Quantification of Blueberry Shoestring Virus RNA and Antigen in its Aphid Vector, *Illinoia pepperi*, During Acquisition, Retention, and Transmission

B. T. Terhune, D. C. Ramsdell, K. L. Klomparens, and J. F. Hancock

First, second, and third authors: Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824. Third author: Director of the Center for Electron Optics, Pesticide Research Center. Fourth author: Department of Horticulture, Michigan State University, East Lansing 48824.

This research was supported in part by USDA grant 86-CRSR-2-2758 and a grant-in-aid from the Michigan Blueberry Growers Association, Grand Junction 49056.

This journal article is in partial fulfillment of the requirements for a masters thesis by the first author. This is a Michigan State Agricultural Experiment Station journal series article.

We wish to thank L. R. Nault, Charles Cress, Peter Callow, and Vicky Rhein for their helpful suggestions and assistance.

Accepted for publication 30 May 1990 (submitted for electronic processing).

ABSTRACT

Terhune, B. T., Ramsdell, D. C., Klomparens, K. L., and Hancock, J. F. 1991. Quantification of blueberry shoestring virus RNA and antigen in its aphid vector, *Illinoia pepperi*, during acquisition, retention, and transmission. Phytopathology 81:1096-1102.

Blueberry shoestring virus (BBSSV) was monitored in late instars of *Illinoia pepperi* by dot-enzyme-linked immunosorbent assay, a silver-enhanced colloidal gold immunosorbent assay, and dot-hybridization. Aphids acquired BBSSV antigen and BBSSV RNA at a rapid rate during the initial 24-h acquisition access period (AAP) from Parafilm sachets containing purified BBSSV, but the acquisition rate leveled off and declined thereafter during subsequent AAPs at intervals of up to 96 h. Over a 4-day AAP, from diseased plants, BBSSV RNA was acquired at concentrations lower than those acquired from artificial sources, according to dot-hybridization data. Levels of BBSSV antigen and RNA retained by aphids on healthy plants declined rapidly 1 day after acquisition, but remained fairly constant during the next 3–4 days. Both BBSSV antigen and RNA were detected in aphid hemolymph after 1- to 4-day AAPs. Aphids were able to transmit BBSSV to blueberry plants for up to 10 days after 1-, 2-, or 3-day AAPs on sachets or on BBSSV-infected plants. Our data suggest persistent transmission of BBSSV by its vector.

Additional keywords: immunoassays, persistent-circulative.

Techniques in immunocytochemistry and molecular biology have been modified to detect plant viruses in their insect vectors (5,7,9,12,13,24). These techniques have also been used to monitor virus levels in the insect vector during acquisition and inoculation feeding periods (6,16,21). In cases when symptom development is delayed several months after transmission of a virus to its host by an insect vector, these techniques provide a complimentary method for assessing a virus-vector relationship. We have examined acquisition, retention, and passage of blueberry shoestring virus (BBSSV) into the hemocoel of its aphid vector, *Illinoia pepperi* MacG., by using dot-enzyme-linked immunosorbent assay (ELISA), silver-enhanced colloidal gold immunosorbent assay (dot-GLISA), and dot-hybridization. We also monitored the transmission of BBSSV by aphids to young, succulent blueberry plants (grown from shoot tissue culture) at daily intervals to corroborate immunocytochemical and dot-hybridization results.

In previous investigations, radio immunoassay (RIA), double antibody sandwich ELISA (DAS-ELISA), and dot-ELISA have been used to detect BBSSV in both host plant and aphid tissues (9,16,24,26,27). By using RIA, Morimoto et al (16) found that *I. pepperi* could acquire BBSSV from artificial and natural virus sources in as little as 6 h, and transmission occurred within 26 h after initiating an acquistion access period (AAP). While this research demonstrated aphid transmission of BBSSV, additional evidence was needed to elucidate the virus-vector relationship. The objectives of this research were to further characterize the virus-vector relationship by examining: 1) BBSSV acquisition and retention by using antigen and RNA assays; 2) the hemocoel for the presence of BBSSV antigen and RNA; and 3) aphid transmission of BBSSV to micropropagated blueberry plants at daily intervals after a 1- to 3-day AAP.

MATERIALS AND METHODS

Aphids. *I. pepperi* colonies were initiated with aphids collected from the Michigan Blueberry Growers test planting at Grand Junction, MI. Gravid females were allowed to deposit ovoviviparae onto moist filter paper in a petri dish. These were tranferred to young, succulent, virus-free *Vaccinium corymbosum* L. 'Jersey.' Colonies were raised on 1- or 2-year-old potted plants. Plants and aphid colonies were maintained at 18–25 C with a 16-h photoperiod. Late instar (third and fourth) and adult aphids were used in all experiments.

Virus purification. All purification procedures were performed at 4 C. BBSSV was purified from blossoms according to Ramsdell (19).

Micropropagated blueberry plants. Micropropagated blueberry plants used in BBSSV transmission experiments were prepared by P. Callow as previously described (3).

Dot-ELISA. Virus and aphid samples were tested by dot-ELISA as described by Banttari and Goodwin (1) with some modifications (27). The IgG fraction of BBSSV-antiserum from rabbit was isolated by ammonium sulfate precipitation and DE-22 cellulose chromatography as described by Clark and Adams (4), and conjugated to alkaline phosphatase (alkaline phosphatase P-0405 Sigma Chemical Co., St. Louis, MO). Nitrocellulose membranes (NCM) (BioRad Co. Inc., Richmond, CA) or nylon membranes (NM) (Hybond-N, Amersham Corp., Arlington Heights, IL) were used as solid support. The NCM was treated as previously described (27). The NM was blocked overnight at 37 C with 10% (w/v) nonfat dry milk. Aphid samples were homogenized in 200 µl of 10 mM Tris-buffer (pH 7.4), containing 15 mM NaCl and 0.05% (v/v) Tween-80 (TBS-T80) plus 1% (w/v) polyvinylpyrrolidone. Virus and aphid samples were spotted onto the membranes with a BioRad blotting manifold (BioRad Co.), and incubated overnight at 4 C.

