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ACQUISITION, RETENTION AND TRANSMISSION OF BLUEBERRY SHOESTRING VIRUS BY ITS APHID VECTOR, ILLINOIA PEPPERI. (MACGILLIVRAY)

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Brian Troy Terhune

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ABSTRACT

ACQUISITION, RETENTION AND TRANSMISSION
OF BLUEBERRY SHOESTRING VIRUS BY ITS APHID VECTOR,
ILLINOIA PEPPERI (MACGILLIVRAY)

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Blueberry shoestring virus (BBSSV) was monitored in late instars of Illinoia pepperi by dot-ELISA, a silver enhanced-colloidal gold linked immunosorbent assay, and dot-hybridization. Aphids acquired both BBSSV-RNA and antigen after a 3 hr acquisition access period (AAP) from Parafilm^{R} sachets containing purified BBSSV, and after a 24 hr AAP on BBSSV-infected blueberry plants. BBSSV-RNA was acquired at higher concentrations than antigen. Levels of BBSSV-RNA and antigen retained by aphids declined rapidly 1 day after acquisition, but low levels persisted 3 to 4 days later. BBSSV-RNA and antigen were retained after a molt, and both were detected in aphid hemolymph after 1 to 4 day AAPs. Aphids were able to transmit BBSSV to blueberry plants 10 days after a 24 hr AAP. Immunocytochemistry with colloidal-gold indicated BBSSV was present in intestinal epithelial cells and accessory salivary glands after a 2 day AAP.

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INTRODUCTION

Blueberry shoestring virus (BBSSV) causes an economically important disease of highbush blueberry, (Vaccinium corymbosum L.), in Michigan (Ramsdell, 1985). The disease was first reported by Varney (1957) in New Jersey. Since then it has been reported in North Carolina (R. Milholland, personal communication, 1983), Washington state (P.R. Bristow and D.C. Ramsdell, unpublished), Michigan (Stretch and Hillborn, 1970), and Nova Scotia, Canada (Lockhart and Hall, 1962). Among all states, BBSSV causes the most severe loss in Michigan's blueberries. Michigan's blueberry crop suffered the largest loss due to BBSSV in 1983. A survey of approximately 1000 acres in Ottawa and Van Buren counties reported the incidence of BBSSV at 0.97% (H. Marlow, personal communication, 1983). The role of the blueberry aphid vector, Illinoia pepperi MacG., was first suggested by Ramsdell, et al. (1979), but aphid control was not implemented until 1982 and 1983. In addition, growers began replacing susceptible blueberry cultivars with resistant ones. As a result, surveys indicated the incidence of infection decreased to 0.1% by 1988 (H. Marlow, personal communication, 1988).

The epidemiology of the disease and aphid vector biology has been studied in the field (Morimoto et al., 1985b) and BBSSV-antigen acquisition and transmission rates by aphids were studied under laboratory conditions. These studies indicated that aphids could acquire BBSSV from artificial and natural virus sources in as little as 6 hr, and transmission occurs in less than 26 hr after initial virus access. Morimoto et al. (1985a) suggested the

relatively short acquisition and transmission times indicated that the aphid transmitted BBSSV in a semi-persistent manner. In later studies, the internal distribution of iodinated-BBSSV was monitored in the aphid following various acquisition access periods (AAP), and the ^{125}I label was found throughout the aphid after a 72 hr AAP (Klomparens et al., 1986). 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. Additional evidence was needed to elucidate the virus-vector relationship.

The objective of this research was to continue characterizing this virus-vector relationship by examining: 1) the rate of BBSSV antigen and RNA acquisition and retention, 2) transstadial passage of BBSSV antigen and RNA (i.e. retention of virus through the molt), 3) passage of BBSSV antigen and RNA into the hemocoel, 4) pre-transmission delay periods (i.e. latent period), and 5) ultrastructural localization of BBSSV in the aphid. Once the virus-vector relationship has been characterized, more efficient chemical and biological control strategies can be designed for use in the field.

LITERATURE REVIEW

Transmission of Plant Viruses by Aphids

Importance of Aphids as Virus Vectors

Understanding how viruses are dispersed is important for the control of virus diseases. Plant viruses are spread or vectored from plant to plant by pollen, seed, nematodes, fungi, parasitic plants, insects or mechanically. Insects have been shown to be one of the major agents of virus dispersal. The Homoptera constitute the largest group of plant virus vectoring insects. This order includes phytophagous insects such as aphids, leafhoppers, scale and whiteflies. Aphids vector more plant viruses than any other group of insects. There are about 4000 species of aphids known, and at least 300 species vector plant viruses. Aphids may be monophagous or polyphagous, and can vector viruses between different plant species. Dispersal of aphid-borne viruses vary depending on which morph is infectious. Alate (winged) morphs carry viruses greater distances than apterate (wingless) morphs (Eastop, 1977).

Differences in virus transmission by aphids also depends on the physical interaction between the virus and the aphid. Various interactions, or modes of transmission were first categorized by Watson and Roberts (1939) as nonpersistent and persistent. These categories were based on the length of time that the aphid remained infectious following virus acquisition. Sylvester (1956) described a third category, semipersistent, which had characteristics of both nonpersistent and persistent virus transmission. Kennedy (1962) later devised another classification scheme based on

the region of virus retention in the aphid. Nonpersistently and semipersistently transmitted viruses were classified as stylet borne, or noncirculative viruses, and persistently transmitted viruses were labeled circulative or propagative viruses. Stylet borne viruses were acquired within minutes, and infectivity was retained for only a few minutes to hours. Virus retention appeared to be localized to the stylets. Circulative and propagative viruses were transmitted by aphids for several days following a latent period. Evidence indicated that the virus circulated through the hemocoel, and was later expelled during salivation. Currently both classification schemes are used, and an additional category, bimodal transmission has been added. The characteristics of nonpersistent, semipersistent, and persistent virus transmission are given in table 1, and will be discussed in detail later.

Table 1. The Characteristics of Transmission for Homoptera-Borne Plant Viruses

Characteristic	Nonpersistent	Semipersistent	Persistent
acquisition	sec/min	min/hr	hr/days
tissue of acquisition	epidermis	epidermis/ phloem	phloem
retention	min/hr	hr/days	days/weeks
retention by molting nymphs	lost	lost	retained
latent period	none	none	required
transmitted following virus injection into hemocoel	no	no	yes

Anatomy of the Aphid

A working knowledge of aphid anatomy is fundamental in discussing the transmission of plant viruses by aphids. The mouthparts, the alimentary canal, and the salivary glands of the aphid are the most important structures involved in the transmission of plant viruses. These structures are shown in the lateral and transverse sections of blueberry aphids in figure 1.

The mouthparts consist of two pairs of flexible stylets protected and guided by the labrum and the labium (Forbes, 1977). The labrum is a short, triangular appendage suspended from the anteclypeus at the base of the head. The labrum covers the base of the labium and the stylet bundle. The inner surface has a groove which guides the stylet bundle. The labium, or proboscis, arises posterior to the labrum, and is extended in a posterior direction beneath the body. The labium is tubular, and it surrounds the stylet bundle. Its telescopic action allows the stylet to emerge from the tip. The stylet bundle consists of two mandibular stylets and two maxillary stylets. The four stylets arise in the head alongside the sucking pump, and converge as they enter the labium. The mandibular stylets contain a central duct which encase two dendrites. The dendrites appear to be sensory organs. The maxillary stylets lie adjacent to one another to form a centrally located food canal. The salivary canal is located entirely in the left maxillary stylet. The food canal empties into the sucking or cibarial pump. The pulsating action of the cibarial pump draws plant sap in when contracted, and expels ingested sap into the foregut when relaxed. The cibarial pump is located between the labium and the tentorial bar in the center of the head. It is lined by a thin flexible chitin wall. Adherence of virus particles to the lining of the food canal is presumed to play a role in nonpersistent virus transmission (Harris, 1977).

The foregut starts as a bulb-shaped pharynx just dorsal to the tentorial bar (Ponsen, 1972 and 1977). The pharynx is approximately 30 microns in diameter at its widest point, and it narrows to 20 microns in diameter as it joins the esophagus. Its lining is thinner than the pump chamber walls. The esophagus is a tube uniform in diameter. It extends into the mesothorax where it opens into the stomach (the midgut). An esophageal valve lies at a junction between the esophagus and the stomach. The wall of the foregut consists of squamous epithelial cells with large nuclei and indistinct cell borders. Both the pharynx and the esophagus have a smooth surface around the lumen, but farther back the walls project into the lumen. This produces a star-shaped lumen in cross section. The chitinous intima which lines the foregut is not attached to the surface of epithelial cells, and it is shed during ecdysis (the molt of the exoskeleton) at the end of each instar. Adherence of virus particles to the intima of the foregut is believed to play a role in semipersistent transmission.

The midgut consists of the stomach and the intestine (Ponsen 1972 and 1977). The entire midgut is made of a single layer of epithelial cells that rest on a basement membrane, the basal lamina. Three epithelial cell types occur in the stomach. The first lies adjacent to the esophageal valve. They are columnar and closely packed. The second cell type comprises most of the midgut. These are large, lobate, pyramidal to polygonal cells. These have free striated borders which protrude into the lumen due to numerous infoldings of the apical plasma membrane. The basal plasma membrane is also highly folded. The third cell type consists of flattened, smaller cells with elongated nuclei. The borders do not protrude into the lumen. These are located in the posterior region of the stomach. The intestinal cells are similar to the second cell type of the

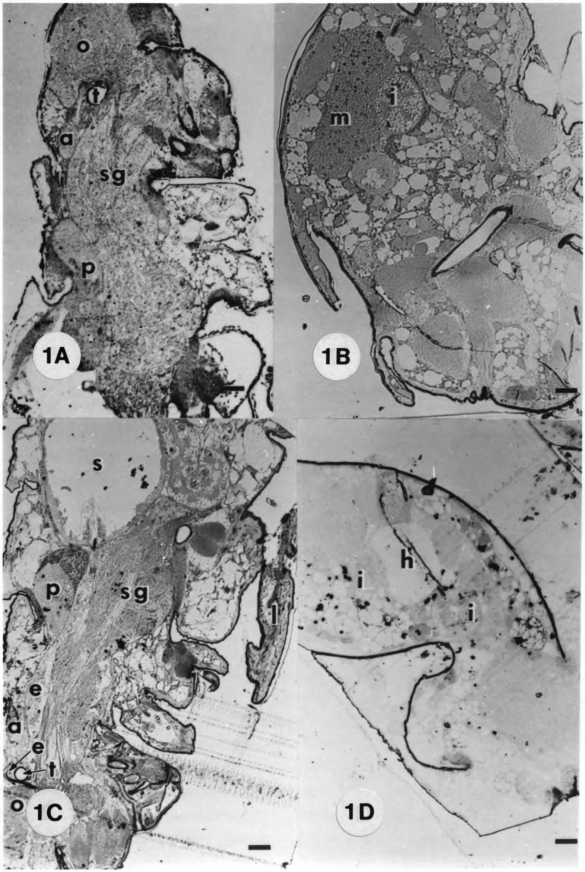
stomach. In cross section, the stomach appears circular with a large lumen, while the intestine is smaller with five cells that have apices projecting into the lumen. These apices almost occlude the intestinal lumen.

The hindgut is centrally located in the posterior region of the abdomen (Ponsen, 1972). It consists of thin-walled, squamous epithelial cells which rest on a basal lamina. The most distinguishing feature is the single row of extracellular microtubule-like structures which line the lumen side of the apical plasmalemma (Gildow, 1985). Gildow (1985) found transcellular passage of an aphid transmissible strain of barley yellow dwarf virus through the epithelial cells of the hindgut, but he did not find the same virions in the epithelial cells of the midgut. He suggested that this was the site of acquisition for persistently transmitted viruses, and that once virus was acquired, it circulated through the hemocoel until it was removed from the hemolymph by the accessory salivary glands.

Three important cell types occur in the hemocoel (body cavity): oenocytes, fat cells, and mycetomes (Ponsen, 1977). Oenocytes are large, polygonal cells situated laterally on either side of the body cavity. They are anchored by membranes originating from connective tissue. Fat cells initially are spherical, nucleated cells which become irregular in shape as lipid deposits accumulate intercellularly. Eventually, the nucleus dissolves, followed by degeneration of the cell membrane. The mycetome consists of two longitudinal masses of cells, or mycetocytes, linked together dorsal to the hindgut. The cytoplasm of the mycetocyte is filled with symbiotic microorganisms. The mycetocytes are enclosed in the mycetome by a nucleated sheath. This sheath disintegrates in adult aphids, and mycetocytes circulate as single cells in the hemocoel. The hemocoel also contains connective tissue, nerve tissues, and tissues associated with the respiratory system.

The salivary system consists of two sets of glands; the large principle salivary glands (PSG) and the smaller accessory salivary glands (ASG) (Ponsen, 1972 and 1977). The ASG have been associated with the persistent transmission of luteoviruses (Ponsen, 1972; Gildow, 1980). The ASG are located immediately posterior to the optic lobe of the nerve ganglion (Gildow, 1987). They consist of two, four-celled glands surrounded by a basal lamina. Below this is the basal plasmalemma which is highly invaginated. The cytoplasm contains secretory vesicles, multivesicular bodies and intercellular canals (canaliculi), but typical golgi do not occur. These intercellular canals are lined with microvilli, and are characteristic of ASG. The PSG differ ultrastructurally from ASG by the relatively low amount of invagination of their basal plasmalemma. The much larger PSG are located above the subesophageal ganglion, posterior to the ASG. Gildow (1980 and 1982) has observed virions from the transmissible strain of barley yellow dwarf virus in the basal lamina of the ASG, and similar virions in intercellular, coated and tubular vesicles.

Figure 1. Light micrographs of Illinoia pepperi. Figures A and C are tangential sections showing lateral views of the aphid head. Figures B and D are tangential sections showing a lateral view of the abdomen. The letters indicate the relative positions of an accessory salivary gland (a), the esophagus (e), the hindgut (h), the intestine (i), the labium (l), a mycetome (m), the optic lobe (o), the stomach (s), the subesophageal ganglion (sg), and the tentorial bar (t); the arrows show the junction between the pharynx and the esophagus and the tentorial bar. Bars = 0.1 mm.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Nonpersistent Virus Transmission

Nonpersistently transmitted viruses require no latent period for transmission. This virus group is generally localized in epidermal cells of the host plant. Aphids acquire the virus from the plant following probes lasting several seconds to a few minutes. Transmission efficiency decreases with increased acquisition periods, while postacquisition starving has been shown to increase transmission efficiency (Harris, 1977). It has been suggested that these observations result from the feeding behavior of aphids; during initial feeding, aphids make a series of short test probes in the epidermal tissue. If the plant tissue is suitable, longer feeding probes are made in which the stylet contacts phloem tissue. Consequently, the aphid is only exposed to epidermal-localized virions during the short test probes. Viruliferous aphids remain infective several minutes to a few hours, and the ability to transmit virus is lost following the molt.

Two hypotheses were used to explain nonpersistent transmission. The first, proposed by Doolittle and Walker (1928), suggested that transmission resulted from stylet contamination. The second hypothesis suggested that virions interacted with the lining of the food canal following ingestion, and were subsequently egested during short sampling periods (Harris, 1977). Bradley and Ganong (1955) gave evidence in support of the first hypothesis. They treated the terminal 15 microns of aphid stylets, following virus acquisition, with ultraviolet radiation or formalin. This rendered aphids nonviruliferous. They suggested that these agents affected transmission by inactivating virus on the stylet. Other investigators have suggested that these treatments may have altered aphid feeding behavior, and consequently, reduced transmission. The first explanation indicated that nonpersistent transmission was stylet-borne. Gamez and Watson (1964) found that viruliferous aphids could

not transmit virus if immediately anesthetized after insertion of the stylet into the plant, but they regained the ability to transmit virus once they recovered. This evidence supported the ingestion-egestion hypothesis based on the assumption that egestion was a conscious activity. Harris and Bath (1973) demonstrated egestion by allowing aphids to acquire carbon black particles from sachets, and then transferred aphids to carbon black-free sachets. Aphids egested, or regurgitated, carbon black particles during the subsequent feeding. Garrett (1973) obtained similar results using ^{32}P-labeled plant sap. Taylor and Robertson (1974) used electron microscopy to localize tobacco etch virus (TEV), a nonpersistent virus, in the distal part of maxillary food canal. This evidence led to suggestions that virions adhered to the lining of the stylet food canal, and were inoculated during egestion in subsequent probes (Harris 1977).

Some nonpersistently transmitted viruses require the aid of a helper component to facilitate transmission. Kassanis (1961) showed that coinfections of either potato virus Y (PVY) or potato virus A (PVA) were necessary for aphid transmission of potato aucuba virus. Hellman et al. (1983) demonstrated that a helper component protein was encoded on the PVY genome, and that this protein was necessary for aphid transmission of potato aucuba virus. More recently, Berger et al. (1986) used autoradiography to localize iodinated TEV acquired by aphid vectors following preacquisition feeding on the helper component necessary for transmission. They found virions accumulated in the stylet and foregut of aphids if they were initially fed helper component. Radiolabel accumulated in the stomach if aphids were not fed helper component. Although helper proteins have not been shown to be necessary for all nonpersistently transmitted viruses, this system indicates that specific adsorption of virus to the lining of the stylet and foregut may play a role in nonpersistent virus transmission.

