueberry test plants in transmission studies, the double antibody sandwich method of ELISA (1) was used to detect BBSSV to ensure their freedom from infection. Flat-bottom polystyrene microtiter plates (Dynatech Laboratories, Alexandria, VA) were used. Anti-BBSSV-γ-globulin was prepared by ammonium sulfate precipitation and DE-22 (Whatman, Ltd., Maidestone, Kent, UK) cellulose column chromatography. The plates were coated with 1 μg of anti-BBSSV-γ-globulin per milliliter in coating buffer (0.05 M sodium carbonate-bicarbonate buffer, pH 9.6) at a volume of 200 μl per well and were incubated for 3 hr at 37 C.

Blueberry plant samples were triturated with a Tissumizer® homogenizer (Tekmar Co., Cincinnati, OH) in 1:10 (w/v) PBS-PVP-OVA-Tween (PBS containing 2.0% polyvinyl pyrrolidone [MW 40,000], [v/v], 0.2% ovalbumin [w/v], and 0.05% Tween-20 [v/v]). After the homogenates were filtered through two layers of cheesecloth, 200-μl aliquots of the samples were added to replicate wells. The plates containing the samples were incubated overnight at 4 C.

Enzyme-conjugate (1:800 [v/v] in PBS-PVP-OVA-Tween) was added at a rate of 200 μl per well and incubated for 4 hr at 37 C. Between each step the plates were flooded with PBS-Tween at least three times to remove any loosely bound or nonabsorbed reactants. One milligram of enzyme substrate (*p*-nitrophenyl phosphate; Sigma Chemical Co., St. Louis, MO), was added per milliliter of substrate buffer (10% [v/v] diethanolamine in distilled water adjusted to pH 9.8 with HCl) and this was added to the plates at a volume of 200 μl per well. After 1 hr of incubation at room temperature, the $A_{405\ nm}$ was measured spectrophotometrically with a MicroELISA® Minireader (Dynatech Laboratories, Alexandria, VA).

The threshold value used for a positive reaction for each plate was the mean $A_{405\ nm}$ value of healthy samples plus three standard deviations. Samples in each test plate with $A_{405\ nm}$ values greater than the threshold were considered positive for BBSSV.

Radioimmunosorbent assay. Partially purified anti-BBSSV-γ-globulin was prepared as previously described for ELISA. The γ-globulin (1 mg/ml) was iodinated by using a modification of the method described by Greenwood and Hunter (7). One milliCurie of $Na^{125}I$, 5 μl of chloramine-T (5 mg/ml in water) and 150 μl PBS (0.01 M phosphate buffer containing 0.15 M sodium chloride and 0.02% sodium azide) were added to 50 μl γ-globulin. The solution was thoroughly mixed and incubated for 15 min on ice. Five μl sodium metabisulfite (5 mg/ml in PBS) was added to stop the reaction; 100 μl 0.5% bovine serum albumin (BSA) (w/v) and 20 μl NaI (20 mg/ml) in PBS were added to act as carriers for the ^{125}I and γ-globulin, respectively.

The mixture was loaded onto a Sephadex G-50 (Sigma) column that had been pre-equilibrated with PBS-BSA. The ^{125}I-γ-globulin was eluted with PBS-BSA and collected in 1-ml fractions. Aliquots of the fractions were counted in a Beckman Biogamma II gamma counter. The protein fractions were pooled and dialyzed three times against PBS.

A modified procedure of the double antibody sandwich system was used for solid-phase radioimmunosorbent assay (RISA) (4). Flexible disposable polyvinyl "V"-bottom microtiter plates (Dynatech) were coated with unlabeled anti-BBSSV γ-globulin (0.5 μg/ml in 0.05 M sodium carbonate buffer, pH 9.6) at a rate of 100 μl per well. The γ-globulin was incubated in the plates for 3 hr at 37 C. After incubation, the plates were washed three times with PBS-BSA or PBS-BSA containing 0.05% Tween-20 (v/v). To test aphids for the presence of BBSSV, individual aphids were triturated with a glass stirring rod with a rounded end in a small glass test tube containing 100 μl PBS-PVP-OVA-Tween. The entire contents of each test tube was transferred with a pasteur pipette to a plate well. These samples were incubated in the plates overnight at 4 C. Approximately 55,000 cpm anti-BBSSV ^{125}I-γ-globulin diluted in PBS-BSA was added to each well at a volume of 100 μl per well. After 4 hr of incubation at room temperature, the nonabsorbed γ-globulin was aspirated out of the wells. The plates were then washed four times with PBS-BSA or PBS-Tween-BSA. To obtain individual wells, the sides of the flexible plates were cut off with scissors and the top was cut off with a stretched hot wire attached to an electric model train transformer. Each well was individually placed into a counting vial and counted by the gamma counter. Samples with counts per minute (CPM) greater than the mean of the healthy sample wells plus three standard deviations were considered positive for BBSSV.