Detection of antigen on nitrocellulose or nylon membranes with a colloidal gold-IgG conjugate. The procedure described by Hsu

(11) was modified to detect BBSSV in aphid homogenates immobilized on nitrocellulose or nylon by using a colloidal gold-anti-BBSSV-IgG conjugate. Colloidal gold (10 nm diameter) was conjugated to IgG as described by Bendayan (2). Membranes and samples were treated as in the dot-ELISA procedure. After incubating samples, membranes were washed for 30 min in TBS-T80 and transferred to a solution containing an anti-BBSSV-IgG-colloidal gold conjugate diluted 1:20 (v/v) in TBS-T80. After incubating 3 h at 23 C, membranes were rinsed for 30 min in buffer. Membranes were transferred to 20 ml of silver enhancer (IntenSE II, Janssen Life Sciences Products, Piscataway, NJ), and incubated 5 min at 23 C. Membranes were rinsed in distilled water and air dried. Positive reactions produced an insoluble, black spot. This technique is referred to as dot-GLISA.

cDNA probe preparation. BBSSV RNA was extracted from purified BBSSV as described by Ramsdell (19), and a cDNA probe was prepared as described by Taylor et al (22). Two to 5 μg of BBSSV-RNA were randomly primed with salmon sperm DNA primers, and a complementary DNA strand was synthesized with four units of avian myeloblastosis virus reverse transcriptase (Amersham Corp.) per microgram of BBSSV RNA. The reaction solution (final volume, 50 μl) contained 0.05 M Tris (pH 8.3), 8 mM $MgCl_2$, 8 mM dithiothreitol, 0.02 μg/ml actinomycin-D, 0.6 mM dATP, 0.6 mM dGTP, 0.6 mM dTTP, 0.2 mM dCTP, 20 μCi [^{32}P]-α-dCTP (Amersham), 12 mM KCl, and 1% Triton X-100. After a 2-h incubation period, incorporated and unincorporated nucleotides were separated on a Sephadex G-50 column. RNA was removed from the purified cDNA-RNA hybrid by boiling in 0.1 N NaOH for 5 min.

Dot-hybridization. Aphids were assayed by a previously described dot-hybridization technique (15,25,28) with modifications (24). All glassware was treated for RNase by baking 4 h at 250 C or soaking in diethylpyrocarbonate-treated distilled water (DEPC) (14); buffers were also DEPC-treated. Individual aphids were homogenized in 50 μl of phosphate buffer, pH 7.4, followed by the addition of 30 μl of 20× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 20 μl of 37% formaldehyde. Virus and aphid samples were heated 15 min at 60 C then chilled on ice. Samples were spotted, then transferred to a plastic bag containing 10 ml of prehybridization solution, consisting of 2× SSC and 4× Denhardt's solution (Denhardt's solution = 0.8% (w/v) BSA, 0.8% (w/v) Ficoll 400, 0.8% (w/v) polyvinylpyrrolidone) and incubated overnight at 65 C. Hybridization was subsequently performed for 18 h at 42 C in a solution consisting of 18.5% (v/v) formamide, 0.1% Denhardt's solution, 1% (w/v) glycine, 1× SSC, 0.2% (w/v) sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer, pH 7.4, 1 mg/ml of salmon sperm DNA, 1–5 × 10^6 dpm/ml [^{32}P]-cDNA probe. Membranes were then washed four times (5 min per wash) in 2× SSC containing 0.1% (w/v) SDS at 23 C followed by two washes (15 min per wash) at 65 C in 0.1× SSC containing 0.1% (w/v) SDS. Air-dried membranes were exposed to X-ray film (Kodak X-Omat AR) with intensifying screens for 48 h at −20 C.

Acquisition of BBSSV by *I. pepperi*. Late instar and adult aphids were allowed to feed on Parafilm sachets containing 50 μg of BBSSV per milliliter of 20% sucrose (w/v), or symptomatic shoots of BBSSV-infected plants. Aphids were removed after AAPs ranging from 30 min to 4 days. Individual aphids were homogenized and assayed for BBSSV antigen and RNA by dot-ELISA, dot-GLISA, and dot-hybridization. Three to four aphids were sampled in at least three replications for each AAP. Autoradiograms and photographic reproductions of membranes from immunoassays were scanned at 550 nm on a Gilford Response II spectrophotometer, and assay absorbance values were converted to nanograms of BBSSV per aphid by using an algorithm describing a dose-response curve (24). The dose-response curves were based on a twofold dilution series of purified BBSSV, and were determined for each assay. The corresponding algorithms were generated by multiple regression analysis on absorbance values using the MSTAT statistical program (17). In addition, the percentage of BBSSV-positive aphids, after exposures for the various AAPs, was recorded. Aphid absorbance values (A_{550} nm) greater than control aphid mean absorbance values + three standard deviations, or two times the mean absorbance value, were considered positive for BBSSV.

Retention of BBSSV by *I. pepperi*. After 1- to 3-day AAPs, viruliferous aphids were transferred to 3- to 5-mo-old, healthy, micropropagated blueberry plants, and allowed to feed for inoculation access periods (IAPs) between 30 min and 4 days. Three to four aphids were tested for BBSSV antigen and RNA by dot-ELISA, dot-GLISA, and dot-hybridization in at least three replicates for each IAP. The percentage of BBSSV-positive aphids and the concentration of BBSSV per aphid were compared over various IAPs.