Semipersistent Virus Transmission

Semipersistently transmitted viruses require longer acquisition periods. Transmission efficiency increases with increased feeding periods. Infectivity is not retained through ecdysis, i.e. molting, (Watson 1960), and viruliferous aphids remain infective several hours to a few days (Harris, 1977). Day and Venables (1961) found that virus could not be recovered from aphid hemolymph, and aphids were not rendered infectious following injection of virus into the hemocoel. Ultraviolet irradiation experiments by Bradley and Sylvester (1962) suggested that the semipersistent virus, sugar beet yellows, was not carried in the distal part of the stylet, but at some other point in the anterior alimentary canal. Murant et al. (1976) localized virus-like particles in thin sections of the foregut of aphids fed on plants infected with the semipersistent virus anthriscus yellows or doubly infected with anthriscus yellows and parsnip yellow fleck viruses. Particles were not found in the food canal or the cibarial pump. This evidence suggests that, unlike nonpersistent viruses, longer acquisition and retention periods may be required for virions to reach the foregut. In addition, longer acquisition and retention periods may be necessary because semipersistent viruses are generally localized in the phloem tissue of the plant.

Bimodal Virus Transmission

Bimodal virus transmission is a combination of nonpersistent and semipersistent transmission. It was first demonstrated by Chalfant and Chapman (1962) with the transmission of cabbage virus B (cauliflower mosaic virus) by the aphid, Brevicoryne brassicae L.. Another aphid vector, Myzus persicae (Sulz.), showed only nonpersistent

transmission, while B. brassicae showed both nonpersistent and semipersistent transmission. Neither aphid was able to transmit purified virus injected into the hemocoel. Lim and Hagedorn (1974 and 1975) also found bimodal transmission of pea seed-borne mosaic virus in one biotype of potato aphid. The current hypothesis suggests that differences in stylet morphology between aphid biotypes may account for differential binding of virions (Lim and Hagedorn, 1977).

Persistent Virus Transmission

Oortwijn Botjes (1920) first described a persistently transmitted virus, potato leaf roll virus. Watson and Roberts (1939) later categorized the relationship as persistent virus transmission. Kennedy (1962) divided persistent transmission into circulative and propagative virus transmission. Propagative virus transmission was distinguished from circulative virus transmission by the ability of propagative viruses to replicate in the aphid vector as well as the host plant. A longer latent period (greater than 14 days) and the ability to pass virus transovarially distinguishes propagative transmission from circulative transmission (Harris, 1977).

Persistent virus transmission is divided into three phases: 1) the virus acquisition phase, 2) the latent phase, and 3) the inoculation phase (Getz et al. 1982). The acquisition phase is characterized by the time required for virus uptake by the aphid vector. Persistently transmitted viruses usually require several minutes to hours for virus acquisition to occur. Unlike nonpersistent viruses, persistent viruses generally move and replicate in the phloem and phloem parenchyma of the plant. This location requires longer feeding probes before aphids can contact these tissues. Consequently, transmission efficiency increases with increased feeding periods (Sylvester, 1980).

The latent phase describes the delay required before transmission can occur. This latent period may last up to 14 days for circulative viruses (Sylvester, 1980; Getz et al., 1982). The inoculation phase is the period following the latent phase. During this period, virus is inoculated into plant tissue during feeding, and it is dependent on the probing behavior of the aphid. This period varies between 1 and 48 hr for different viruses (Sylvester, 1980). The combination of these three phases affects the length of time that virus transmission is retained.

The first evidence for a circulative relationship resulted from experiments by Day (1955) and Heinze (1955). They rendered aphids capable of virus transmission by injecting virus directly into the hemocoel. This work suggested that persistent viruses passed from the alimentary canal into the hemocoel. Nault (1964) later demonstrated that virus transmission was retained through ecdysis (molt). During a molt, the chitin layer of the foregut is shed. If the virus was adhering to the lining of the foregut, transmission would be lost, unless the virus passed through the foregut into the midgut. Once virions entered the hemocoel, they could move through the hemolymph to the salivary glands. If virions invaded the salivary glands, they could be expelled back into the plant during salivation.

The first ultrastructural studies using transmission electron microscopy (TEM) revealed the presence of virions in fat bodies and the gut lumen of viruliferous aphids (Shikata et al. 1966). Harris et al. (1975) later localized virions in gut epidermal cells of aphids following acquisition of pea enation mosaic virus, PEMV. When aphid transmissible strains of PEMV were injected into the hemocoel, virions accumulated in the basal lamina of both principle and accessory salivary glands, but they accumulated in the plasmalemma of the accessory salivary gland only. No virions were found in the salivary glands

when non-aphid transmissible strains of PEMV were injected into the hemocoel. Gildow (1980 and 1982) found that aphid transmitted luteoviruses accumulated in accessory salivary glands following injection of virus into the hemocoel, and he specifically identified particles in the basal lamina using ferritin immunochemistry. He later localized virions in the hindgut epithelial cells when aphids were fed transmissible virus strains, but virions did not accumulate in epithelial cells when aphids were fed non-transmissible virus strains. These results indicated that virions were acquired through gut epithelial cells, while unacquired virus was excreted. It also indicated that specific cell receptors interacted with transmissible virus strains.

In a recent review of persistent transmission of luteoviruses, Gildow (1987) suggested that upon acquisition of virus from infected plants, virus passes through the foregut and the midgut into the hindgut where the virus is endocytosed into hindgut epithelial cells in coated vesicles. Virus is then transported through the cell in smooth vesicles, and exocytosed into the hemocoel. Once in the virus circulates through the hemolymph to the accessory salivary gland, virions are endocytosed into the accessory salivary gland in coated vesicles. Coated vesicles fuse to intercellular canals, and expel virions into the salivary duct.

Methods of Virus Detection in Aphid Vectors

Reliable virus assays are essential to investigate virus-vector interactions. The first assays relied on aphid transmission experiments to determine retention times. These assays are very reliable, but may be ineffective if transmission efficiency is low or if a suitable assay plant does not exist. Eventually electron microscopy was used to localize virions in the aphid internally, but initially,

these virions could only be identified based on size. Since then, immunochemical staining using ferritin or gold probes has been used to identify virus-like particles in ultrastructural studies. Gildow (1982) used a technique in which anti-viral antibodies were injected into the hemocoel of viruliferous aphids followed by a second injection with goat anti-rabbit antibodies conjugated to ferritin. The disadvantage of this technique is that it could not localize intracellular virions. Immunogold labeling has been used by several researchers to localize virions in situ in plant tissues (Garzon et al., 1982; Lin and Langenberg, 1983; Tomenius et al., 1983; Ammar et al., 1985; Garnier et al., 1986; Giunchedi and Pollini, 1988), but it has not been successfully used to localize virions in aphid vectors. The successful application of this technique to aphids would allow intracellular, as well as intercellular, localization of virions. Autoradiography has been applied in light microscopy and in electron microscopy to detect iodinated virions in aphids (Berger et al., 1986; Klomparens et al., 1986). The disadvantage with this technique is that altering the capsid protein may interfere with any possible interactions with membrane receptors.

Gera et al. (1978) first used ELISA to detect cucumber mosaic virus in individual aphids. Since then ELISA has been used to monitor virus levels in individual aphids (Clark et al., 1979; Tamada and Harrison, 1981; Gillett et al., 1982), but these studies found that levels of virus acquired by individual aphids for a fixed access period were extremely variable, and the levels of virus per aphid were frequently below the detection endpoint of the assay. Radioimmunosorbent assay (RIA) was successfully used by Morimoto et al. (1985a) to detect BBSSV in individual aphids. This technique uses the double-antibody sandwich ELISA protocol with an iodinated secondary antibody as a label. Gillett et al. (1982) compared detection levels of ELISA with the radio-immunosorbent assay (RIA), and found

RIA to be a more sensitive and reliable technique than ELISA. Dot-ELISA uses a nitrocellulose or nylon membrane for a solid support instead of polystyrene, and it has been shown to be much more sensitive than other ELISA techniques when used to detect virus in plant samples (Bantarri, 1985; Urban, 1987). Attempts to use dot-ELISA to detect virus in aphids have been unsuccessful due to the high background absorbance responses from non-viruliferous aphids (Berger and Pirone, 1986; Urban, 1987). Recently, complimentary DNA (cDNA) probes, have been used to determine viral RNA levels in aphids (Jayasena et al., 1984). This technique can be used to monitor viral nucleic acid movement and distribution regardless of the presence of the coat protein.

Transmission of Blueberry Shoestring Virus (BBSSV) by Illinoia pepperi (MacG.)

Description of BBSSV

BBSSV is an isometric particle 27 nm in diameter. It has a sedimentation coefficient of 120 s, and a bouyant density in CsCl of 1.392 g/cm^3. It consists of 20% RNA and 80% protein. The RNA has a molecular weight of 1.45 x 10^6 daltons, and the protein subunits have a molecular weight of approximately 30,000 daltons. Purified virus aggregates at room temperature, but aggregation can be reversed at 4 C. It is stabilized by divalent metal cations (Ramsdell, 1979a). BBSSV is serologically unrelated to other viruses (Lesney and Ramsdell, 1976).

BBSSV is not transmissible to herbaceous hosts (Lesney et al. 1978), and it has been reported only in highbush blueberry [*Vaccinium corymbosum* L. (Varney, 1957)] and lowbush blueberry [*V. angustifolium* Ait. (Lockhart and Hall, 1962)]. BBSSV has been transmitted to blueberry by rub inoculation of purified virus and by chip budding (Lockhart

and Hall, 1962; Schulte, 1983). It is vectored by the blueberry aphid, Illinoia pepperi (MacG.) (Ramsdell, 1979b; Morimoto, 1985). BBSSV appears to most closely related to be the sobemovirus group based on its physical and chemical properties (Ramsdell, 1979a).

Typical BBSSV-caused symptoms on blueberry plants are strapped or crescent shaped leaves, oak-leaf patterns on leaves and red vein-banding on leaves. Reddish streaks generally develop on current and 1-year-old stems. Occasionally, flower petals are pink, and immature berries may develop a premature reddish-purple cast. The berry yield on infected plants is significantly reduced. A 4 year latent period usually occurs before symptoms develop (Ramsdell, 1979b). BBSSV has been identified by TEM, ELISA, and cDNA probes in epidermal, mesophyll, phloem and xylem plant tissues. The highest virus concentrations occur in root tissues (Hartmann, 1973; Urban, 1987).

BBSSV has been observed on the following highbush blueberry cultivars: Burlington, Coville, Earliblue, Jersey, June, Rancocas, Rubel, and Weymouth (Ramsdell, 1979b). Field observations have indicated that Burlington and Jersey have the highest incidence of infection, while Bluecrop has the lowest. Hancock et al. (1986) mechanically inoculated 28 cultivars of blueberry with purified BBSSV. They found 60-80 % of most cultivars tested positive for the virus by ELISA. Bluejay and Burlington had the lowest incidence of infection; 44 and 38 %, respectively. Bluecrop had an 80% infection rate, but it rarely shows symptoms in the field indicating the cultivar may be tolerant to infection by the virus. Wild blueberry populations, both highbush and lowbush, in Michigan have tested positive for BBSSV, but they also rarely show symptoms.

The Biology of *Illinoia pepperi* (MacG.)

Morimoto and Ramsdell (1985b) demonstrated that BBSSV was vectored from bush to bush by the blueberry aphid, *Illinoia pepperi* (MacG.). Taxonomically, this genus belongs to the subfamily, Aphidinae (Wilson, 1910). Aphidinae is divided into three tribes; Trichosiphini, Aphidini, and Macrosiphini. The genus *Illinoia* has been placed in the Macrosiphini tribe, which also includes *Amphorophora*, *Marcosiphina*, and *Myzus*. *Illinoia* is holocyclic (all morphs occur on a single host species) and monophagus (rarely oligophagous). Sexual forms are found only in autumn. They have no association with ants, and they do not cause galls on plants. Eggs, laid during spring and summer, diapause until the following spring. *Illinoia* have five morphological forms: apterous fundatrix, apterous viviparous females, alate viviparous females, apterous oviparous females, and alate males (Gilbert and Gutierrez, 1973). Four instars and an adult stage occur during the parthenogenic cycle. Both red and green morphs occur (Elsner, 1982). *Illinoia pepperi* (MacG.) has been shown to be distributed on the southwest side of Michigan (Giles, 1966). Adults overwinter in leaf litter and on the base of the bush. Seven or more parthenogenic generations may occur per year. The reproduction base temperature is 3.4 C. The number of degree days per instar increases with each successive instar. The generation time is 10 days at 23 C (Elsner, 1982).

The blueberry aphid prefers to feed on the underside of blueberry leaves, but alternate host plants are used during crowded conditions. Acceptable host plants include: red oak, black gum, common winterberry, holly and Prunus sp.. These alternate hosts do not appear to be significant, i.e. no lasting populations were found on these plants (Elsner, 1982). They occur on both natural and cultivated blueberry populations, but tend to have the highest populations on the

Spartan, Darrow, Lateblue, Coville and Jersey cultivars of highbush (Hancock et al., 1982).

Transmission of BBSSV by Illinoia pepperi (MacG.)

Previous research investigated several aspects of the relationship between BBSSV and its aphid vector. Analysis of maps from BBSSV-infected fields from 1958 and 1959 indicated a non-random, bush to bush spread pattern had occurred (Lesney et al. 1978). Morimoto et al. (1985a) demonstrated 5 to 28% transmission of BBSSV to 1-year-old healthy blueberry plants by aphids following a 24 hr virus acquisition access period (AAP) on BBSSV-infected plants and a 1 to 192 hr inoculation access period (IAP). They demonstrated that aphids could acquire detectable levels of BBSSV after 12 or 6 hr AAPs on Parafilm^{R} sachets containing BBSSV or BBSSV-infected plants, respectively. BBSSV levels varied from 0.15 to 11.3 ng per aphid. Epidemiological studies by Morimoto and Ramsdell (1985b) found that populations of alate and apterae aphids were greatest on blueberry during June. The percentage of BBSSV-positive aphids ranged from 0 to 30% throughout the growing season. Klomparens et al. (1986) used autoradiography to monitor the movement of ^{125}I-labeled BBSSV through Illinoia pepperi following acquisition of virus from sachets. After 72 hr, the radioactive label was distributed throughout the entire aphid. There were several explanations for this result: 1) BBSSV circulated through the aphid in a persistent manner, 2) the virus had degraded in the alimentary canal, and only the ^{125}I was circulating through the aphid, or 3) iodination of BBSSV may have altered the capsid protein, and resulted in enhanced cellular uptake. Although these results were not conclusive, the evidence indicated that BBSSV was not transmitted by Illinoia pepperi in nonpersistent manner.

METHODS AND MATERIALS

Aphids

Illinoia pepperi colonies were initiated with aphids acquired from the Michigan Blueberry Growers test plot at Grand Junction, MI. Aphid colonies were raised on 1 to 2-year-old, virus-free *V. corymbosum* L. cv. Jersey plants. Plants and aphid colonies were maintained at 18-25 C on a 16 hr 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 (1979). Blossoms were taken from shoots symptomatic for BBSSV from plants in the field, as described by Ramsdell (1979). One hundred grams of infected blueberry blossoms were homogenized for 3 to 5 min in 0.1 M potassium phosphate buffer containing 5 mM thioglycolic acid and 10 mM 2-mercaptoethanol, pH 7.0, in a Waring blender. Thirty-five ml of Triton X-100 (8% v/v) were slowly added to the homogenate, and stirred for 2 hr. Chloroform and n-butanol (5% v/v each) were added, and stirred 15 min. The mixture was squeezed through four layers of cheesecloth. The filtrate was centrifuged at 7.5K for 15 min. in Sorvall GSA rotor. Polyethylene glycol (M.W. 6000, 8% (w/v) and NaCl (0.1 M) were added to the upper aqueous phase, and

stirred 4 to 6 hr. The mixture was centrifuged for 30 min at 10K in a Sorvall SS-34 rotor. The pellet was resuspended in 10% of the starting volume with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiotreitol (P-DTT). The solution was allowed to stand 4 to 6 hr, and then centrifuged at 10K for 20 min. The supernatant was ultracentrifuged for 2 hr at 36K in a Beckman 40 rotor. The pellet was resuspended in 0.3 ml P-DTT buffer per tube, and allowed to stand overnight. The resuspended solution was layered on 0 to 30% linear sucrose gradients (0.3 ml/gradient). Gradients were prepared with P-DTT in SW41 tubes, and centrifuged in Beckman SW41 rotor for 90 min at 38K. Gradients were fractionated with an Isco Density Gradient Fractionator equipped with an ultraviolet analyzer (254 nm wavelength). Fractions containing virus were diluted 3x with 50 mM potassium phosphate buffer, pH 7.0, and ultracentrifuged for 3 hr at 36K in a Beckman 40 rotor. Pellets were resuspended with a glass rod in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.0, per tube, and allowed to stand overnight.

Micropropagated Blueberry Plants

Micropropagated blueberry plants used in BBSSV transmission experiments were prepared by P. Callow as described in P. Callow et al. (1989). Softwood cuttings were taken from greenhouse grown highbush blueberry cv. Jersey. The tissue was surfaced disinfested by 1) a 15 sec soak in 10% commercial bleach followed by a 5 sec dip in 95% ethanol and three rinses in sterile deionized water, or 2) if tissue was very "soft", it was dipped in 95% ethanol for 10 sec followed by three rinses in sterile deionized water. Sterile buds were placed on one-half strength Murashige-Skoog salts containing 27.84 mg/L $FeSO_4.7H_2O$, 37.24 mg/L

Na_2EDTA, 0.4 mg/L thiamine-HCl, 5 mg/L 6-(gamma, gamma-dimethylallylamino)-purine, 100 mg/L myo-inositol, 0.35% agar (w/v), 20 g/L sucrose; pH was adjusted to 5.0 with NaOH. Explants were subsequently subcultured to fresh medium every 4 wk. Plants were grown in Magenta GA-7 vessels or 250 ml glass Erlenmyer flasks in a culture room at 26 C under a 16 hr photoperiod with a light intensity of 25-40 uMol m^{-2} sec^{-1}. When shoots reached 2 to 3 inches in height, they were potted in Canadian peat moss, and enclosed in plastic bags to induce root formation. After 3 to 5 wk, plants were individually potted.

