MOVEMENT AND DISTRIBUTION OF BLUEBERRY SHOESTRING VIRUS (BBSSV) IN HIGHBUSH BLUEBERRY CV. JERSEY

by

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ABSTRACT

MOVEMENT AND DISTRIBUTION OF BLUEBERRY SHOESTRING VIRUS (BBSSV) IN HIGHBUSH BLUEBERRY CV. JERSEY

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Blueberry shoestring virus causes an economically important disease of highbush blueberry in Michigan. Annual crop loss due to this disease is estimated at 3 to 4 million dollars. Fifty percent of the 18,000 acres of blueberries under cultivation in Michigan are of the cultivar Jersey which is highly susceptible to this disease. Shoestring disease is vectored by the blueberry aphid, Illinoia pepperi, and spread is bush to bush.

BBSSV antigen was found in berries, seeds, stems, blossoms, leaves, and roots from symptomatic, diseased bushes using ELISA and dot-blot immunoassay. It was determined that dot-blot immunoassay was four times more sensitive than ELISA in dilution series tests. Dot-blot immunoassay and a cDNA probe, made to viral RNA, were equally sensitive in detecting purified virus diluted in buffer (0.3 pg) and healthy leaf sap (3.0 pg). These assays were also similar in detecting virus in leaves, blossoms, and stems (xylem and phloem). Dot-blot immunoassay was unable to detect virus in aphids fed on

symptomatic, diseased leaves and purified virus in sachets, due to the endogenous enzymes of the aphid.

Fluorescent antibody studies of symptomatic, diseased leaves and stems revealed BBSSV antigen in all cell types except phloem in leaves and in tracheary elements of stems. Aphid-and rub-inoculation of BBSSV into test plants showed that progression of BBSSV antigen was random, beginning in the vascular tissue at 1 to3 days post-inoculation. The viral antigen spread outward to the mesophyll and palisade parenchyma cells by day 7 post-inoculation. Viral antigen reached the epidermal cells by day 14 post-inoculation.

Grafting and girdling experiments revealed that long distance transport of BBSSV is through both the xylem and the phloem. Viral antigen found in leaves above girdles provided evidence for xylem transport. Viral antigen was also found in leaves from stems that were not grafted, which demonstrated phloem transport through the root system. Evidence of xylem and phloem transport along with dot-blot immunoassay/ cDNA probe data on stems showed that BBSSV was moving in the form of whole virus.

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LITERATURE REVIEW

LITERATURE REVIEW

Blueberry shoestring virus (BBSSV)

Blueberry shoestring disease was first reported by Varney in 1957(50) as a probable virus-caused disease of cultivated blueberries in New Jersey. Since that time, blueberry shoestring has been found in Michigan(46), Washington state (P. Bristow and D. Ramsdell, unpublished data), North Carolina (27), and Nova Scotia(25).

Shoestring disease is characterized by red streaks on current, 1- and 2-year old stems. This streaking sometimes disappears as the season progresses. Blossoms may also exhibit pink shading or streaks on the petals and pedicels may be red streaked.Leaves may be strapped or shoestring-shaped, which is a reduction in the leaf lamina. Leaves are sometimes curled or crescent shaped due to irregular reduction in the leaflamina. Leaves may also exhibit red streaks along the midrib and secondary veins resulting in an oak-leaf pattern. Immature berries on infected bushes may develop a premature reddish-purplish cast(34).

The virus is known to infect the highbush blueberry cultivars June, Jersey, Burlington, Rancocas, Earliblue, Weymouth, Rubel, and Blueray in the field. Coville and Blueray have also been infected by graft inoculation(51). Bluecrop has been infected by rub inoculation in a greenhouse situation but has been found infected in the field only once (15). Wild highbush and lowbush blueberries (*Vaccinium angustifolium* Ait. and *V. myrtilloides* L.) also harbor the

virus(14,25,51).

Lesney et al.(23) attempted to transmit the causal virus to 44 herbaceous indicator plant species but was unsuccessful. Shoestring has been shown to be graft transmissible by chip budding(40,50) and also mechanically transmissible by rub inoculation to other blueberries(23,40).

Blueberry shoestring virus is an isometric virus with a diameter of 27 nm. It contains one species of single stranded RNA, molecular weight 1.45 X 10^6 daltons, that is estimated to be 20% of the total particle weight. The protein subunits, 180 in all, comprise the remaining 80% of the particle weight and the molecular weight of each subunit is 30,000 daltons(33,34).BBSSV has been tentatively placed in the sobemovirus group according to it's similar physical and chemical properties. However, BBSSV has an aphid vector and other members of the sobemovirus group have beetle vectors(3).

The virus is transmitted by the blueberry aphid, Illinoia pepperi (MacG.). Ferritin labelled virions in I. pepperi salivary glands suggest that the virus is circulative and passage of virions through the gut wall suggests that it is semipersistent(32). An acquisition access period of 24 hours and an inoculation access period of 1 hour also suggests a semipersistent relationship(27).

Mathematical analysis of field mapping data indicates bush-to-bush spread of BBSSV(23). Aphids are also carried down the row in mechanical harvestors(52). Aphids have been reported to overwinter as eggs in the field, in the leaf litter

and on bushes(27).

Michigan is the largest producer of blueberries in the United States with 18,000 acres under cultivation. This acreage carries a cash value of over 40 million dollars (J. Nelson, personal communication). Fifty-six percent of the totalacreage of commercial plantings is of the cultivar Jersey. `Jersey' seems to be one of the most susceptible cultivars and is estimated to have a loss of 3 to 4 million dollars per year to this virus(35). Loss is due to reduced yield and removal of bushes.

Control measures for shoestring consist of removal of diseased bushes, use of virus-free plants, control of the aphid vector through well-timed sprays, and cleaning of harvestors and other equipment between harvests of different fields.

In 1973, Hartmann et al.(16) conducted an ultrastructural study of tissues infected with BBSSV. In transmission electron micrographs they found virus-like particles(VLP) in leaf epidermal cells, in small vacuoles in the palisade cells, in the spongy mesophyll, and in the xylem parenchyma of the leaf. Both root xylem cells and leaf epidermal cells contained crystalline arrays of particles. Larger masses of VLP were found in root than in leaf cells. Nearly all cytoplasmic cells of the root xylem contained some VLP. VLP were found near plasmodesmata, but not in them. It has been suggested that the connections are of a sufficient diameter to allow passage of VLP. No work was reported on stem tissue.

Intra- and Intercellular Movement of Virus

Intracellular movement of plant viruses has not been widely studied. It has been suggested, since viral particles are not motile on their own, that cyclosis is the general force behind virus movement. Esau(11) found that tobacco mosaic virus(TMV) particles were entrapped in nuclei during cell division. This may suggest cyclosis or microtubules associated with the spindle fibers as locomotive forces. In another study, deZoeten and Gaard(8) looked at the pore complexes in the nuclear membrane of nuclei in leaf palisade and mesophyll cells of several plant species commonly used in plant virus research. They found that southern bean mosaic virus(SBMV) particles could move through nuclear pores into nuclei and suggested the influence of diffusion pressure.

Intercellular movement is mainly by the means of cytoplasmic streaming through plasmodesmata as first reported by Livingston in 1935(24). Sheffield(42) found that guard cells lacked plasmodesmatal connections to adjacent epidermal cells and inclusion bodies were never found in these cells. Wu et al.(54) found callose depositions sealed off plasmodesmata between infected bean cells and noninfected cells, suggesting a host resistance mechanism. Plasmodesmata have also been suggested as the route of virus movement in tobacco and carrot callus tissue(44). Esau(10,11) suggested that plasmodesmata are the "sole pathway" of cell to cell movement.

More recent studies have shown that virus infection could lead to modifications of certain plasmodesmata in order to accomodate transport of virus particles. Kitajima and Lauritis(20) reported that the modified plasmodesmata had larger openings with no endoplasmic reticulum association. In some cases the cell wall formed papilla at the openings of the plasmodesmata, extending the channel further into the cell. Half-transformed plasmodesmata were also observed where one end was enlarged and contained virions and the other end was normal.

Nishiguchi et al.(31) and Taliansky et al.(47) reported that a temperature sensitive strain of TMV was able to spread to and infect other cells at 22^{0}C but not at 32^{0}C unless it was assisted by a temperature resistant virus. They suggested that the lower temperature and the helper virus initiate an "unknown" modification of host cells allowing the virus to move. These modifications have not been examined at the electron microscope level, but could be similar to Kitajima and Lauritis' findings.

Shalla(41), in 1959, calculated that plasmodesmata were large enough for virus to pass through. Since then there have been two classic reports of virions found in plasmodesmata utilizing electron microscopy. Esau et al.(12) found beet yellows virus, a flexuous rod-shaped virus, and deZoeten and Gaard(8) found tomato ringspot virions, an icosahedral virus, in plasmodesmata.

Long Distance Transport of Virus

Long distance transport is thought to occur mainly in the phloem and, in a few reported cases, in the xylem. Transport in the phloem is mainly through the sieve tubes. Some viruses which have insect vectors are directly injected into the phloem and can move quickly from there. One example is beet curly top virus, vectored by the beet leafhopper(1).

Rates of movement out of a leaf have been measured as high as 1 inch per minute(1). Comparisons of vector and mechanically inoculated rates of movement have also been done. Bennett(4) found that beet mosaic virus, when introduced by it's aphid vector, moved a distance of 10 cm out of a leaf in 20 minutes, whereas it took 54 hours to move the same distance when rub inoculated. The rapid rate of the former method was attributed to the aphid introducing the virus directly into the phloem. Other examples of rates of movement are 7 inches per hour with TMV in tomato leaflets(21), 40 cm in 2 hours with maize streak virus(45), beet yellows virus at 30 cm per hour(4), and 8 cm per hour with potato virus X (5).

Direction of movement of virus has been investigated by a number of researchers. Samuels(37) working with TMV in tomato found that the virus moved downward toward the roots first, then upward via the roots to the shoot tip. He suggested that this downward movement was the general case unless there was developing fruit a little above the inocula-

tion point. Capoor(5) had the same general results but did not believe that the virus occurring in the upper parts had come from the roots. Kunkel(21) found that upon reaching the stem, TMV frequently traveled both upward and downward simultaneously, but also frequently downward only or upward only. Robb(36), working with tomato aucuba mosaic virus, also recorded simultaneous upward and downward movement of virus. There was only one report of upward movement of virus as the initial movement. Maduewesi and Hagedorn(26), working with Wisconsin pea streak virus, reported that the virus moved upward initially, passing some nodes, then moved down to the roots. They also reported some simultaneous up and down movement.

Long distance transport of virus has also been related to direction of food transport. Most of the aforementioned researchers found evidence of movement toward fruit, suggesting a sink. C.W. Bennett has done most of the definitive work in this area. In 1937(2), working with curly top virus in tobacco and sugarbeet, plants were grown in the light and in the dark or shoots were defoliated. Virus was found to move out of plants or shoots in the light, but not out of darkened shoots or etiolated plants. When shoots were returned to the light, virus moved out in 24 to 72 hours. For shoots in the light, virus moved out in 4 hours. Virus did not move out of inoculated shoots on etiolated plants for the 21 day study period.

In 1940, working with TMV in both tobacco and tomato plants, Bennett found translocation of virus hastened in an

acropetal direction if the top of the plants was removed or defoliated. He also did some ringing experiments and found that passage of virus was delayed or non-existent even after 300 days(3). In 1960, working with three viruses, Bennett found that defoliation of shoots accelerated translocation of beet yellows, beet mosaic, and curly top virus which he associated with transportation of carbohydrates(4).

Esau et al.(12) stated that the presence of "developing fruit trusses" modified the direction of movement of virus because an upward movement was observed. This upward movement paralleled an experiment using ^{14}C-labelled sugar. The authors suggested that recorded velocities of virus translocation are similar to those established for transport of organic solutes in the phloem.

There have been very few reports of virus found in the xylem. Early reports often included Pierce's disease and phony peach, whose infectious agents have now been classified as xylem-limited fastidious prokaryotes. Schneider and Worley (38,39), working with southern bean mosaic virus (SBMV) in Pinto bean, found that SBMV moved upward in the water stream. However, the viruswould not normally be found in the vascular system of this local lesion host. In these experiments, SBMV was introduced directly into steamed regions of bean stems or by approach grafting Black Valentine bean, a systemic host, to Pinto bean and then steaming the Pinto bean. The virus then moved from parenchyma cells into tracheary elements and upward, moving long distances or into the phloem and was not able to

pass the steamed region. Their stated purpose for this research was to show that if the virus could reach the vascular system, it was then able to move and infect other cells, contrary to the results with superficial mechanical inoculation.

Another possible xylem association is with lettuce necrotic yellows virus (LNYV) (6). Virions of LNYV were found in young xylem cells of leaf veins, but not in the phloem, and was also recovered from xylem sap in the stem. The virus was not limited to the xylem as particles were also observed in mesophyll and epidermal cells and leaf hairs. Virus has also been found in natural guttation water suggesting transport in the xylem(18).

Jones(19) suggested that the slow pattern of movement of potato mop top virus is consistent with transport in the xylem. He also proposed that the defective mutants PM1 and PM2 may move by the same route.

Along with where, or in what tissues viruses are transported, the question arises as to what form the virus is in. Little information has been reported regarding the form of the virus translocated. However, with the availabiltity of new molecular hybridization techniques, this should become an easier and important task. TMV has been found to be infective as nucleic acid only and there are defective strains of TMV which have no protein coat. Zech(55) proposed that infectious TMV moving over long distances is not whole virus, but "smaller non-infective cleavage products".

Siegal et al.(43), working with defective TMV strains,

stated that virus moving long distances must be whole virus as the defective TMV strains, which consisted of nucleic acid only, moved only short distances. A more recent paper suggests that in TMV infected plants a virus-specific informosome-like ribonucleoprotein(vRNP) is produced. It is further suggested that vRNP "serves as a transport form of virus genetic material". The authors also related coat protein to the systemic spread of the virus(9).

Fluorescent Antibody Detection of Viruses in Plant Tissues

In the 1930's, it was discovered that introducing chemical groups into the antibody molecule did not destroy its specificity. Coons et al.(7) made an anti-*Pneumococcus* antibody, conjugated it to fluorescein and found it to be specific. They wanted to be able to look at localized antigen-antibody reactions, *in situ*, to establish that this localized action does produce a given disease. They were successful, but the conjugation procedure and the resulting antibody-fluorescein isothiocyanate molecule needed to be further perfected. The authors also suggested the use of fluorescent antibody for the demonstration of viruses in tissues and cells.

Most of the fluorescent antibody work with plant viruses was done in the 1960s and 1970s. The emphasis in these works has been on location, distribution, and replication of virus. Nagaraj and Black(30) found wound tumor virus in the

pseudophloem of the induced tumor in infected sweet clover plants. Mumford and Thornley(28) confirmed that curly top virus is restricted to phloem by looking at infected bean, tomato, sugarbeet, and tobacco leaf sections with fluorescent antibody. Citrus tristeza virus(CTV) was found in the phloem of the vascular bundles from the pericarp of fruit. CTV was also found in the vascular bundles of stem bark, green twigs, and petioles. This work all correlated with transmission electron microscope evidence of CTV location (49).

Gingery(13) favored using an immunofluorescence test for the presence of maize chlorotic dwarf virus because of its speed, reliability, and sensitivity. In this case the author ground the tissue and looked at the extracts instead of sections, which would save time. This work was also done before ELISA (enzyme linked immunosorbent assay) testing became routine.

Distribution of virus in plant tissues has been looked at even less. Worley and Schneider(53) used a fluorescent antibody technique to follow the progression of SBMV in an inoculated leaf and the opposite uninoculated leaf of a bean plant. They found that the virus progressed from cell to cell in the inoculated leaf, but was observed throughout the mesophyll in the uninoculated leaf with no visible progression.

Others have looked at progression; not as to which cell type is infected, but how many cells are infected. Lei and Agrios(22) used a fluorescent antibody technique to look at the number of cells infected with maize streak virus in re-

sistant and susceptible corn lines. They found that infection in the susceptible line spread faster and toward the stem rather than the radial, slow spread found in the resistant line.

Fluorescent antibody studies have also been used to look at synthesis of virus and where this occurs. Thornley and Mumford(48) found CTV virus antigen in the nucleus early on, and later found that it increased in the nucleus and spread progressively into the cytoplasm.

With TMV there is some controversy in the fluorescent antibody studies as to where the virus replicates. Hirai and Hirai(17), working with both whole virus and TMV-RNA, found that the nucleus was the first organelle in which TMV protein was detected. Six hours after infection TMV protein was also found in the cytoplasm. However, Nagaraj(29) found TMV protein only around the chloroplasts in the mesophyll. He reported no antigen around or in the nucleus.

Objectives

The objectives of this research were to study BBSSV in the plant after infection has occurred, to determine in what cell types and tissues BBSSV is located, and to dertermine the possible route of virus transport. First, detection systems will be compared for their sensitivity in detecting BBSSV antigen and RNA. Second, fluorescent antibody staining will be used to monitor distribution and progression of the virus in fresh tissue sections. And finally, grafting and girdling techniques will be used to determine long distance

transport of the virus.

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CHAPTER II

LOCALIZATION OF BLUEBERRY SHOESTRING VIRUS (BBSSV) IN HIGHBUSH BLUEBERRY CV. JERSEY

INTRODUCTION

Blueberry shoestring virus (BBSSV) has been studied in aphids and plants by transmission electron microscopy techniques. It has been found in the aphid salivary glands as well as the gut wall(21). Virus-like particles (VLP) were found in leaf epidermal, palisade, mesophyll, and xylem parenchyma cells(10). VLP were also found in large masses in cytoplasmic cells of the roots.

Blossoms are routinely used as a source of virus for purification(22) and symptomatic blueberry leaves are used as virus-positive controls in immunoassays. It is also known that the virus can be transmitted by grafting chip buds onto healthy plants(26,29). This suggests that virus is in the bud tissue. Other tissue and cell types of blueberry have not been examined to determine whether they harbor the virus.

The purpose of the following experiments was to 1) determine the location of BBSSV by testing various blueberry tissues, 2) to determine the sensitivity of enzyme-linked immunosorbent assay(ELISA), dot-blot immunoassay, and a cDNA probe to detect BBSSV in purified preparations and blueberry tissues, and 3) to determine what host cell types BBSSV invades.

MATERIALS AND METHODS

Sample Preparation for ELISA and Dot-blot Immunoassay
(Buffers can be found in Appendices A and B)

Leaves - Leaves were weighed, cut into small pieces, and ground in extraction buffer 1:10(w/v) with a Tekmar tissuemizer (Tekmar Company, Cincinnati, Ohio 45222). Samples were strained through one layer of cheesecloth and kept cold until plated.

Stems - Stems were placed in water to ease separation of phloem from xylem. Outer bark/phloem was cut away with a razor blade and chopped into small pieces. The xylem/cortex was also chopped into small pieces with a razor blade. All samples were then treated as above.

Roots - Roots were washed, dried, weighed, and cut into small pieces before dilution and grinding as above.

Berries & Seeds -Seeds were hand separated from berries, washed, and ground in spot plate wells in 2 ml of extraction buffer. Berries were ground in extraction buffer (1:2 w/v) and treated as above.

Antiserum Production

A polyclonal antiserum to BBSSV was prepared by K. Morimoto(15), lyophilized, and stored at -20^{0}C.

Gamma Globulin Purification

Anti-BBSSV gamma globulin was purified by the procedure of Clark and Adams(5). One ml of antiserum was diluted with 9 ml distilled water and 10 ml of saturated ammonium sulfate were added dropwise while stirring the antiserum. The solution was left for 30-60 minutes at room temperature. The solution was then centrifuged at 6,000 rpm in a Beckman No. 40 rotor for 5 minutes and the precipitate retained. The precipitate was resuspended in 2 ml of half-strength phosphate buffered saline(PBS, pH 7.4). The solution was dialyzed three times against 500 ml 1/2-strength PBS including once overnight.