Detection of BBSSV antigen and RNA in aphid hemolymph. Hemolymph samples were withdrawn from five aphids following 1-, 2-, 3-, and 4-day AAPs on either sachets containing 50 μg of BBSSV per milliliter of 20% (w/v) sucrose or blueberry plants symptomatic for shoestring disease. Samples were collected by probing the exoskeleton on the dorsal side of the abdomen with

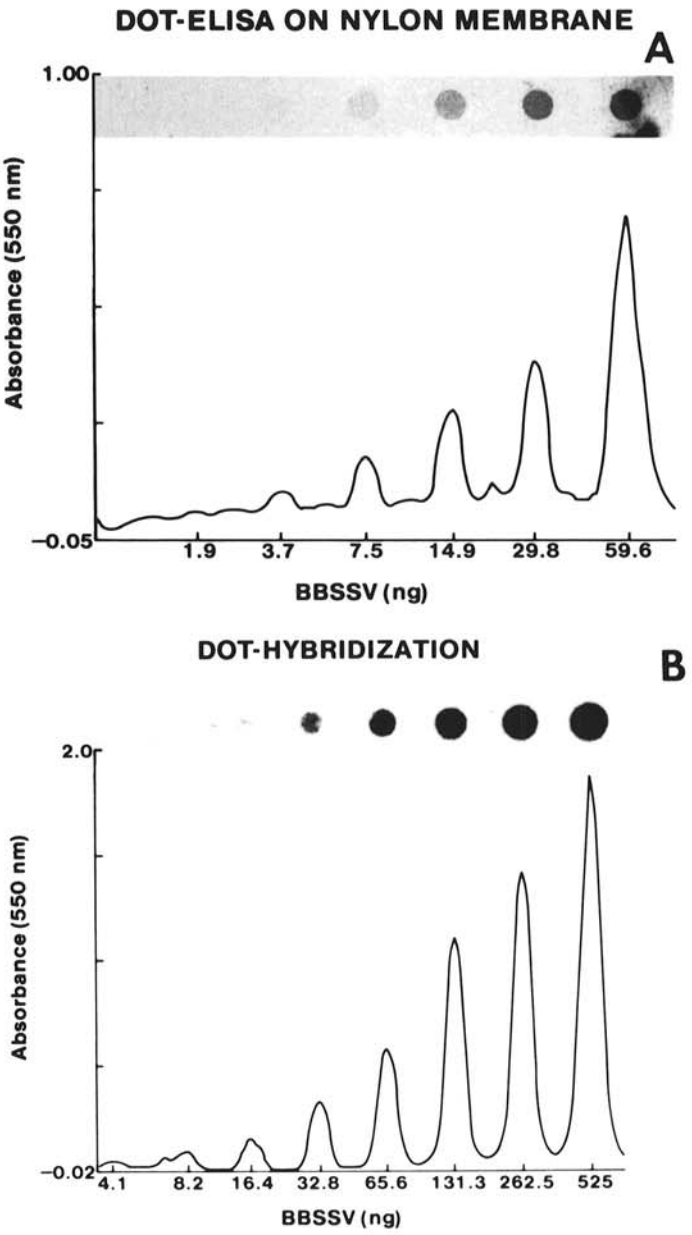

Fig. 1. Absorbance peaks derived from a 550 nm scan of spots on **A**, a 10.1 × 12.7 cm (4 × 5 in.) color transparency reproduction of a dot-enzyme-linked immunosorbent assay (ELISA) on nylon membrane, and **B**, a dot-hybridization autoradiogram. The corresponding spots, which represent a twofold dilution series of purified blueberry shoestring virus, have been included immediately above the peaks.

a finely drawn glass needle. Once the needle pierced the exoskeleton, hemolymph (approximately 0.1–0.5 μl) was withdraw by capillary action. Hemolymph samples from five aphids were combined with 200 μl of 10 mM sodium phosphate buffer (pH 7.0) and assayed by dot-ELISA or dot-hybridization. The residual aphid bodies were ground in the appropriate assay buffer and assayed also.

Aphid transmission of BBSSV to healthy micropropagated blueberry plants. Groups of 15 aphids were transferred from the virus source to a caged, 3- to 5-mo-old, healthy micropropagated blueberry plant (cv. Jersey), and allowed to feed for 24 h. After this period, the group of aphids was transferred to a new healthy blueberry plant and allowed to feed for 24 h. The transfers were repeated over a 10-day period or until all of the aphids were dead. The blueberry plants were sprayed with Piramor aphicide and kept under a 16-h photoperiod at 20–30 C. Then, shoots and roots (2–5 g each) were assayed by dot-ELISA on NCM at 3, 6, and 12 mo after inoculation. This experiment was replicated three times for aphids initially fed on sachets containing BBSSV, and twice for aphids initially fed on plants symptomatic for shoestring disease. Blueberry plant samples with absorbance values greater than the mean for healthy blueberry plant samples plus three standard deviations were considered positive for BBSSV.

RESULTS

Quantification of BBSSV concentrations in *I. pepperi*. Absorbance scans of spots on NCM and NM resulting from dot-ELISA and dot-hybridization assays, respectively, of a twofold dilution series of purified BBSSV, are shown in Figure 1A and B. The peak heights, measured at 550 nm, were used as one of the parameters in the dose-response curve. A similar series was used in each assay to determine the BBSSV dose-response curve. It is important to note that the nature of the BBSSV RNA detected in aphids (i.e., virion-associated or free RNA) remains unclear. For the purposes of this study, BBSSV RNA refers to RNA detected by dot-hybridization assay and does not imply its association with or without the virus coat protein.

Acquisition of BBSSV by *I. pepperi*. When aphids fed on an artificial source of BBSSV, (i.e., sachets) significant levels of BBSSV were detected by the immunoassays and dot-hybridization after 0.5- or 3-h AAPs, respectively (Fig. 2). After a 30-min AAP, the percentage of aphids positive for BBSSV antigen increased from 10 to 75% after a 24-h AAP. Then, the percentage of positive aphids decreased, ranging between 39 and 72%, during subsequent AAPs. The percentage of BBSSV RNA-positive aphids increased from 10% after a 3-hr AAP to 63% after a 24-h AAP, and ranged between 50 and 62% during subsequent AAPs. When aphids were given a 4-day AAP to BBSSV-infected blueberry plants 62–71% of the aphids were positive for BBSSV RNA; blueberry antigen levels were below the positive threshold.