Immunoglobulin Purification

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 (1977). One ml of crude serum was added to 9 ml distilled water. Ten ml of saturated ammonium sulfate was then added slowly to crude serum while stirring. After incubating 1 hr at room temperature, the precipitate was collected by a low speed centrifugation at 5K for 5 min in a Sorvall SS-34 rotor. The precipitate was resuspended in 2 ml of 1/2 strength phosphate buffered saline [PBS, containing 15 mM phosphate and 8% (v/v) NaCl], and dialyzed three times against 500 ml 1/2 strength PBS. The IgG was passed through a 6 ml bed of DEAE-22 cellulose (Sigma Chemical Co., St. Louis, MO 63178) which was pre-equilibrated in 1/2 strength PBS. Two ml fractions were collected from a column monitored for absorbance at 280 nm. The fractions containing IgG were combined, and the concentration was adjusted to 1 mg/ml ($E^{0.1\%}_{280\ nm} = 1.4$).

Conjugation of Alkaline Phosphatase to IgG

One ml, or 1 mg of purified anti-BBSSV IgG was mixed with 2 mg of alkaline phosphatase (Alkaline Phosphatase P-0405 Sigma Chemical Co., St. Louis, MO 63178). Two ul of 25% glutaraldehyde were added to the solution, and allowed to incubate 4 hr at room temperature. Excess glutaraldehyde was removed by dialyzing the mixture against three changes of 1/2 strength PBS overnight at 4 C. After dialysis, bovine serum albumin (BSA) was added [5 mg/ml (w/v)]. The conjugate was stored at 4 C.

Conjugation of Colloidal-Gold to IgG

Ten nm diameter colloidal gold was conjugated to IgG as described by Bendayan (1984). One hundred and twenty ug of BBSSV IgG was dialyzed overnight against three changes of 2 mM borate buffer, pH 9.0. Ten ml of colloidal gold G10 (Janssen Life Science Products, 2430 Olen, Belgium) was adjusted to pH 9.0 with 0.2 M K_2CO_3. Dialyzed IgG was added to the colloidal gold, and stirred for 2 min. BSA was added to 1% (w/v). The mixture was centrifuged for 30 min at 25.5K in a Beckman type 40 rotor, and the supernatant was aspirated off. The red sediment portion of the pellet (conjugated gold-IgG) was resuspended in 1.5 ml of 10 mM PBS pH 7.3 containing 0.02% polyethylene glycol (MW 20,000). The solution was decanted leaving the black portion (unconjugated gold particles) of the pellet. The suspension was layered on a 10 to 30% glycerol gradient, prepared in 10 mM PBS pH 7.3. Gradients were centrifuged for 45 min at 21K in a Beckman SW41 rotor. The upper 1/3 of the gradient was collected for use.

Double-Antibody Sandwich ELISA

Aphid samples were homogenized in phosphate buffered saline, pH 7.4, containing 2% (w/v) polyvinylpyrrolidone and 0.2% (w/v) ovalbumin. Homogenates were assayed using DAS-ELISA as described by Clark and Adams (1977). Immulon I plates (Dynatech Co., Alexander, VA 22021) were coated with anti-BBSSV IgG, diluted 1:1000 (v/v) in extraction buffer, and incubated for 4 hr at 37 C. The plates were rinsed, and then incubated with samples overnight at 4 C. The plates were rinsed again, and coated with an IgG-alkaline phosphatase conjugate, diluted 1:800 (v/v), and incubated for 3 hr at 37 C. The plates were given a final rinse. The substrate, p-nitrophenyl phosphate was diluted to 1 mg/ml in 0.97% diethanolamine substrate buffer, pH 9.6, and added to the plates. The reaction was allowed to incubate for 15 to 30 min at room temperature. Absorbances of reactions were measured at 405 nm with a Dynatech ELISA-plate reader.

Dot-ELISA

Virus and aphid samples were tested by dot-ELISA (dot-blot immunosorbent assay) as described by Banttari et al. (1985). Nitrocellulose membranes (NCM) or nylon membranes (NM) were coated with a 1:1000 (v/v) dilution of anti-BBSSV IgG, and incubated 4 hr at room temperature. Membranes were washed six times (5 min/wash) in Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 80 (TBS-T80). The NCM was blocked 1.5 hr at 37 C with 3% BSA (w/v). The NM was blocked overnight at 37 C with 10% non-fat dry milk (w/v). Aphid samples were homogenized in TBS-T80 plus 1% polyvinylpyrrolidone (w/v). Virus and aphid samples were spotted onto the membranes, using a BioRad blotting manifold (BioRad Co., Richmond, CA 94804), and incubated overnight at

4 C. Membranes were washed 30 min in TBS-T80, and transferred to a solution containing an anti-BBSSV IgG-alkaline phosphatase conjugate diluted 1:800 (v/v) in TBS-T80. After incubating 3 hr at 23 C, membranes were rinsed 30 min. A substrate solution containing 0.06% (v/v) AS-MX phosphate and 3 mg/ml fast red TR salt (Sigma Chemical Co., St. Louis, MO 63178) was prepared 5 min before use. Membranes were transferred to the substrate solution, and allowed to incubate 15 to 60 min at room temperature. Positive reactions developed an insoluble, red pigment.

Detection of Antigen on Nitrocellulose or Nylon Membranes Using A Colloidal Gold-IgG Conjugate

The procedure described by Hsu (1984) was modified to detect BBSSV in aphids immobilized on nitrocellulose or nylon (Hybond-N, Amersham Corporation, Arlington Heights, Ill. 60005) using a colloidal gold/anti-BBSSV IgG conjugate. Nitrocellulose membranes (NCM) or nylon membranes (NM) were coated 4 hr at room temperature in sodium carbonate buffer, pH 9.6, containing anti-BBSSV IgG diluted 1:1000 (v/v). Membranes were washed six times (5 min/wash) in Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 80 (TBS-T80). The NCM was blocked 1.5 hr at 37 C with 3% BSA (w/v). The NM was blocked overnight at 37 C with 10% non-fat dry milk (w/v). Aphid samples were homogenized in TBS-T80 containing 1% polyvinylpyrrolidone (w/v). Virus and aphid samples were spotted on membranes, using a BioRad blotting manifold, and incubated overnight at 4 C. Membranes were washed 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 hr at 23 C, membranes were rinsed 30 min. Membranes were transferred to 20 ml of silver enhancer (IntenSE II, Janssen

Life Sciences Products, Piscataway, NJ 08854), and incubated 5 min at 23 C. Membranes were rinsed in distilled water, and air dried. Positive reactions developed an insoluble, black spot. This technique was referred to as dot-GLISA.

cDNA Probe Preparation

BBSSV RNA was extracted from purified BBSSV as described by Ramsdell (1979), and a cDNA probe was prepared as described by Taylor et al. (1976). Two to 5 ug of BBSSV-RNA were randomly primed with salmon sperm DNA primers, and a complementary DNA strand was synthesized with 4 units AMV-reverse transcriptase/ug BBSSV RNA. The reaction solution contained 0.05 M Tris (pH 8.3), 8 mM $MgCl_2$, 8 mM dithiothreitol, 0.02 ug/ml actinomycin-D, 0.3 mM dATP, 0.3 mM dGTP, 0.3 mM dTTP, 0.1 mM dCTP, 20 uCi (alpha-^{32}p) dCTP (Amersham), 12 mM KCl, and 8 mM Triton X-100. After a 2 hr incubation period, incorporated and unincorporated nucleotides were separated by gel-filtration on Sephadex G-50. RNA was removed from the purified cDNA-RNA hybrid by boiling in 0.1 N NaOH for 5 min.

Dot-Hybridization

All glassware was treated for RNase by baking 4 hr at 250 C or soaking in diethylpyrocarbonate (DEPC); buffers were also DEPC-treated. Aphid samples were homogenized in 50 ul phosphate buffer, pH 7.4, followed by the addition of 30 ul 20x SSC (SSC = 3 M NaCl and 0.3 M sodium citrate, pH 7.0) and 20 ul of 37% formaldehyde. Virus and aphid samples were heated 15 min at 60 C then chilled on ice. Samples were spotted on NCM for 30 min, and then baked for 2 hr at 80 C in vacuo. Membranes were prehybridized overnight at 65

C in a solution containing 2x SSC, 40% Denhardt's solution [Denhardt's = 0.8% (w/v) BSA, 0.8% (w/v) Ficoll 400, 0.8% (w/v) polyvinylpyrrolidone] and 1.5 mg salmon sperm DNA/cm^2 membrane. Three by 5 inch membranes were hybridized overnight at 65 C in 15 ml of 50% (v/v) formamide (37% stock solution), 1% (w/v) glycine, 5x SSC, 0.2% (w/v) sodium dodecyl sulfate (SDS), 0.05 M phosphate buffer, pH 7.4, 0.1% Denhardt's solution, 1 mg/ml salmon sperm DNA, 1 to 5 x 10^6 dpm/ml ^{32}P cDNA probe. Membranes were then washed four times (5 min/wash) in 2x SSC containing 0.1% SDS (w/v) at 23 C followed by two washes (15 min/wash) at 65 C in 0.1x SSC containing 0.1% SDS (w/v). Air-dried membranes were exposed to X-ray film 48 hr at -20 C (Maule, et al., 1983; Thomas, 1980; White et al., 1982).

Quantification of Dot-ELISA, Dot-hybridization and Immunogold Assays

In dot-ELISA, fast red TR salt precipitates on the membrane, producing an insoluble red spot which varies in intensity depending on virus concentration. To measure spot intensity, membranes were photographed on 4 x 5 inch color film (1:1 reproduction). The resulting transparencies were scanned for percent light absorbed at 550 nm on a Gilford Response II scanning spectrophotometer. Spectrophotometer measurements were reported as percent absorbance. Autoradiographs were also scanned at 550 nm for percent light transmission. Two-fold serial dilutions of purified virus were spotted on membranes (three to five spots/dilution) to establish a dose-response curve. Regression analysis of response values generated polynomial models which were used to estimate the concentration of BBSSV in aphids.

Effect of Aphid Homogenate on BBSSV Detection

Aphid-amended and non-amended virus dilution series were compared using DAS-ELISA, dot-ELISA on NCM and NM, dot-GLISA on NCM and NM, and dot-hybridization on NCM. Five separate virus preparations were serially diluted to a final concentration ranging from 0.22 to 2.50 ng BBSSV/ml, using the appropriate assay extraction buffer. Individual aphids, raised on healthy blueberry plants, were homogenized in 200 ul of each virus dilution. Aphid-amended and non-amended virus dilutions were assayed using the four assay techniques. Absorbance responses were plotted against virus concentration, and treatment differences were compared by the Student's T-test.

Each technique was also evaluated for detection limit of purified BBSSV, background absorbance resulting from aphid homogenate alone, and detection of BBSSV in aphids following acquisition access periods from 24 to 96 hr on Parafilm^{R} sachets containing 20% (w/v) sucrose plus 50 ug/ml BBSSV. The average detection endpoints (3 to 5 replicates per assay) were compared by Duncan's multiple range test. To compare differences between aphids positive for BBSSV and aphids negative for BBSSV by each assay, the sum total of absorbances from aphids given access to BBSSV were divided by the sum total of absorbances from aphids not given access to BBSSV (i.e. positive to negative ratio). In addition, the percentage of positive aphids was determined as those aphids given access to BBSSV whose absorbance was greater than the mean absorbance for aphids not given access to BBSSV plus three standard deviations of that mean.

Acquisition of BBSSV Antigen and RNA by Illinoia pepperi

Late instar and adult aphids were allowed to feed on Parafilm^{R} sachets, containing 50 ug BBSSV/ml in 20% sucrose (w/v), or symptomatic shoots of BBSSV-infected plants. Aphids were removed after various acquisition access periods (AAP), and individual aphids were assayed for BBSSV antigen and RNA by dot-ELISA, dot-GLISA and dot-hybridization. Three to four aphids were sampled in one to eight replications for each acquisition access period (AAP). Assay absorbance values were converted to ng BBSSV per aphid (using the purified virus dose-response models), and plotted against the AAP. The percentage of positive aphids (i.e. greater than the healthy mean plus three standard deviations) was also compared against the length of the AAP.

Retention of BBSSV Antigen and RNA by Illinoia pepperi

Late instar and adult aphids were allowed to feed on Parafilm^{R} sachets containing 50 ug BBSSV/ml in 20% sucrose (w/v) or symptomatic shoots of BBSSV-infected plants for 1 to 4 days. Viruliferous aphids were transferred to healthy blueberry plant, and allowed to feed for inoculation access periods (IAP) between 15 min and 4 days. Three to four aphids were sampled for BBSSV antigen and RNA by dot-ELISA, dot-GLISA and dot-hybridization with three to eight replications for each inoculation access period (IAP). Concentrations of BBSSV and the percentage of positive aphids were compared during over various IAPs.

To monitor retention of BBSSV following a molt, aphids were allowed to feed on Parafilm^{R} sachet and plant virus sources for 1 to 4 days, and individual aphids were transferred to excised, healthy blueberry leaves suspended

above moist filter paper in Petri dishes. Leaves were checked daily for the presence of a shed exoskeleton. Following a molt, the aphid was immediately assayed by dot-ELISA or dot-hybridization or stored at -20 C and assayed a few days later. Assay absorbance responses were converted to ng BBSSV per aphid using purified virus dose-response models.

Detection of BBSSV Antigen and RNA in Aphid Hemolymph

Late instar and adult aphids were allowed to feed on Parafilm^{R} sachets containing 50 ug BBSSV/ml in 20% sucrose (w/v) or symptomatic shoots of BBSSV-infected plants for 1 to 4 days. Hemolymph samples were withdrawn from the abdomen of five aphids following each day of AAP. Samples were acquired by probing the exoskeleton on the dorsal side of the abdomen with a finely drawn glass needle. Once the needle pierced the exoskeleton, hemolymph (approx. 0.1 to 0.5 ul) was withdraw by capillary action. Hemolymph samples from five aphids were expelled into 200 ul of 0.01 M phosphate buffer (pH 7.0), and assayed by dot-ELISA or dot-hybridization. The remaining aphid was ground in the appropriate assay buffer, and assayed also.

Aphid Transmission of BBSSV to Healthy Blueberry Plants Following Daily Serial Transfers

Late instar and adult aphids were allowed to feed on Parafilm^{R} sachets containing 50 ug BBSSV/ml in 20% (w/v) sucrose or symptomatic shoots of BBSSV-infected plants for 1 to 4 days. Groups of 15 aphids were transferred from the virus source to caged, 3 to 5 mo-old, healthy micropropagated blueberry plants (cv. Jersey), and allowed

to feed for 24 hr. Following this period, the group of aphids was transferred to a new healthy blueberry plant, and allowed to feed for 24 hr. 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 hr photoperiod at 65 to 75 C for 6 mo. Then, 2 to 5 g of shoots or roots were assayed by dot-ELISA on NCM. Blueberry plant samples with absorbance values greater than the mean for healthy blueberry plant samples plus three standard deviations were considered positive for BBSSV.

Preparation of Plant and Aphid Samples for Ultrastructural Examination

The fixation and embedding techniques used here were described previously (Gildow, 1982; Spurr, 1969). Aphids were immersed in fixative, and bisected transversely with a razor blade or the exoskeleton was pierced with a fine glass needle. Fixative consisted of 1% formaldehyde (w/v), 2% glutaraldehyde, 0.01% $CaCl_2$ (w/v), and 0.05% sodium azide (w/v) made in 0.01 M potassium phosphate buffer (pH 7.0). Samples were fixed 2 hr at 4 C, rinsed two times (15 min per rinse) in phosphate buffer, and fixed 1 hr in 1% osmium tetroxide (v/v). Samples were rinsed two times in distilled water, and stained overnight in 2% aqueous uranyl acetate (w/v). Samples were rinsed in distilled water two times, and dehydrated in an ethanol series (25, 50, 75, 95, and 100%) over a 4 hr period. Samples were dehydrated in absolute ethanol for 24 hr, and infiltrated in Spurr's epoxy resin (Spurr, 1969) over a 3 day period. The resin consisted of 10 g vinyl cyclohexene dioxide, 6 g diglycidyl ether of propylene glycol, 26 g of nonenyl succinic anhydride and 0.4 g of dimethylaminoethanol. For light

microscopy, 1-um thick sections were cut with glass knives on a Porter-Blum MT-2 ultramicrotome. Sections were mounted on glass slides by heating overnight at 60 C, and stained for 1 min at 60 C with 1% toluidine blue (w/v) in 1% sodium borate (w/v). Sections were mounted with a drop of xylene-thinned permount medium. The permount medium was spread with a cover glass, and polymerized by heating at 60 C for 48 hr. For transmission electron microscopy (TEM), sections 60 to 80 nm thick were cut with a Dupont^{R} diamond knife. Sections were picked up on formvar and carbon-coated grids, and stained 5 min with 2% lead citrate (w/v) followed by a 1 min rinse in 0.02 N NaOH, and a 1 min rinse in distilled water. Grids were examined at 60 kV with a Philips 201 transmission electron microscope.