Kinetics of BBSSV acquisition by aphids feeding from symptomatic leaf tissue and from purified virus in sachets. To test the kinetics of virus acquisition by *I. pepperi*, groups of 30 aphids (late-instar nymphs and apterous adults) were allowed acquisition access feeding periods (AAFPs) of 10 min, 1, 6, 12, 24, 48, and 72 hr on symptomatic BBSSV-infected leaves on detached shoots in a vase of water and purified BBSSV in 20% sucrose (w/v) in 0.05 M phosphate buffer, pH 7.2, contained in Parafilm® sachets (9,10). Aphids were individually tested for the presence of BBSSV by RISA.

Effect of aphid homogenates on the sensitivity of BBSSV detection by RISA. Purified BBSSV was diluted in a twofold dilution series with extraction buffer in concentrations ranging from 1.5 to 200 ng/ml (based upon $E_{260\ nm}^{0.1\%} = 5.2$). Individual apterous late-instar nymph or apterous adult aphids were ground directly in 100 μl each of the purified virus dilutions (three replicates per virus dilution) using a blunted glass rod in a small glass test tube. These aphid-amended virus dilutions were tested versus nonaphid-amended virus dilutions by using RISA. Samples with radioactivity counts greater than the mean plus three standard deviations of extraction buffer were considered BBSSV-positive. The experiment was repeated twice.

Variability of BBSSV uptake by individual aphids. To determine the extent of variability of the amount of BBSSV uptake by aphids, 53 aphids (late-instar nymphs and apterous adults) were allowed an AAFP of 24 hr on infected symptomatic blueberry shoots. Individual aphids were prepared for RISA as described previously and then assayed to determine the relative amount of radioactivity contained in each aphid.

Relative aphid acquisition of BBSSV from symptomatic versus symptomless infected blueberry leaves. To compare relative quantities of virus uptake by the vector from symptomatic versus symptomless infected leaves, aphids were removed from BBSSV-infected leaves or shoots with severe shoestring disease symptoms and from symptomless leaves and shoots on the same infected plants after a 24-hr AAFP. Eighty-eight aphids (late-instar nymphs and apterous adults) that had fed on symptomatic and 92 aphids that had fed on symptomless infected tissue, were individually tested for the presence of BBSSV by RISA.

Inoculation access feeding period studies. Groups of late-instar nymphs and adult apterous blueberry aphids were allowed 24-hr AAFPs on symptomatic BBSSV-infected shoots in a vase of water. Aphids were transferred to potted healthy 1-yr-old rooted cultivar Jersey blueberry cuttings in groups of 15 for inoculation access periods (IAFPs) of 1, 6, 12, 24, 48, 96, or 192 hr. At the end of each IAFP the aphids were removed and the test plants were sprayed with Pirimicarb (5,6-dimethyl-2-dimethylamino-4-pyrimidinyl, dimethylcarbamate). After 6 mo of incubation in the greenhouse, the test plants were put into a dark cold room (4 C) to satisfy a dormant period. After a dormant period of at least 1,000 hr the test plants were moved to the greenhouse. Leaf samples taken from new growth after dormancy was broken were tested for BBSSV infection by using ELISA. This experiment was done twice. For the first experiment, there were 15 replicate test plants per IAFP treatment and for the second experiment, there were seven. The plants were arranged in a randomized complete block experimental design on the greenhouse bench.

RESULTS

Effect of aphid homogenate on the sensitivity of BBSSV detection by RISA. The effect of adding aphid homogenate to extraction buffer containing a range of purified BBSSV concentrations was negligible up to a virus concentration of 5 ng per aphid (Fig. 1). There was a slight quenching effect (about 12%) by added aphid homogenate at higher concentrations of BBSSV,

but these levels were out of the usual range of BBSSV concentrations found in vector aphids. RISA detected purified BBSSV concentrations as low as 0.15 ng per aphid when aphid extract was present.