Quantified dot-ELISA results indicated that the mean concentration of BBSSV increased most rapidly during the first 6 hr of the AAP, rising from 1.4 ng of BBSSV per aphid after a 30-min AAP to 7.0 ng of BBSSV per aphid after a 6-hr AAP (Fig. 3). During the following 90 hr of the AAP, mean BBSSV levels ranged from 2.9 to 6.8 ng of BBSSV per aphid with some individual aphids acquiring as much as 41 ng after a 24-hr AAP. Logarithmic regression analysis indicated a moderate increase in antigen acquisition during the latter part of the AAP. The dot-hybridization assay indicated that the mean concentration of BBSSV acquired from sachets increased from 5.3 ng of BBSSV per aphid after a 0.5-hr AAP to 20.2 ng of BBSSV per aphid after a 24-hr AAP. Then, the level declined to 10.7 ng per aphid after a 48-hr AAP, followed by an increase to 18.7 ng per aphid after a 96-hr AAP. Regression analysis on dot hybridization data showed a continuous increase in BBSSV levels up to a 96-hr AAP. After a 4-day AAP on infected plants, the mean concentration of BBSSV RNA ranged from 0.8 to 3.6 ng per aphid.

Retention of BBSSV by *I. pepperi*. In general, the immunoassays and dot-hybridization assays detected BBSSV antigen (at least 4 days) and BBSSV RNA (up to 3 days) in aphids after 1- to 3-day AAPs on an artificial source of BBSSV (Fig. 4). The percentage of aphids positive for BBSSV antigen decreased from 71 to 23% after a 4-day IAP. The percentage of aphids positive for BBSSV RNA decreased from 71 to 20% during a 3-day IAP. When aphids were given a 1- to 3-day AAP on BBSSV-infected blueberry plants, 10% retained BBSSV RNA after a 3-day IAP on healthy blueberry plants. Aphids were also assayed for BBSSV antigen, but failed to acquire detectable virus levels from source plants.

Dot-ELISA data showed that the mean concentration of

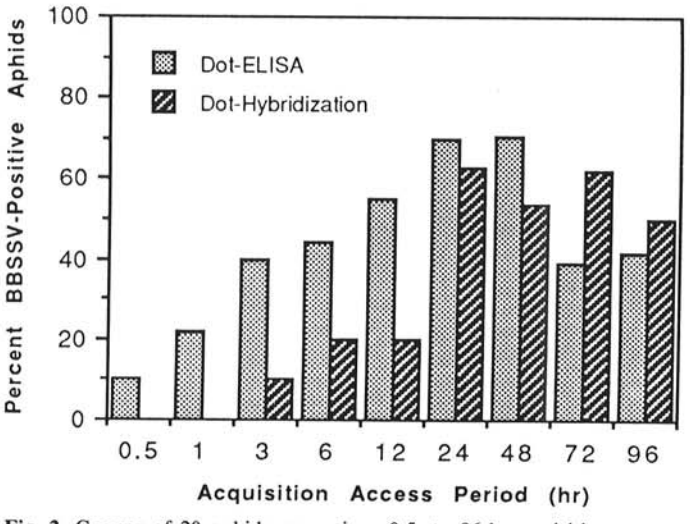

Fig. 2. Groups of 20 aphids were given 0.5- to 96-h acquisition access periods (AAPs) on Parafilm sachets containing 50 μg of blueberry shoestring virus (BBSSV) per milliliter of 20% (w/v) sucrose and then tested by silver-enhanced colloidal gold immunoassay, dot-enzyme-linked immunosorbent assay, and dot-hybridization assays. The percentages of BBSSV-positive aphids for each type of assay are shown in the bar graphs. Positive threshold values were determined from mean absorbance values from three to five aphids (maintained on healthy blueberry plants) plus three standard deviations or two times the mean.

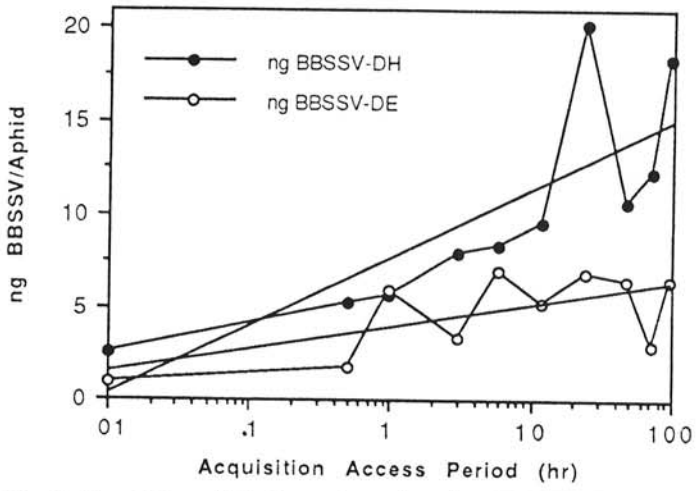

Fig. 3. Acquisition of blueberry shoestring virus (BBSSV) following various acquisition access periods (AAPs) on Parafilm sachets containing 50 μg/ml of purified BBSSV in 20% (w/v) sucrose. BBSSV levels were detected as described in Figure 2. Dose-response curves from a twofold dilution series included in each assay were used to convert absorbance values from acquisition experiments (as in Fig. 2) to nanogram concentrations of BBSSV per aphid. Data points represent mean BBSSV concentrations per aphid (nine to 20 aphids per data point) and lines without data points represent regression lines for dot-enzyme-linked immunosorbent assay (ELISA)/silver-enchanced colloidal gold immunoassay (GLISA) ($\hat{Y} = 3.86 + 1.20 \log X$; $r^2 = 0.43$) and dot- hybridization ($\hat{Y} = 7.55 + 3.67 \log X$; $r^2 = 0.64$). DH = dot-hybridization; DE = dot-ELISA/dot-GLISA.