Immunogold Detection of BBSSV in Samples Embedded in Spurr's Resin

The technique used for labeling virions in thin sections was previously described by Ammar and Nault (1985). To determine the concentrations of anti-BBSSV IgG and protein A-colloidal gold (pAg) which would result in the highest density of gold particles per virion, formvar and carbon-coated grids were floated on a drop of purified BBSSV [diluted 1/100 (v/v)] for 2 min. Grids were blotted with Whatman filter paper, and placed on 1/50, 1/100 or 1/500 dilutions (v/v) of immunoglobulin for 4 hr at 23 C or overnight at 4 C. Grids were rinsed by placing them on a drop of 0.01 M potassium phosphate buffer (pH 7.0) for 5 min, and placed on 1/10, 1/100 or 1/500 dilutions (v/v) of pAg (Polysciences, Inc., Warrington, PA 18976) for 30 min at 37 C, 1 hr at 23 C or 4 hr at 23 C. Grids were rinsed for 30 sec with a stream of distilled water, and stained with 2% ammonium molybdate (w/v). Labeling was quantified by

counting the number of gold particles labeling each virion. Thick and thin sections of samples were etched for 30 min with saturated sodium metaperiodate, and then rinsed with distilled water for 30 sec. Gold labeling was performed as described above with a 1/50 dilution (v/v) of anti-BBSSV IgG for 4 hr at room temperature and a 1/10 dilution (v/v) of pAg for 4 hr at room temperature. Thin sections were stained with 2% lead citrate (w/v) for 5 min, and rinsed 2 min on a drop of 0.2 M NaOH followed by 2 min on distilled water. Thick sections were stained with the silver enhancer (used for dot-GLISA) for 5 min, and rinsed with distilled water.

RESULTS

Detection of Blueberry Shoestring Virus (BBSSV) by Dot-ELISA, Silver Enhanced Dot-GLISA, and Dot-Hybridization

Nanogram quantities of both the BBSSV-antigen and the BBSSV-RNA were routinely detected by the assays used for these studies. The immunoassays, dot-ELISA and silver-enhanced colloidal gold-linked immunosorbent assay (dot-GLISA) were more sensitive than the RNA assay, dot-hybridization, and they were capable of detecting picogram quantities of BBSSV. Dot-hybridization had a BBSSV detection endpoint of 1.93 ng. The BBSSV detection endpoint for the immunoassays varied dependent upon the type of membrane that was used as a solid support. Dot-ELISA had a detection endpoint of 0.94 ng when nitrocellulose membrane (NCM) was used as a solid support, and 1.37 ng when nylon membrane (NM) was utilized. Dot-GLISA gave similar results, 0.77 ng on NCM and 1.78 ng on nylon. The BBSSV detection endpoint of double-antibody sandwich ELISA (DAS-ELISA) was compared with dot-hybridization, dot-ELISA and dot-GLISA (Table 2). A multiple range test indicated that dot-ELISA and dot-GLISA on NCM were the most sensitive assays, while DAS-ELISA and dot-hybridization were the least sensitive. The sensitivity of dot-GLISA was greatly reduced if the silver enhancement procedure was omitted. The red spots, which resulted from colloidal gold labeling, were barely visible, but became dark black spots following silver enhancement. Membranes could not be incubated in the silver

enhancer for more than 5 min, or a black background developed. To improve the response, membranes were reincubated in a fresh solution of silver enhancer for 5 min.

To determine the specificity of the assays, brome mosaic virus (BMV) and southern bean mosaic virus (SBMV) were tested along with BBSSV. Neither the antigen nor the RNA assay reacted with BMV or SBMV. All three assays detected BBSSV in homogenized blossoms, stems and leaves from BBSSV-symptomatic highbush blueberry, cv. Jersey, but they did not react with the same tissues from healthy blueberry.

Table 2. The Blueberry Shoestring Virus Detection Endpoint for DAS-ELISA, Dot-ELISA on Nylon and Nitrocellulose (NCM) Membranes, Silver-Enhanced Dot-GLISA on Nylon and Nitrocellulose Membranes and Dot-Hybridization on NCM

Assay[a]	Mean Detection Endpoint (ng BBSSV)[b]	
dot-ELISA on NCM	0.94	a
dot-ELISA on nylon	1.37	b
dot-GLISA on NCM	0.77	a
dot-GLISA on nylon	1.78	c
DAS-ELISA	2.01	d
dot-hybridization on NCM	1.93	d

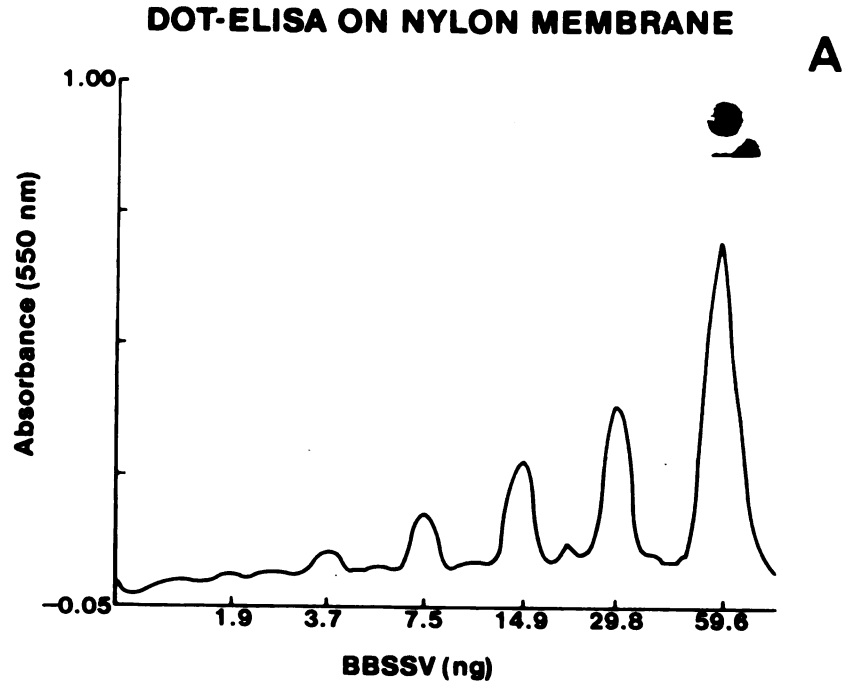
[a] DAS-ELISA = double-antibody sandwich - enzyme-linked immunosorbent assay. Dot-ELISA = enzyme-linked immunosorbent assay on nitrocellulose or nylon membrane. Dot-GLISA = silver-enhanced, colloidal gold immunosorbent assay on nitrocellulose or nylon membrane.

[a] Values derived from assays (replicated four times) of a two fold dilution series of purified BBSSV with three samples per dilution. Dilutions ranged from 0.22 to 158 ng BBSSV. Letters indicate significant differences between means [Duncan's multiple range test ($p = 0.05$)].

Quantification of BBSSV

Spot intensities from all three assays were quantified spectrophotometrically to determine the concentration of BBSSV in aphid and plant samples containing unknown quantities of BBSSV and for statistical verification. Autoradiograms from dot-hybridization could be directly scanned on a transmission spectrophotometer. The nitrocellulose and nylon membranes were too dense to allow light transmission, and required the use of a reflectance spectrophotometer or translucent replicas. Four by five inch photographic color negatives enabled a membrane, containing 96 spots, to be reproduced without changing the size of the spots. Each assay included a two-fold dilution series of a known concentration of purified BBSSV. The mean absorbance responses (three samples per dilution) were plotted against the concentration of BBSSV. The resulting dose-response curve was analyzed by multiple regression using Log X and $(\text{Log X})^2$ transformations of the dose values (i.e. BBSSV concentration). This gave a polynomial model which was used to estimate concentrations of BBSSV in aphid and plant samples. Figures 2A and 2B show typical scans of a BBSSV dilution series from a dot ELISA reproduction and a dot-hybridization autoradiogram, respectively. The increase in peak height represented an increase in spot intensity; the spots were included at the top of the figure. The resulting dose-response curves (Figures 3A and 3B) indicated a curvilinear model best described the absorbance response over a logarithmic increase in BBSSV concentration.

Figure 2. **Absorbance peaks derived from a 550 nm scan of spots on A) a 4 by 5 inch color transparency reproduction of a dot-ELISA on nylon membrane, and B) a dot-hybridization autoradiogram. Spots, shown at the top of each scan, were produced when a two-fold blueberry shoestring virus (BBSSV) dilution series was assayed.**


DOT-ELISA ON NYLON MEMBRANE
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1.00
-0.05
Absorbance (550 nm)
1.9
3.7
7.5
14.9
29.8
59.6
BBSSV (ng)

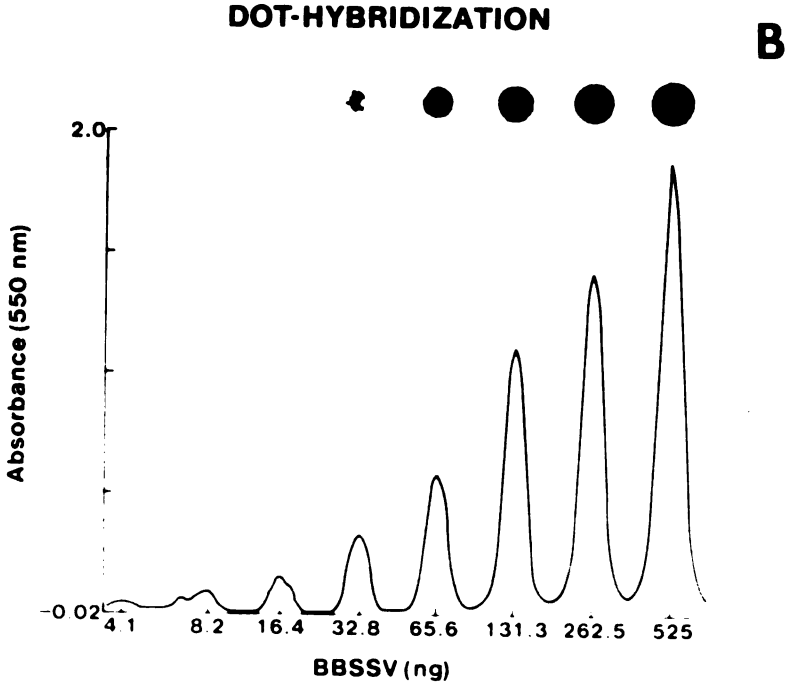

DOT-HYBRIDIZATION
B
2.0
-0.02
Absorbance (550 nm)
4.1
8.2
16.4
32.8
65.6
131.3
262.5
525
BBSSV (ng)

Figure 2

Figure 3. The dose-response curves generated by regression analysis of absorbance responses (550 nm) resulting from a two-fold blueberry shoestring virus dilution series assayed by A) dot-ELISA on nylon membrane ($Y = -.007 + .05X + .23X^2$, $r^2 = .99$), and B) dot-hybridization on nitrocellulose ($Y = .16 - .42X + .35X^2$, $r^2 = .99$).

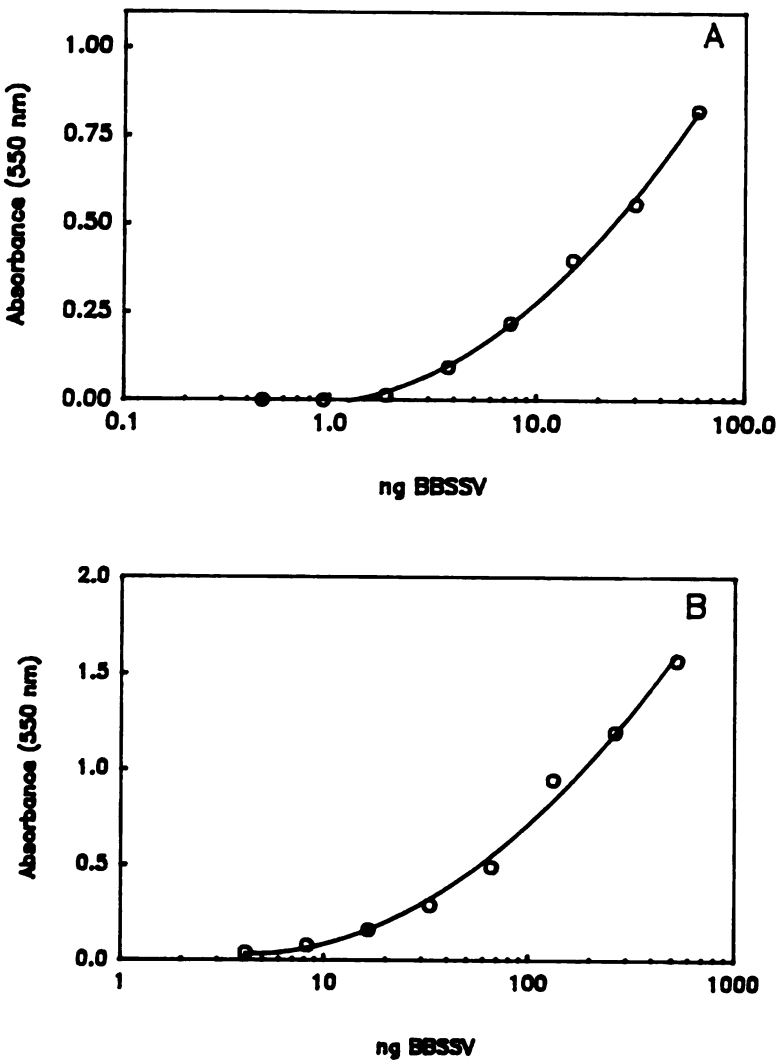

A
1.00
0.75
0.50
0.25
0.00
Absorbance (550 nm)
0.1
1.0
10.0
100.0
ng BBSSV
B
2.0
1.5
1.0
0.5
0.0
Absorbance (550 nm)
1
10
100
1000
ng BBSSV

Figure 3

Detection of BBSSV in Illinois peppers

DAS-ELISA, dot-ELISA, dot-GLISA and dot-hybridization were examined for 1) the effect of aphid homogenate on the detection of BBSSV, 2) the ability to detect BBSSV acquired by aphids during feeding, and 3) the response of aphids in the absence of BBSSV. In dot-ELISA, dot-GLISA and dot-hybridization, aphid homogenate amendments added to purified BBSSV decreased the absorbance responses 40 to 60% (Figures 4A, 4B, 5A, 5B and 6B). Aphid homogenate amendments added to purified BBSSV increased absorbance responses in DAS-ELISA approximately 12% (figure 6A). This indicated that the BBSSV concentration would be underestimated when viruliferous aphids were assayed by dot-ELISA, dot-GLISA and dot-hybridization.

Dot-ELISA on NM was the most discriminating assay for detecting BBSSV ingested by aphids. Seventy-nine percent of the aphids allowed to feed on sachets containing BBSSV tested positive by this assay with a 14:1 positive aphid absorbance-to-negative aphid absorbance ratio (Table 3). Dot-GLISA on NCM also detected BBSSV in viruliferous aphids at a high frequency (72%), but with less distinction between viruliferous and non-viruliferous aphids (a positive-to-negative ratio of 5:1). Other variations of dot-ELISA and dot-GLISA resulted in both lower BBSSV detection frequencies in aphids, and they had a lower positive-to-negative aphid ratio (Table 3). Due to their high detection frequencies, both Dot-ELISA on nylon and dot-ELISA on NCM were used in experiments to monitor BBSSV levels in aphids.

Table 3. Detection of Blueberry Shoestring Virus (BBSSV) in Aphids Fed on Sachets Containing BBSSV using DAS-ELISA, Dot-ELISA on Nylon Membrane and Nitrocellulose Membrane (NCM), Silver-Enhanced Dot-GLISA on Nylon Membrane and NCM, and Dot-Hybridization on NCM

Assay[a]	% Positive Aphids[b]	Ratio of Positive to Negative Aphid Absorbance Responses[c]
dot-ELISA on NCM	53	11:1
dot-ELISA on nylon	79	14:1
dot-GLISA on NCM	72	5:1
dot-GLISA on nylon	45	3:1
DAS-ELISA	25	5:1
dot-hybridization	57	3:1

[a] DAS-ELISA = double-antibody sandwich - enzyme-linked immunosorbent assay. Dot-ELISA = enzyme-linked immunosorbent assay on nitrocellulose or nylon membrane. Dot-GLISA = silver-enhanced, colloidal gold immunosorbent assay on nitrocellulose or nylon membrane.

[b] Positive aphids had absorbance responses (A_{550} nm) greater than the mean + 3 standard deviations. Results given are the mean number of positive aphids divided by the total number of aphids assayed x 100.

[c] The positive to negative ratio was determined by dividing the sum of absorbance values for positive aphids by the sum of the absorbance values for negative aphids.

Figure 4. **The effect of aphid homogenate on detection of blueberry shoestring virus (BBSSV) using dot-ELISA on A) nitrocellulose and B) nylon membrane: Data points represent mean A 550 nm values at each virus concentration from a 2-fold serial dilution (n = 5); bars represent standard error.**

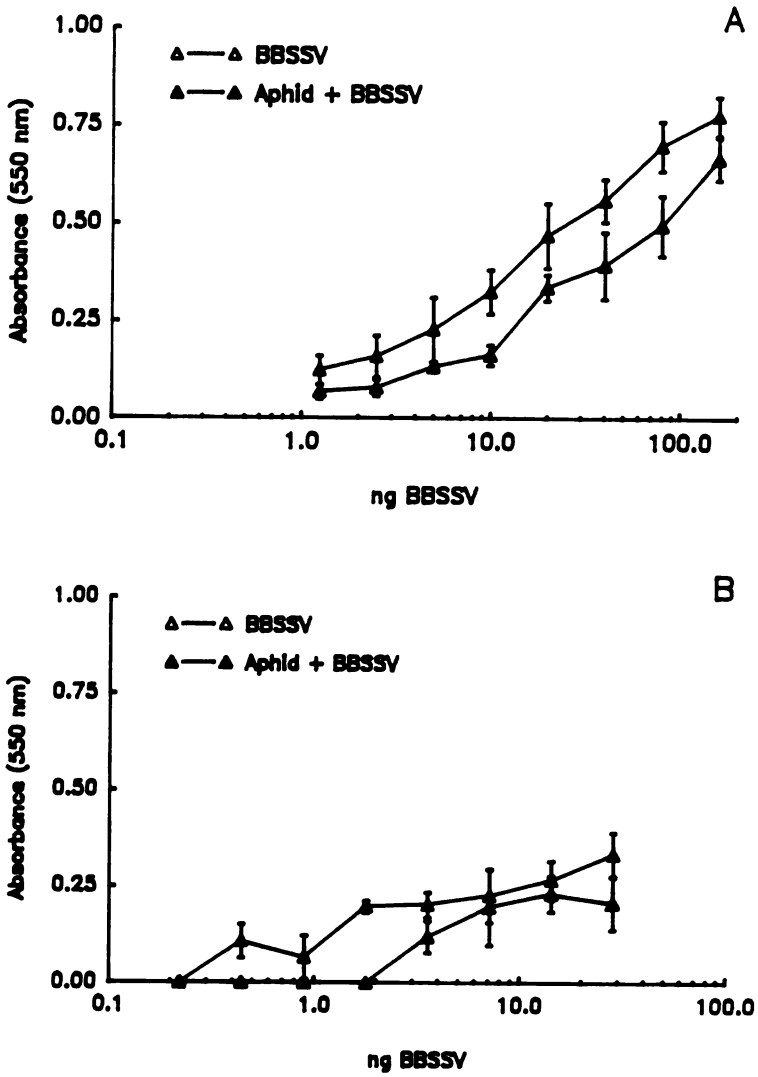

A
1.00
0.75
0.50
0.25
0.00
Absorbance (550 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
ng BBSSV
B
1.00
0.75
0.50
0.25
0.00
Absorbance (550 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
ng BBSSV

Figure 4

Figure 5. The effect of aphid homogenate on detection of blueberry shoestring virus (BBSSV) by a silver-enhanced colloidal gold-dot immunoassay on A) nitrocellulose and B) nylon membrane: Data points represent mean A 550 nm values at each virus concentration from a 2-fold serial dilution (n = 5); bars represent standard error.