Variability of BBSSV uptake by individual aphids. Based upon a standard curve for RISA versus BBSSV concentrations (Fig. 1), individual aphids that had fed for 24 hr on symptomatic tissue contained between 0.15 ng and 11.3 ng of BBSSV. It is apparent that aphids vary considerably in the relative amount of BBSSV

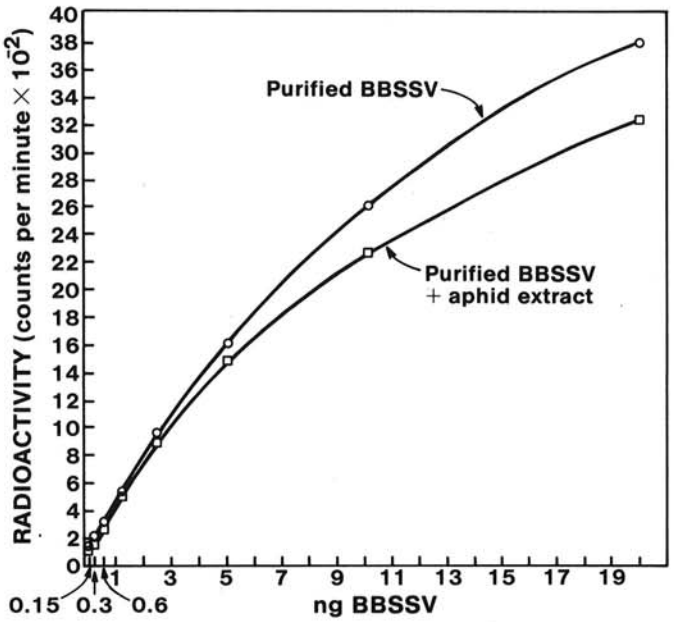

Fig. 1. Effect of added extract of aphids (*Illinoia pepperi*) upon detection of blueberry shoestring virus (BBSSV) by radioimmunosorbent assay (RISA). Individual aphids were ground in 100 μl of RISA extraction buffer containing a known amount of purified BBSSV. Nonaphid-amended, purified BBSSV at the same concentrations was compared.

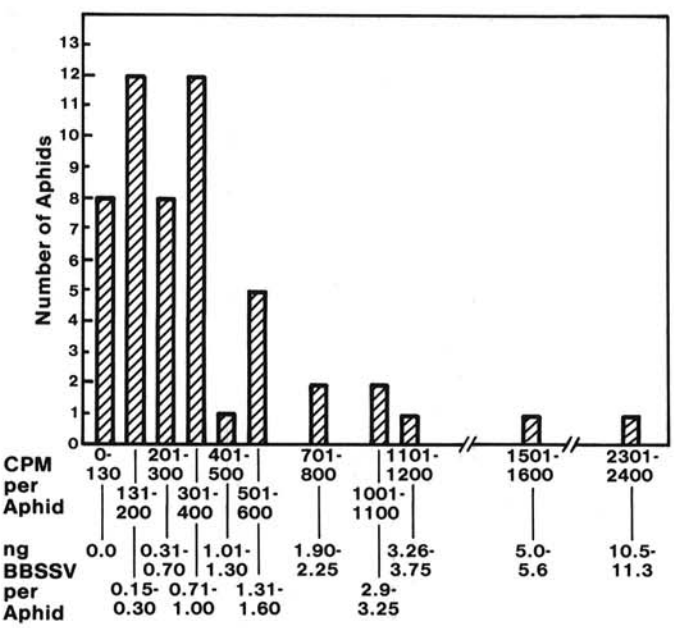

Fig. 2. Variability of uptake of blueberry shoestring virus (BBSSV) by individual aphids (*Illinoia pepperi*) from shoestring-diseased blueberry leaves after a 24-hr acquisition access feeding period prior to radioimmunoassay. The first bar (0–130 counts per minute [CPM]) represents nonBBSSV-containing aphids as determined by 2× the mean value of culture aphids fed on healthy blueberry.