BBSSV antigen per aphid, which fed on sachets, decreased from 4.8 ng per aphid to 0.8 ng per aphid after a 3-day IAP (Fig. 5). Dot-hybridization results indicated that aphids retained a mean concentration of 2.2 ng of BBSSV after a 3-day IAP (Fig. 5) with some individual aphids retaining as much as 4.1 ng of BBSSV. After acquisition access to BBSSV-infected blueberry plants, the mean concentration of BBSSV per aphid, based on dot-hybridization results, decreased by approximately 60% during the initial 24-h inoculation feeding period, decreasing from 4.9 to 2.0 ng. Concentrations ranged from 1.25 to 2.0 ng of BBSSV per aphid between the second and fourth day IAP. Some individual aphids retained as much as 6 ng of BBSSV after 3 days of inoculation feeding.

Detection of BBSSV antigen and RNA in the aphid hemocoel. The immunoassays and dot-hybridization assays showed that both BBSSV antigen and RNA were present in the hemolymph. In two experiments, aphid hemolymph samples tested positive for antigen after AAPs of 1, 2, 3, and 4 days on sachets containing BBSSV (Table 1). Forty to 100% of the residual aphid bodies tested positive for BBSSV antigen. Aphid hemolymph samples also tested positive for BBSSV RNA after AAPs of 1, 3, and 4 days, and 40–100% of the residual aphid bodies tested positive. Dot-hybridization indicated the concentration of BBSSV in the hemolymph samples ranged from 1.0 to 10.1 ng per aphid with a mean of 2.1 ng of BBSSV per aphid. The immunoassays indicated the concentration of BBSSV in the hemolymph samples ranged from 0.1 to 7.9 ng per aphid with a mean of 1.5 ng of BBSSV per aphid. In a third experiment, aphids were given AAP on symptomatic blueberry plants. Whereas BBSSV was not detected in hemolymph samples or residual aphid bodies by the immunoassays, BBSSV was detected in both hemolymph and

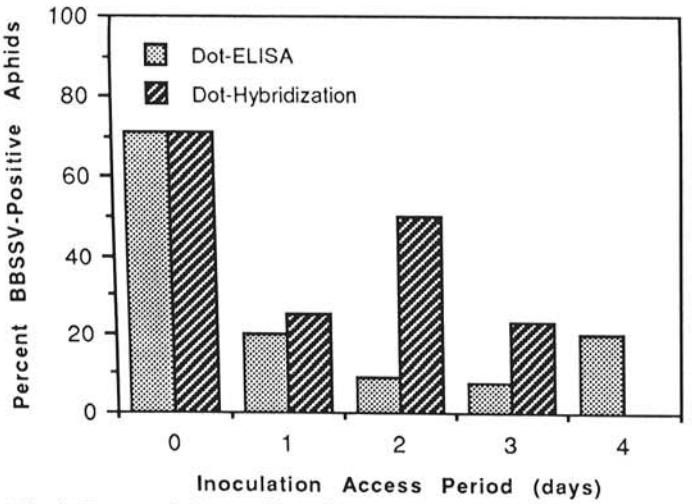

Fig. 4. Groups of nine to 20 aphids were given 1- to 3-day acquisition access periods on Parafilm sachets containing 50 μg of blueberry shoestring virus (BBSSV) per milliliter of 20% (w/v) sucrose, and then allowed to feed on healthy blueberry plants for 1- to 4-day inoculation access periods. Aphids were tested by silver-enhanced colloidal gold immunosorbent assay (dot-GLISA), dot-enzyme-linked immunosorbent assay (ELISA), and dot-hybridization. The dot-GLISA and the dot-ELISA results were combined to determine the percentage of aphids positive for BBSSV antigen.

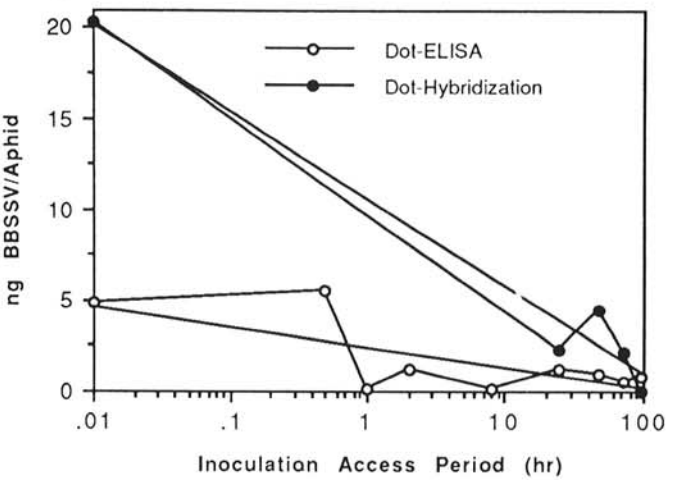

Fig. 5. Assay results from Figure 4 were quantified spectrophotometrically from photographic reproductions based on dose-response curves from a twofold dilution series of purified blueberry shoestring virus (BBSSV) included in each assay. Data points represent mean BBSSV concentration for groups of nine to 20 aphids, and lines without data points represent regression lines for dot-enzyme-linked immunosorbent assay (ELISA) ($\hat{Y} = 2.14 - 1.11$ logX; $r^2 = 0.51$) and dot-hybridization ($\hat{Y} = 3.12 - 0.88$ logX; $r^2 = 0.96$).

TABLE 1. Detection of blueberry shoestring virus (BBSSV) antigen and RNA in the hemolymph of *Illinoia pepperi* after acquisition access periods on Parafilm sachets containing purified BBSSV or on BBSSV-infected blueberry plants.