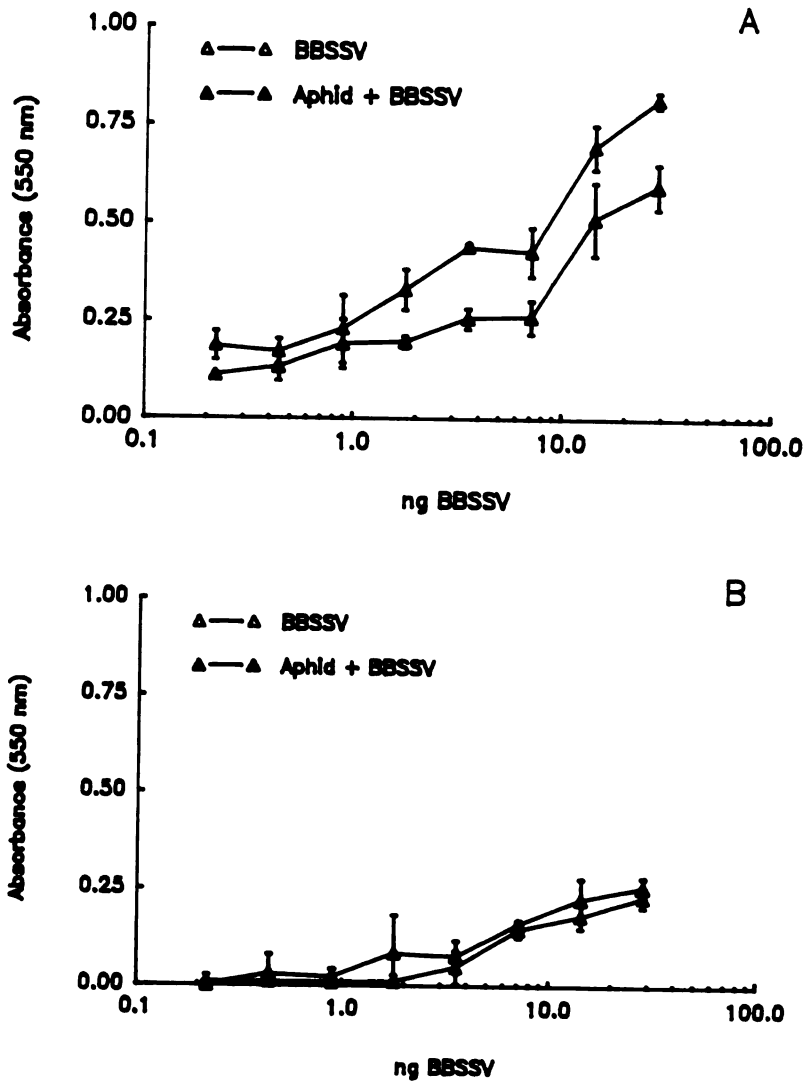

A
1.00
0.75
0.50
0.25
0.00
Absorbance (550 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
ng BBSSV
B
1.00
0.75
0.50
0.25
0.00
Absorbance (550 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
ng BBSSV

Figure 5

Figure 6. The effect of aphid homogenate on detection of blueberry shoestring virus (BBSSV) by A) DAS-ELISA, and B) dot-hybridization: Data points represent mean A 550 nm values at each virus concentration from a 2-fold serial dilution (n = 5); bars represent standard error.

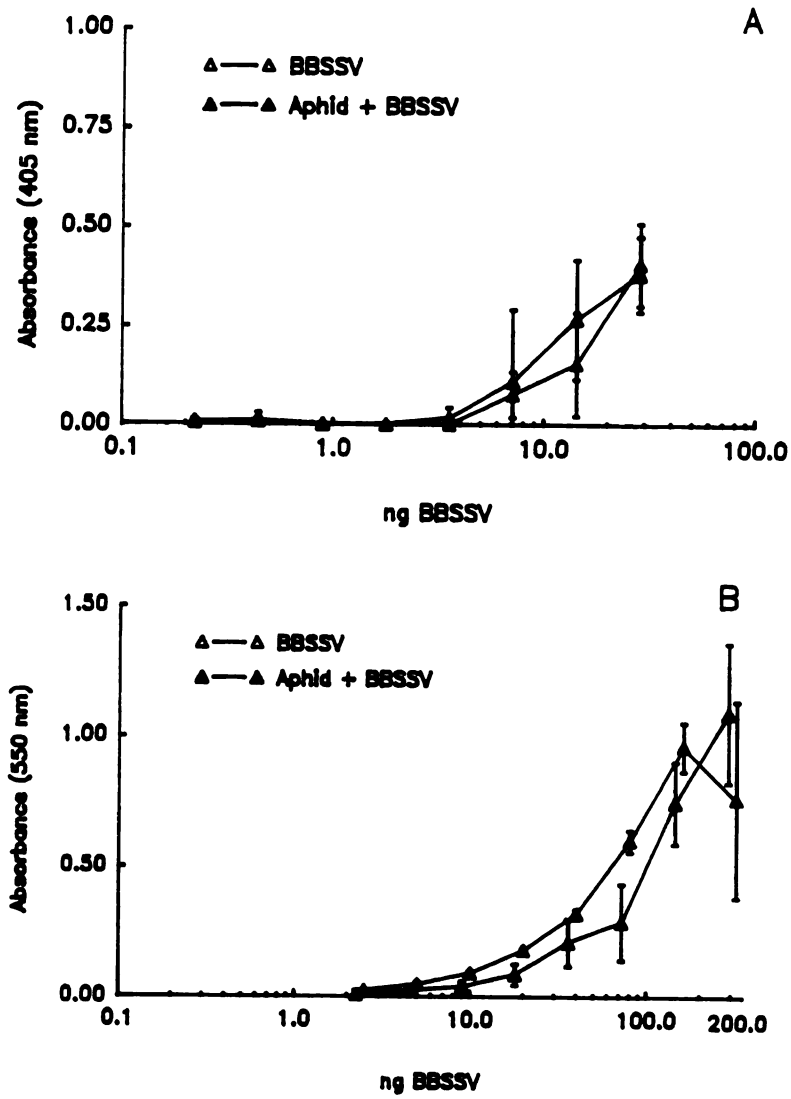

A
1.00
0.75
0.50
0.25
0.00
Absorbance (405 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
ng BBSSV
B
1.50
1.00
0.50
0.00
Absorbance (550 nm)
BBSSV
Aphid + BBSSV
0.1
1.0
10.0
100.0
200.0
ng BBSSV

Figure 6

Acquisition of BBSSV by Illinoia pepperi

Blueberry aphids were assayed for BBSSV antigen and RNA following acquisition access periods (AAP), ranging from 15 min to 120 hr on Parafilm^{R} sachets containing 50 ug purified BBSSV/ml 20% sucrose or BBSSV-infected blueberry plants. Significant levels of BBSSV-antigen were acquired after a 15 min AAP on sachets (Figure 7A). Significant levels of BBSSV-RNA were acquired after a 3 hr AAP on sachets. The percentage of aphids positive for BBSSV-antigen increased from 20% after a 15 min AAP to 82% after a 24 hr AAP. Then, the percentage decreased (ranging between 39% and 70%) during the following 3 day AAP. The percentage of BBSSV-RNA-positive aphids increased from 10% after a 3 hr AAP to 63% after a 24 hr AAP, and remained constant during the following 3 day AAP (ranging from 50% to 62%). The immunoassays indicated that the average concentration of BBSSV increased from 1.4 ng BBSSV/aphid after a 15 min AAP to 7.0 ng BBSSV/aphid after a 6 hr AAP (Figure 8A). Then, the average level of BBSSV did not increase during the next 90 hr AAP (ranging from 2.9 ng to 6.8 ng BBSSV per aphid), but the levels of BBSSV between individual aphids varied with some aphids acquiring as much as 41 ng after a 24 hr AAP. Regression analysis on antigen acquisition data indicated that BBSSV levels remained constant after 12 hr of virus acquisition. The RNA assay, dot-hybridization, indicated that the average concentration of BBSSV acquired from sachets increased from 7.9 ng BBSSV/aphid after a 3 hr AAP to 20.2 ng BBSSV/aphid after a 24 hr AAP. Then, the level declined to 10.7 ng/aphid after a 48 hr AAP, followed by an increase to 18.7 ng/aphid after a 96 hr AAP. Results from dot-hybridization also varied between individual aphids, with some aphids acquiring as much as 44 ng BBSSV after a 24 hr AAP. Regression analysis on RNA acquisition data showed a continuous increase in BBSSV levels up to a 96 hr AAP.

When aphids were given access to BBSSV-infected blueberry plants, significant levels of BBSSV-antigen were not acquired until after a 24 hr AAP, but significant levels of BBSSV-RNA were acquired after a 1 hr AAP (Figure 7B). The percentage of aphids positive for BBSSV antigen following AAPs on infected plants was generally lower than for aphids given AAPs to BBSSV on sachets. In contrast, the percentage of aphids positive for BBSSV-RNA, after a 24 hr AAP, was similar for both sources of the virus. Examination of aphids for the average concentration of BBSSV per aphid indicated both antigen and RNA levels acquired for infected plants were less than 4.0 ng/aphid. Regression analysis indicated both RNA and antigen levels slowly increased during a 96 hr AAP (Figure 8B).

Figure 7. The percentage of aphids positive for blueberry shoestring virus (BBSSV) following acquisition access periods on A) Parafilm^{R} sachets containing purified BBSSV in sucrose or B) BBSSV-infected blueberry plants. Results from the colloidal gold immunosorbent assays were included with the dot-ELISA results.

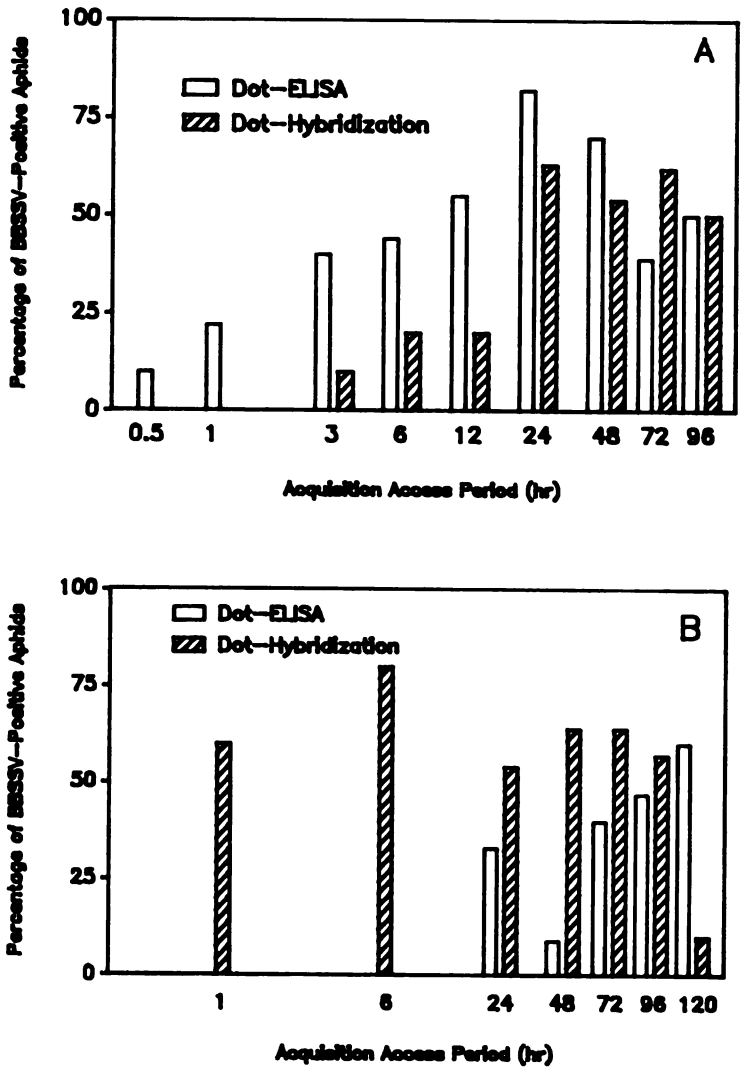

A
Dot–ELISA
Dot–Hybridization
Percentage of BBSSV–Positive Aphids
100
75
50
25
0
0.5
1
3
6
12
24
48
72
96
Acquisition Access Period (hr)
B
Dot–ELISA
Dot–Hybridization
Percentage of BBSSV–Positive Aphids
100
75
50
25
0
1
6
24
48
72
96
120
Acquisition Access Period (hr)

Figure 7

Figure 8. Acquisition of blueberry shoestring virus (BBSSV) following various acquisition access periods on A) Parafilm^{R} sachets containing BBSSV, or B) BBSSV-infected blueberry plants; points represent mean BBSSV concentrations/aphid, and lines without data points represent regression lines on A) dot-ELISA ($Y = 4.2 + 2.58X - 1.03X^2$, $r^2 = .73$) and dot-hybridization ($Y = 6.16 + 4.21X + 0.39X^2$, $r^2 = 0.83$), and B) dot-ELISA ($Y = 0.49 + 0.11X$, $r^2 = 0.42$) and dot-hybridization ($Y = 2.28 + 0.32X$, $r^2 = 0.54$).

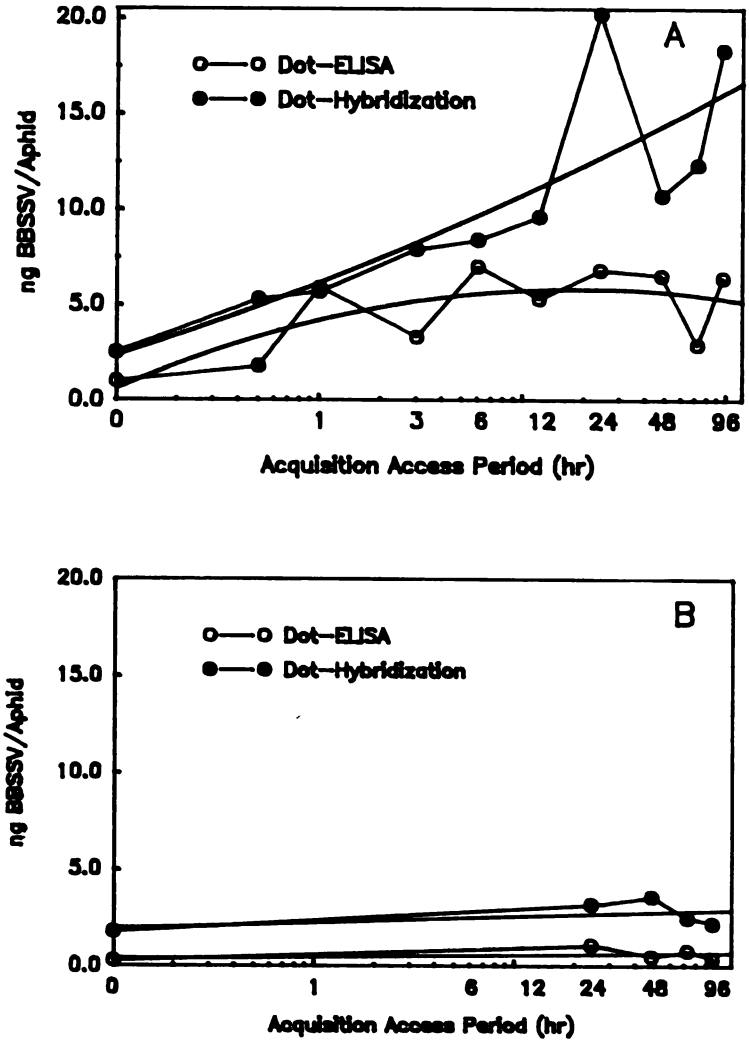

A
o—o Dot–ELISA
●—● Dot–Hybridization
ng BBSSV/Aphid
20.0
15.0
10.0
5.0
0.0
0
1
3
6
12
24
48
96
Acquisition Access Period (hr)
B
o—o Dot–ELISA
●—● Dot–Hybridization
ng BBSSV/Aphid
20.0
15.0
10.0
5.0
0.0
0
1
6
12
24
48
96
Acquisition Access Period (hr)

Figure 3

Retention of BBSSV by Illinoia pepperi

Blueberry aphids were given 1 to 3 day AAPs to BBSSV on sachets, and then transferred to 3 to 5 mon-old, healthy, micropropagated highbush blueberry plants, cv. Jersey. After various feeding periods on healthy plants, aphids were assayed for BBSSV antigen and RNA. In general, aphids tested positive for both BBSSV-antigen 4 days after acquisition and BBSSV-RNA 3 days after acquisition. The percentage of aphids positive for BBSSV-antigen decreased from 71%, immediately following the AAP on sachets, to 20% on the fourth day of post-acquisition feeding (Figure 9A). The percentage of aphids positive for BBSSV-RNA decreased from 71%, immediately following the AAP of virus from sachets, to 20% on the third day of post-acquisition feeding. The immunoassays showed that the mean concentration of BBSSV per aphid decreased from 4.8 ng/aphid to 0.8 ng/aphid (Figure 10A). Some individual aphids retained as much as 2.2 ng BBSSV after 3 days of post-acquisition feeding. Dot-hybridization assay indicated that the average concentration of BBSSV per aphid decreased from 20.3 ng/aphid to 2.2 ng/aphid. Individual aphids retained as much as 4.1 ng BBSSV after 3 days of post-acquisition feeding.