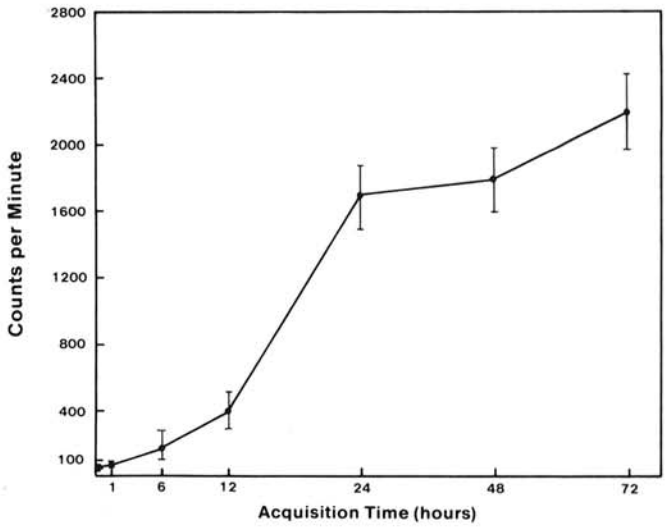

Fig. 3. Kinetics of BBSSV uptake by individual aphids (*Illinoia pepperi*) that fed on Parafilm® sachets containing purified BBSSV in 20% sucrose for varying acquisition access feeding periods prior to radioimmunosorbent assay.

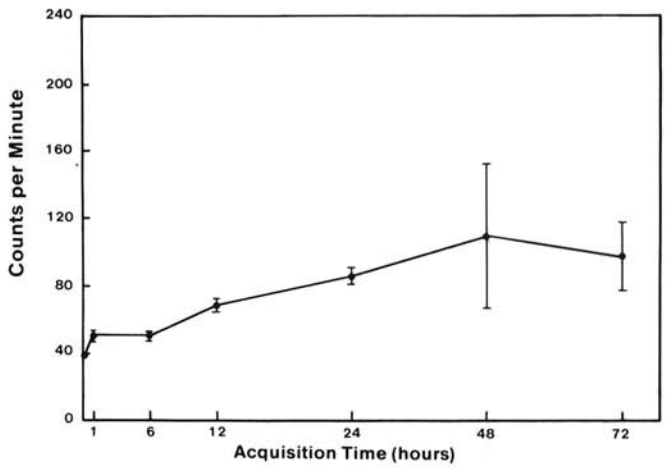

Fig. 4. Kinetics of uptake of blueberry shoestring virus (BBSSV) by individual aphids (*Illinoia pepperi*) that had fed on BBSSV-infected blueberry leaves for varying acquisition access feeding periods prior to radioimmunosorbent assay.

TABLE 1. Transmission of blueberry shoestring virus (BBSSV) from infected highbush blueberry to cultivar Jersey highbush blueberry by *Illinoia pepperi*[a]

Inoculation access feeding time (hr)	Healthy 1-yr-old rooted cuttings infected (mean %)[b]
1	28
6	5
12	28
24	10
48	14
96	11
192	11

[a] Fifteen late-instar or adult apterae were given an acquisition access feeding on BBSSV-infected plants for 24 hr prior to transferring them to each of 22 1-yr-old rooted cuttings of cultivar Jersey for the indicated inoculation access feeding time.

[b] The data are the mean of two experiments involving 15 and 7 test plants, respectively. Radioimmunosorbent assay (RISA) was used to determine whether test plants were infected. RISA values for infected plants ranged from 133 to 327 cpm. Plants were considered diseased if cpm values exceeded the mean plus three standard deviations of healthy controls.

taken up per individual (Fig. 2). The most frequent amount taken up per individual ranged from 0.15 to 1.0 ng of virus.

Kinetics of BBSSV acquisition by aphids from symptomatic leaf tissue and from purified virus in sachets. The rate of virus acquisition by aphids allowed varying AAFPs to purified BBSSV in 20% sucrose contained in sachets increased significantly ($P = 0.05$) between 12 and 24 hr (Fig. 3). Thereafter the curve leveled off.

The virus acquisition curve was similar when aphids were allowed varying AAFPs on symptomatic diseased blueberry shoots (Fig. 4). However, in this case, the curve was less steep. The first significant ($P = 0.05$) virus uptake occurred between 6 and 12 hr. The curve became asymptotic at about 48 hr. It should be noted that the sachets contained an artificially high concentration of BBSSV and that this would probably account for the steeper curve for virus acquisition from sucrose compared to that from infected tissues.