The dialysate was then filtered through a 5 cm bed of DE 22 cellulose in a 10 ml pipette pre-equilibrated with 1/2-strength PBS. The gamma globulin was washed through the column and 2 ml fractions collected. The fractions were read at A_{280nm} in a Gilford spectrophotometer and diluted to give a reading of 1.4 O.D. units (about 1 mg/ml). Aliquots of 1 ml were then stored at $-20^{o}C$.

Preparation of Enzyme Conjugate

Anti-BBSSV gamma globulin was conjugated separately to alkaline phosphatase and horseradish peroxidase by the procedure of Clark and Adams(5). Two mg of alkaline phosphatase (Sigma no. P-4502 Type VII) or horseradish peroxidase (Sigma no. P-6140 Type X) were centrifuged in a No. 40 rotor at 6,000 rpm for five minutes and the precipitate retained. The precipitate was then dissolved in 1 ml of a 1mg/ml fraction of

purified gamma globulin. The solution was dialyzed three times against 500 ml PBS, once overnight. Glutaraldehyde was added to make a final concentration of 0.05%(v/v) and mixed well. The mixture was allowed to stand at room temperature for four hours. The glutaraldehyde was removed by dialyzing three times against 500 ml PBS with 0.01% (w/v) sodium azide added as a preservative. Bovine serum albumin was added at a final concentration of 5mg/ml and the conjugate was stored at $4^{0}C$.

Enzyme-Linked Immunosorbent Assay(ELISA)(Appendix A)

The double-antibody sandwich method of ELISA was used to detect BBSSV antigen in blueberry tissue(5). Flat bottom, polystyrene plates (Dynatech Laboratories, Alexandria, VA 22314) were coated with purified anti-BBSSV gamma globulin diluted in coating buffer (0.05M sodium carbonate-bicarbonate buffer, pH 9.6) 1:1000 at a rate of 200 ul per well. The plate was placed in a plastic bag, sealed, and incubated for four hours at $37^{0}C$.

Wells were washed three times at three minute intervals with phosphate buffered saline containing Tween 20 (PBS-Tween). Samples were prepared and added to wells at the rate of 200 ul per well. Plates were again sealed in plastic bags and refrigerated overnight ($4^{0}C$).

Wells were washed as above and alkaline phosphatase conjugate was diluted in extraction buffer, 1:800 (v/v), and added to wells at a rate of 200 ul per well. The plate was sealed in a plastic bag and incubated at $37^{0}C$ for three

hours. The plate was rinsed with PBS-Tween three times and substrate,p-nitophenol phosphate (Sigma), was dissolved in substrate buffer (10% diethanolamine, pH 9.8) and added to wells at a rate of 200 ul per well. After 15 and 45 minute incubations at room temperature, the A_{405nm} values were read spectrophotometrically with a microELISA minireader (Dynatech Laboratories, Alexandria, VA 22314).

The threshold value for positive virus identification was the mean A_{405nm} value plus three standard deviations of healthy control samples. Samples greater than the threshold value were considered positive.

Dot-blot Immunoassay (Appendix B)

The method of Bantarri and Goodwin(2) was used with one modification, the addition of 1% (w/v) polyvinylpyrrolidone (PVP) to the grinding (extraction) buffer.

Nitrocellulose membrane (NCM - BioRad 0.45um) was labelled with a pencil and placed, with forceps, in distilled water for 15 seconds. The NCM was handled with forceps or when wearing gloves. NCM was then transferred to a pyrex dish containing 60 ml coating buffer (0.05M sodium carbonate-bicarbonate, pH 9.6) containing a 1:1000(v/v) dilution of anti-BBSSV-IgG. The NCM was left covered at room temperature for four hours. After four hours, the IgG-coating solution was replaced by a wash buffer, tris-buffered saline with 0.05% Tween 80 (TBS-T80), and placed on a gyratory shaker. The wash solution (60 ml) was changed six times in 30 minutes. After the last wash, 60 ml of TBS-T80 plus 3% BSA(w/v) were

added to block remaining available binding sites. The pyrex dish was placed in a plastic bag, sealed, and incubated at 37^{0}C for 1.5-2 hours.

The NCM was removed from the BSA solution and placed on a Bio-Rad Bio-dot^{R} apparatus (Bio-Rad Laboratories, Richmond, CA, 94804). A vacuum was applied to eliminate leaking of samples from well to well.

Samples were applied to the wells of the dot-blot apparatus at rates of 100, 200, or 400 ul per well. The apparatus was placed in a plastic bag, sealed, and placed in the refrigerator overnight (4^{0}C).

The next day the apparatus was disassembled, the NCM was washed with distilled water to remove sample debris, and placed in TBS-T80 in a pyrex dish on the shaker for another 30 minute wash series. A conjugate of anti-BBSSV-IgG and alkaline phosphatase or horseradish peroxidase was added to 60 ml of TBS-T80 at a dilution of 1:800 v/v (75 ul in 60 ml) which replaced the wash solution. The dish was covered and left at room temperature for three hours. After three hours, the NCM was again washed for 30 minutes on a shaker with TBS-T80.

Substrate was prepared during the last wash. Substrate consisted of 0.2M tris-HCl buffer, pH 8.2. Fifteen ml of buffer were mixed with 90 mg fast red TR salt (Sigma) and filtered (6 mg/ml of buffer). Nine ml of the tris buffer were added to 6 ml of naphthol AS-MX phosphate solution (Sigma) to make a final concentration of 0.1%(v/v). The two

solutions were mixed together in a clean pyrex dish and the NCM added. Color development was stopped after 45 minutes to one hour by rinsing with distilled water. The NCM was air dried on filter paper and placed in an envelope to prevent cracking. Samples were judged to be either positive(+) if a red to pink dot appeared or negative(-) if no dot appeared.

When using horseradish peroxidase, a substrate stock was prepared. The stock contained 3 mg/ml of 4-chloro-1-napthol in methanol (75 mg in 25 ml methanol stored in a brown bottle at 4^{o}C). During the last rinse of the NCM, 5 ml of stock solution were added to 25 ml TBS-T80 plus 10 ul of 30% hydrogen peroxide (final concentration of 0.01% v/v). The 30 ml of prepared substrate were added to a clean pyrex dish and the NCM placed in the substrate for 4 hours. Virus-positive dots were purple.

Virus Purification(Appendix C)

BBSSV was purified from diseased blossoms by the method of Ramsdell(22) with some minor adjustments. All procedures were carried out at 0-4^{o}C. Three hundred grams of blossoms were homogenized in a cold Waring blender in 900 ml of a 0.1M sodium phosphate buffer containing 0.01M 2-mercapto-ethanol and 0.005M thioglycolic acid, for 3 to 5 minutes. Triton X-100 was added slowly to the homogenate (8% v/v) as it stirred and was left to stir for 2 hours.

The homogenate was then squeezed through cheesecloth and the amount of supernatant determined. Chloroform (10% v/v) and butanol (10% v/v) were added to the supernatant and stir-

red for 15-30 minutes. The mixture was centrifuged in an IEC No. 872 rotor at 10K rpm for 15 minutes (low speed centrifugation). The upper aqueous phase was separated from the organic phases and 8%(w/v) polyethylene glycol (M.W. 6,000) and 0.1M NaCl were added while stirring. The mixture was left stirring for 4 to 6 hours and then given a low speed centrifugation(10K rpm) for 30 minutes. Pellets were resuspended with a glass rod using at least 10% of the starting volume with a 0.05M sodium phosphate buffer containing 0.001M dithiothreitol, pH 7.0 (P-DTT). This mixture was left for 30 minutes or overnight in the cold.

The mixture was given a low-speed centrifugation(10K rpm) for 30 minutes and the supernatant retained. The supernatant was ultracentrifuged in a Beckman No. 30 rotor at 28K rpm for 2.5 hours. Pellets were resuspended in 0.3 ml of P-DTT per tube and left in the cold overnight. Linear log sucrose gradients were prepared with P-DTT buffer and left to equilibrate overnight.

Sucrose gradients were loaded with 0.3 ml of the virus preparation and centrifuged in a Beckman SW-41 rotor at 38K rpm for 90 minutes ($4^{O}C$). Gradients were fractionated and the single virus band collected using an ISCO density gradient fractionator and UV analyzer (Instrumentation Specialties Co., Lincoln, NE 68504). The sucrose fractions containing buffer were diluted 1:3(v/v) with 0.05M sodium phosphate buffer, pH 7.0, slowly mixed by overturning the tube, and centrifuged in a Beckman No. 40 rotor at 36K rpm for 4 hours. Pellets were resuspended in 0.5ml of the 0.05M sodium

phosphate buffer per tube using a glass rod and allowed to stand overnight.

Virus concentration was determined by taking readings of an 0.1ml aliquot of the virus preparation at A_{260nm} in a Gilford spectrophotometer, adjusting for dilution, and dividing by the extinction coefficient for BBSSV ($E^{0.1\%}_{260nm} = 5.2$).

cDNA Probe Preparation

A cDNA probe was prepared by Tim Lynch using BBSSV RNA purified from infected blossoms(22). The random primer method of cDNA preparation, as described in the Amersham cDNA kit (Amersham Corporation, Arlington Heights, IL 60005) was utilized. The three fragments, representing 85% of the genome, were inserted into the BamH1 site of the plasmid pBR322. Competent E. coli cells, strain JM109, were transformed with the recombinant plasmids and grown in a selective medium.

Hybridization of cDNA Probe with BBSSV RNA

All solutions mentioned in this section are found in Appendix D.

Approximately 1 ug of an E. coli preparation with an insert was nick translated following the proceduresupplied with a BRL nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD 20877). This resulted in the labelling of BBSSV cDNA with ^{32}P.

Samples were prepared the same as for dot-blot immunoassay. The NCM was also prepared as in the dot-blot procedure

up to the first wash after addition of samples. The NCM was then air dried and transferred to a Seal-a-Meal^{R} bag containing 3X SET buffer (1 ml to wet filter). Prehybridization solution(14) was added to the 3X SET, 10-20 ml, and incubated at 42^{0}C for 1 hour. Prehybridization mix was poured off and 10 ml of hybridization mix(14) plus 10^{6}dpm/ml ^{32}P-labeled cDNA, for a standard size filter, were added to the bag containing the NCM and incubated at 42^{0}C overnight. The NCM was washed three times, five minutes each, at room temperature with 2X SET containing 0.1% sodium dodecyl sulfate (SDS). The NCM was then washed two times, 20 minutes each, at 42^{0}C in 0.12X SET containing 0.1% SDS(14). The NCM was air dried and exposed to X-ray film (Kodak X-Omat AR) with intensifying screens for two days at -70^{0}C.

Sample Preparation for a Comparison of ELISA with Dot-blot Immunoassay

Diseased and healthy leaf samples were divided in half (0.8 g and 1.0 g respectively) and ground in their respective buffers for ELISA and dot-blot immunoassay (1:10 w/v). Samples were filtered through cheesecloth and dilutions were made from the 1:10 (w/v) first dilution to give final dilutions of 1:100, 1:400, 1:800, 1:1200, 1:1600, 1:3200, 1:5000, 1:7500, and 1:10,000 (v/v). Samples were plated at a rate of 200 ul per well for ELISA and 200 and 400 ul per well for dot blot immunoassay. Both assays were allowed 45 minutes to develop after addition of substrate to keep the assays as equal as possible.

Sample Preparation for cDNA, Dot-blot Immunoassay Comparison

Purified Virus Dilutions

The starting purified concentration of BBSSV was 0.6 mg/ml. Eighty-three microliters of this preparation (=50 ug) were added to 332 ul of TBS-T80 plus 0.5% PVP buffer to give a final virus concentration of 6 ug/50 ul. A 50 ul aliquot was diluted with 450 ul of buffer (1:10 v/v). The dilutions of 50 ul in 450 ul of buffer were carried out seven more times to give final virus quantities of 600 ng, 60 ng, 6 ng, 600 pg, 60 pg, 6 pg, 0.6 pg, and 0.06 pg. Each sample consisted of 450 ul. Samples were divided in half; 225 ul were added to each of two wells. Therefore, final virus quantities added to the wells were 300 ng, 30 ng, 3 ng, 300 pg, 30 pg, 3 pg, 0.3 pg, and o.03 pg. This dilution series was repeated using healthy plant sap, diluted 1:10 (w/v) with TBS-T80 plus PVP buffer as the diluent. Final virus quantities were the same as above.

Leaves and Blossoms

Samples consisted of 1.0 g each of healthy and diseased blueberry leaves and 2.0 g of diseased blossoms. Samples were diluted 1:10 (w/v) with TBS-T80 plus PVP buffer and ground with a tissuemizer. Samples were strained through cheesecloth and further diluted. The dilution series was set up in two ways. The first series consisted of dilutions of 1:10, 1:300, 1:600, 1:1000, 1:1200, 1:2400, 1:7200, 1:12,000, 1:16,000, and 1:20,000 (v/v). The dilutions 1:300 and 1:1000 were not plated. Samples wereplated at a rate of 200 ul per

well with two wells per sample. The second dilution series consisted of 1:10, 1:100, 1:1000, 1:10,000, 1:20,000, 1:30,000, 1:40,000, 1:50,000, and 1:60,000 (v/v). The dilution 1:100 was not plated. Samples again were plated at a rate of 200 ul per well with two wells per sample.

Stems

Stems were cut from diseased bushes in the field on August 29, 1986. The stems were placed in plastic bags and stored in a coldroom (4^{0}C).

Five diseased stems and three healthy stems were placed in water on September 24, 1986 and stripped of the outside bark (including phloem). The inner layers, xylem and cortex, were cut into small pieces. Phloem (0.2 g) and xylem (0.5 g) were ground in 5 ml of TBS-T80 plus PVP buffer (1:25 and 1:10 (w/v) respectively), strained, and plated onto nitrocellulose paper in a dot-blot apparatus at a rate of 200 ul per well.

Aphids

Blueberry aphids (*Illinioa pepperi*) were placed on diseased and healthy plants for an acquisition feeding and left for approximately one week. Aphids were also fed on sachets containing 20% sucrose (75 ul) and PBS buffer (75 ul) or 20% sucrose (75 ul) and 75 ul of a virus preparation that contained 300 ug/ml (final virus quantity was 22.5 ug of virus per sachet). Ten aphids were placed on the sucrose/ buffer sachet and 10 aphids were fed on the sucrose/BBSSV sachet for 48 hours.

Aphids were ground in 250 ul of TBS-T80 buffer in a spot plate well. The number of aphids ground together was varied

to test the ability of the assay to detect virus in single aphids or groups of aphids. Six samples of aphids fed on diseased material consisted of one, one, one, two, three, and four aphids. Four samples of aphids fed on sachets consisted of one, one, and two aphids ground together, and one aphid diluted 1:10, 1:20, 1:40, 1:80, and 1:160 (v/v) assuming the first dilution of the aphid plus 250 ul of buffer was 1:2 (w/v). Aphid samples were plated at a rate of 100 ul per well.

Cryostat Sectioning

An IEC CTF microtome-cryostat (IEC, Needham Heights, MA 02194) was cooled to -20^{O}C before sectioning. A thin layer of distilled water was placed on a stub and allowed to freeze. Tissue-tek^{R} (Miles Scientific, Naperville, IL 60566), a cryoprotectant, was layered onto the stub and a small piece of leaf, root, or stem positioned in the cryoprotectant. Tissue-tek^{R} was layered onto the specimen until it was completely covered. Sections were cut with a single-edge, teflon coated, injector-type razor blade and then placed on a slide or into phosphate buffered saline (PBS, pH 7.0). Sections were kept cold until stained.

Fluorescent Antibody Staining

Fluorescent antibody staining of BBSSV in leaves, stems, and roots was done by following the procedures of Mumford and Thornley(16) and Malin et al.(13). After trial and error, an indirect staining procedure was adapted to overnight staining

at 4°C instead of the reported 0.5 to 1 hour at 37°C.

Dilutions of anti-BBSSV-IgG and of sheep anti-rabbit-IgG conjugated to fluorescein isothicyanate (FITC) were tested to determine the best working dilutions. Dilutions of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024 were paired with dilutions of 1:16, 1:32, 1:64, and 1:128 of FITC. It was determined that IgG at a dilution of 1:256 and FITC at a dilution of 1:32 were the best working dilutions.

Preimmune rabbit serum was used as a control as well as anti-tobacco ringspot virus-IgG (TRSV-IgG; TRSV is another virus which infects blueberry). TRSV was used only on material from the greenhouse, as material from the field could possibly be infected with TRSV. Healthy plant material was also used as a control.

Cryostat sections were placed in spot plate wells containing 400 ul of anti-BBSSV-IgG, preimmune serum, or anti-TRSV-IgG, at a dilution of 1:256(v/v) in 0.85% NaCl, with a small paintbrush. The spot plates were placed in a covered tray and then into a coldroom (4°C) overnight. Sections were washed three to four times with phosphate buffered saline (PBS, pH 7.0) at 10 minute intervals by drawing out and replacing solutions with a pasteur pipette.

Sections were then stained with sheep anti-rabbit FITC at a dilution of 1:32(v/v) in PBS and left overnight in the cold. Sections were again washed three to four times with PBS and mounted in PBS on glass slides. Coverslips were sealed and slides placed in a moist chamber at 4°C until observed.

Sections were observed with a Zeiss Universal Research photomicroscope equipped for epifluorescence (450-490nm excitation filter, 520nm barrier filter). Kodachrome 64 film at exposures of 1.5 minutes (400X), 3 minutes (250X), or 4 minutes (100X) was used to record observations.

RESULTS

Distribution of BBSSV in Blueberry Tissues

In all blueberry tissues tested, roots, seeds, berries, and stems, BBSSV antigen was present. High concentrations of virus were present in both berries and seeds represented by the high ELISA values taken five minutes after substrate addition (Table 2.1). The dot-blot immunoassay confirmed these findings.

Stems had the highest ELISA values and dot-blot positive red dots of any tissue (Table 2.2). After only five minutes, ELISA values were over 1.00 (A_{405nm}). Both xylem and phloem were positive with essentially no difference between them.

Blossoms and leaves have also been found as positive sources of the virus.

Roots were also found to be a source of virus. Table 2.3 represents the ELISA and dot-blot immunoassay results from eight symptomatic, diseased plants and five healthy plants. All eight diseased plants contained BBSSV antigen in their roots while none of the healthy plants showed posiitive reactions in either immunoassay. Schulte(26) also found BBSSV antigen in the roots.

TABLE 2.1 DETERMINATION OF THE PRESENCE OF BBSSV IN BLUEBERRIES AND SEEDS USING ELISA AND DOT-BLOT IMMUNOASSAY

Source Material[a]		Dot-blot[b]	ELISA(A_{405nm})[c]
Berries	1	+	1.250
	2	+	1.120
	3	+	1.100
	4	+	0.750
	5	+	0.590
	6	+	0.860
	7	+	1.280
Healthy[d] Control	1	-	0.025
Seeds	1	+	0.600
	2	+	0.820
	3	+	0.940
	4	+	0.920
	5	+	1.000
	6	+	0.770
	7	+	0.990
Healthy[e] Control	1	-	0.050

[a]Samples were taken from seven diseased bushes and one healthy bush.

[b]Samples were dot-blot positive(+) or negative(-) and were based on two replicates.

[c]ELISA values are the average of two readings taken five minutes after adding the enzyme substrate. The threshold established were values above the mean plus three standard deviations of healthy controls.

[d]Mean A_{405nm} reading of healthy berries = 0.025.
Mean plus three standard deviations = 0.046.

[e]Mean A_{405nm} reading of healthy seeds = 0.050.
Mean plus three standard deviations = 0.130.

TABLE 2.2 DETERMINATION OF THE PRESENCE OF BBSSV IN DISEASED STEMS USING ELISA AND DOT-BLOT IMMUNOASSAY

Source Material[a]		Dot-blot[b]		ELISA(A_{405nm})[c]	
		Xylem	Phloem[d]	Xylem	Phloem
Diseased	1	+	+	1.690	1.650
	2	+	+	1.300	1.000
	3	+	+	1.380	0.920
	4	+	+	1.220	1.180
	5	+	+	1.420	1.010
	6	+	+	1.190	1.010
	7	+	+	1.070	1.240
Healthy[e]	1	-	-	0.040	0.055
	2	-	-	0.040	0.040
	3	-	-	0.025	0.035
	4	-	-	0.030	0.035

[a]Samples included stems from seven diseased bushes and four healthy bushes.