Type of BBSSV material to be assayed[a]	Source of virus used for aphid acquisition access[b]	Source of sample assayed[c]	Acquisition access period[d] (days)			
			1	2	3	4
Antigen	S	Aphid	2/5	5/5	4/5	2/3
		Hemolymph	+	+	+	+
Antigen	S	Aphid	4/5	4/5	3/5	3/5
		Hemolymph	+	+	−	+
Antigen	P	Aphid	0/5	0/5	0/5	0/5
		Hemolymph	−	−	−	−
RNA	S	Aphid	3/5	4/5	5/5	5/5
		Hemolymph	+	−	+	+
RNA	S	Aphid	2/5	4/5	2/5	NT[e]
		Hemolymph	−	−	+	NT
RNA	P	Aphid	2/5	1/5	3/5	3/5
		Hemolymph	−	−	+	−

[a] Aphids were assayed for BBSSV antigen by dot-enzyme-linked immunosorbent assay or silver-enhanced dot colloidal gold immunosorbent assay, or BBSSV RNA by dot-hybridization following various acquisition access periods on Parafilm sachets containing 50 μg of BBSSV per milliliter of 20% (w/v) sucrose or on plants symptomatic for shoestring disease.

[b] S = aphids were given a 24-hr acquisition access to purified BBSSV on Parafilm sachets; P = aphids were given 24-h access to BBSSV-infected blueberry plants.

[c] Hemolymph samples were withdrawn by piercing the exoskeleton on the dorsal side of the abdomen with a finely drawn glass needle. Hemolymph samples from five aphids were combined and tested for BBSSV by the immunoassays or dot-hybridization following various acquisition access periods on a virus source. The remaining aphid was also assayed for BBSSV.

[d] The numerator indicates the number of BBSSV-positive aphids; the denominator, the total number of aphids assayed (these aphids were the source of hemolymph for assay); + = hemolymph was positive for BBSSV antigen or RNA; − = hemolymph was negative for BBSSV antigen or RNA.

[e] NT = not tested.

bodies by dot-hybridization (Table 1).

Transmission of BBSSV to blueberry plants by *I. pepperi* after successive transfers. The number of aphids per group that survived the entire 10-day period ranged from 0 to 10 aphids. Aphids transmitted the virus to a healthy blueberry plant on days 1, 2, 3, 7, 8, and 10 after a 1-day AAP to purified BBSSV in sachets and on days 1, 4, 5, and 8 after a 1-day AAP on infected plants (Fig. 6A). After a 2-day AAP to purified BBSSV in sachets, aphids transmitted the virus to plants on days 4, 5, 6, 8, and 9. After a 2-day AAP on diseased plants, the virus was transmitted to healthy plants on days 2, 4, 6, and 8 (Fig. 6B). Aphids given a 3-day AAP on sachets, transmitted BBSSV to healthy plants on days 1, 2, 3, and 4, whereas those given a 3-day AAP on diseased plants transmitted BBSSV to healthy plants on days 1–7, and 10 (Fig. 6C). Overall, aphids were able to transmit BBSSV throughout the 10-day period. Nonviruliferous aphid controls did not transmit BBSSV on test plants. Aphid mortality was greater after an AAP on sachets than on plants, presumably because the sucrose solution did not sustain them as well as the plant sap.

DISCUSSION

Two important factors for determining virus transmission by aphids are the length of time required for virus acquisition and the length of time that transmission can occur (i.e., the persistence of transmission). The acquisition and retention periods associated with aphid transmission of viruses are generally elucidated through transmission experiments. Whereas transmission experiments are easily performed with herbaceous host plants, they are more difficult to perform on woody host plants. Symptom development on blueberry caused by BBSSV requires a 4-yr incubation period before symptom development; virus infections are not detectable by ELISA until after a 12-mo incubation period. There are no known herbaceous host plants for BBSSV, but the advent of fast growing, succulent, micropropagated blueberry plants facilitated transmission experiments. These plants still required a 6-mo incubation period before BBSSV could be detected by dot-ELISA (26).

An indirect approach, involving ELISA, has been used to assess virus acquisition and retention periods for persistently transmitted viruses (6,21). These studies used ELISA to assay individual aphids for virus content following AAPs and IAPs. This method did not indicate the transmissibility of virions following acquisition and retention, but results positively correlated with previous transmission experiments (i.e., significant virus antigen levels were retained by aphids over several days), and aphids also remained infective during this period. We used this same approach to determine the acquisition and retention periods of BBSSV by *I. pepperi*, but dot-ELISA or dot-GLISA were used to detect BBSSV antigen, and dot-hybridization was used to detect BBSSV RNA.

Previous studies have used ELISA to detect viruses in individual and groups of aphids and have achieved a virus detection threshold of about 5.0 ng of virus (6,9). Because this is a relatively low level of sensitivity, it is likely that the ELISA would not detect low virus concentrations in aphids unless aphids were assayed as a group. The sensitivity of ELISA has been improved by the introduction of a different solid phase support, nitrocellulose membrane (NCM) (1). This technique, dot-ELISA, was used by Urban (26) to detect as little as 3.0 pg of BBSSV in infected blueberry samples, but attempts to use this assay to detect virus in the aphid were unsuccessful due to the high background absorbance responses from nonviruliferous aphids near the detection limit of the assay. Later research demonstrated that background absorbances could be reduced by using nylon membranes with longer blocking periods, but assay sensitivity was limited by quenching or masking due to components within the aphid (24). When purified BBSSV preparations were mixed with nonviruliferous aphid homogenates, the sensitivity of dot-ELISA and dot-GLISA were reduced 10–40%, whereas the sensitivity of dot-hybridization was reduced 5–10% (23,24). Differences in the degree of quenching between these techniques could account for discrepances in the concentrations of BBSSV detected by the immunoassays compared to dot-hybridization.