Relative aphid acquisition of BBSSV from symptomatic versus symptomless infected blueberry leaves. A total of 6/88 (6.8%) aphids that had fed on symptomatic infected tissue were positive for BBSSV by RISA, while 6/92 (6.5%) that fed on symptomless infected tissue were positive for BBSSV. Therefore, it is possible for blueberry aphids to acquire BBSSV with equal facility from infected tissue whether or not it is showing symptoms.

Inoculation access feeding period studies. The results of two IAFP experiments were combined. With the constant AAFP of 24 hr, prior to transfer of the aphids to test plants for varying IAFPs, transmission occurred to healthy test bushes within a 1-hr IAFP and at all IAFPs tested (Table 1). The percentage of infected test plants varied from as little as 5% to a maximum of 28% over the range of IAFPs tested. This is the first published report of experimental transmission of BBSSV from blueberry to blueberry by *I. pepperi*.

DISCUSSION

BBSSV is taken up relatively slowly from infected blueberry leaf tissue; the uptake is comparable to that of potato leaf roll virus (PLRV) (15) which, like BBSSV, is nonsap-transmissible and in low titer in its host. With RISA we were able to reliably detect as little as 0.15 ng of BBSSV per aphid, even though uptake by individuals was variable. With PLRV, uptake was variable and batches of 10–20 *Myzus persicae* (Sulz.) were required for a reliable test by ELISA. In the case of pea enation mosaic virus (PeMV) (2), uptake by *Acyrthosiphon pisum* (Harris), was also variable from infected pea plants, but the level of virus in the aphids was up to 30-fold higher than was BBSSV in *I. pepperi*. PeMV uptake was asymptotic after 16 hr. This was similar to BBSSV, which became asymptotic at between 24 and 48 hr; uptake was probably in equilibrium with excretion by the aphids for both viruses. This contrasts with a linear increase of PLRV uptake by *M. persicae* from potato over a 5-day IAFP (15).

I. pepperi, given a 24-hr AAFP, inoculated BBSSV to blueberry test plants in as little as 1 hr of IAFP. Increasing IAFPs of 1, 6, 12, 24, 48, 96, and 192 hr all resulted in BBSSV infection of test plants, but did not result in an increased percentage of infected test plants. Percentage of infected plants was quite variable over the IAFP time course. This could have been due in part to the great variability of virus uptake by individual aphids and possibly by their erratic feeding on the test plants.

Petersen's (10) work has shown that BBSSV appears in the salivary gland of *I. pepperi* after 12- and 24-hr IAFPs, using rabbit anti-BBSSV IgG indirectly tagged with goat anti-rabbit ferritin and viewed in the TEM. This information indicates that BBSSV is circulative in *I. pepperi*. The lack of sap-transmissibility, plus the acquisition and inoculation kinetics and the appearance of virions in the salivary gland in as little as 12 hr suggests that BBSSV may have a circulative, persistent relationship with its aphid vector (8). Recent work done with ferritin-labled rabbit anti-PLRV-IgG and antibarley yellow dwarf virus-IgG has shown labeling of these virions in the accessory salivary glands of *M. persicae* (5). Both of these luteoviruses have a persistent and circulative virus-vector relationship. Unfortunately, retention tests cannot be conducted, because there is no known nonhost of BBSSV upon which *I. pepperi* will feed and survive.

The information reported herein is for apterous third- and fourth-instar nymphs and apterous adults. It is not known whether earlier instar nymphs are capable of vectoring BBSSV. However, in the field, young nymphs move very little and would thus be less effective in spreading the virus compared to the more active older nymphs and adults. We also know that up to 25% of alatae collected from infected bushes in the field contained BBSSV as detected by RISA (9). The relative vector efficiency of alateae is not known.

The spread of BBSSV in the field has been observed to be relatively slow; it may take 15–20 yr before 50% of the plants in a 16-ha (40 acre) field are infected. Until growers were recently informed that *I. pepperi* is a vector of BBSSV they did not try to control aphid populations effectively. The knowledge that the virus-vector relationship is not nonpersistent and more probably circulative-persistent, suggests that the spread of BBSSV may be curtailed with effective aphicides. Insecticidal control measures should be applied at the first sign of aphid population buildup in the spring and continued as needed when populations start to rebuild. Field epidemiology research has been done with BBSSV and *I. pepperi* to define further refinement of control strategies. This will be reported separately.

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