[b]Samples were dot-blot positive(+) or negative(-) and were based on two replicates.

[c]ELISA values are the average of two readings taken five minutes after adding the enzyme substrate. The threshold established were values above the mean plus three standard deviations of healthy controls.

[d]Samples consisted of 0.5 g of xylem tissue and 0.2 g of phloem tissue.

[e]Mean A_{405nm} reading of healthy xylem = 0.034.
Mean plus three standard deviations = 0.056.
Mean A_{405nm} reading of healthy phloem = 0.041.
Mean plus three standard deviations = 0.069.

TABLE 2.3 DETERMINATION OF BBSSV IN ROOTS FROM SYMPTOMATIC DISEASED PLANTS USING ELISA AND DOT-BLOT IMMUNO-ASSAY

Source Material[a]		Dot-blot[b]	ELISA(A_{405nm})[c]
Diseased	1	+	0.215[e]
	2	+	0.105
	3	+	0.300[e]
	4	+	0.235[e]
	5	+	1.185[e]
	6	+	0.640[e]
	7	+	0.645[e]
	8	+	1.180[e]
Healthy[d]	1	-	0.055
	2	-	0.055
	3	-	0.080
	4	-	0.095
	5	-	0.100

[a]Samples included roots from eight diseased bushes and five healthy bushes.

[b]Samples were dot-blot positive(+) or negative(-) and were based on two replicates.

[c]ELISA values are the average of two readings taken 30 minutes after adding enzyme substrate.

[d]Mean A_{405nm} reading of helthy roots = 0.065.
Mean plus three standard deviations = 0.177.

[e]Values above the threshold limit of the mean plus three standard deviations of healthy controls.

Comparison of ELISA with Dot-blot for Detection of BBSSV

Dot-blot immunoassay of diseased leaf samples was sensitive at a dilution endpoint of 1:7500 (v/v), and ELISA was sensitive at a dilution endpoint of 1:1600 (v/v) (Table 2.4). This represents a four-fold difference in sensitivity. These results represent only a difference between dot-blot immunoassay and ELISA and not the limits of detection each system because the titer of virus in leaf samples varies.

Comparison of a cDNA Probe with Dot-blot Immunoassay for the Detection of BBSSV

The dot-blot immunoassay and cDNA probe were equal in their ability to detect BBSSV in all samples. In purified virus dilutions, BBSSV was detected at a quantity of 0.3 pg in buffer and 3.0 pg in healthy leaf sap (Figures 2.1). ELISA has been reported to detect 5.0 ng of virus in buffer and sometimes as little as 2.5 ng (6).

Both dot-blot and the cDNA probe were able to detect virus in symptomatic leaf tissue and blossoms down to a dilution of 1:30,000 (v/v). Healthy sap did not seem to cause any background problems in either system. Results were not always repeatable with leaves and blossoms because of the varying virus concentrations in samples from test to test. Reported are the best results or detection of the highest dilutions that were achieved at any time (Figure 2.1).

TABLE 2.4 COMPARISON OF ELISA WITH DOT-BLOT IMMUNOASSAY FOR DETECTION OF BBSSV IN LEAVES

Sample	Dilution(v/v)	Dot-blot[a]	ELISA(A_{405nm})[b]
Diseased	1:10(w/v)	+	1.750[d]
	1:100	+	1.725[d]
	1:400	+	0.630[d]
	1:800	+	0.335[d]
	1:1200	+	0.250[d]
	1:1600	+	0.160[d]
	1:3200	+	0.080
	1:5000	+	0.085
	1:7500	+	0.070
	1:10,000	-	0.070
Healthy[c]	1:10(w/v)	-	0.050
	1:100	-	0.065
	1:400	-	0.060
	1:800	-	0.050
	1:1200	-	0.040
	1:1600	-	0.045
	1:3200	-	0.050
	1:5000	-	0.045
	1:7500	-	0.050
	1:10,000	-	0.050

[a]Samples were dot-blot positive(+) or negative(-) and were based on two replicates.

[b]ELISA values are the average of two readings taken 45 minutes after adding the enzyme substrate.

[c]Mean A_{405nm} reading of healthy leaves = 0.050. Mean plus three standard deviations = 0.071.

[d]Values above the threshold limit of the mean plus three standard deviations of healthy controls.

Figure 2.1 Dot-blot immunoassay (left) and cDNA probe autoradiogram (right) of purified virus, leaf, and blossom samples. Quantities of purified virus in buffer (VB) and healthy leaf sap (VS) were (from top to bottom) 300 ng, 30 ng, 3 ng, 300 pg, 30 pg, 3 pg, 0.3 pg, and 0.03 pg respectively. Dilutions of diseased blossoms (DB) and healthy (HL) and diseased leaves (DL) were (from top to bottom) 1:10(w/v), 1:1000, 1:10,000, 1:20,000, 1:30,000, 1:40,000, 1:50,000, and 1:60,000 (v/v) respectively. Also included is a buffer (B) control row.

Figure 2.2 Dot-blot immunoassay (left) and cDNA probe autoradiogram (right) of blueberry stem xylem and phloem tissue and aphids fed on purified BBSSV. Healthy phloem (HP) and healthy xylem (HX) extracts were loaded into the top three wells of the dot-blot apparatus. Diseased phloem (DP) and diseased xylem (DX) extracts were loaded into the five wells below the healthy samples. Healthy aphid (HA) and diseased aphid (DA) samples (from top to bottom) 1 aphid, 1 aphid, 1 aphid, 2 aphids, 3 aphids, and 4 aphids per well, respectively. Also included is a buffer (B) control row.

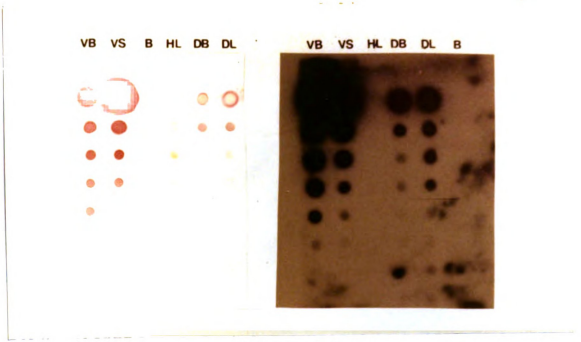

Figure 2.1

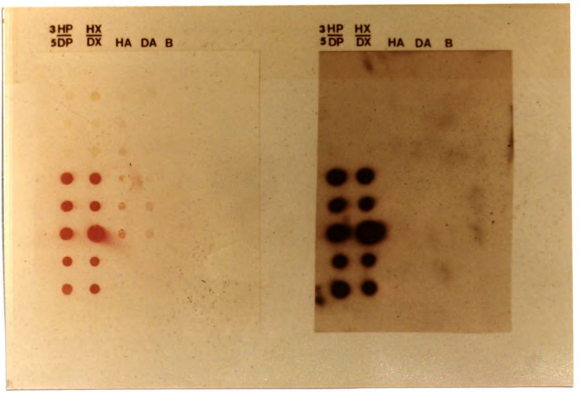

Figure 2.2

Diseased stems were divided into phloem and xylem and both tissues were dot-blot and cDNA probe positive, therefore both contained coat protein as well as RNA (Figure 2.2). These results verify that whole virus is found in both the xylem and the phloem.

Samples of aphids fed on sachets of purified virus and aphids fed on symptomatic leaves did not give conclusive results (Figure 2.2). Both healthy and diseased aphids were positive in dot-blot assays using alkaline phosphatase and horseradish peroxidase. An autoradiogram of an NCM identical to the alkaline phosphatase NCM showed no positives using the probe. Diluting the samples appeared to help in dot-blot, but the results were not confirmed by the cDNA probe. Therefore, all positive results in aphids were thought to be caused by endogenous enzyme systems of the aphid.

Fluorescent Antibody Study of Infected Cell Types

An apple green to yellow fluorescence, representing the presence of BBSSV antigen, was found in most cell types of symptomatic leaves. Fluorescence was found in the upper epidermis and palisade layers (Figures 2.3 and 2.4) along with the spongy mesophyll (Figure 2.4). Fluorescence was also seen in the xylem elements of secondary veins (Figure 2.6) as well as in the midrib of the leaf (Figure 2.7). Healthy control leaves showed no reaction to the second antibody-FITC conjugate (see Figures 2.5, 2.9, and 2.11 which are the healthy controls for Figures 2.4, 2.8, and 2.10, respectively).

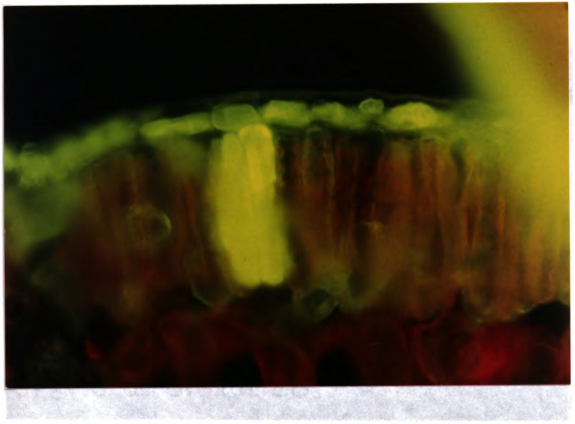

Figure 2.3 **Fluorescent antibody staining of BBSSV in two palisade cells in a section of a symptomatic, diseased leaf (250X).**

Figure 2.4 Fluorescent antibody staining of BBSSV in palisade and mesophyll cells in a section of a symptomatic, diseased leaf (250X).

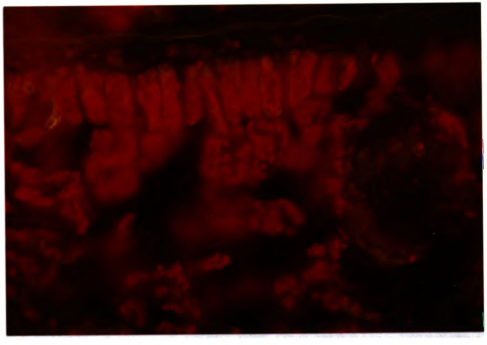

Figure 2.5 Healthy blueberry leaf section indirectly stained with pre-immune serum followed by sheep-anti-rabbit FITC as a control (250X).

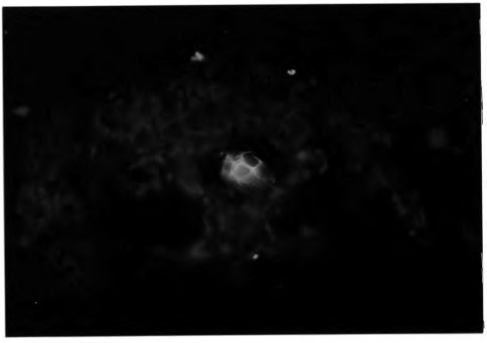

Figure 2.6 Secondary vein xylem fluorescence in a cross-section of a diseased leaf (250 X).

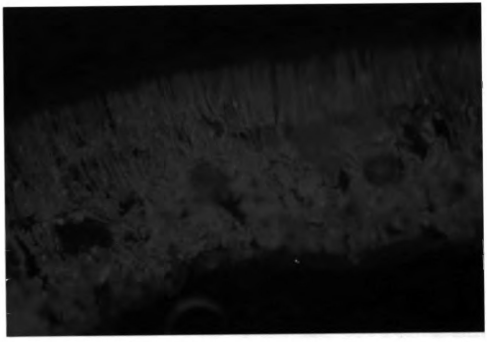

Figure 2.7 Diseased blueberry leaf section indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbit FITC as a control (250 X).

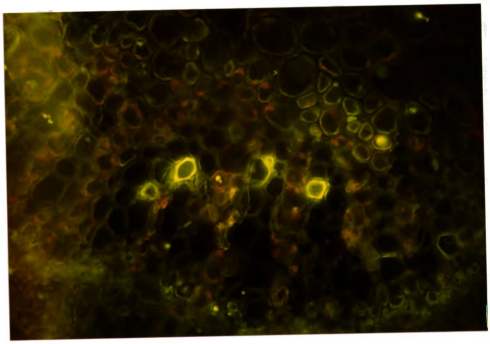

Figure 2.8 **Fluorescence of xylem elements in the midrib of a section of a blueberry leaf infected with BBSSV (250X).**

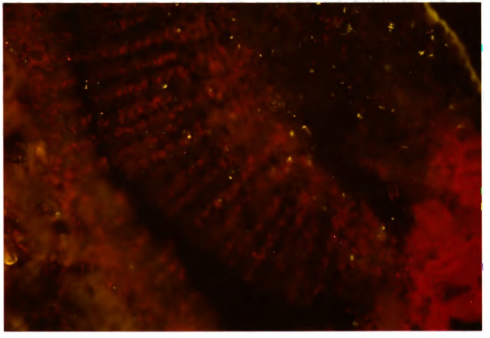

Figure 2.9 **Diseased blueberry leaf section indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbit FITC as a control (250 X).**

In diseased stem sections, fluorescence was found in the xylem of cross-sections (Figure 2.10). Healthy stems exhibited only autofluorescence due to the high lignin content of the cell walls (Figure 2.11). Longitudinal sections revealed fluorescence in the xylem elements (Figure 2.12) and at the end wall of a xylem element (Figure 2.13). Fluorescence was also seen in a few cortex parenchyma cells.

Root sections either did not turn out well or did not exhibit specific viral fluorescence. Root sections were compressed, fell apart, or were too thick to use for fluorescence microscopy.

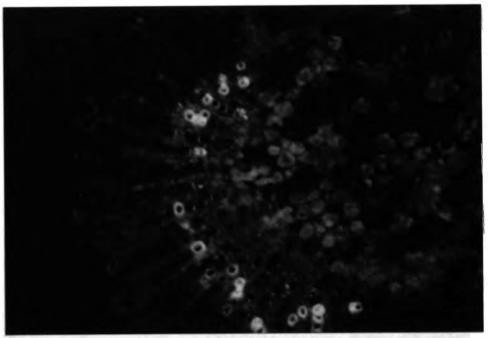

Figure 2.10 Symptomatic, diseased 'Jersey' blueberry stem cross-section indirectly stained with anti-BBSSV IgG andsheep-anti-rabbit FITC. Only xylem elements arestained (100X).

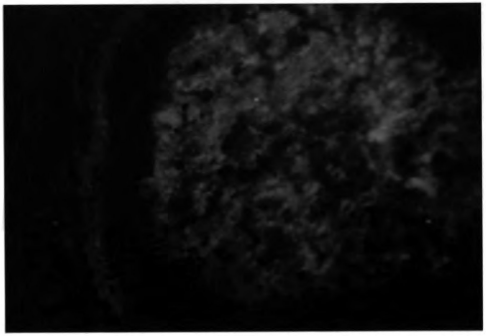

Figure 2.11 Healthy 'Jersey' blueberry stem cross-section containing no specific fluorescence from the indirect FITC stain. Note auto-fluorescence of cell walls and cortex (100X).

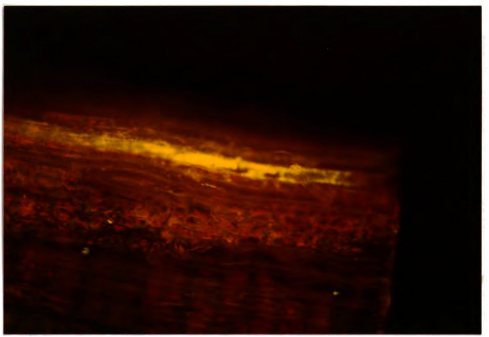

Figure 2.12 **A longitudinal section of a BBSSV-infected blueberry stem exhibiting fluorescence in the xylem elements (100X).**

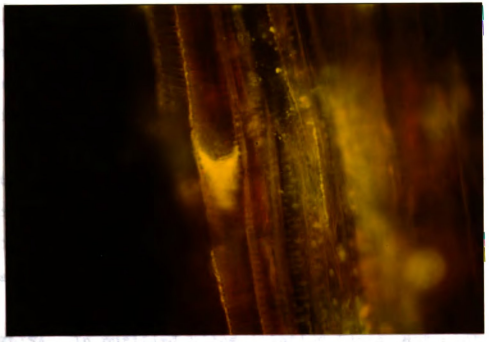

Figure 2.13 **A longitudinal section of a BBSSV-infected blueberry stem exhibiting fluorescence at the end wall of a xylem vessel element (250X).**

DISCUSSION

BBSSV was found in all tissue types tested. BBSSV was detected in berries, seeds, stems, blossoms, roots, and leaves by both ELISA and dot-blot immunoassay. Seeds from diseased bushes were planted after holding the fruit for three months at $4^{0}C$. Most of the seeds failed to germinate with only three seedlings recorded after 1 year (ca. 1%). Since so few seeds germinated, the seedlings were not tested for BBSSV and they did not exhibit symptoms. Lesney et al. (12) planted 400 seeds each from diseased and healthy source bushes. From the 400 planted seeds only 65 from diseased bushes germinated after 2 months. After 10 months no virus symptoms were reported. They reported no evidence for seedborne transmission of the virus.

Comparison of ELISA with dot-blot immunoassay gave similar results except for dilutions of diseased leaf sap (Table 2.4). Dot-blot immunoassay was four times more sensitive than ELISA. In purified virus dilution tests, dot-blot immunoassay appeared to be 1000 times more sensitive with a lower detection limit of 0.3 pg compared to the reported lower detection limit of 2.5 ng of virus in ELISA tests (6). Bantarri and Goodwin (2) also found dot-blot immunoassay to be more sensitive than ELISA for detection of potato viruses S, X, and Y. It should be noted that dot-blot immunoassay tests may not give more sensitivity than ELISA and only comparative tests will determine the more sensitive test.

The dot-blot immunoassay and cDNA probe tests were found to be equal in sensitivity for the detection of BBSSV (Figure 2.1). There were an equal number of "dots" in each test. It is interesting to note only a ten-fold difference between detection of virus in buffer and virus in healthy plant sap. Healthy leaf sap thickens as it stands and could possibly trap the virus or interfere with its reaching the nitrocellulose membrane. This is especially true when samples are left standing overnight as in the tests done here. However, this did not seem to cause any problems in this research.

Leaf and blossom material were also compared regarding detection limits (Figure 2.1). Again it is necessary to point out that the titer of virus varies in plant tissues. For this reason, determining the consistent endpoint of detection is not easy and not always possible. As reported, the highest dilution detected was 1:30,000(v/v). This value is three times more sensitive than the earlier reported value of 1:7500 in the ELISA/dot-blot comparison.

Figure 2.2 shows the results of stem material tested by dot-blot immunoassay and the cDNA probe. Once again they were equally sensitive. More important was the discovery that virus in both xylem and phloem is whole virus rather than just viral antigen. The dot-blot immunoassay confirms the presence of coat protein. It has not been reported, to my knowledge, what happens to coat protein after the virus "uncoats" for replication. It may be possible for some of the material to be carried in the vascular system and subsequently detected. With the use of the cDNA probe, it was determined

that RNA was also present and associated with the coat protein because of the antibody trapping method used. Use of the cDNA probe also offers a test not based on immunoassays, which were used almost exclusively in this research. Combining these tests adds strength to the conclusions of whole virus in the vascular system.

Dot-blot immunoassay and the cDNA probe were also used to detect BBSSV in the aphid vector. Both alkaline phosphatase (AP) and horseradish peroxidase (HRP) enzyme detection systems were used. Healthy and diseased aphid samples were positive for AP (Figure 2.2) and HRP. Dilution techniques, the addition of diethyl dithiocarbamic acid (DIECA) to the grinding buffer (4), and the deletion of polyvinylpyrrolidone, which stabilizes enzymes, did not alter results. The endogenous enzyme systems of the aphid interferes with immunoassays utilizing similar enzymes. This has also been reported by Berger et al. (3). The researchers incubated a nonviruliferous aphid thin section with AP histochemical substrate and found evidence of the endogenous enzyme.