Aphids were also assayed for BBSSV-RNA retention following a 1 to 3 day AAP on infected blueberry plants. Aphids tested positive for BBSSV-RNA after 3 days post-acquisition access feeding (Figure 9B). The percentage of positive aphids was 10% immediately following acquisition, and decreased to only 8.7% after 3 days of post-acquisition feeding. The mean concentration of BBSSV/aphid decreased by about 50% after the first day of post-acquisition feeding (Figure 10B), and the concentration ranged from 3.1 ng BBSSV/aphid to 2.5 ng BBSSV/aphid day the next 2 days (Appendix, Table A4). Some individual aphids

retained as much as 6 ng BBSSV after 3 days of post-acquisition feeding. Aphids were also assayed for BBSSV antigen, but they failed to acquire significant virus levels from the source plants.

Figure 9. The percentage of aphids positive for blueberry shoestring virus (BBSSV) after various post-acquisition feeding periods; A) aphids were given access to BBSSV on Parafilm^{R} sachets, B) aphids were given access to BBSSV-infected blueberry plants.

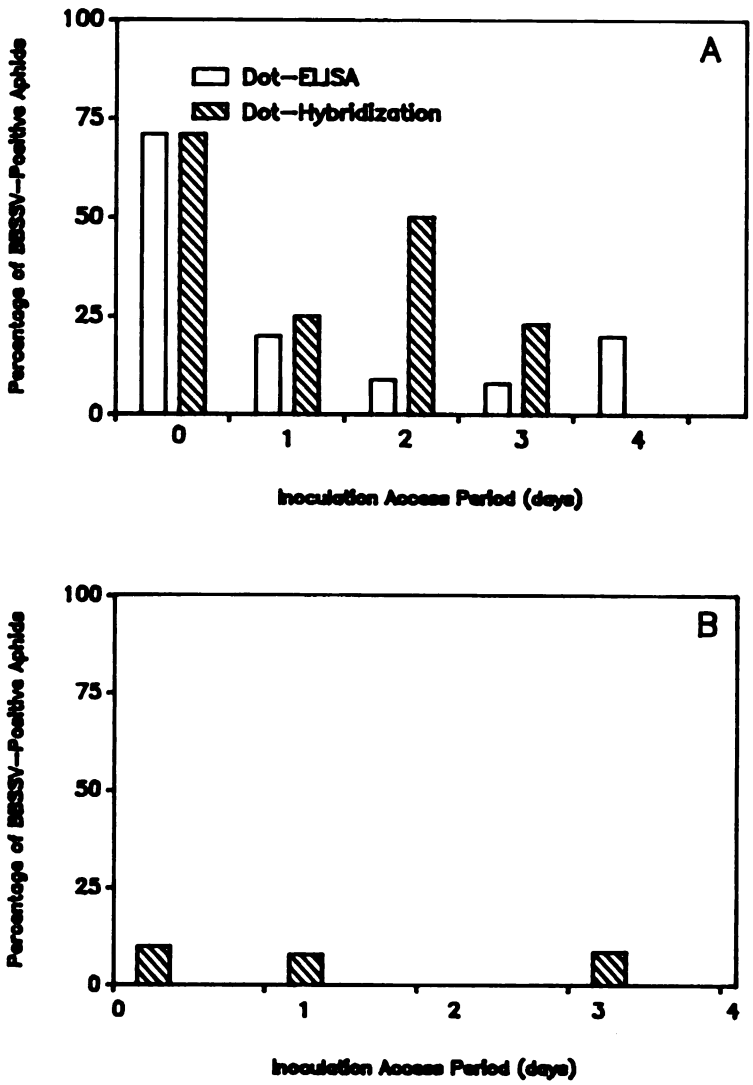

A
Dot--ELISA
Dot--Hybridization
Percentage of BBSSV-Positive Aphids
100
75
50
25
0
0
1
2
3
4
Inoculation Access Period (days)
B
Percentage of BBSSV-Positive Aphids
100
75
50
25
0
0
1
2
3
4
Inoculation Access Period (days)

Figure 9

Figure 10. The mean concentration of blueberry shoestring virus (BBSSV) retained by aphids after various post-acquisition feeding periods; A) aphids were given access to BBSSV on Parafilm^{R} sachets, or B) aphids were given access to BBSSV-infected blueberry plants; points represent ng BBSSV/ aphid and lines without data points represent regression lines for A) dot-ELISA ($Y = 2.07 - 2.4X + 0.92X^2$, $r^2 = 0.80$) and dot-hybridization ($Y = 1.2 - 7.37X + 0.95X^2$, $r^2 = 0.99$), and B) dot-hybridization ($Y = 4.9 - 3.22X + 0.74X^2$, $r^2 = 0.98$).

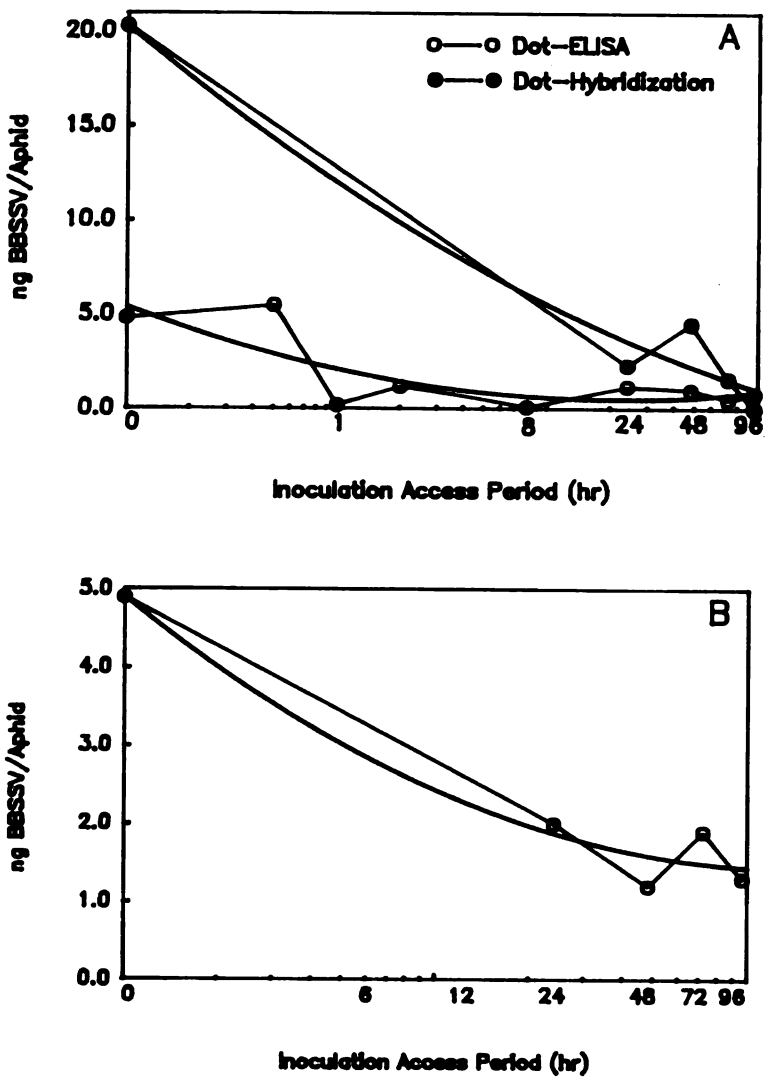

20.0
15.0
10.0
5.0
0.0
ng BBSSV/Aphid
o—o Dot-ELISA
●—● Dot-Hybridization
A
0
1
8
24
48
96
Inoculation Access Period (hr)
5.0
4.0
3.0
2.0
1.0
0.0
ng BBSSV/Aphid
B
0
6
12
24
48
72
96
Inoculation Access Period (hr)

Figure 10

Retention of BBSSV by Illinoia pepperi Through the Molt

To determine if BBSSV was restricted to the foregut or passed to the alimentary canal, individual aphids were assayed for BBSSV antigen and RNA following the molt (ecdysis) between instars. During a molt, the lining of the foregut is shed along with the rest of the exoskeleton. Aphids were given a 1, 2, or 3 day AAP on sachets containing BBSSV, and were assayed after the exoskeleton was shed. Aphids typically molted 1 to 3 days after virus acquisition. Significant levels of both antigen and RNA were found regardless of the number of days following virus acquisition (Table 4). The level of BBSSV retained by aphids which had molted were similar to the level of BBSSV retained by aphids which had not molted (Table 5).

Table 4. Retention of Blueberry Shoestring Virus (BBSSV) Antigen and RNA by Illinoia pepperi Following Ecdysis

BBSSV Assay[a]	Acquisition Access Period (days)	No. Aphids Positive/No. Aphids Negative at the Indicated Days Post-Acquisition Access to BBSSV[c]		
		1	2	3
antigen	1	2/8	4/19	1/12
antigen	2			1/5
RNA	1	1/4	1/3	1/6
RNA	2	0/1	0/1	1/3
RNA	3	2/3	0/2	3/3

[a] Aphids were assayed for BBSSV-antigen by dot-ELISA or silver-enhanced dot-colloidal gold linked immunosorbent assay. Aphids were assayed for BBSSV-RNA by dot-hybridization

[b] Aphids were allowed to feed on Parafilm[R] sachets containing purified BBSSV for 1 to 3 days.

[c] Individual aphids were allowed to feed on an excised highbush blueberry leaf placed in moist Petri dishes following BBSSV acquisition. Petri dishes were then monitored daily for the presence of a shed exoskeleton (i.e. ecdysis or molt). Aphids were then assayed for BBSSV antigen or RNA. Tabular results indicate the number of aphids positive for BBSSV out of the total number of aphids assayed.

Table 5. Concentration of Blueberry Shoestring Virus (BBSSV) in Aphids Following the Molt

Post-Acquisition Feeding (days)[a]	BBSSV (ng) Mean	sd.
0	7.86	13.01
<u>No Molt</u>		
1	1.88	3.27
2	1.48	6.32
3	0.54	1.08
<u>After Molt</u>		
1	0.92	2.10
2	1.94	3.25
3	0.58	1.57
No Acquisition	0.24	0.65

[a] Aphids were given a 24 hr acquisition access period to purified BBSSV in Parafilm^{R} sachets, and individual aphids were placed on excised blueberry leaves in moist petri dishes. Petri dishes were monitored daily for a shed exoskeleton, and new instars were assayed by dot-ELISA or dot-hybridization for BBSSV. "No Molt" aphids did not molt following the acquisition access period. Values indicate the mean concentration of BBSSV from 3 to 33 aphids.

Passage of BBSSV into the Hemocoel

Hemolymph samples from five aphids were withdrawn and combined following AAPs of 1, 2, 3, and 4 days. Then, samples were assayed for BBSSV antigen and RNA. The remaining aphid, from which the hemolymph sample was taken, was also assayed for BBSSV antigen and RNA. Evidence from the immunoassays and dot-hybridization indicated that both BBSSV antigen and RNA were present in the hemolymph. Aphid hemolymph samples tested positive for antigen after AAPs of 1, 2, 3, and 4 days (Table 6). Forty to 100% of the remaining aphid samples also tested positive for BBSSV-antigen. Aphid hemolymph samples tested positive for BBSSV-RNA after AAPs of 1, 3, and 4 days. Forty to 100% of the remaining-aphid samples also tested positive. Dot-hybridization indicated the concentration of BBSSV in the hemolymph samples ranged from 0.26 to 14.35 ng per sample with a mean of 21.27 $\pm$ 28.19 ng BBSSV per sample. The immunoassays indicated the concentration of BBSSV in the hemolymph samples ranged from 0.09 to 7.87 ng per sample with a mean of 7.69 $\pm$ 13.16 ng BBSSV per sample.

Table 6. Detection of Blueberry Shoestring Virus (BBSSV) Antigen and RNA in the Hemocoel of Illinoia pepperi Following Acquisition Access Periods on Parafilm® Sachets Containing Purified BBSSV or BBSSV-Infected Blueberry Plants

BBSSV Assay[a]	Sample[b]	Acquisition Access Period (days)[c]			
		1	2	3	4
Antigen	aphid	2/5	5/5	4/5	2/3
	hemolymph	+	+	+	+
Antigen	aphid	4/5	4/5	3/5	3/5
	hemolymph	+	+	-	+
RNA	aphid	3/5	4/5	5/5	5/5
	hemolymph	+	-	+	+
RNA	aphid	2/5	4/5	2/5	
	hemolymph	-	-	+	

[a] Aphids were assayed for BBSSV-antigen by dot-ELISA or silver-enhanced dot-colloidal gold linked immunosorbent assay. Aphids were assayed for BBSSV-RNA by dot-hybridization.

[b] Hemolymph samples were withdrawn by piercing the exoskeleton on the dorsal side of the abdomen with a fine-drawn glass needle. Hemolymph samples from five aphids were combined, and assayed for BBSSV. The remaining aphid was also assayed for BBSSV.

[c] The numerator indicates the number of positive aphids , the denominator 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.

Transmission of BBSSV to Blueberry Plants by Illinoia pepperi Following Successive Transfers

Groups of 15 blueberry aphids were given AAPs of 1 to 5 days to purified BBSSV on sachets or BBSSV-infected blueberry, and were then successively transferred to 10 healthy, 3 to 5 mo-old, micropropagated blueberry plants (cv. Jersey) at daily intervals. Plants were grown for 1 yr in a greenhouse at 65 to 75 C with a 16 hr photoperiod. Root and shoot samples were assayed for BBSSV antigen 3, 6 and 12 mo after inoculation. The number of aphids per group that survived the entire 10 day period ranged from 0 to 10 aphids. The results in Figure 11A show that aphids transmitted BBSSV to blueberry plants on each of the 10 days, except day 6 and 9, after a 24 hr AAP. Figure 11B shows that aphids transmitted on days 1, 2, 3, 7, 8 and 10 after a 24 hr AAP to purified BBSSV on sachets. Aphids transmitted BBSSV to blueberry plants 1, 4, 5 and 8 days after a 24 hr AAP on BBSSV-infected blueberry plants (cv. Jersey). Only root samples tested positive for BBSSV antigen (Appendix, Table A5), and plants did not become symptomatic. Longer AAPs did not affect transmission; aphids were able to transmit BBSSV throughout the 10 day period. When non-viruliferous aphids were placed on blueberry plants, no transmission occurred, and when blueberry plants (cv. Bluecrop) were rub-inoculated with purified BBSSV, they tested positive for BBSSV antigen after 6 months and 1 yr of incubation.

Figure 11. Transmission of blueberry shoestring virus (BBSSV) to healthy, micropropagated blueberry plants by aphids; aphids were given a 24 hr acquisition access period to BBSSV on sachets or BBSSV-infected blueberry plants, and then successively transferred at daily intervals for 10 days to new plants; A) shows the days when aphids, given access to BBSSV in either sachet or plant, transmitted BBSSV; B) compares transmission with regard to virus access source.