When these techniques were used to monitor acquisition of BBSSV by *I. pepperi*, virus was detected after short AAPs (30–60 min) on sachets. Fargette et al (6) found similar acquisition kinetics for the persistently transmitted pea enation mosaic virus (PEMV); levels of virus increased up to a 16-h AAP, but remained constant with longer AAPs. They suggested that after 16 h, virus ingestion and excretion were at equilibrium. *I. pepperi* had a similar pattern for the acquisition of BBSSV antigen from artificial sources, with maximum BBSSV concentrations acquired after a 12-h AAP. Subsequent decreases in BBSSV levels may have reflected changes in feeding behaviour. The dot-hybridization assays de-

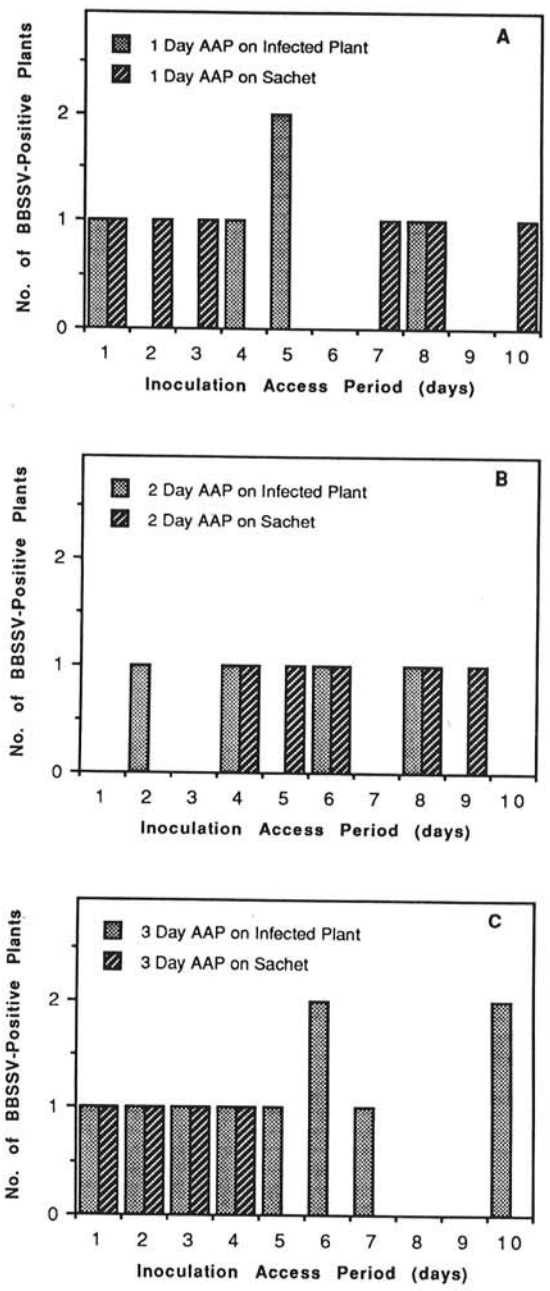

Fig. 6. Transmission of blueberry shoestring virus (BBSSV) to healthy, micropropagated blueberry plants by aphids. Aphids were given **A,** a 1-day acquisition access period (AAP) to purified BBSSV in sucrose in Parafilm sachets or BBSSV-infected plants, or **B,** a 2-day AAP to the same, or **C,** a 3-day AAP to the same. Then, 15 aphids were successively transferred at daily intervals for 10 days to each of 10 healthy test plants. Each bar represents three plants assayed after aphid inoculation by aphids initially fed on sachets or two plants assayed after aphid inoculation by aphids initially fed on plants symptomatic for shoestring disease.

tected BBSSV at higher concentrations after the various acquisition periods than did the immunoassays. The most likely reason for this difference would have been due to the differences in quenching effect of aphid components on the assays. It is doubtful that these higher levels were due to replication of BBSSV in the aphid because similar decreases in virus concentrations during IAPs were detected by both the immunoassays and dot-hybridization.

I. pepperi also acquired BBSSV from infected blueberry plants after 24-h AAPs, but the amount of BBSSV acquired was relatively low compared to acquisition from artificial sources of virus. In a similar experiment, Morimoto et al (16) suggested that the relatively low acquisition rate of BBSSV from plants may have been due to unequal distribution of virus throughout the plant. Investigations into the distribution of BBSSV in blueberry by transmission electron microscopy (10) and immunocytochemistry (26) support this claim.

Retention experiments showed that significant levels of BBSSV were retained at least 4 days after acquisition from sachets, although, BBSSV concentration dropped 75–90% during the initial 24-h inoculation feeding period. Possibly, much of the ingested virus was expelled or digested during this period. The immunoassays indicated that BBSSV levels between 0.5 and 1.2 ng were retained after this period, whereas dot-hybridization results indicated that up to 4.5 ng of BBSSV was retained. The BBSSV retention pattern was similar to the retention pattern of PEMV by its aphid vector (6), in which a 43% drop in virus content per aphid was observed during the first 24 h of post-acquisition feeding. The level of PEMV continued to decrease until the sixth day of post-acquisition feeding. After day 6, the level of virus remained constant for another 4 days. The initial decrease in retention for BBSSV was greater than for PEMV, but significant levels of BBSSV were present for periods that might be expected for persistent viruses. The presence of BBSSV in hemolymph samples suggests that virus had passed through gut epithelial cells, and movement of BBSSV through the hemocoel would be synonymous with other persistent viruses (8). In previous experiments, the internal distribution of iodinated BBSSV was monitored in the aphid after various AAPs, and [^{125}I]-label was found throughout (13). Again, the distribution of the [^{125}I]-label may have indicated that BBSSV circulated through the aphid in a manner similar to other persistently transmitted viruses, but the effect of iodination on viral integrity and transcellular passage of the virus was ambiguous. Furthermore, no transmission tests were done in that study.

In an effort to demonstrate circulative transmission, BBSSV was injected into the hemocoel of *I. pepperi*, followed by IAPs on healthy blueberry plants. However, these experiments failed, due to 100% mortality of aphids. Results from transmission experiments (i.e., aphids transmitted BBSSV up to 10 days after acquisition) were also similar to PEMV transmission times (20) and provide the best evidence that BBSSV is a persistently transmitted virus, but additional evidence is still needed to determine if a relatively short latent period (less than 24 h) is required before transmission. Such a latent period has been demonstrated for PEMV (18) in which a 12–18 h period after acquisition was necessary before transmission of the virus by the aphid occurred.