The cDNA probe did not detect the virus in sachet fed aphids. The virus may have aggregated and was thus not acquired by the aphids. However, there was a high concentration of virus per volume and it should have been picked up by the aphids. There may have been other factors interfering with the detection of virus by the cDNA probe such as trapping of the virus in the aphid body or some substance coating the virus such that the RNA was not exposed.

Use of cDNA probes in plant virology is varied. Researchers have used them for determining sequence homology between different RNA species of the same virus (7,8,24). cDNA probes have also been used to identify strains of citrus tristeza virus (25), and Australian bean yellow mosaic virus (1) as well as differences between members of the tobamovirus group (20).

A most interesting and practical use for cDNA probes in plant virology is in indexing for virus. Harrison et al. (9) were able to detect tobacco rattle virus in narcissus plants and potato tubers using nucleic acid hybridization. However, they had to extract the RNA from plant tissues which is a time consuming process. Palukaitis et al. (19) and Owens and Diener (18) used a cDNA probe to detect avocado sunblotch viroid and potato spindle tuber viroid, respectively. In the former case, a large sample size, 50 grams, and partial nucleic acid extraction were required, while in the latter case clarified plant sap and a small sample size, 0.1 to 0.2 grams, were sufficient. Sela et al. (27) were also able to detect virus, TMV, in small plant leaf samples by cDNA hybridization techniques. The important features of the use of cDNA probes for indexing would be: 1) sensitivity of the probe and 2) ease of sample preparation. Both the first and second features have been shown for BBSSV; the probe is sensitive and no special preparation of samples is necessary.

The future utilization of cDNA probes for indexing will be with nonradioactive systems of detection for reasons of

safety and reagent longevity. Two systems are currently being developed. The use of biotin labeled probes have been described by Leary et al. (11). They suggest the use of a biotin/avidin/AP system and describe this as a less time consuming method. They also reported that biotin-labeled DNA exhibited lower nonspecific binding to nitrocellulose than radioactively labeled DNA. Renz and Kurz (23) described a system whereby they crosslinked a protein to single stranded DNA. The protein is crosslinked with a synthetic polymer, such as polyethyleneimine, containing many primary amino groups. If the protein is an enzyme, such as AP or HRP, a color reaction will appear when the proper substrate is added. Both methods are currently being developed for mammalian systems and would be useful in indexing procedures depending upon specificity and sensitivity.

Fluorescent antibody techniques have been used by a number of researchers to determine location of virus antigen in cells. Nagaraj and Black (17) found wound-tumor virus in the pseudophloem of the tumor produced and in a few thick-walled cells in the xylem region. Tsuchizaki et al. (28) found fluorescence in the pericarp of fruit infected with citrus tristeza virus. Fluorescence was also observed in the phloem cells of the vascular bundles in the stem bark, green twigs, and petioles. All fluorescence was correlated with transmission electron microscope studies. Mumford and Thornley looked at the location of sugarbeet curly top virus in bean, sugarbeet, tobacco, and tomato (16). Specific fluores-

cent antibody staining was only found in phloem cells of stems or petioles of all four test species.

Blueberry plants infected with BBSSV and exhibiting symptoms were tested by a fluorescent antibody technique to determine cellular location of the virus. Most cell types in the leaves were found fluorescing. From the distortion caused by this disease it is easy to see that most cell types could be affected.

Stems contained only xylem fluorescence. Some phloem or outer bark fluorescence was expected due to the red-streaking symptom on shoots. Phloem cells and outer bark cells tested positive for BBSSV by both ELISA and dot-blot immunoassay indicating presence of viral antigen in these tissues. It is possible that fluorescence was missed because many stem sections separated at the cambium layer, although these sections were observed, if possible, even when parted. Separation at the cambium layer may be due to instability of this tissue and the force of mechanical shearing.

Root sections were not as good as leaf and stem sections or did not exhibit fluorescence. This could be explained by the lack of symptoms on roots of diseased plants. It is easy to visually pick well infected leaf or stem material, but this not possible for roots. It is possible that roots sectioned did not contain virus.

In summary, dot-blot immunoassay and the cDNA probe were equally sensitive for the detection of BBSSV in all tissue types. ELISA detection is slightly less sensitive. The

virus antigen was found in berries, seeds, stems (xylem and phloem), blossoms, leaves, and roots. The latter three tissues have already been reported by others to contain BBSSV antigen. The virus antigen was also found in all cell types of leaves except phloem, and in the xylem of stems utilizing a fluorescent antibody detection system.

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CHAPTER III

MOVEMENT OF BLUEBERRY SHOESTRING VIRUS IN APHID- AND MECHANICALLY-INOCULATED HIGHBUSH BLUEBERRY

INTRODUCTION

Mechanical transmission of viruses in woody plants has been reviewed by Fulton (7). He discussed a number of viruses that could be transmitted between woody hosts including cocoa swollen-shoot virus, plum line pattern virus, and infectious variegation virus of citrus. However, herbaceous hosts seem to be more susceptible and provide a higher concentration of virus than isfound in the woody plant host. This discovery has lead to more woody plantviruses being characterized through the use of herbaceous hosts.

Many studies of vector transmission and mechanical inoculation stop at the point of showing the virus has been transmitted by noting visible symptoms, immunoassay testing, etc., or at the point of finding the vector and proving transmission. Other studies look at cells after symptoms appear by using electron microscopic techniques (5). To better understand the sequence of events after the plant has been exposed to the infectious agent, i.e. the virus, it is necessary to look atcell types affected and how the virus moves from the infection point.

Blueberry shoestring virus (BBSSV) is vectored by the blueberry aphid Illinoia pepperi MacG. (11). No herbaceous host has been found for BBSSV, although 44 have been tested (11). The virus is also not readily mechanically transmissible to other blueberry plants (14). This may be due to high tannin concentrations or oxidizable phenolic compounds in the leaves (6). BBSSV also has a latent period of four

years such that visible symptoms may not occur and immunoassays may or may not work (D.C. Ramsdell, personal communication).

The objectives of this research were to: 1) study the movement of BBSSV by simulating natural vector transmission conditions and 2) to study the movement of BBSSV after artificial inoculation methods.

MATERIALS AND METHODS

In 1984 and 1985, 2-year-old `Jersey' blueberry plants were inoculated with BBSSV in the greenhouse by either the blueberry aphid, Illinoia pepperi MacG., or by rub-inoculation of leaves with purified virus.

Aphid-Inoculation

Blueberry aphids were obtained from a field that did not contain bushes with shoestring disease and was not near a field with infected bushes. Aphidswere transferred to three healthy, ELISA tested, `Jersey'blueberry plants and to four symptomatic, diseased bushes fora 48-hour acquisition access period. Aphids were then transferred to a 2.5 cm diameter cage consisting of a hair clip, tygon tubing, fine mesh nylon, and string (Figure 3.1).Three to five aphids per cage were used in 1984 and seven toten aphids per cage in 1985. The cages were placed on tenleavesof healthy plants, with aphids on the adaxial (underside) of leaves only. Three bushes had aphids fed on healthy tissue and 10 bushes had aphids fed on symptomatic BBSSV infected tissue.

Figure 3.1 Apparatus used to cage aphids on leaves. The cage consisted of a hair clip, tygon tubing, fine mesh nylon screen, and a string.

Aphids were allowed a 48-hour inoculation access period after which time aphids were removed from plants and killed or tested by dot-blot immunoassay (see Chapter II Materials and Methods). Before removing cages, leaves were marked with a black waterproof marker, outlining the cage location. This experiment was done in 1984 and repeated in 1985. Plants were maintained in separate greenhouses. Plants were also allowed a vernalization period each year, at least 1000 hours less than 40^{0}F in a coldroom in 1984 and a cold greenhouse in 1985, and tested in subsequent years.

Mechanical Inoculation

BBSSV was purified from blossoms using the method of Ramsdell (see Chapter II Materials and Methods). In 1984, BBSSV was rub-inoculated onto the adaxial (top) side of leaves of 15 2-year-old `Jersey' blueberry bushes using carborundum as an abrasive and a sponge to apply the inoculum. Inoculum levels of purified virus were: 0.25 (five plants), 0.025 (five plants), and 0.0025 mg/ml (five plants). After inoculation of each group, theleaves were rinsed with water. Three plants were rub-inoculated with sodium phosphate buffer (0.05M, pH 7.0) as controls.

In 1985, ten 2-year-old plants were rub-inoculated with purified virus at a concentration of 0.25 mg/ml only (14). Again, three healthy plants were rub-inoculated with buffer as controls. Plants were maintained in separate greenhouses and given vernalization periods each year.

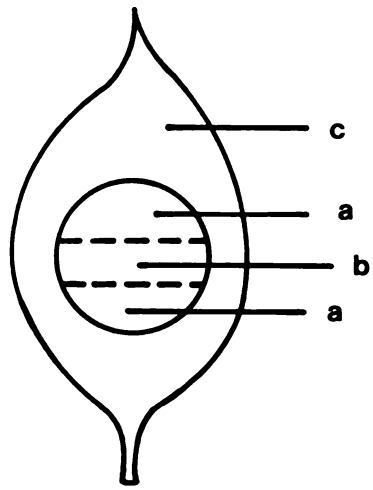
Sampling and Testing of Leaf Tissue for Detection of BBSSV

Leaves from both aphid- and rub-inoculated plants were tested at intervals of 1, 3, 14, 21, 35, 56, 77, and 98 days post-inoculation in 1984 and 1, 3, 7, 14, 21, 35, and 63 days post-inoculation in 1985. Leaves were sectioned with a razor blade (Figure 3.2) and tested using indirect fluorescent antibody staining and ELISA or dot-blot immunoassay (see Chapter II Materials and Methods). For aphid-inoculated leaves, two portions were tested by immunoassay techniques to determine whether BBSSV moved outside of the area inoculated by aphids and also whether the area close to sectioned and stained material contained detectable amounts of virus. With rub-inoculated leaves the different areas were tested only to determine if detectable amounts of virus were present.

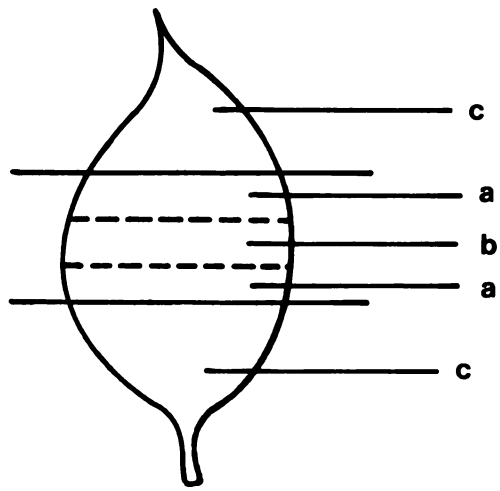
All procedures for ELISA and dot-blot immunoassay were followed as outlined in Chapter II except for sample preparation. Leaf samples were shredded and placed in spot plate wells with 400 ul of the appropriate extraction buffer for ELISA or dot-blot immunoassay. Samples were ground using glass rodswith flattened, saucer-shaped ends. An additional 400 ul ofbuffer were added to the smaller samples (see sample a in Figure 3.2) and 1.2 ml of buffer were added to the largersamples (see sample c in Figure 3.2). Samples were pipetted without straining into microtiter plates for ELISA or a dot-blot apparatus at the rate of 400 ul per well with two wells per sample.

Figure 3.2 (a) Line drawing of an aphid-inoculated leaf showing the location of an aphid cage. Sections a and crepresent parts of the leaf tested by immunoassay. Section b was tested by indirectfluorescent antibody staining.

Figure 3.2 (b) Line drawing of a rub-inoculated leaf showingsections used for testing. Sections a and c were tested by immuno-assay methods and section b was tested by indirect fluorescent antibody staining.



A. Aphid-Inoculated Leaf



B. Rub-Inoculated Leaf

Samples used for fluorescent antibody staining were sectioned using a cryostat to obtain approximately 100 sections per sample. The sections were placed in cold phosphate buffered saline (PBS, pH 7.0) and indirectly stained with anti-BBSSV-IgG or pre-immune serum and then with sheep-anti-rabbit fluorescein isothiocyanate (FITC) and observed with an epifluorescent microscope (see Chapter II Materials and Methods). It was necessary to cut at least 100 sections per sample due to the loss of sections during solution changes. Slides consisted of 30 to 50 sections per sample mounted in PBS and kept in a moist chamber at 4^{0}C. Slides were observed as soon as possible after mounting.

Leaf samples from the same plants were taken once per month in subsequent years, three to four leaves per sample, and processed as outlined in the dot-blot immunoassay section of Chapter II to determine if BBSSV was still detectable.

RESULTS

Aphid-Inoculated Plants

ELISA and Dot-blot Immunoassay Results

In 1984 there were no positive samples when tested by the ELISA method. After the first vernalization period the blossoms on plants four, five, seven, and eight were positive by the dot-blot immunoassay system. There were no positive leaf samples in 1985 or 1986 (Table 3.1).

Plants inoculated in 1985 had no positive results in 1985 and only one sample was positive in 1986 (Table 3.2).

TABLE 3.1 ELISA AND DOT-BLOT IMMUNOASSAY RESULTS FOR 'JERSEY'BLUEBERRY PLANTS INOCULATED WITH BBSSV IN 1984 BY THE BLUEBERRY APHID ILLINOIA PEPPERI

Source Material[a]		Year Tested - Results[b]		
		1984[c]	1985[d]	1986[d]
Diseased	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	+(A)[e]	-
	5	-	+(A)[e]	-
	6	-	-	-
	7	-	+(A)[e]	-
	8	-	+(A)[e]	-
Healthy	1	-	-	-
	2	-	-	-
	3	-	-	-

[a]Source material consisted of eight plants with aphids fed on symptomatic, diseased tissue and three plants with aphids fed on healthy tissue. Acquisition and inoculation periods were both 48 hours.

[b]Results are based on ELISA tests for 1984 and dot-blot immunoassay for 1985 and 1986 and recorded as positive(+) or negative(-).

[c]In 1984, plants were sampled 1, 3, 14, 21, 35, 56, 72, and 98 days post-inoculation; one leaf per sample (see Figure 3.2a).

[d]In 1985 and 1986, plants were sampled monthly (April(A)-August(AU)). Samples consisted of three to four leaves per plant.

[e]Blossom samples.

TABLE 3.2 DOT-BLOT IMMUNOASSAY RESULTS FOR 'JERSEY' BLUEBERRY PLANTS INOCULATED WITH BBSSV IN 1985 BY THE BLUEBERRY APHID *ILLINOIA PEPPERI*

Source Material[a]		Year Tested - Results[b]	
		1985[c]	1986[d]
Diseased	1	-	-
	2	-	+ June
	3	-	-
	4	-	-
	5	-	-
	6	-	-
	7	-	-
	8	-	-
Healthy	1	-	-
	2	-	-
	3	-	-

[a]Source material consisted of eight plants with aphids fed on symptomatic, diseased tissue and three plants with aphids fed on healthy tissue. Aquisition and inoculation periods were both 48 hours.

[b]Results are based on dot-blot immunoassay and are recorded as positive(+) or negative(-).

[c]In 1985 plants were sampled 1, 3, 7, 14, 21, 35, and 63 days post-inoculation; one leaf per sample (see Figure 3.2a).

[d]In 1986 plants were sampled monthly (May - September). Samples consisted of three to four leaves per plant.

The one positive sample showed up in only one month of testing and is thought to be a false positive.

Fluorescent Antibody Study

In 1984, day 1 post-inoculation samples were showing some vascular tissue fluorescence (Figure 3.3a). It appeared to be mostly xylem, but it was difficult to determine if some phloem fluorescence was being masked. By day 14 palisade cells were fluorescing (Figure 3.4). This meant that the virus had traversed from the adaxial to abaxial side of the leaf. Mesophyll cells were also exhibiting fluorescence (Figure 3.5) while the controls had no specific fluorescence (Figure 3.6). At Day 21 there were a number of lower epidermis cells fluorescing (Figures 3.7a and 3.8a) and by Day 77, an increase in the number of upper epidermal cells fluorescing was evident (Figures 3.9a and 3.10).

In 1985, vascular fluorescence was observed first and throughout the study mostly in secondary veins (Figure 3.11a) with no specific fluorescence seen in any of the controls. Mesophyll cells were only observed to be fluorescing on days 1, 3, and 7 post-inoculation. Day 21 samples exhibited vascular fluorescence along with single palisade cells (Figures 3.12a and 3.13a). By Day 35 vascular fluorescence was extensiveand appeared to be mainly in the xylem (Figure 3.14a). Sectionsof leaves from Day 35 also exhibited fluorescence in the lowerepidermis andpalisadeand upper epidermis (Figures 3.15 and 3.16, respectively). Day 63 samples exhibited an increasedamount of fluorescence similar to Day 35.

Figure 3.3 (a) Aphid-inoculated leaf 1 day post-inoculation, exhibiting fluorescence in a secondary vein. (100X). 1984

Figure 3.3 (b) Healthy blueberry leaf control (100X). 1984

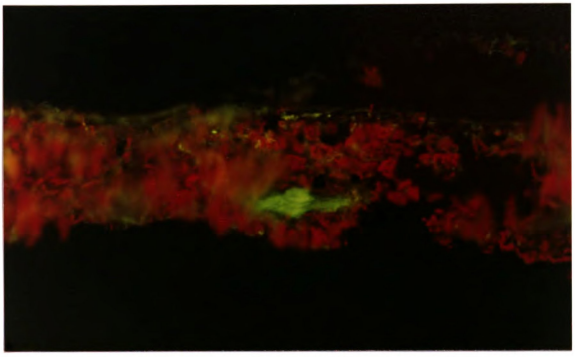


Figure 3.3 (a)

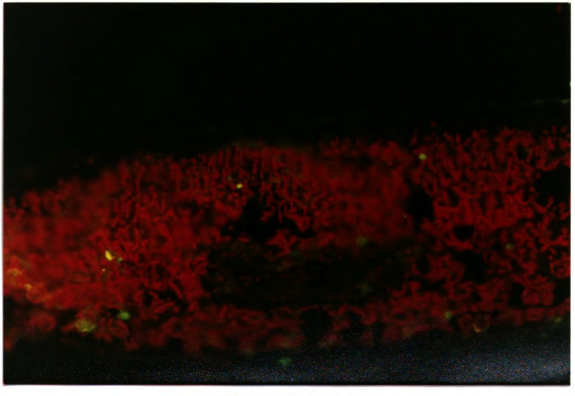


Figure 3.3 (b)

Figure 3.4 One palisade cell fluorescing in a leaf cross section, inoculated by aphids, 14 days post-inoculation (100X). 1984

Figure 3.5 Mesophyll cells fluorescing in a leaf inoculated by aphids, 14 days post-inoculation (100X). 1984

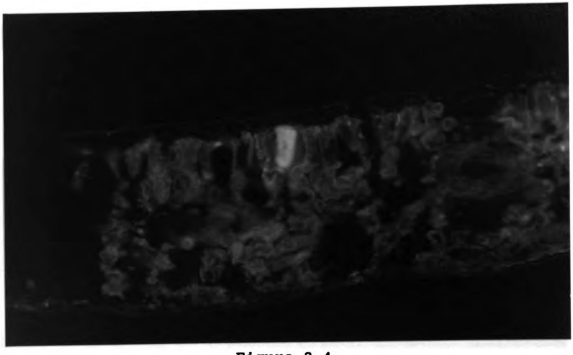


Figure 3.4

Figure 3.5

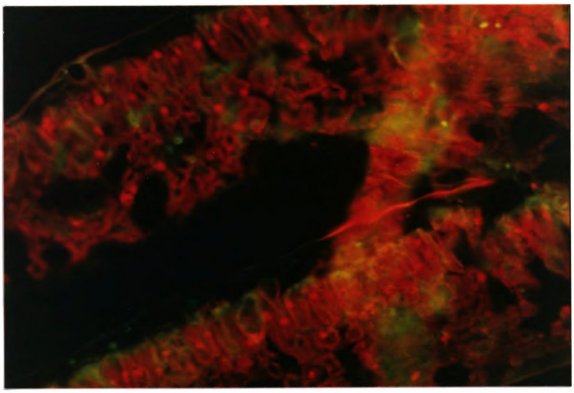


Figure 3.6 Healthy blueberry leaf cross section used as a control (100X). 1984

Figure 3.7 (a) Aphid inoculated leaf exhibiting fluorescence in the lower epidermal cells (100X, day 21 postinoculation). 1984