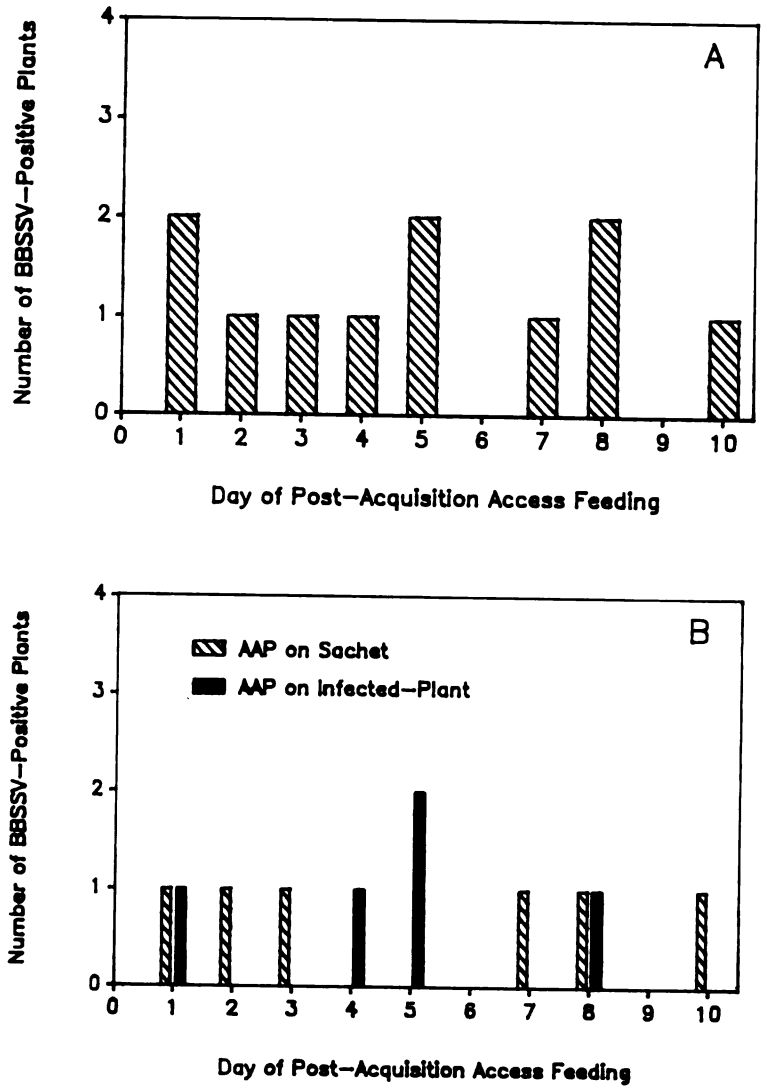

A
Number of BBSSV-Positive Plants
0
1
2
3
4
0 1 2 3 4 5 6 7 8 9 10
Day of Post-Acquisition Access Feeding
B
AAP on Sachet
AAP on Infected-Plant
Number of BBSSV-Positive Plants
0
1
2
3
4
0 1 2 3 4 5 6 7 8 9 10
Day of Post-Acquisition Access Feeding

Figure 11

Detection of BBSSV with Colloidal Gold Immunocytochemistry

In order to determine the optimal concentrations of anti-BBSSV IgG and protein A-colloidal gold (pAg) that were required to effectively label BBSSV, formvar-carbon coated copper grids were incubated on a drop of purified BBSSV followed by incubation with various concentrations of IgG and pAg at different times and temperatures (Table 7). The highest number of gold particles per virion resulted when grids were incubated in a 1/100 dilution (v/v) of 1 mg/ml anti-BBSSV-IgG in 0.01 M phosphate buffer, pH 7.0, for 4 hr at 23 C followed by an incubation in a 1/10 dilution (v/v) of pAg in 0.01 M phosphate buffer, pH 7.0, for 4 hr at 23 C. Twenty nm diameter gold particles labeled BBSSV at 0.86 gold particles per virion under optimal conditions. Under the same conditions, 5 nm and 10 nm colloidal gold completely encircled virions with more than ten gold particles per virion (Figure 12A, 12B and 12C). Substituting anti-blueberry leaf mottle virus IgG for anti-BBSSV IgG eliminated any specific labeling (Figure 13A). Deleting the IgG labeling step also eliminated any specific labeling (Figure 13B). In addition, brome mosaic virus and southern bean mosaic virus were not specifically labeled by this procedure (Figure 13C). Purified BBSSV was placed in 1% agarose, and subjected to the fixation and embedding procedure used for aphids. Electron microscopy of thin sections of agar-embedded BBSSV showed localized areas of labeling, although virions appeared to be disrupted by the procedure. Blueberry shoots, symptomatic for BBSSV, were also fixed, embedded and labeled by this procedure. Electron microscopic examination of thin sections did not reveal virions in plant tissues, and specific labeling with colloidal gold was rarely observed.

Table 7. Optimal Conditions for Colloidal Gold Labeling of Purified Blueberry Shoestring Virus (BBSSV)[a]

Dilution of Protein A-Gold and Incubations Conditions[b]	Dilution of Anti-BBSSV IgG and Incubation Conditions					
	4 hr @ 23C			overnight @ 23C		
	1/50	1/100	1/500	1/50	1/100	1/500
1/10 for 30 min @ 37C	<0.01					
1 hr @ 23C						
1/10	0.29	0.20	<0.01			
1/100	<0.01	<0.01	0.00			
4 hr @ 23C						
1/10	0.56	0.86	0.68	0.67	0.55	0.82
1/100	0.27	0.37	0.43	0.35	0.35	0.36
1/500	0.24	0.16		0.30	0.20	

[a] Numbers in the body of the table signify the ratio of gold particles to virions.

[b] Formvar and carbon coated grids were incubated 5 min on a drop of purified BBSSV diluted 1/100 in pH 7.0 phosphate buffer (0.05 M). Then grid were incubate on a drop of anti-BBSSV antibody (dilution, time and temp. designated in table), rinsed, and incubated on a drop of 20 nm diameter protein A-gold (dilution, time, and temp. designated in table). Values indicate the mean number of gold particles per virion in three replications.

Figure 12. Detection of purified blueberry shoestring virus (BBSSV) using A) 5 nm, B) 10 nm, and C) 20 nm protein A-gold; bar = 100 nm.

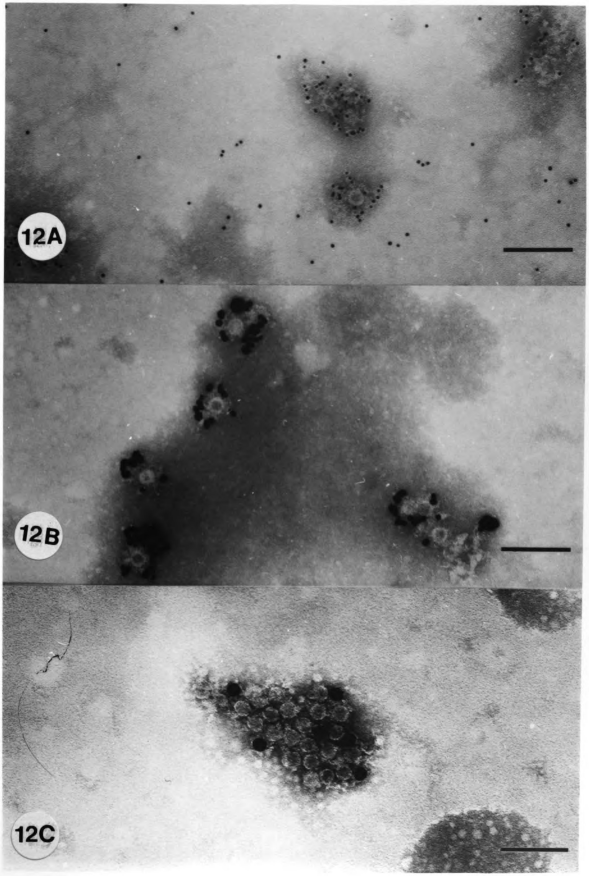
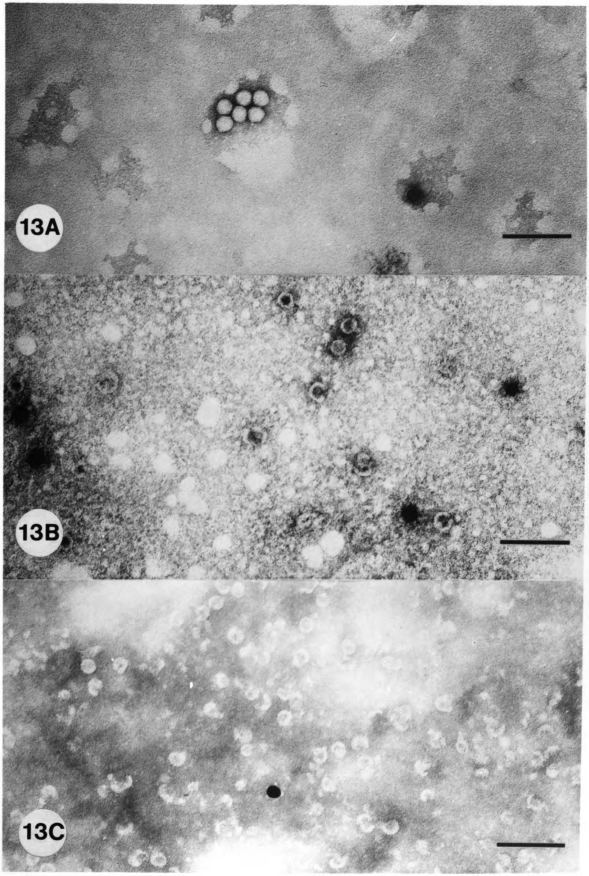

12A
12B
12C

Figure 13. The specificity of protein A-gold; A) purified blueberry shoestring virus (BBSSV) labeled with protein A-gold but no anti-BBSSV IgG, B) BBSSV labeled with anti-BBLMV IgG and protein A-gold, and C) southern bean mosaic virus labeled with anti-BBSSV IgG and protein A-gold; bar = 100 nm.


13A
13B
13C

Detection of BBSSV in Thick and Thin Sections of Viruliferous Aphids by Colloidal Gold Immunocytochemistry

Thick (1 um) and thin (60-100 nm) sections of viruliferous and non-viruliferous blueberry aphids were stained with anti-BBSSV IgG followed by 10 or 20 nm diameter colloidal gold-Protein A (pAg) conjugates. Light microscopy revealed specific labeling of the intestinal cells and salivary glands of viruliferous aphids (Figure 14), but not in non-viruliferous aphids. Electron microscopy did not conclusively reveal BBSSV in any aphid tissues; although, virus-like particles between 27 and 30 nm in diameter were observed in the intestinal epithelial cells of viruliferous aphids (Figure 15A). Both viruliferous and non-viruliferous aphids appeared to be infected by a much larger virus; virus-like particles, 50 nm in diameter, were found in the hindgut lumen, intestinal epithelial cells, and in lysosomes in the hemocoel (Figure 15B and 15C).

Figure 14. Detection of blueberry shoestring virus (BBSSV) in thick sections of aphids; aphids were given 1 to 3 day acquisition access periods to BBSSV on sachets; A) a thin section of an intestinal epithelial cell, silver-enhanced-gold labeling of B) intestinal epithelial cells and C) an accessory salivary gland; D) an electron micrograph of an accessory salivary gland; a = accessory salivary gland, ap = apical plasmalemma, b = basal membrane, bp = basal plasmalemma, c = canaliculi, i = intestine, s = stomach, sv = secretory vesicle, arrow shows silver enhancer; bars in A and D = 100 nm; bars in B and C = 0.1 mm.

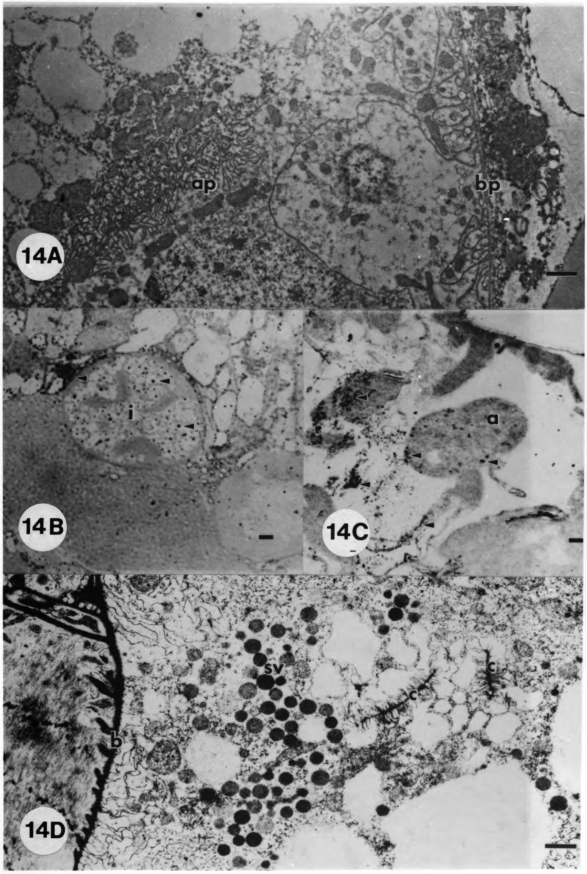
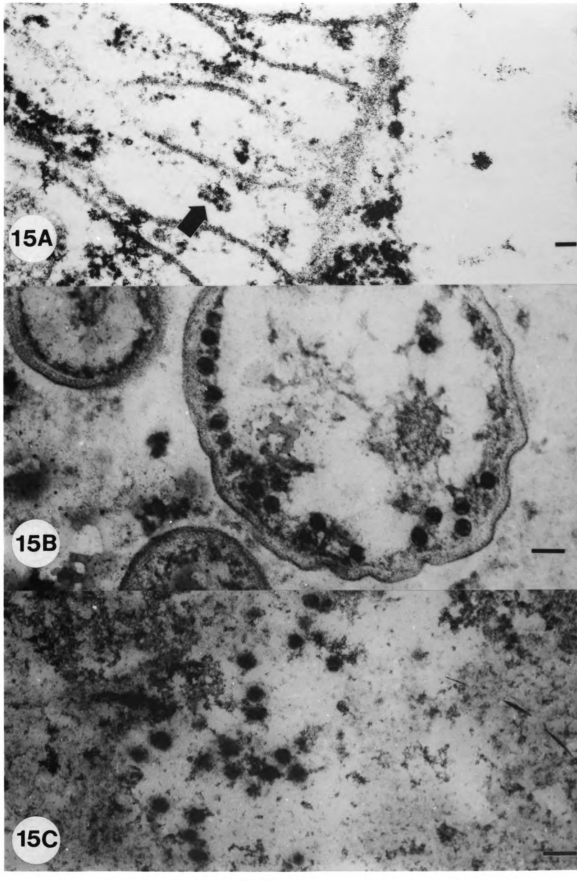

ap
bp
14A
i
14B
a
14C
sv
c
c
b
14D

Figure 15. Virus-like particles (VLPs) present in intestinal epithelial cells (A and C) and a lysosomal vesicle in the hemolymph (B). The arrow points to four VLPs 27 nm in diameter in an epithelial cell; bar = 100 nm.


15A
15B
15C

DISCUSSION

The two most important factors for determining virus transmission by aphids are 1) the length of time required for virus acquisition and 2) the length of time that transmission can occur (i.e. the persistence of transmission). Determining these factors is important for virus control strategies. Insecticides may effectively control the spread of persistently transmitted viruses, but if a short delay (e.g. a few hours) occurs before an insecticide kills the aphid, the aphid may have already spread a nonpersistent virus to another plant. Two studies found that the use of insecticides to control the nonpersistently transmitted, maize dwarf mosaic virus, actually increased the incidence of virus infections by increasing aphid feeding activity (Onazi and Wilde, 1974; Seifers and Harvey, 1989). Although this may not be characteristic of all nonpersistently transmitted viruses, it is important to realize that the time involved in virus acquisition and retention by its aphid vector should be considered when developing virus control strategies.

The acquisition and retention periods associated with aphid transmission of viruses are generally elucidated through transmission experiments. Aphids are given various acquisition access periods (AAPs) on a virus source followed by inoculation access periods (IAPs) on healthy test plants; after an incubation period, plants are either examined for symptom development or assayed for virus to determine the time required for virus acquisition and transmission. While these transmission experiments were easily performed with herbaceous host plants, they were more difficult to perform on woody host plants. Symptom development on blueberry

caused by BBSSV requires a 4 yr latent period, and virus infections are not detectable by ELISA until after a 6 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. An indirect approach, involving ELISA, has been used to determine virus acquisition and retention periods for persistently transmitted viruses (Tamada and Harrison, 1981; Fargette et al., 1982). 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 infectious during this period. This same approach was used here to determine the acquisition and retention periods of BBSSV by Illinoia pepperi, but dot-ELISA or dot-GLISA were used to detect BBSSV-antigen, and dot-hybridization was used to detect BBSSV-RNA.

Previous studies found that ELISA achieved a virus detection endpoint of 5.0 ng in individual aphids (Fargette et al., 1982; Gillett et al., 1982). Other reports indicated that individual aphids carry virus levels (0.01 ng) well below the ELISA detection endpoint (Tamada and Harrison, 1981); therefore, groups of several aphids would be required to accurately determine virus content. The sensitivity of ELISA was improved by the introduction of a different solid phase support, nitrocellulose membrane (NCM) (Banttari and Goodwin, 1985). This technique, dot-ELISA, was used by Urban (1987) 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 non-viruliferous aphids. Results presented have shown that this problem could be avoided by using other types of membranes, more efficient blocking, and by accurate quantification of assay responses.

Dot-ELISA, silver-enhanced dot-GLISA and dot-hybridization were reliable techniques for monitoring concentrations of BBSSV antigen and BBSSV genomic RNA. Although dot-ELISA on NCM was a sensitive technique for the detection of purified BBSSV, dot-ELISA on nylon membrane (NM) and dot-GLISA on NCM were more suitable techniques for the detection of BBSSV in aphids due to the increased resolution between BBSSV-positive and negative aphids. Dot-hybridization was less sensitive, but it may have been a more accurate indicator of the presence of infectious virus particles.

The lowest aphid background levels were obtained with dot-ELISA on nylon membrane. In the enzyme-linked immunoassays, aphid background levels may have been the result of an enzymatically active aphid component (e.g. endogenous alkaline phosphatase) binding non-specifically to the membrane. The blocking procedure used for nylon membrane may have blocked unbound membrane sites more efficiently than the other assay blocking procedures. In addition, the nylon membrane contained positively charged binding sites, while NCM contained uncharged binding sites. Under standard assay conditions, the nylon would have repelled aphid components that had a low ionization equilibrium. Background responses were also prevalent when non-viruliferous aphids were assayed by the colloidal gold-linked immunosorbent assay. Background levels were probably due to non-specifically bound endogenous metal ions which would induce nucleation of the silver-enhancer. More efficient blocking might have reduced background. In dot-hybridization, background levels could be reduced by using a

shorter hybridization period (12 hr) at a higher temperature (65 C).

BBSSV concentrations in aphids were determined by quantifying assay responses. The quantification technique described here utilized a transmission spectrophotometry detection system to assess numerical values for assay spot intensities. This technique required the use of color transparencies for light transmission as an alternative to more expensive reflectance spectrophotometers. Four by five inch transparencies were the most suitable size because entire blots could be reproduced on one negative, and baseline (A550 nm = 0.0) regions were easily located. Threshold values, based on non-viruliferous aphid absorbance responses, were used to distinguish between positive and negative aphid absorbances. Resulting dose-response curves were S-shaped; absorbance values increased gradually at very low BBSSV concentrations, and rose sharply at BBSSV concentrations above the detection endpoint. Absorbance values reached a plateau as membrane binding sites became saturated at high BBSSV concentrations. Generally, the bottom portion of the curve was used to generate first and second order polynomial models. These models underestimated BBSSV concentrations in aphids due to the quenching effect of aphid homogenate on detection. Corrected dose-response curves could be generated by using a virus dilution series amended with aphid homogenate.