In conclusion, the transmission tests with *I. pepperi* suggest that BBSSV is persistent-circulative in nature, but it remains unclear whether transmission occurs within the 24-h period following acquisition or if BBSSV replicates within the aphid. The significance of immunoassay and dot-hybridization results are uncertain; while there appears to be a correlation between antigen and RNA levels and transmission, additional evidence is needed to demonstrate their role in transmission. As for the nature of the RNA detected in aphids, previous attempts to transmit BBSSV by feeding aphids purified BBSSV RNA were unsuccessful (*unpublished data*). Viral RNA was quickly degraded by ribonucleases in the aphid's body. Therefore, it was most likely that the RNA detected in the research data reported here, was virion-associated. We will examine these questions in more detail in future investigations.

LITERATURE CITED

1. Banttari, E. E., and Goodwin, P. H. 1985. Detection of potato viruses S, X, Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (dot-ELISA). Plant Dis. 69:202-205.
2. Bendayan, M. 1984. Enzyme-gold electron microscopic cytochemistry: A new affinity approach for the ultrastructural localization of macromolecules. J. Electron Micros. Tech. 1:349-372.
3. Callow, P., Haghigi, K., and Hancock, J. 1989. In vitro shoot regeneration on leaf tissue from micropropagated highbush blueberry. HortScience 24:373-375.
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. 34:457-483.
5. Clark, R. G., Converse, R. H., and Kojima, M. 1979. Enzyme-linked immunosorbent assay to detect potato leaf roll virus in potato tubers and viruliferous aphids. Plant Dis. Rep. 64:43-45.
6. Fargette, D., Jenniskens, M., and Peters, D. 1982. Acquisition and transmission of pea enation mosaic virus by the individual pea aphid. Phytopathology 72:1386-1390.
7. Gera, A., Loebenstein, G., and Raccah, B. 1978. Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay. Virology 86:542-545.
8. Gildow, F. E. 1985. Transcellular transport of barley yellow dwarf virus into the hemocoel of the aphid vector *Rhopalosiphum padi*. Phytopathology 75:292-297.
9. Gillett, J. M., Morimoto, K. M., Ramsdell, D. C., Baker, K. K., Chaney W. G., and Esselman, W. J. 1982. A comparison between the relative abilities of ELISA, RIA and ISEM to detect blueberry shoestring virus in its aphid vector. Acta Hortic. 129:25-29.
10. Hartmann, J. X., Bath, J. E., and Hooper, G. R. 1973. Electron Microscopy of viruslike particles from shoestring-diseased highbush bluberry, *Vaccinium corymbosum* L. Phytopathology 63:432-436.
11. Hsu, Y. H. 1984. Immunogold for detection of antigen on nitrocellulose paper. Anal. Biochem. 142:221-225.
12. Jayasena, K. W., Randels, J. W., and Barnett, O. W. 1984. Synthesis of a complementary DNA probe specific for detecting subterranean clover red leaf virus in plants and aphids. J. Gen. Virol. 65:109-117.
13. Klomparens, K. L., Peterson, M. A., Ramsdell, D. C., and Chaney, W. G. 1986. The use of scanning electron microscope autoradiography to localize the blueberry shoestring virus in its aphid vector. J. Electron Micros. Tech. 4:47-50.
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. 525 pp.
15. Maule, A. J., Hull, R., and Donson, J. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6:215-224.
16. Morimoto, K. M., Ramsdell, D. C., Gillett, J. M., and Chaney, W. G. 1985. Acquisition and transmission of blueberry shoestring virus by its aphid vector *Illinoia pepperi*. Phytopathology 75:709-712.
17. MSTAT. Version 4.0. Department of Crop and Soil Sciences. Michigan State University, East Lansing.
18. Osborn, H. T. 1935. Incubation period of pea mosaic in the aphid, *Macrosiphum pisi*. Phytopathology 25:160-177.
19. Ramsdell, D. C. 1979. Physical and chemical properties of blueberry shoestring virus. Phytopathology 69:1087-1091.
20. Simons, J. N. 1954. Vector-virus relationships of pea-enation mosaic and the pea aphid *Macrosiphum pisi* (Kalt.). Phytopathology 44:283-289.
21. Tamada, T., and Harrison, B. D. 1981. Quantitative studies on the uptake and retention of potato leafroll virus by aphids in the laboratory and in the field conditions. Ann. Appl. Biol. 98:261-276.
22. Taylor, J. M., Illmensee, R., and Summers, J. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta 442:324-330.
23. Terhune, B. T. 1989. Acquisition, retention and transmission of blueberry shoestring virus by its aphid vector, *Illinoia pepperi* (MacG.). M.S. thesis. Michigan State University, East Lansing. 170 pp.
24. Terhune, B. T., and Ramsdell, D. C. 1987. Detection and quantification of bluberry shoestring virus in *Illinoia pepperi* (MacG.) using dot-ELISA and dot-hybridization. Acta Hortic. 236:117-128.
25. Thomas, P. S. 1980. Hybridization of denatured RNA in small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77:5201-5205.
26. Urban, L. A. 1987. Movement and distribution of blueberry shoestring virus (BBSSV) in highbush cv. Jersey. Ph.D. thesis. Michigan State University, East Lansing. 170 pp.
27. Urban, L. A., Ramsdell, D. C., Klomparens, K. L., Lynch, T., and

Hancock, J. F. 1989. Detection of blueberry shoestring virus in xylem and phloem tissues of highbush blueberry. Phytopathology 79:488-493.

28. White, B. A., and Bancroft, F. C. 1982. Cytoplasmic dot hybridization: Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. 257:8569-8572.