Figure 3.7 (b) Healthy blueberry leaf control (100X). 1984

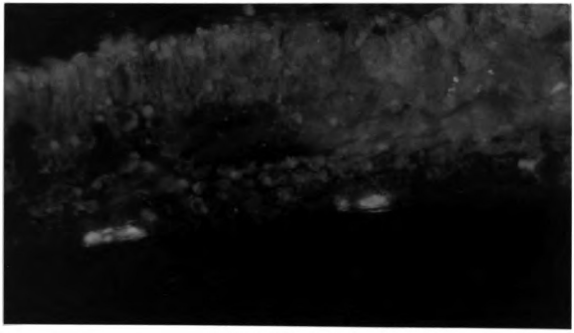


Figure 3.7 (a)

Figure 3.7 (b)

Figure 3.8 (a) Aphid-inoculated leaf exhibiting mesophyll cell fluorescence. (100X, day 35 post-inoculation). 1984

Figure 3.8 (b) Healthy blueberry leaf control (100X). 1984

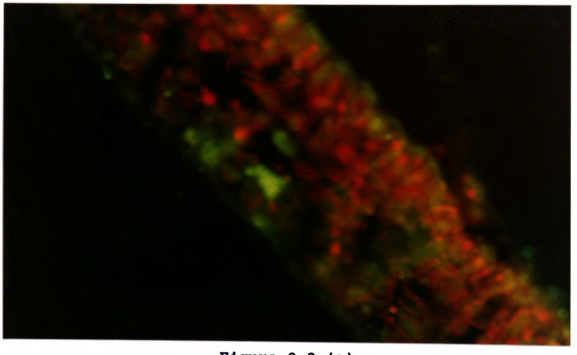


Figure 3.8 (a)

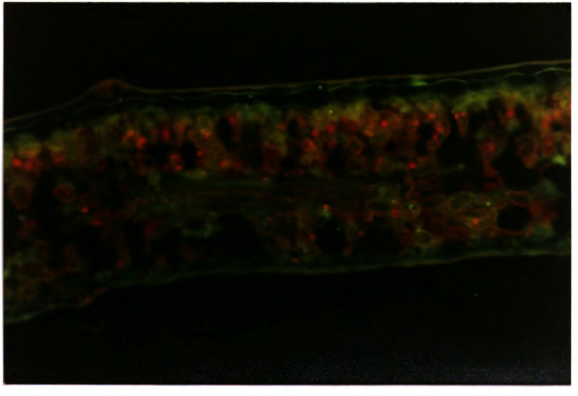


Figure 3.8 (b)

Figure 3.9 (a) Aphid-inoculated leaf exhibiting fluorescence in the upper epidermis. (100X, day 77 post-inoculation). 1984

Figure 3.9 (b) Healthy blueberry leaf control (100X). 1984

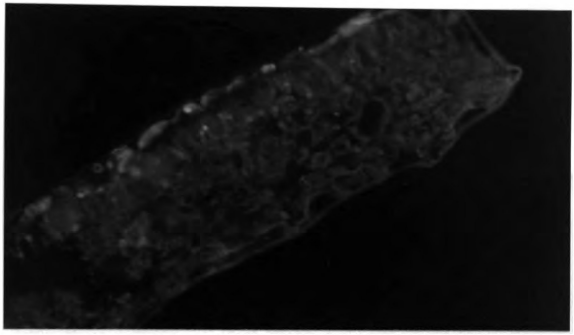


Figure 3.9 (a)

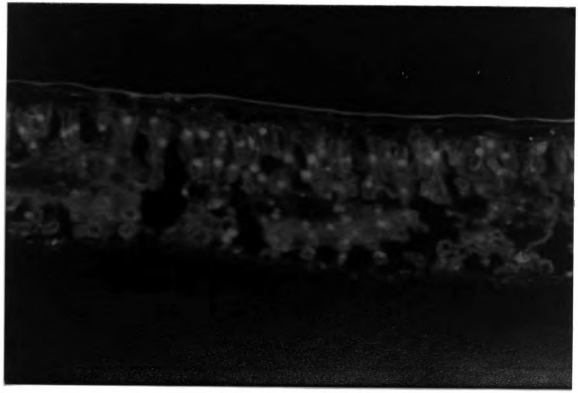


Figure 3.9 (b)

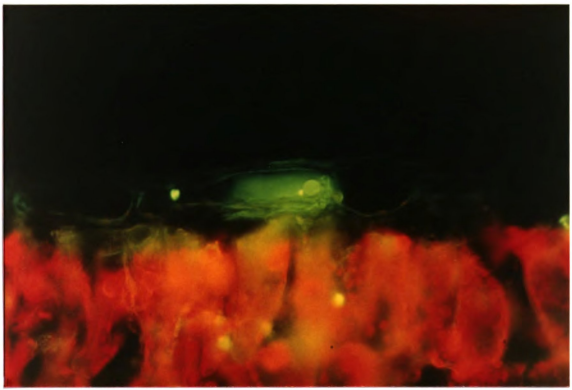


Figure 3.10 Upper epidermal fluorescence in an aphid-inoculated blueberry leaf (250X, day 77 post-inoculation). 1984

Figure 3.11(a) Fluorescence of a longitudinal secondary vein from a blueberry leaf inoculated with BBSSV byaphids, 14 days post-inoculation (100X). 1985

Figure 3.11(b) Healthy blueberry leaf control (100X). 1985

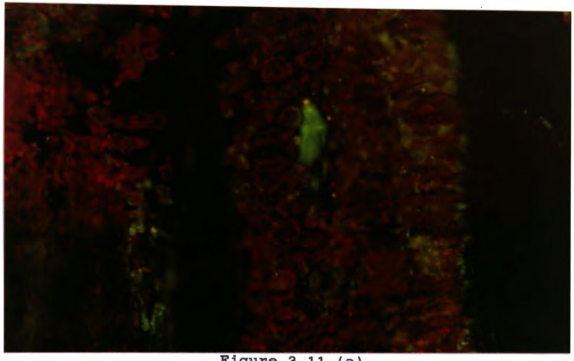


Figure 3.11 (a)

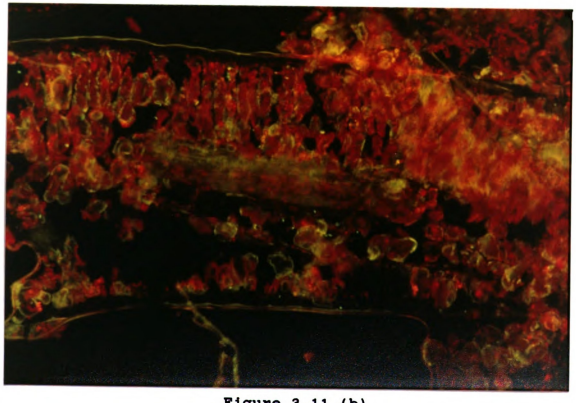


Figure 3.11 (b)

Figure 3.12 (a) Fluorescence of vascular material and a palisade cell in a leaf inoculated with BBSSV by aphids, 21 days post-inoculation (100X). 1985

Figure 3.12 (b) Healthy blueberry leaf control(100X). 1985

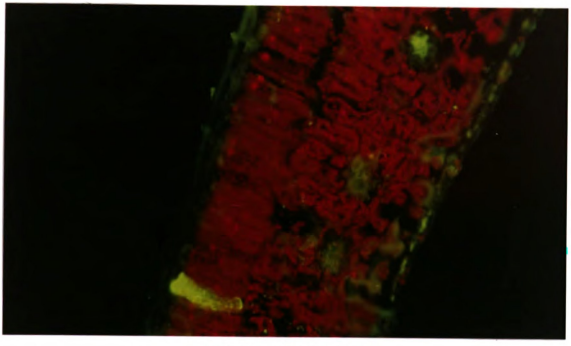


Figure 3.12 (a)

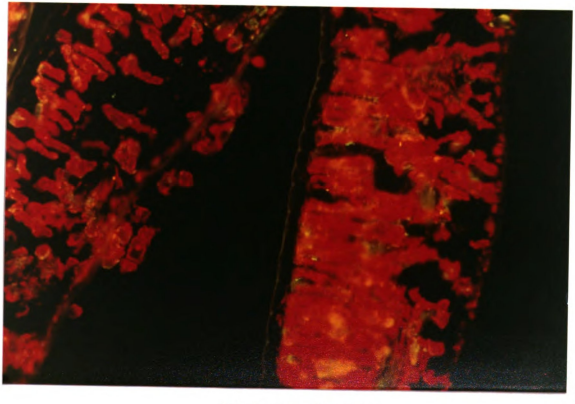


Figure 3.12 (b)

Figure 3.13 (a) Vascular fluorescence in a leaf inoculated with BBSSV by aphids, 21 days post-inoculation (250X). 1985

Figure 3.13 (b) Diseased leaf indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbit FITC used as a control (250X). 1985

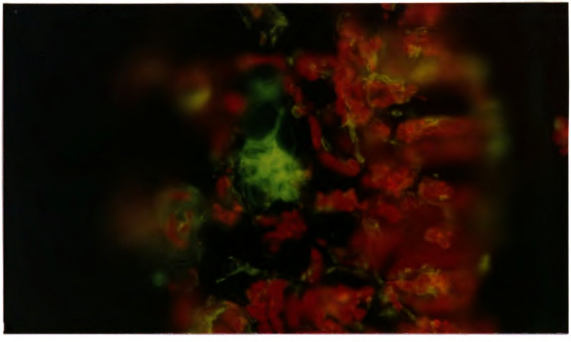


Figure 3.13 (a)

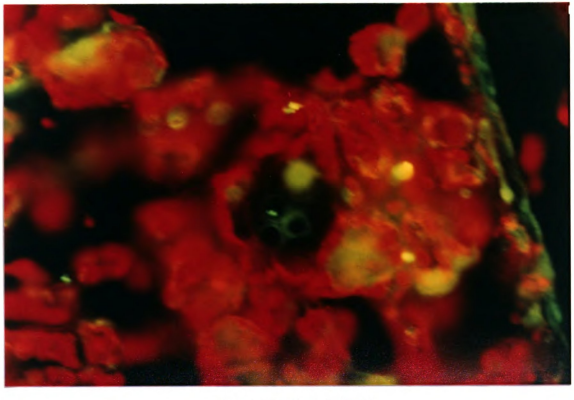


Figure 3.13 (b)

Figure 3.14 (a) Longitudinal section through a secondary vein exhibiting fluorescence in a leaf inoculated by aphids, 35 days post-inoculation (250X). 1985

Figure 3.14 (b) Diseased leaf indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbit FITC, used as a control (250X). 1985

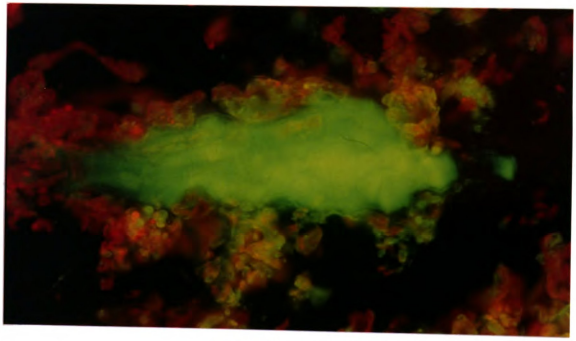


Figure 3.14 (a)

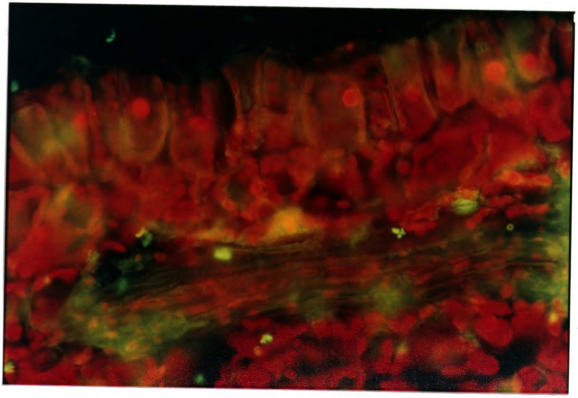


Figure 3.14 (b)

Figure 3.15 Two lower leaf surface epidermal cells of a leaf inoculated with BBSSV by aphids, fluoresecing on day 35 post-inoculation (250X). 1985

Figure 3.16 Palisade cells of a leaf, inoculated with BBSSV by aphids, with prominent red nuclei and smaller chloroplasts 35 days post-inoculation (250X). 1985

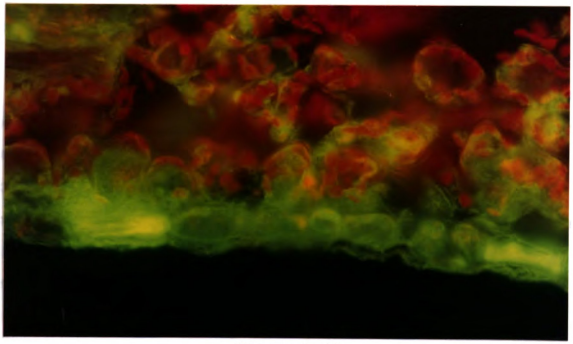


Figure 3.15

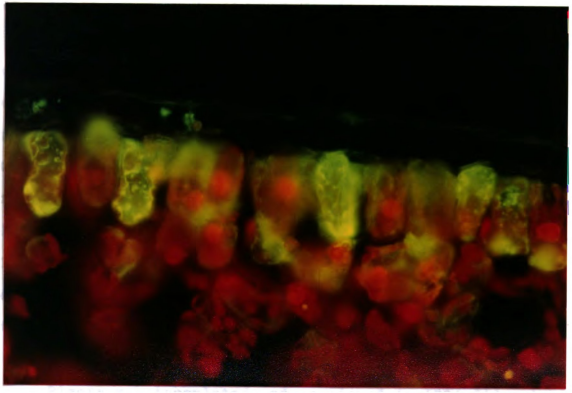


Figure 3.16

Rub-Inoculated Plants

ELISA and Dot-blot Immunoassay

Plants inoculated in 1984 had only two borderline ELISA positive samples in that year. These samples were from plant No. 2 inoculated with 0.0025 mg/ml on day 35 post-inoculation and plant No. 5 inoculated with 0.25mg/ml on day 56 post-inoculation (Table 3.3). In 1985 there was an increase in the number of positive samples, but the same plants were rarely positive from month to month. In 1986, plant No. 1 inoculated with 0.25 mg/ml in 1985, had symptoms of red-streaking on seven of the new shoots. This was the only plant to exhibit symptoms in this study.

Plants inoculated in 1985 were tested by dot-blot immunoassay and samples from two plants had positive samples on day 35 post-inoculation. Plants number one and eight were positive forBBSSV in samples taken from the ends of leaves. On day 63, two different plants were positive for BBSSV (plants four and five). Both the samples taken from the middle of the leaf and from the outer portions of the leaf were positive. In 1986, after a vernalization period, none of the plants exhibited visible symptoms or were dot-blot immunoassay positive (Table 3.4).

Fluorescent Antibody Study

Plants rub-inoculated and sectioned in 1984 did not exhibit much fluorescence until Day 56 post-inoculation. Controls exhibited no specific fluorescence. On Day 14, there was mostly mesophyll fluorescence. By Day 21, there was some

TABLE 3.3 ELISA AND DOT-BLOT IMMUNOASSAY RESULTS FOR 'JERSEY' BLUEBERRY PLANTS MECHANICALLY INOCULATED WITH PURIFIED BBSSV IN 1984.

Source Material[a]		Year Tested - Results[b]		
		1984[c]	1985[d]	1986[d]
Diseased	0.25(1)	-	+(M,JL,S)	+(JL,S)
	0.25(2)	-	+(M,S)	-
	0.25(3)	-	-	-
	0.25(4)	-	-	-
	0.25(5)	C(Day 56)	-	-
	0.025(1)	-	-	-
	0.025(2)	-	+(JN)	-
	0.025(3)	-	-	-
	0.025(4)	-	-	-
	0.025(5)	-	+(JN)	-
	0.0025(1)	-	-	-
	0.0025(2)	C(Day 35)	+(A)	-
	0.0025(3)	-	+(A)	-
	0.0025(4)	-	+(JN)	-
	0.0025(5)	-	-	-
Healthy	1	-	-	-
	2	-	-	-
	3	-	-	-

[a]Source material consisted of 15 plants rub-inoculated with 0.25, 0.025, or 0.0025 mg/ml of purified BBSSV, five plants per group, and three plants rub-inoculated with sodium phosphate buffer (0.05M, pH 7.0) as controls.

[b]Results are based on ELISA tests for 1984 and dot-blot immunoassay for 1985 and 1986. Results are recorded as positive(+) or negative(-).

[c]In 1984 plants were sampled 1, 3, 14, 21, 35, 56, 72, and 98 days post-inoculation. The C represents samples taken from the outer portions of the leaf (see Figure 3.2b).

[d]Samples were taken once per month in 1985 and 1986 (April (A), May(M), June(JN), July(JL), August(AU), September(S)) for dot-blot immunoassay.

TABLE 3.4 DOT-BLOT IMMUNOASSAY RESULTS FOR 'JERSEY' BLUEBERRY PLANTS MECHANICALLY INOCULATED WITH PURIFIED BBSSV IN 1985.

Source Material[a]		Year Tested - Results[b]	
		1985[c]	1986[d]
Diseased	1	C(Day 35)	-
	2	-	-
	3	-	-
	4	A,C(Day 63)	-
	5	A,C(Day 63)	-
	6	-	-
	7	-	-
	8	C(Day 35)	-
Healthy	1	-	-
	2	-	-
	3	-	-

[a]Source material consisted of eight plants rub-inoculated with 0.25 mg/ml purified BBSSV and three plants rub-inoculated with sodium phosphate buffer (0.05M, pH 7.0) as controls.

[b]Results are based on dot-blot immunoassay and are recorded as positive(+) or negative(-).

[c]Leaf samples were taken 1, 3, 7, 14, 21, 35, and 63 days post-inoculation; one leaf per sample (see Figure 3.2b). The A represents a sample taken from the middle of the leaf and the C represents a sample of the outer portion of the leaf.

[d]Samples were taken once per month (May - August); three to four leaves per sample.

lower epidermal fluorescence along with what appeared to be organelle fluorescence. By Day 35, mesophyll fluorescence had increased to its highest level (Figure 3.17a). Prominent nuclei are noted in the palisade cells of Figure 3.18. Occasionally a nucleus was found to be fluorescing (Figures 3.19 and 3.20a). When switching to a filter for observing sections stained with rhodamine isothiocyanate (RITC) the outline of the nucleus is more defined (Figure 3.20b).

Day 56 samples also exhibited many mesophyll (Figure 3.21a) andlower epidermal cells fluorescing. Day 77 samples exhibited mostly lower epidermal (Figure 3.22) and upper epidermal fluorescence (Figure 3.23). No specific fluorescence was noted in the controls (Figure 3.24).

Plants rub-inoculated in 1985 exhibited fluorescence throughout the leaf except for the lower epidermis on Day 1 post-inoculation. Day 3 samples had the only example of fluorescencein the midrib xylem and parenchyma cells (Figure 3.25). Day 7 samples had many fluorescing mesophyll cells (Figure 3.26a). Also found on day 7 were vascular tissue (Figure 3.27), palisade cells (Figure 3.28), and upper epidermal cells fluorescing (Figure 3.29a).