The immunoassays and the dot-hybridization assay indicated that only short periods of time (15 to 60 min) were required for BBSSV acquisition from sachets by Illinoia pepperi. This type of acquisition is characteristic of semipersistent virus transmission, but these data did not indicate transmissibility of acquired BBSSV. Fargette et al. (1982) found similar acquisition kinetics for the persistently transmitted pea enation mosaic virus (PEMV); levels of virus increased up to 16 hr AAP, but remained

constant with longer AAPs. They suggested that after 16 hr, virus ingestion and excretion were at equilibrium. Illinoia pepperi had a similar acquisition pattern for BBSSV-antigen only; acquisition and excretion were at equilibrium after a 12 hr AAP. BBSSV-RNA levels continued to increase up to 4 days AAP; the maximal concentration of BBSSV-RNA acquired was about 60% higher than BBSSV-antigen. Unequal uptake of BBSSV-antigen and RNA may have resulted from greater degradation rate of BBSSV antigen. Alternatively, enzymes in the alimentary canal may have masked or degraded antigen more readily than RNA. In vitro, aphid homogenate reduced detection of BBSSV antigen and RNA 40 to 60%. This may have been the result of masking or enzymatic degradation. If enzymes were involved in RNA and antigen degradation, RNases may have been compartmentalized In vivo, excluding them from BBSSV-RNA, while enzymes degrading antigen were present in extracellular matrices. A third possibility would have been the unequal acquisition of RNA and antigen, i.e. BBSSV-RNA was more readily acquired than antigen. This explanation appears to have been confirmed by the continued increase in RNA levels during acquisition, whereas antigen levels remained constant after 12 hr. Hemolymph assays determined that the maximum level of RNA in the hemocoel was 45% higher than the maximum level of antigen in the hemocoel. These results suggest that more BBSSV-RNA passed from the gut into the hemocoel, possibly due to the presence of BBSSV-specific cell receptors on epithelial cells of the the alimentary canal.

Illinoia pepperi also acquired BBSSV from infected blueberry plants after short AAPs (1 to 6 hr), but fewer aphids acquired BBSSV from infected blueberry plants than from sachets, and they acquired less BBSSV. Similar results were reported earlier by Morimoto et al. (1985a). Differences in amounts of acquisition of BBSSV RNA and antigen from infected blueberry were similar to the

differences in acquisition of BBSSV RNA and antigen from sachets. Sixty-six to 82% more RNA was acquired than antigen. This may have resulted from higher levels of RNA than antigen in the infected plant. It may have also resulted from unequal uptake, masking or degradation of the two BBSSV components.

Persistently and semipersistently transmitted viruses require longer periods for acquisition than nonpersistently transmitted viruses because they usually accumulate in the phloem tissues of the plant, while nonpersistent viruses accumulate in epidermal cells of the plant. These tissues are less accessible to the aphid, and longer feeding periods are required before aphids can acquire phloem limited viruses. Urban (1987) used fluorescent antibodies against BBSSV to localize BBSSV in epidermal, mesophyll, and xylem tissues of infected blueberry, and grafting experiments indicated that BBSSV moved through phloem and xylem tissues. Hartmann et al. (1973) found high concentrations of BBSSV in xylem elements of BBSSV-infected blueberry plants. Morimoto et al. (1985a) suggested the lower acquisition rate may have been due to unequal distribution of BBSSV throughout the plant. These differences may have also resulted from a higher concentration of BBSSV in the xylem. The presence of BBSSV in vascular tissues indicated that BBSSV was more closely related to semipersistent or persistent viruses rather than nonpersistent viruses.

Retention experiments showed that significant levels of BBSSV were retained at least 4 days after acquisition from sachets. Levels dropped by 75 to 90% during the first 24 hr of post-acquisition feeding. This may have indicated that most of the unacquired virus was expelled during this period. The level of BBSSV retained after this period ranged from 0.5 to 1.2 ng of BBSSV-antigen and 0.0 to 4.5 ng of BBSSV-RNA. The higher level of RNA retained supports the hypothesis that more RNA was acquired than antigen. The

BBSSV retention pattern was similar to the retention pattern of PEMV by its aphid vector (Fargette et al. (1982). They found a 43% drop in virus content per aphid during the first 24 hrs of post-acquisition feeding. The level of virus 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 longer than expected for semipersistent viruses. The similarity in retention patterns between aphids which had acquired BBSSV from sachets and aphids which had acquired BBSSV from infected plants indicated that the artificial feeding system was representative of the natural feeding system.

In addition, significant BBSSV antigen and RNA levels were retained following the molt, and there were no significant differences in the level of BBSSV in aphids which did molt compared to aphids which did not molt. This suggested that detectable levels of BBSSV were not adhering to the lining of the foregut, and that most of the BBSSV had passed to the posterior portion of the alimentary canal. The presence of BBSSV antigen and RNA in hemolymph samples indicated that BBSSV passed from the lumen of the mid or hind-gut, through gut epithelial cells and into the hemocoel. These results were more characteristic of persistently transmitted viruses than semipersistently transmitted viruses.

To corroborate virus monitoring experiments, aphids were examined for their ability to transmit BBSSV during each day of a 10 day IAP. When aphids were given 24 hr AAP on infected plants or sachets containing BBSSV, they transmitted virus to plants during each of the 10 days except days 6 and 9. Initial investigations on transmission of BBSSV by aphids during undisturbed feeding indicated BBSSV could be transmitted to blueberry in as little as 26

hr from the initial virus access (Morimoto et al., 1985a), but it did not indicate the requirement of a latent period. Successive serial transfers also indicated that aphids could transmit BBSSV within the first 48 hr following initial virus access, and they continued to transmit virus up to 10 days after virus acquisition. Increased AAPs had no effect on transmission of BBSSV. Although the acquisition periods were not short enough to determine if a latent period exists, these data suggested that a possible latent period would be less than 48 hr. This type of transmission pattern was similar to the transmission pattern for PEMV. PEMV required a 12 to 18 hr latent period (Osborn 1935), and transmission was retained for 6 to 15 days following a 24 hr AAP (Simons, 1954).

Ultrastructural studies have shown that persistent viruses circulate through their aphid vectors. Recent reviews suggest that persistently transmitted viruses are acquired by cell receptors on mid- or hindgut epithelial cells, and non-transmitted viruses remain in the alimentary canal. Transmitted viruses then pass transcellularly by endo- and exocytosis into the hemocoel. Transmitted viruses in the circulating hemolymph can contact and pass into that accessory salivary gland where they can be expelled into the plant during salivation. Initial ultrastructural studies identified virions by size, but the recent use of immunocytochemistry is more definitive for virion identification. Attempts to use immunocytochemistry to localize BBSSV in thin sections of viruliferous Illinoia pepperi were unsuccessful; gold labels appeared to be randomly distributed throughout the section. Intestinal epithelial cells and salivary glands in thick sections of viruliferous aphids were occasionally labeled. This suggested that the intestine was a possible site for BBSSV acquisition, but a thorough examination of the labeling specificity is still required. The lack of association

between the virions and the gold label may have resulted from disruption of BBSSV-antigenicity during the fixation and embedding procedure. The absence of a virion-gold label association could also be explained by the differences in acquisition and retention of BBSSV-RNA and antigen. The gold labeling system detected BBSSV antigen, if BBSSV-RNA was prevalent in the aphid, it would not have been detected in thick and thin sections.

To summarize, BBSSV antigen and RNA were acquired and retained in a manner similar to other circulative viruses. BBSSV antigen and RNA were also retained following the molt, and both passed into the hemolymph within a 24 hr period. Transmission experiments suggested that there was a positive correlation between BBSSV content and the retention of transmission by Illinoia pepperi. Differences in the content of RNA and antigen indicated that acquisition and retention of these two components were not equivalent. Although gold labeling was never found in association with virions in aphids by transmission electron microscopy, light microscopy of gold labeled thick sections from aphids indicated that BBSSV may have been acquired by transcellular passage through intestinal epithelial cells; then, circulated through the aphid, and accumulated in accessory salivary glands, but virions were not positively identified. These experiments suggested that the BBSSV-Illinoia pepperi relationship was persistent in nature, but they do not exclude the possibility of a bimodal relationship involving both semipersistent and persistent virus transmission.

In future experiments, transmission experiments should be repeated following shorter AAPs to determine the length of the latent period. Transmission should also be examined following the molt. These two experiments would determine the possible role of semipersistent transmission in the BBSSV-Illinoia pepperi relationship. In addition, the specificity of the gold labeling technique should be

examined, and the antigen component of BBSSV should be determined by examining labeling following protease and RNase digestion. This would determine if the capsid protein were more antigenic. Finally, the gold labeling procedure should be performed on unfixed hemolymph samples and salivary glands using probes against both BBSSV antigen and RNA. This would detect differences in transcellular passage of RNA and antigen through gut epithelial cells, and determine if BBSSV RNA can be transmitted independent of the coat protein.

APPENDIX

APPENDIX

Table A1. The Concentration of Blueberry Shoestring Virus (ng BBSSV) in Illinoia pepperi Following Various Acquisition Access Periods on Sachets Containing BBSSV or BBSSV-Infected Blueberry Plants

Assay[a]	Acquisition Access Period (hr)[b]												
	0	.25	.5	1	2	3	4	6	8	12	24	48	72	96
Es	5.7		4.2	6.3		5.3		8.4		9.4	23.5	19.7	5.1	12.6
Es	1.3		1.0	5.5		3.7		5.5		7.7	10.2			
Es	0.0									0.3	0.3	0.3		
Es	0.0									3.8	3.2			
Es	0.2	1.4	0.8		1.1	0.8	0.4		0.5		7.5			
Es	0.7										3.2	5.2	4.7	6.1
Es	0.0										4.7	4.0	1.6	0.6
Es	0.2		1.1								0.9			
Hs	6.6		4.5	5.5		9.9		9.2		11.5	25.3	12.1	7.6	5.5
Hs	6.0		6.1	5.8		5.8		7.5		7.7	8.8	7.5		
Hs	0.0										28.9	29.0	31.1	31.1
Hs	0.0										5.0	5.1	2.8	
Hs	0.0										33.0	0.0	7.5	
Ep	0.5							0.4		0.6	0.5	0.7		
Ep	0.0										0.1	0.0		0.3
Ep	0.6										0.7	0.7	0.8	0.6
Ep	0.2										3.1	0.6		0.4
Hp	0.7			0.8			0.9				0.8	0.8	0.8	0.8
Hp	4.6										6.9	4.4	5.9	4.9
Hp	0.2										2.0	5.5	0.7	0.9

[a] The symbols in this column represent the assay method and the virus source; E = dot-ELISA or colloidal gold immunosorbent assay was used to assay aphids for BBSSV-antigen; H = dot-hybridization was used to assay aphids for BBSSV-RNA; s = aphids given acquisition access periods (AAPs) on sachets containing virus source, and p = aphids given AAPs on BBSSV-infected plants.

[b] Groups of three to five aphids were given various AAPs on Parafilm[R] sachets containing BBSSV or on BBSSV-infected blueberry plants. Then individual aphids were homogenized, and assayed for BBSSV antigen or RNA. This table gives the mean concentration of BBSSV (ng) for each group of aphids.

Table A2. Concentration of Blueberry Shoestring Virus (ng BBSSV) in Illinoia pepperi Following Various Acquisition Access Periods (AAP) on Sachets Containing BBSSV or BBSSV-Infected Blueberry Plants: Cumulative Results

Acquisition Access Period (hr)	Sachet Virus Source				Plant Virus Source[a]			
	Antigen		RNA		Antigen		RNA	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd[b]
0	1.0	0.7	2.5	1.5	0.3	0.1	1.8	0.4
0.25	1.4							
0.5	1.8	0.8	5.3	0.8				
1	5.9	0.4	5.7	0.2			0.8	
2	1.1							
3	3.3	1.3	7.9	2.1				
4	0.4						0.9	
6	7.0	1.5	8.4	0.9	0.4			
8	0.5							
12	5.3	2.0	9.6	1.9	0.6			
24	6.8	2.7	20.2	5.6	1.1	0.7	3.2	1.8
48	6.5	3.4	10.7	5.0	0.5	0.2	3.6	1.4
72	2.9	1.2	12.3	6.4	0.8		2.5	1.7
96	6.4	3.5	18.3	12.8	0.4	0.1	2.2	1.4
120					0.3	0.2	22.3	

[a] Concentrations of BBSSV, determined in several assays, were combined for each acquisition access period (see Table A1) and averaged. Aphids were fed on either Parafilm[R] sachets containing BBSSV or BBSSV-symptomatic blueberry.

[b] Aphids were assayed for BBSSV-antigen by dot-ELISA or silver-enhanced dot-colloidal gold linked immunosorbent assay. Aphids were assayed for BBSSV-RNA by dot-hybridization; sd = standard deviation.

Table A3. Concentration of Blueberry Shoestring Virus (ng) Retained by Viruliferous Illinoia pepperi Following Various Feeding Access Periods on Healthy Blueberry Tissue

Assay[a] Method	Virus[b] Source	Days AAP	Post-Acquisition Feeding Period (hr)[c] 0	.5	1	2	8	24	48	72	96	120
DE	sachet	1	3.7					0.6	1.5	0.5	0.4	
DG	sachet	1	2.3					0.3	0.0	0.0		
DG	sachet	1	4.7					0.0	0.0			
DG	sachet	1	3.2					1.5	0.0	0.4		
DE	sachet	2	4.1					1.4	1.1	1.6	1.1	
DE	sachet	2	3.2					1.6	0.2	0.2		
DG	sachet	2	7.9					0.0	4.7			
DG	sachet	2	3.8	5.5	0.2	1.2	0.1	0.5	0.6			
DE	sachet	3	10.3					4.5	1.1			
DE	plant	2	0.6					0.8	0.6	0.6	0.6	
DH	plant	1						0.0	2.3	2.5		
DH	plant	2	2.6					7.8	2.5	2.6	2.6	
DH	plant	3	2.5					2.8	2.7	2.8	2.5	
DH	plant	3	4.9					2.0	1.2	1.9	1.3	
DH	plant	4	2.5					2.5	2.9	2.7	2.9	2.5
DH	sachet	1	33.0						1.4	1.2	0.0	0.0
DH	sachet	3	7.5					2.3	7.6	3.2		

[a] DE = dot-ELISA was used to assay aphids for BBSSV; DG = dot/colloidal gold immunosorbent assay was used to assay aphids for BBSSV-antigen; DH = dot-hybridization was used to assay aphids for BBSSV-RNA.

[b] Aphids were initially given acquisition access periods (AAP) on either Parafilm[R] sachets containing BBSSV or on BBSSV-infected blueberry plants prior to the post-acquisition feeding periods.

[c] Three to five aphids were allowed to feed on healthy blueberry plants following the BBSSV access period. Then individual aphids were homogenized, and assayed for BBSSV antigen or RNA. The mean concentration of BBSSV (ng) is given in the table.

Table A4. Concentration of Blueberry Shoestring Virus Retained by Viruliferous Illinoia pepperi Following Various Feeding Access Periods on Healthy Blueberry Tissue: Cumulative Results

Post-Acquisition Access Period (hr)	Sachet BBSSV Source				Plant BBSSV Source[a]			
	Antigen		RNA		Antigen		RNA	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd[b]
0	4.8	0.9	20.3	18.0	0.6		3.1	0.6
0.5	5.5							
1	0.2							
2	1.2							
8	0.1							
24	1.2	0.5	2.3		0.8		3.0	1.3
48	1.0	0.5	4.5	4.4	0.6		2.4	0.3
72	0.5	0.3	2.2	1.0	0.6		2.5	0.2
96	0.8	0.4	0.0		0.6		2.3	0.4
120			0.0				2.5	

[a] Concentrations of BBSSV (ng), determined in several assays, were combined for each post-acquisition access period (see Table 4) and averaged. Aphids were fed on either Parafilm[R] sachets containing BBSSV or BBSSV-symptomatic blueberry, on which aphids were given access to BBSSV.

[b] Aphids were assayed for BBSSV-antigen by dot-ELISA or silver-enhanced dot-colloidal gold linked immunosorbent assay. Aphids were assayed for BBSSV-RNA by dot-hybridization; sd = standard deviation.

Table A5. Transmission of Blueberry Shoestring Virus (BBSSV) by Illinoia pepperi to Healthy, Micropropagated Blueberry Plants Following Ten Consecutive Transfers

BBSSV Source	Acquisition Access Period (days)	Day of Post-Acquisition Feeding[a] 1	2	3	4	5	6	7	8	9	10
plant	1	+			+	+					
plant	1					+			+		
sachet	1	+	+	+							+
sachet	1							+	+		
plant	2		+		+		+		+		
sachet	2				+			0	0	0	0
sachet	2					+	+		+	+	
plant	3		+	+	+		+	+			+
plant	3	+				+	+				+
sachet	3	+		+	+		0	0	0	0	0
sachet	3		+		0	0	0	0	0	0	0
plant	4						+		+	+	
sachet	4			+	0	0	0	0	0	0	0
sachet	4										
sachet	5	+									

[a] Groups of 15 aphids were given acquisition access periods to BBSSV in Parafilm[R] sachets or infected blueberry plants, and were transferred to healthy, micropropagated blueberry plants. After a 24 hr inoculation access period, aphids were transferred at daily intervals to ten new blueberry plants (i.e. 1 plant/day). + = roots were positive for BBSSV 6 months to 1 year after aphid inoculation; 0 = no plants inoculated.

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