Day 14 samples exhibited mainly vascular and upper epidermal fluorescence which continued throughout the study. There was also a section with possible nuclear fluorescence, but it was not as clearlydefined as in the 1984 study. By Day 35, vascular and epidermal fluorescence had reached its

Figure 3.17 (a) Fluorescence of mesophyll cells in a leaf rub-inoculated with purified BBSSV, 35 days post-inoculation (100X). 1984

Figure 3.17 (b) A healthy control section from a leaf rub-inoculated with buffer (100X). 1984

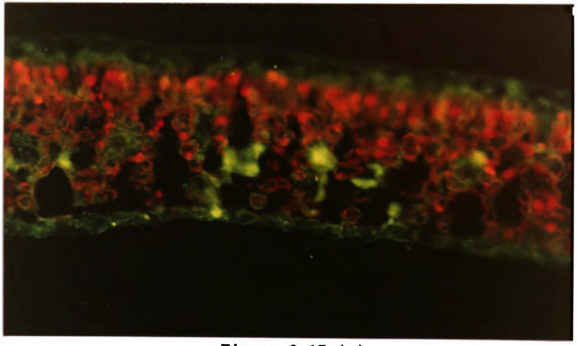


Figure 3.17 (a)

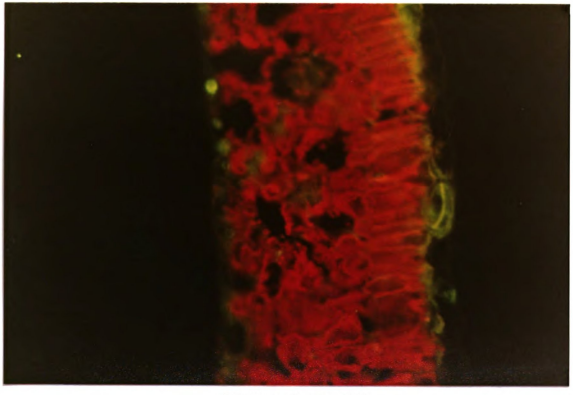


Figure 3.17 (b)

Figure 3.18 Prominent red nuclei in a leaf rub-inoculated with BBSSV in 1984 (250X).

Figure 3.19 Nuclear fluorescence in a palisade cell of a leaf rub-inoculated with purified BBSSV, 56 days post-inoculation (250X). 1984

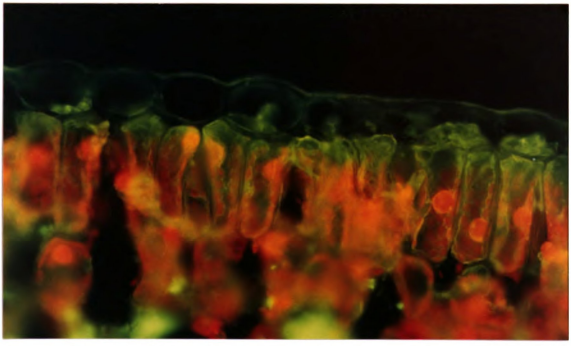


Figure 3.18

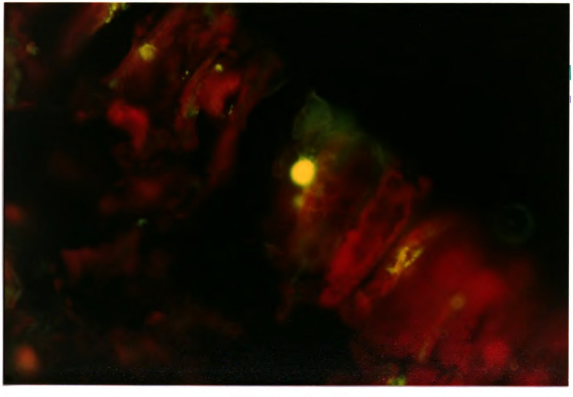


Figure 3.19

Figure 3.20 (a) Fluorescing nuclei in palisade cells of a leaf rub-inoculated with purified BBSSV, 56 days post-inoculation. Note middle palisade cell specifically (250X). 1984

Figure 3.20 (b) Same section as in (a) with the rhodamine isothiocyanate filter. Note outline of nuclei (250X). 1984

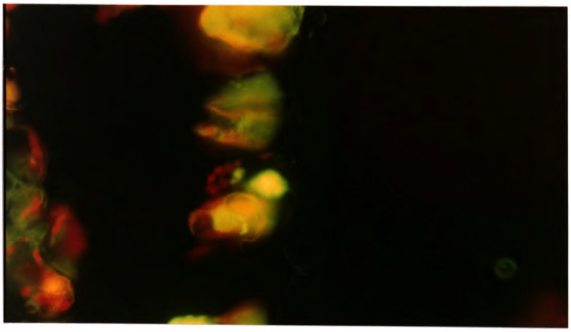


Figure 3.20 (a)

Figure 3.20 (b)

Figure 3.21 (a) Fluorescing mesophyll cells in a leaf rub-inoculated with BBSSV, 56 days post-inoculation (100X). 1984

Figure 3.21 (b) Healthy control leaf rub-inoculated with buffer (100X). 1984

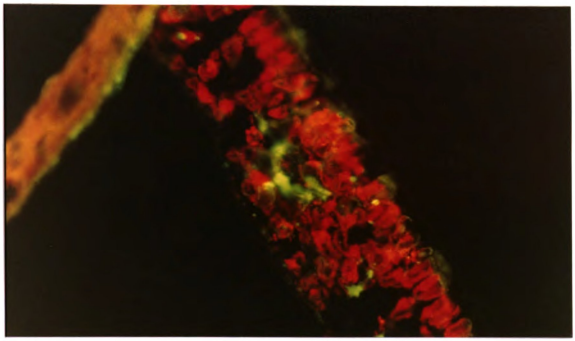


Figure 3.21 (a)

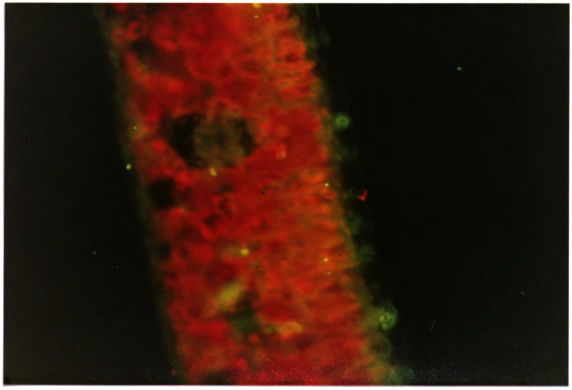


Figure 3.21 (b)

Figure 3.22 Lower epidermal fluorescence in a cross section of a leaf rub-inoculated with BBSSV, 77 days post-inoculation(100X). 1984

Figure 3.23 Upper epidermal cell fluorescence in a cross section of a leaf rub-inoculated with BBSSV, 77 days post-inoculation(100X). 1984

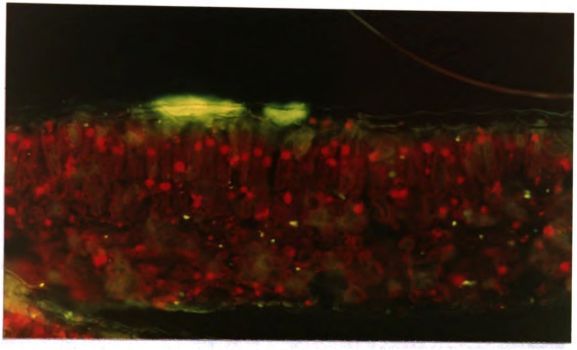


Figure 3.22

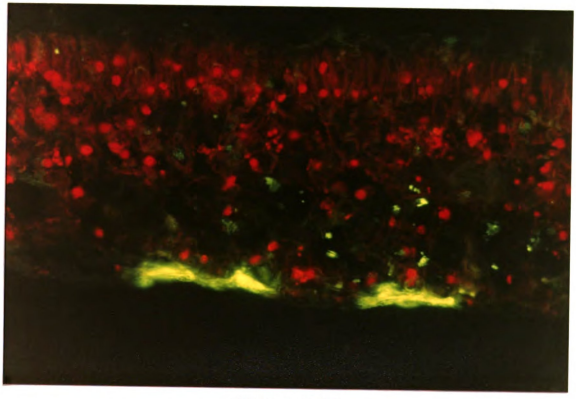


Figure 3.23

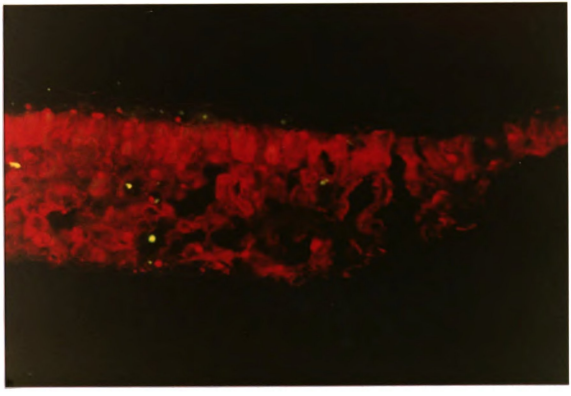


Figure 3.24 Healthy control leaf rub-inoculated with buffer (100X). 1984

Figure 3.25 (a) Fluorescence of midrib xylem and parenchyma cells ina leaf rub-inoculated with BBSSV, 3 days post-inoculation (100X). 1985

Figure 3.25 (b) Healthy control leaf rub-inoculated with buffer (100X). 1985

Figure 3.25 (a)

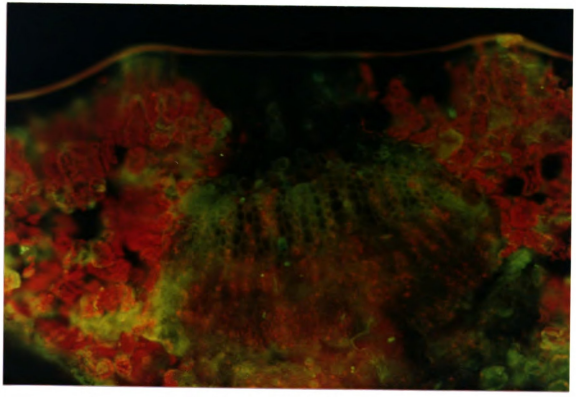


Figure 3.25 (b)

Figure 3.26 (a) Fluorescing mesophyll cells from a leaf rub-inoculated with BBSSV, 7 days post-inoculation (250X). 1985

Figure 3.26 (b) Control leaf section indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbitFITC (100X). 1985

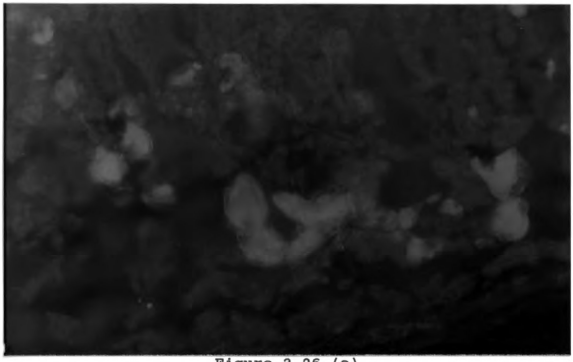


Figure 3.26 (a)

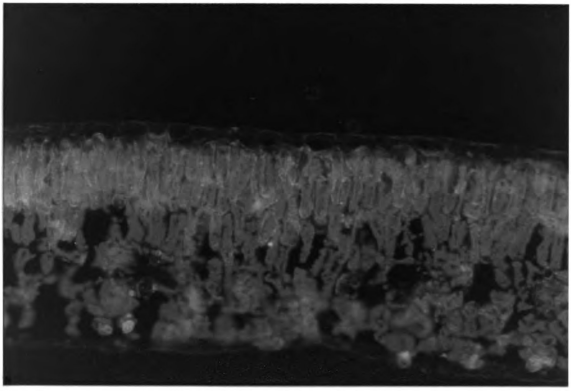


Figure 3.26 (b)

Figure 3.27 Vascular fluorescence in a leaf rub-inoculated with BBSSV, 7 days post-inoculation (100X). 1985

Figure 3.28 Fluorescence of palisade cells of leaf a rub-inoculated with BBSSV, 7 days post-inoculation (250X). 1985

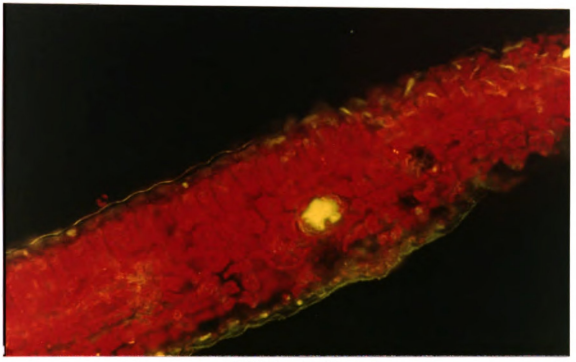


Figure 3.27

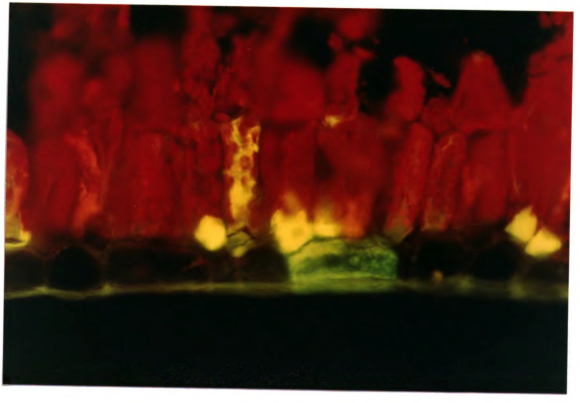


Figure 3.28

Figure 3.29 (a) Upper epidermal fluorescence in a leaf rub-inoculated with BBSSV, 7 days post-inoculation (100X). 1985

Figure 3.29 (b) Cross section of a healthy control leaf rub-inoculated with buffer (100X). 1985

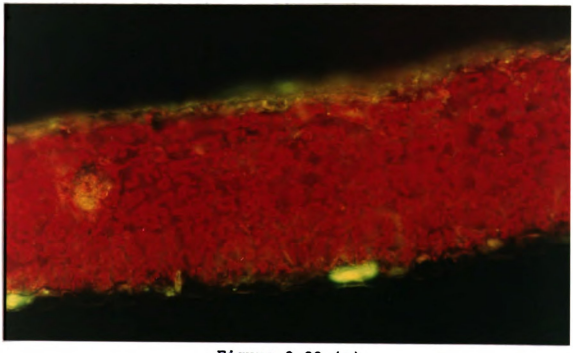


Figure 3.29 (a)

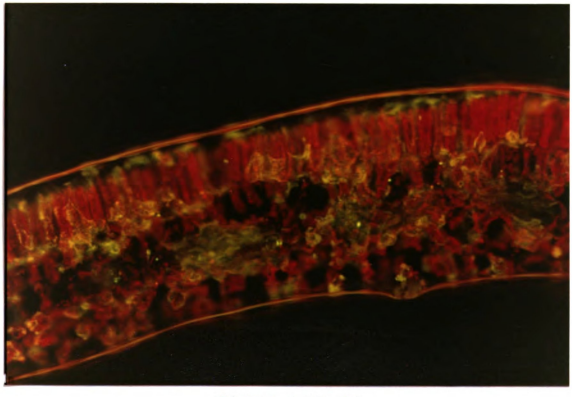


Figure 3.29 (b)

peak (Figure 3.30a) whichcontinued through day 63. Day 35 samples also exhibited some fluorescence in palisade cells.

Figure 3.30 (a) Upper epidermis and vascular fluorescence in a blueberry leaf rub-inoculated with BBSSV, 35 days post-inoculation (250X). 1985

Figure 3.30 (b) Healthy control leaf section indirectly stained with pre-immune rabbit serum followed by sheep-anti-rabbit FITC (250X). 1985

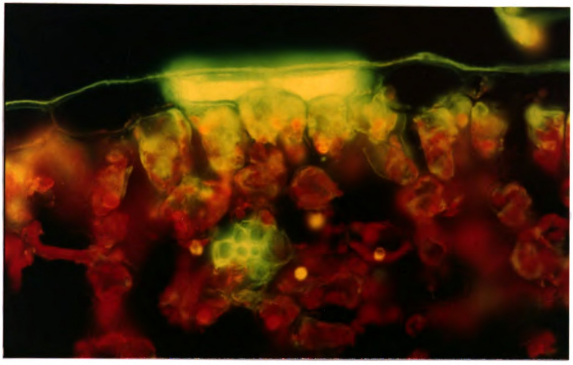


Figure 3.30 (a)

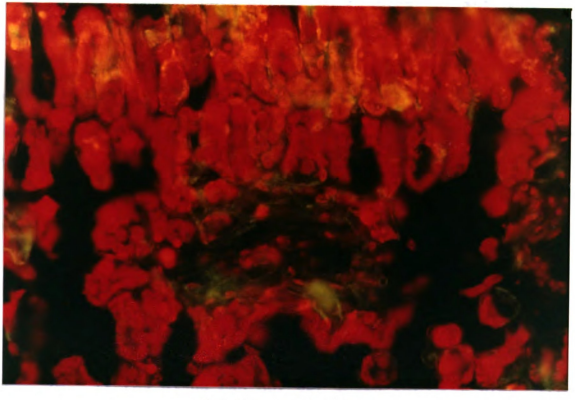


Figure 3.30 (b)

DISCUSSION

In both aphid- and rub-inoculated leaves, immunoassay tests were not sensitive enough to detect virus antigen in plants during early infection periods. Detections were few and irregular with few plants repeatedly positive. ELISA and dot-blot immunoassay are usually sensitive tests for BBSSV detection, with the ability to detect as low as 2.5 ng and 0.3 pg of virus, respectively (see Chapter II results). However, it does not seem unusual that these tests were not useful in this experiment, since many of the sections stained with a fluorescent antibody revealed only one infected cell (Figure 3.4). Detection of virus antigen in a single cell has been found by other researchers utilizing fluorescent antibody techniques (12,13,16).

The immunoassay tests were to be used for determining when the virus had moved beyond the inoculation boundaries in aphid-inoculated plants. They were also used as a comparison with the sensitivity of fluorescent antibody staining. The immunoassays were not successful because there probably was not enough virus to detect along with the small sample size. In subsequent years, when the virus had time to build titer, the tests may have failed to reveal viral antigen because there was none there. At each stage in the year of inoculation, leaves were removed, thus removing much of the inoculum. If the virus had not yet moved into the stem, then there would not have been the chance for accumulation and

detection. The viral infection may have been confined to the leaves inoculated, even though many of these leaves showed vascular fluorescence. Some virus-positive plants were expected in aphid-inoculated plants for this reason. In rub-inoculated plants, more positives were expected because at least twice as many leaves were inocualted as used for testing and would have remained on the plant longer.

Worley and Schneider (16) used a fluorescent anti-bodystain to determine progressive distribution of southern bean mosaic virus in bean leaves. They found that the virus spread from the epidermal cells of one surface to the other surface in a chain and lateral spread followed later. In the opposite uninoculated leaf, the virus spread through the mesophyll first. They did not explain why the virus spreads in this manner. It is possible, since they were using a systemic host, that the mesophyll cells are the major cells for replication after translocation through the phloem.

Fluorescent antibody detection techniques were employed in this study to follow the progression of viral infection in blueberry leaves inoculated with BBSSV. Only general patterns of spread can be discussed here because greenhouse conditions were not held constant.

Aphid-inoculated plants were typical of those under field conditions where aphids feed mostly on the lower leaf surface (4). In both 1984 and 1985, the first tissue to fluoresce was vascular tissue. This was expected as aphids feed primarily in the phloem cells of vascular tissue. How-

ever, what was not expected was fluorescence of xylem elements Phloem cells were not observed fluorescing, but their fluorescence may have been missed in the many samples or masked by the xylem fluorescence. The direction of spread seemed to be random, radiating out from the vascular tissue to mesophyll cells and the palisade parenchyma. The time differences of the spread of infection between 1984 and 1985 were probably due to varied greenhouse temperatures and the condition of the plants. If you compare the 1985 data with the typical rates of virus translocation reported in the literature review, the faster spread of virus seems more typical. Also, some of the leaf sections from 1984 dried out before being observed and may have contained some fluorescing cells.

The epidermal cells were the last to be observed fluorescing. The lower epidermis was found fluorescing before the upper epidermis. This may have been from aphid probing and the inability of virus to invade and replicate as well in epidermal cells compared to other cell types. Late in the study, fluorescence increased in epidermal cells where the virus may have been confined. Some autofluorescence in the lower epidermis of mature, older leaves was also found. If fluorescence was seen in both controls and samples late in the study it was regarded as autofluorescence. It would be interesting to look at the number of fluorescing epidermal cells in resistant and susceptible cultivars of blueberry to determine if the virus is confined here.

In the rub-inoculated plants, it was surprising to see how quickly the virus reached vascular tissue. By day 3 post-inoculation in 1985, midrib xylem was fluorescing and fluorescence continued in vascular tissue throughout the study. In 1984, vascular fluorescence was first observed on day 14 post-inoculation and also continued throughout the study. The 1984 samples seemed to be about a week behind the progression of the 1985 samples, but were still similar. After vascular fluorescence, mesophyll cells were observed fluorescing. Next were the epidermal layers with the lower epidermis showing fluorescence in 1984 and the upper epidermis in 1985. Epidermal fluorescence was expected early in this study since the virus was rub-inoculated into the upper leaf surface. There was also more epidermal fluorescence in rub-inoculated samples compared with aphid-inoculated samples, most likely because of the inoculation procedures used. Again, the pattern of spread seemed to be random, in all directions, starting with the vascular tissue and mesophyll and moving toward both epidermal layers.

Viruses move from cell to cell in several ways. The main route in these studies of intercellular transport was probably through plasmodesmata. Shalla (15) determined the size of plasmodesmata and reported that they were of sufficient size to allow passage of tobacco mosaic virus in the form of whole virus. Hartmann et al. (8) found virus-like particles near plasmodesmata in BBSSV-infected root cells. They concluded that the plasmodesmata in blueberry root cells were of a sufficient diameter for virus to enter.

It is likely that BBSSV could travel in the plasmodesmata of cells in leaf tissue also and is probably the main transportation route between cells.

If the plasmodesmata of leaf and stem cells were of a smaller diameter than in root cells, it may still be possible for BBSSV to move in this manner. Kitajima and Lauritis (10) observed modifications in the size of plasmodesmata of zinnia leaf cells when infected with dahlia mosaic virus. Their photomicrographs show what appear to be half transformed plasmodesmata, where one end is enlarged and contains virions and the other end is of normal size. They reported that these transformations occurred in all types of leaf cells and there was no special distribution pattern of the modification.

Another possible mode of virus transport between cells is the division of cells and redistribution of virus. Chandra and Hildebrandt (1,2) were able to show TMV infected cells involved in mitosis and cytokinesis with the end result being random distribution of virus. TMV did not seem to alter cell division even when large amounts of virus were present. It seems possible for most viruses, especially single component, small isometric viruses like BBSSV, to be transported in this manner.

It is possible that BBSSV replicates and assembles on or near the nucleus. Fluorescing nuclei were noted in several cells. De Zoeten and Gaard (3) reported that the diameter of the nuclear pores was large enough to allow pas-

sage of icosahedral plant viruses between 25 and 30nm. They also observed, with transmission electron microscopy, southern bean mosaic virus (SBMV) in the nuclei of infected cells. Blueberry shoestring virus, being a small icosahedral virus, could possibly move through nuclear pores and replicate in the nucleus, although it has never been reported in this organelle.

TMV has also been reported as replicating and assembling in the nucleus. Hirai and Hirai (9) injected living hair cells with TMV and looked for the presence of viral coat protein using a fluorescent antibody stain. Six hours after injection the viral protein was detected in the nucleus. As the time after infection increased, the fluorescence was more apparent in the cytoplasm.

It would be interesting to look at the replication of BBSSV in blueberry cells an added step. Now that a cDNA probe has been made to detect BBSSV RNA (see Chapter II), a replication cycle could be studied by simultaneously using the cDNA probe with biotin/avidin/FITC and the anti-BBSSV-IgG indirect staining procedure used in this study. By using both, at the same time, on separate sections, the time of replication and coating could be determined by which test was detecting fluorescence. Also, the location of replication could be determined.

There are advantages and disadvantages concerning the use of fluorescent antibody stains for viral detection. It is a sensitive and reliable assay, but precautions and complete understanding of the system being examined are necessary. In Figure 3.24, a control section, there are "flecks" of yel-

low stain. After much thought and looking at many sections, these seem to be some of the FITC that has settled out of solution. FITC will go into solution atpH 7.0. Blueberry tissues are highly acidic and probably become even more acidic when sectioned. Ground blueberry leaves in a pH 7.0 phosphate buffer (0.1M) changed the pH of the solution to 3.0. With this knowledge and the fact that flecks are seen on the slides where no sections are present as well as in empty spaces in sections, it is safe to assume this is not specific fluorescent antibody staining.

Another problem encountered is autofluorescence of plant tissues. Blueberry tissues are extremely autofluorescent. In this study sections were mounted in a phosphate buffer solution because other mounting media also fluoresced. This gives the researcher less time to examine fresh sections before they dry out. Also, leaf sections taken from older, mature leaves fluoresce in the lower epidermis even without staining with a fluorescent dye. However, with proper controls and a knowledge of the system, fluorescent antibody techniques enable a rapid, sensitive assay.

In summary, ELISA and dot-blot immunoassay were not sensitive enough assays to detect BBSSV antigen during early infection periods. Fluorescent antibody staining techniques revealed that the progression of viral antigen in aphid-inoculated plants was from vascular tissue to the mesophyll and palisade parenchyma layers and finally to the epidermal layers. Progression in rub-inoculated plants was similar except for some early upper epidermal fluorescence due to the

location of inoculation.

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CHAPTER IV

MOVEMENT OF BLUEBERRY SHOESTRING VIRUS IN HIGHBUSH BLUEBERRY `JERSEY' AS DETERMINED BY GRAFTING AND GIRDLING TECHNIQUES

INTRODUCTION

The first unofficial reports of graft transmission of viruses are from the seventeenth century when Dutch tulip growers discovered that by grafting bulbs together they could get the prized white streaking or "breaking" of solid colored tulips(13). In the late 1800's, E.F. Smith did extensive research on peach yellows virus and pointed out what growers had known for many years: that yellows was transmitted by budding(12).

Grafting is used commercially for plant propagation. It has been found that this can also lead to the spread of virus. Plum pox virus was originally detected by grafting suspected diseased material onto peach(24). Citrus cultivars used commercially were found infected with citrus tristeza virus only when a sweet orange scion was grafted onto a sour orange rootstock. This grafting method is now used as a means of indexing for citrus tristeza virus(4).

Graft transmission of viruses is usually artificial, i.e. a person is doing the grafting. However, natural root grafts between plants are sometimes found and have been suggested for the transmission of carnation mosaic virus and apple mosaic virus(24).

Chip-budding, a form of grafting involving the transfer of a bud plus some vascular material, has been used widely to transfer virus. In small fruits, chip-budding has been used to transmit virus to grape, raspberry, gooseberry, and blueberry(8). Varney(25) and Schulte(23) have transmitted BBSSV

by grafting chip buds onto healthy plants. The following experiments were designed to utilize chip-budding of BBSSV diseased buds onto healthy plants as a rapid means of infecting material without debilitatingthe plant as wedge grafts could.

BBSSV is transmitted by the blueberry aphid Illinoia pepperi (MacG.) in nature(19). Being aphid vectored, BBSSV is hypothesized to be phloem translocated. The objective of this study was to determine if BBSSV moves in the xylem, phloem, or both, utilizing chip-budding and girdling techniques.

MATERIALS AND METHODS

Stems from diseased bushes in Eastmanville, Michigan, to be used as a budwood source of BBSSV-infected `Jersey' blueberry, were cut on February 19th, 1985, placed in plastic bags with moist paper towels, and stored in a coldroom ($4^{o}C$) until grafting time.

Fifty, 3-year-old potted `Jersey' blueberry plants were obtained from John W. Nelson, South Haven, Michigan. Plants chosen had at least two main stems. All plants were tested by the ELISA method of Clark and Adams (see Chapter II Methods and Materials) and were found to be negative for BBSSV. Stems from the coldroom were placed in water, in the laboratory, overnight to "loosen" wood for chip-budding.

Chip-budding took place on the 13th, 15th, 16th, 19th, and 20th of April, 1985. Chip buds were cut from both healthy and diseased stems using budding knives. Two knives

were used to avoid contamination. Before cutting buds, the size of the stems were matched to insure match-up of vascular tissue. Chip buds of diseased material were placed on healthy plants, three to four per plant, spaced 1.5 cm apart on a single stem, and wrapped with Parafilm^{R} to allow air flow to the wood for better healing conditions(15). All buds were placed on the same stem. Thirty plants received diseased buds. After three days, 10 plants were girdled above the chip buds, 10 plants were girdled below the chip buds, and 10 plants were not girdled (Figure 4.1). Girdles were approximately 1.5 cm in width. Plants were designated GA-1 through GA-10 for those girdled above, GB-1 through GB-10 for those girdled below, and NG-1 through NG-10 for those with no girdle.

Healthy chip buds were placed on 10 healthy plants, three to four per plant, and buds were wrapped with Parafilm^{R} as above (Figure 4.2). Three days later, three plants were girdled above the chip buds, three plants were girdled below chip buds, and four plants were not girdled. Plants were designated as above.

Healthy and diseased plants were placed in separate greenhouses. Ten additional plants were chip-budded with diseased buds and girdled above or girdled below the buds on the same day as budding. Buds were again wrapped with Parafilm^{R}. Plants in these two groups were designated GA-1* through GA-5* and GB-1* through GB-5*, respectively.

Diseased and healthy chip buds were also tested by ELISA (see Chapter II Materials and Methods) to insure that the

Figure 4.1 Location of grafted chip buds and girdling variations used to determine movement of BBSSV in the vascular system.

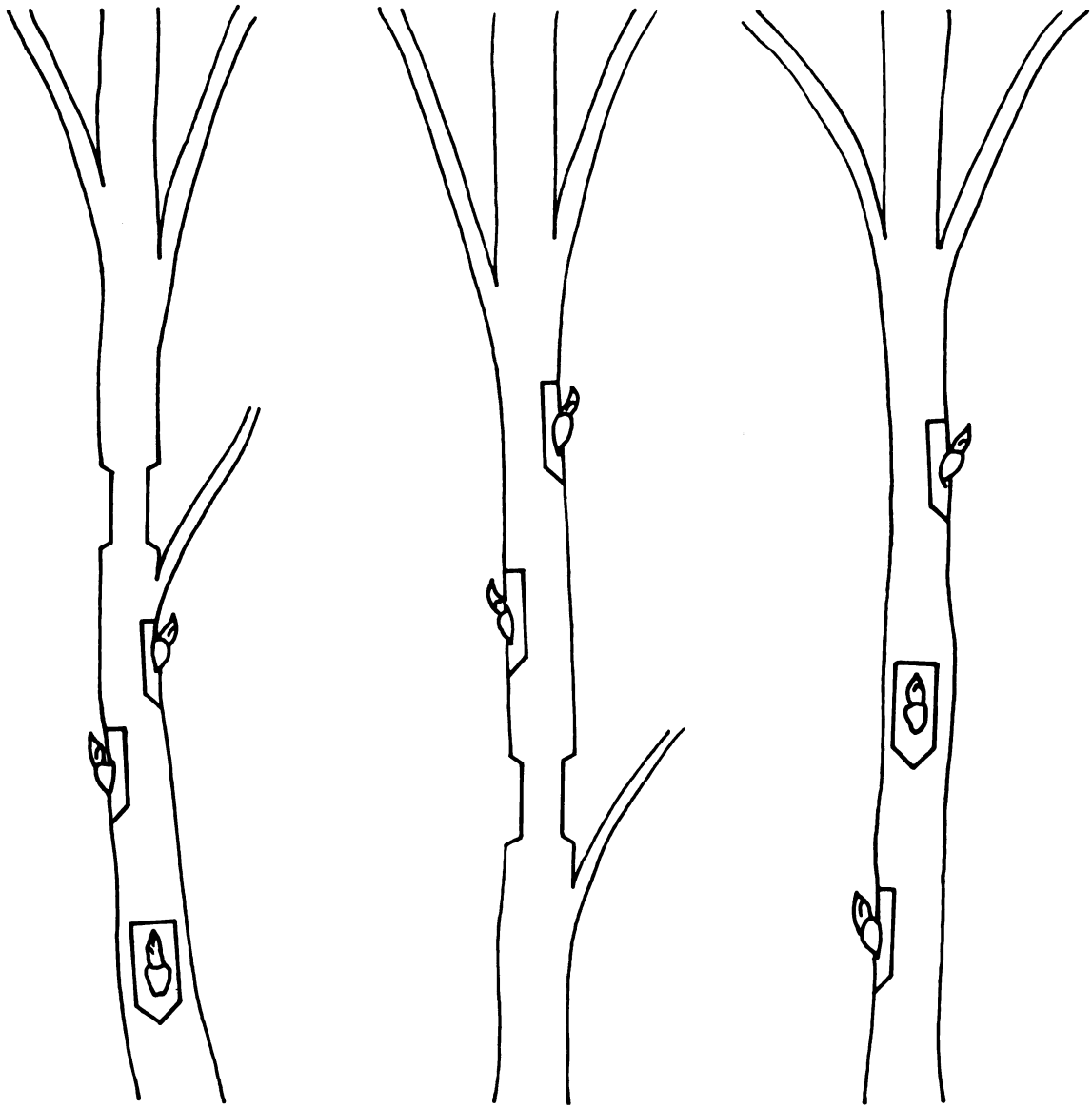
GIRDLED ABOVE
GIRDLED BELOW
NO GIRDLE

Figure 4.2 Chip-bud held in place by Parafilm^{R}.

material used was actually diseased or healthy. Both terminal and lateral buds were tested from six different stems.

On May 1st, 1985, the first chip buds were unwrapped. By May 21st all buds were unwrapped. Testing leaves by the dot-blot immunoassay method of Bantarri and Goodwin(1) began the 7th of May (approximately 1 month after chip-budding) and plants were tested monthly until October (see Chapter II Materials and Methods). Samples consisted of three leaves picked randomly from the shoot with chip buds and diluted 1:10 (w/v) with virus extraction buffer.

Girdles on plants were checked periodically throughout the 2-year study and callus regrowth tissue was removed with a clean razor blade. Plants were placed in a coldframe in October for a vernalization period of at least 1000 hours at less than $40^{o}C$.

In December 1985, chip-budded plants were transferred to a greenhouse that was being maintained at $30-40^{o}F$. Root samples of all plants were taken in December 1985,and January and April of 1986, and tested for BBSSV antigen using the dot-blot immunoassay procedure. An additional root sampling in May 1986 included only plants that were grafted three days after chip-budding. Root samples were approximately 1 gram in weight pruned from the root mass and diluted 1:10(w/v) in extraction buffer, except inMay when 10 grams of roots were tested. In this case, the bottom 2 inches of the root mass was cut off with a pruning saw.

Plants were moved into a greenhouse being maintained at $70-85^{o}F$ in April 1986. Leaf sampling was started in May and

continued on a once a month basis until September. In 1986, plant material above girdles was dead, therefore samples were taken from below girdles and from stems with no chip buds. Three leaves per plant were sampled and diluted 1:10(w/v) with extraction buffer, ground, and analyzed by the dot-blot immunoassay procedure (see Chapter II Materials and Methods).

Visible symptoms of BBSSV infected plants were recorded and photographed as they apppeared in 1986.

Roots were sampled in November 1986 from plants girdled the same day as chip-budded, and tested for the presence of BBSSV antigen with the dot-blot immunoassay procedure.

RESULTS

Both buds that were expanding, i.e. breaking dormancy, and dormant buds were ELISA tested. Both terminal and lateral buds were tested. The terminal buds that were expanding and those that were dormant tested positive for the virus. Only the dormant, lateral buds were positive (Table 4.1).

Chip-budding was successful (healed and green) in a range of 47% to 100% (Table 4.2). Data were taken one week after unwrapping the buds. Later in the season some buds dried out and/or fell off.

In 1985, leaf samples were taken above and below the girdles or above and below chip buds on those plants with no girdling. The first dot-blot positive sample for BBSSV antigen was in June, but only for one plant. In successive months more plants were dot-blot positive. This was checked in July

TABLE 4.1 DETERMINATION OF THE PRESENCE OF BLUEBERRY SHOESTRING VIRUS IN BUDS FROM STEMS USED FOR CHIP-BUDDING.

Source Material[a]		ELISA values (A_{405nm})[b]
Healthy[c] (Expanding)	terminal bud 1	0.010
	terminal bud 2	0.015
	terminal bud 3	0.010
	lateral bud 1	0.015
	lateral bud 2	0.035
	lateral bud 3	0.075
Diseased	terminal bud 1	0.205[e]
	terminal bud 2	0.150[e]
	terminal bud 3	0.175[e]
	lateral bud 1	0.075
	lateral bud 2	0.055
	lateral bud 3	0.065
Healthy[d] (Dormant)	terminal bud 1	0.055
	terminal bud 2	0.085
	terminal bud 3	0.060
	lateral bud 1	0.075
	lateral bud 2	0.085
	lateral bud 3	0.085
Diseased	terminal bud 1	0.855[e]
	terminal bud 2	0.190[e]
	terminal bud 3	0.270[e]
	lateral bud 1	0.275[e]
	lateral bud 2	1.450[e]
	lateral bud 3	1.265[e]

[a] Each group consists of buds from three stems.

[b] ELISA values are the average of two readings taken 15 minutes after adding the enzyme substrate.

[c] Mean A_{405nm} reading of healthy expanding buds = 0.027. Mean plus three standard deviations = 0.103.

[d] Mean A_{405nm} reading of healthy dormant buds = 0.075. Mean plus three standard deviations = 0.115.

[e] Values above the threshold limit of the mean plus three standard deviations of healthy controls.

TABLE 4.2 NUMBER OF SUCCESSFUL(HEALED AND GREEN) CHIP BUDS DETERMINED ONE WEEK AFTER PARAFILM[R] WAS REMOVED[a].

	Girdled Above	Girdled Below	No Girdling
Healthy[b]	8/9 89%	11/12 92%	13/13 100%
Girdled[c] same day	10/15 67%	9/19 47%	
Girdled[d] three-days after budding	32/34 94%	31/40 78%	30/36 83%

[a] Parafilm[R] was used to hold chip buds in place. It had to be removed to prevent girdling of the stems.

[b] Healthy plants consisted of three plants girdled above the chip buds, three plants girdled below the chip buds and four plants with no girdling.

[c] Ten plants girdled either below the chip buds(5) or above the chip buds(5) on the same day they were budded.

[d] Plants with diseased buds and girdled three days after budding consisted of 10 plants girdled above the chip buds, 10 plants girdled below the chip buds, and 10 plants with no girdling.

by indirect fluorescent antibody staining of a leaf from a positive sample (see Chapter II Materials and Methods). Figure 4.3 shows extensive staining of BBSSV in the palisade and mesophyll areas. By September, five plants were dot-blot positive above the girdle suggesting virus movement in the xylem. These plants were GA-5, GA-6, GA-8, GA-9, and GB-2 (Table 4.3).

Root samples were negative for BBSSV antigen in December 1985, and January and April of 1986 when plants were dormant. In May, two plants, GB-2 and NG-6, had dot-blot positive root sample. In November 1986, root samples from the girdled same-day-as-budded group had three plants with dot-blot positive root samples. These plants were GA-1*, GA-4*, and GA-5*. Root samples were not taken from the other plants due to the severe root pruning they received in May. However, plant GA-9 had developed a stem late in the season, from the crown area, that had all strap-shaped leaves. This suggests that virus was in the crown or roots.

In 1986, a number of plants showed symptoms of BBSSV infection (Table 4.4). Symptoms included red-streaked stems, red vein-banding, crescent shaped leaves, and strapped leaves.

Leaves tested in 1986 were taken from below the girdle on stems with chip buds, above the chip buds on stems with no girdling, and from stems other than those budded. During dormancy all stem material above the girdling died. Monthly dot-blot tests showed the same results for all stems with chip buds. As the season progressed, stems with no chip buds began

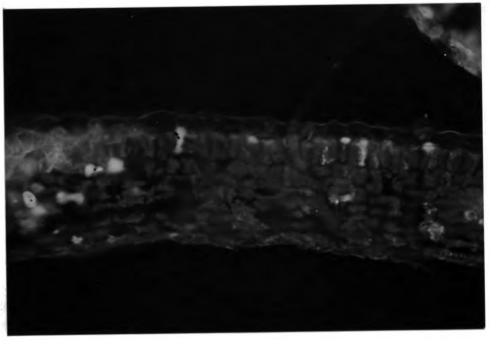

Figure 4.3 Fluorescent palisade and mesophyll cells representing BBSSV antigen in a blueberry leaf section three months after grafting (100X).

TABLE 4.3 DOT-BLOT IMMUNOASSAY OF LEAVES FROM BLUEBERRY PLANTS BUDDED WITH BBSSV INFECTED BUDS AND WITH GIRDLE VARIATIONS - 1985.

Sample[a]	May	June	July	July	Sept. B	Sept. A[b]	Sept.(NG)
GA - 1	-[c]	-	-	-	+	-	
GA - 2	-	-	+[c]	-	+	-	
GA - 3	-	-	-	-	-	-	
GA - 4	-	-	-	-	-	-	
GA - 5	-	-	+	+	+	+	
GA - 6	-	-	+	-	+	+	
GA - 7	-	-	-	+	+	-	
GA - 8	-	-	-	+	+	+	
GA - 9	-	-	+	+	+	+	
GA - 10	-	-	+	+	+	-	
GB - 2	-	-	-	-	+	+	
NG - 1	-	-	-	-			+
NG - 2	-	-	-	-			+
NG - 3	-	-	+	+			+
NG - 4	-	-	-	-			+
NG - 5	-	-	-	-			-
NG - 6	-	-	-	-			+
NG - 7	-	-	-	-			+
NG - 8	-	-	-	+			+
NG - 9	-	-	-	-			+
NG - 10	-	-	-	-			+
GA - 1*	-	-	+	+	+	-	
GA - 2*	-	-	+	+	+	-	
GA - 3*	-	-	+	+	+	-	
GA - 4*	-	+	+	+	+	-	
GA - 5*	-	-	-	-	+	-	

[a] Samples consisted of three leaves taken randomly from each plant. Groups included girdled above (GA 1-10) or girdled below (GB 1-10) three days after budding, no girdling(NG 1-10), and girdled above(GA 1-5*) or girdled below (GB 1-5*) the same day as budded.

[b] Samples were taken below the girdle (B) and above the girdle (A) on each plant.

[c] = Samples were dot-blot positive(+) or negative(-).

TABLE 4.4 PLANTS EXHIBITING SYMPTOMS OF BLUEBERRY SHOE-STRING VIRUS DISEASE ONE YEAR AFTER CHIP BUDDING.

Plant number	Symptom
GA - 1[a]	red-streaked stems
GA - 5	red-streaked stems
GA - 8	red-streaked stems
GA - 9	red-streaked stems, strapped leaves
GA - 10	red-streaked stems
GA - 1*[b]	red-streaked stems
GA - 2*	red-streaked stems, strapped leaves, crescent shaped leaves
GA - 4*	red-streaked stems
NG - 3[c]	red-streaked stems
NG - 4	red-streaked stems

[a] Plants girdled above chip buds three days after they were budded.

[b] Plants girdled above chip buds the same day they were budded.

[c] Plants chip budded but not girdled.

TABLE 4.5 DOT-BLOT IMMUNOASSAY(1986) OF LEAVES FROM BLUEBERRY PLANTS BUDDED WITH BBSSV INFECTED BUDS AND GIRDLING VARIATIONS FROM 1985.

Sample[a]	April		May		June		July		Aug.	
	S	O[b]	S	O	S	O	S	O	S	O
GA - 1	-[c]	-	-	-	+	-	+	+	+	-
GA - 2	+	-	+	-	+	-	-	-	+	-
GA - 3	-	-	-	-	-	-	-	-	-	-
GA - 4	-	-	-	-	-	-	-	-	-	-
GA - 5	+	-	+	-	+	-	?	-	+	+
GA - 6	+	-	+	-	+	-	+	-	-	-
GA - 7	+	-	-	-	+	-	+	-	+	+
GA - 8	+	-	+	-	-	-	N	N	-	-
GA - 9	+	-	+	-	+	+	+	-	+	-
GA - 10	+	-	?	-	-	-	N	N	N	N
NG - 1	-	-	+	-	+	-	+	-	+	+
NG - 2	-	-	-	-	+	+	+	?	+	+
NG - 3	+	-	+	-	-	-	-	-	+	-
NG - 4	-	-	+	-	-	-	?	-	-	-
NG - 5	-	-	-	-	-	-	+	-	-	-
NG - 6	+	-	+	-	-	-	+	+	+	+
NG - 7	+	-	-	-	-	-	+	-	+	-
NG - 8	+	-	+	-	+	-	+	-	-	-
NG - 9	-	-	-	-	-	-	N	N	N	N
NG - 10	-	-	-	-	-	-	+	-	-	-
GA - 1*	+	-	+	-	+	-	+	+	+	+
GA - 2*	+	-	+	-	+	+	+	+	+	+
GA - 3*	+	-	-	-	+	-	+	-	+	-
GA - 4*	+	?	+	+	+	+	+	+	+	+
GA - 5*	+	-	+	-	+	-	+	-	+	-

[a] Samples consisted of three leaves taken randomly from each plant. Groups included girdled above chip buds (GA 1-10) and girdled below chip buds(GB 1-10) three days after budding, budded but no girdling(NG 1-10), and girdled above chip buds(GA 1-5*) and girdled below chip buds(GB 1-5*) the same day as budded.

[b] Samples were taken from the same stem as budded(S) and sample was taken from stem other than the one budded(O).

[c] Samples were dot-blot positive(+), negative(-), questionable(?), or no sample was taken(N). Missing samples were due to dead plants or brown leaves.

to show positive results for BBSSV antigen (Table 4.5) mainly in the group of plants girdled the same day as they were chip-budded. Other groups may not have exhibited this phenomenon due to the debilitating effect of large root samples taken from them in May. Groups GB and GB* contained no virus positive samples in 1986.

DISCUSSION

The time it takes for a virus to pass a graft union is an important consideration when doing graft transmission and girdling experiments. Fridlund (11) reported that three ilarviruses were able to pass through graft unions in Prunus spp. within 72 hours with 100% transmission. He also reported that it took longer for other viruses; up to 150 hours. Bennett (2) reported transmission of sugar beet curly top virus to Turkish tobacco in 5 days while it took only 2 days for transmission of cucumber mosaic virus. Because transmission times vary for different viruses, and it was not known how long it would take for transmission of BBSSV, the experiments were set up in two different ways: 1) with girdling done 3 days after chip-budding and 2) with girdling done the same day as chip-budding. In this way , if the plants were physiologically affected by the girdling such that chip buds would not heal properly, there would be plants that were given 3 days to establish some connection before girdling took place. However, girdling did not seem to adversely affect the plants in the year of girdling, as evidenced by the number of successful chip buds (Table 4.2) and vigor of plants.

Fridlund (9) also reported that the time required for 100% graft transmission of prunus necrotic ringspot virus (PNRSV) was reduced with each successive inoculation. He tried a combination of one to five grafts per tree and a 56 hour inoculation period. With one graft he achieved 15%

transmission; with five grafts he achieved 86% transmission. In the reported experiments, three or four chip buds were placed on a plant to achieve a higher rate of successful grafts as well as a higher rate of inoculation or transmission.

It was also reported that some of the chip buds in these experiments dried up and/or fell off. This does not seem to be problematic, given that the contact period of the diseased buds with the healthy stems was about a month. Fridlund (9) and Kunkel (17) did experiments where the grafted chip buds were removed after various time intervals. Fridlund's experiments ran only 4 days and he found he needed only 56 hours of contact to achieve transmission. Kunkel also considered the time of year, age, and condition of the tree inoculated and reported that the shortest transmission times occurred in the spring, which was when the chip-budding was done in this study.

Fridlund (10) has also experimented with temperature and how it affects virus transmission. He reported an optimum temperature of 30^{0}C for the transmission of PNRSV, using a range of temperatures from 18 to 46^{0}C.

In this research it was assumed safe to run the experiments with BBSSV in a greenhouse with a temperature range of 70 to 85^{0}F sincethis is adequate for blueberry growth and would not harm the virus (19).

The fact that it may have taken less than 3 days to achieve some transmission of BBSSV could explain the dot-blot immunoassay positive root sample of plant GB-2. Since the

plant was girdled below the chip buds 3 days after their placement, the virus may have been transmitted and moved downward in the phloem stream past the point of future girdling. This would have enabled the virus to continue down to the roots. This situation occurred with only one out of ten plants, but is still highly probable as an explanation of a positive root sample.

Because BBSSV is aphid-vectored, it was hypothesized that the virus moves in the phloem. From this study it is thought that BBSSV moves in both the phloem and the xylem. Evidence of phloem transport consisted of: 1) the initial transmission from the grafted chip-bud, 2) results of immunoassays on leaves from new stems immediately below the girdles, 3) positive root samples, and 4) leaves from stems containing no chip buds producing positive immunoassay results.

Initial transmission from phloem was assumed because buds chosen for grafting were always near a red streak on the stem and buds tested positive in dot-blot immunoassay tests. It is also possible that the buds themselves, and not the wood, were positve for virus or that the virus was transmitted from parenchyma cells. Because no timing of the transmission was done, it is hard to determine the actual tissue the virus came from. Fridlund (11) suggested that the rate of transmission is linked to the route of transmission. He stated that the fastest rate would occur through meristematic cells because these tissues would unite first. Second would be the cells behind the meristem where mature cells would unite.

The slowest rate of transmission would be through phloem cells which would require more time for connection.

New stems were produced directly below the girdle on all plants. In the case where girdles were placed above the grafts, leaves from the new stems were found positve for BBSSV by dot-blot immunoassay. The new stem presented a sink for carbohydrates and most of the virus was probably initially drawn to this area. Dot-blot immunoassay showed positive samples only below the girdles and above grafts until September. Because of the strong sink force, virus may not have been moving into other cell types leading to the xylem until later in the season when the stem directly below the girdle had established itself and was sending carbohydrates back down the main shoot to the roots.

It had been shown by Hartmann et al. (14), using electron microscope techniques, that virus-like particles were in root sections of diseased blueberry plants. It was also shown, in a study in Chapter II, that symptomatic, diseased plants had dot-blot immunoassay positive roots. The failure to find virus in root samples in this study early on may have been due to the small sample taken and/or low accumulation of virus in the roots. When 10 grams of roots were sampled in May 1986, the virus may have already moved out of the roots or there may not have been a detectable amount of virus in the roots. It may be that the initial accumulation of virus is in thecrown area and buds and not in the root extremities, as weresampled.

The virus positive root samples taken in October of 1986 (plants GA-1*, GA-4*, and GA-5*) could have been due to a larger increase in virus being translocated from the stem or a wider distribution of the virus in the root system. Unlike annual plants, the roots of woody perennials are stronger sinks for carbohydrates in the early spring and autumn when they grow earlier and later, respectively, than the shoots or stems (16). At these times, the carbohydrates being produced are channeled to the roots via the phloem. At the same time, virus may be moving passively in the carbohydrate stream. Many viruses are believed to move passively in the phloem at rates up to 30 cm/hr (3).

When virus reaches the roots, it may multiply in the parenchyma cells and is probably immobilized there until a stronger sink is developed, i.e. the stem. The virus can then be translocated out with the food by an exchange of water and photosynthates between parenchyma cells and sieve elements. This exchange also occurs in reverse with sugars being drawn into parenchyma cells from sieve elements and thereby delivering virus as well as food (7). The exchange between sieve elements and their contiguous parenchyma cells would provide transport to the entire plant, especially to new growing areas.

The plants GA-1*, GA-2*, GA-4*, GA-5, GA-7, NG-1, NG-2, and NG-6 were all dot-blot immunoassay positive in leaves taken from a stem that was not grafted. These results imply that the virus moved down to the roots from a grafted stem

and then up into a different stem. Since all plants were kept free of the aphid vector of BBSSV, this was the only possible route of the virus. Also, plant GA-9 produced a new stem from the crown area in 1986 that exhibited the characteristic red stem streaking and strap-shaped leaves of a BBSSV-infected plant. The virus had to come from the roots to infect this developing stem.

Xylem transport is more difficult to prove conclusively. The girdling experiments revealed four plants with dot-blot immunoassay positive samples in 1985 above the girdle and above the diseased chip buds. Since no phloem transport could occur in an upward direction from the diseased chip buds due to the removal of phloem tissue, and the fact that girdles were kept free of callus regrowth, the virus had to be moving in the xylem or xylem parenchyma. The late season virus positive samples may argue in favor of translocation through the xylem parenchyma. It would take longer for virus to travel from intact cell to intact cell. However, xylem transport of virus may have been limited early in the season by the fact that the stem just below the girdlewas acting as a strong sink. Virus has also been detected by fluorescent antibody staining in the xylem elements of both stems and leaves (Chapters II and III) to further support that this virus is xylem transported.

Also in Chapter II, results from xylem material, which would include xylem parenchyma, tested by both dot-blot immunoaasay and a cDNA probe to BBSSV RNA, establish that both protein coat and RNA were present. Because BBSSV is not sap

transmissible to any herbaceous plant yet tested (18) and it is transmissible with some difficulty to other blueberries (23) it is difficult to determine if the virus in the xylem is infective. Normally one would grind up the tissue and attempt sap transmission to another plant, preferably herbaceous, to demonstrate infectivity. It can only be assumed that the virus in this case was infective because it was whole virus. If it is true that the xylem contains whole, infective virus, this may be one explanation of why this disease is so devastating.

Vessel length has been investigated in the highbush blueberry, Vaccinium corymbosum L., by Zimmermann and Jeje (26). They reported that 85% of the vessels were in the 0-to-10 cm length class, 6.5% in the 10-to-20 cm class, and the rest were longer, some as long as 1.3-to-1.4 meters. These long vessels provide a continuous "tube" to to the top of the shrub. This wouldprovide a direct route for the virus into developing leavesas well as those already expanded.

When BBSSV infects leaves in the bud stage or during early expansion, the leaves become strap-shaped. If the leaves are already expanded, they become crescent-shaped or red-veined. There are also symptomless, diseased leaves which may have attained virus after full expansion and hardening, creating a less welcoming environment for viral replication and destruction. The first two instances may argue for either phloem or xylem transport, as both would be possible at this stage of growth. But, the latter case

argues for xylem transport. If we assume that virus does travel in the food stream, the direction of carbohydrate transport in a mature leaf would be out of the leaf toward new leaves whereas water would continue to be supplied to the mature leaf.

The question arises as to how the virus gets into the xylem from surrounding parenchyma. Schneider (20) suggested that viruses may reach conducting xylem elements by first invading immature cells. As the cells differentiate, membranes degenerate, and virus may move into the mature conducting cells. This is not inconceivable, when one considers the feeding behavior of blueberry aphids. According to Elsner (6) they prefer succulent stems and are often found feeding at shoot tips. They could be probing and feeding in immature xylem cells and thus introduce BBSSV into the xylem as well as the phloem. It would be interesting to look at shoot tips fed on by viruliferous aphids to determine where the virus is introduced.

The question also arises as to the ability of the virus to leave tracheary elements and infect other cells. Schneider and Worley (22) found symptoms in bean plants, infected with southern bean mosaic virus (SBMV), at a distance from the infection point after it's introduction into tracheary elements. They suggested that SBMV could pass into uninjured cells from the xylem and cause disease. This does not answer the question of how the virus moves out of the xylem, but it is evidence that it can.

Another possible route of virus translocation in the xylem is through lateral transport. Water is laterally transported from one tracheary element to another through pits. If the pores in the pit membrane were large enough to allow passage of virus, this would help to distribute virus to many vessels. This may be one reason for the irregular distribution of this disease in a blueberry bush.

Only two other viruses have been documented as moving in the xylem. Chambers and Francki (5) observed lettuce necrotic yellows virus in young xylem cells of leaf veins. Infective virus was also recovered from xylem sap in the stem. The virus was not confined to the xylem; it was also found in mesophyll cells, epidermal cells, and leaf hairs. Schneider and Worley (21, 22) found that SBMV could travel in the xylem when a local lesion host was approach-grafted to a systemic host and then the stem was steamed to kill phloem cells. However, the local lesion host would not be a natural host for xylem movement. They were speculating on what could happen if the virus was able to get into the water stream.

The lack of evidence for xylem translocation of other viruses may be due to a number of factors. Some researchers may not look at the xylem as a possibility for transport. Others may think they are artifacts of the technique used or that the technique has not been perfected to prove viral residence in xylem. Still others may be looking for support-ingevidence to report conclusively that a virus is in the xylem. The reported experiments should lend ideas to those

lookingat possible virus transport in the xylem and also the phloem.

In summary, viral antigen was found in leaves above girdles suggesting xylem transport of the virus. Viral antigen was also found in leaves from ungrafted stems adjacent to those that contained grafted, diseased buds suggesting phloem transport through the roots. This evidence of xylem and phloem transport, along with earlier data from dot-blot/cDNA probe studies suggests that the virus is moving in the form of whole virus.

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APPENDICES

APPENDIX A

ELISA Solutions

Coating Buffer

1.59 g sodium carbonate
2.93 g sodium bicarbonate
1 liter distilled water; pH 9.6

Wash Solution (PBS-Tween)

1.15 g sodium phosphate, dibasic
0.20 g potassium chloride
0.20 g potassium phosphate, monobasic
8.00 g sodium chloride
1 liter distilled water; pH 7.4
0.50 ml Tween 20
0.20 g sodium azide (optional)

Extraction Buffer

1 liter PBS-Tween
2.0 g ovalbumin
20.0 g polyvinylpyrrolidone

Substrate Buffer

97.0 ml diethanolamine bring up to 1 liter with distilled water; pH 9.8

0.20 g sodium azide (optional)

Substrate

p-nitrophenyl phosphate

APPENDIX B

Dot-blot Immunoassay Solutions

TBS-T80 (0.1M)

0.388 g tris base
2.644 g tris-HCl
add 2 liters of distilled water; pH 7.4

18.0 g sodium chloride
1.0 ml Tween 80

Extraction Buffer

TBS-T80 1 liter
10.0 g polyvinylpyrrolidone

Substrate Buffer (Tris-HCl (0.2M); pH 8.2)

3.54 g Tris-HCl
3.34 g tris base
add 250 ml distilled water

Coating Buffer

1.59 g sodium carbonate
2.93 g sodium bicarbonate
add 1 liter distilled water; pH 9.6

APPENDIX C

Virus Purification Reagents

Virus Buffer (0.1M ; pH 7.0)

8.95 g sodium phosphate, dibasic
5.40 g sodium phosphate, monobasic plus water
0.7 ml 2-mercaptoethanol (0.01M)
0.46 g thioglycolic acid (0.35 ml, 0.005M)
make up to 1 liter with distilled water

P-DTT (0.05M ; pH 7.0)

2.16 g sodium phosphate, dibasic
1.35 g sodium phosphate, monobasic plus water
0.077 g dithiothreitol (0.001M DTT)
make up to 500 ml with distilled water

Phosphate Buffer (0.005M ; pH 7.0)

0.432 g sodium phosphate, dibasic
0.270 g sodium phosphate, monobasic plus water
make up to 100 ml with distilled water

APPENDIX D

cDNA Probe Hybridization Reagents

Prehybridization Solution

50% formamide
5X Denhardt's
5X SET
50 mM sodium phosphate; pH 6.5
1% glycine
10 mg calf thymus DNA

SET (20X)

3.0M sodium chloride
0.4M Tris-HCl; pH 7.8
20 mM EDTA

Denhardt's (50X)

5.0 g Ficoll
5.0 g polyvinylpyrrolidone
5.0 g bovine serum albumin
make up to 500 ml with distilled water
filter and store in 25 ml aliquots at -20^{o}C

Hybridization Solution

50% (v/v) formamide
1X Denhardt's
5X SET
20 mM sodium phosphate, pH 6.5
2 mg calf thymus DNA