Etiology of Blueberry Shoestring Disease and Some Properties of the Causal Virus

M. S. Lesney, D. C. Ramsdell, and Mike Sun

Graduate Research Assistant, Associate Professor, and Research Associate of Plant Pathology, respectively, Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824.

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ABSTRACT

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Blueberry shoestring virus (BBSSV) was purified from frozen infected blossoms of highbush blueberry, *Vaccinium corymbosum* 'Jersey', using polyethylene glycol precipitation and differential centrifugation. After two cycles of centrifugation on a linear sucrose gradient the $A_{260/280}$ ratio was 1.47. The purified virus was inoculated to healthy 'Jersey' seedlings and caused typical disease symptoms within 5 or 6 mo. Attempts to transmit the virus to 44 herbaceous indicator species were unsuccessful. Blueberry shoestring virus is an isometric, apparently single-component virus approximately 24 nm in diameter with an estimated sedimentation coefficient of 78.5S (± 3.1). An antiserum to BBSSV with an homologous titer of 1:256 failed to react with 20 other isometric plant viruses. Antisera to 23 other isometric plant viruses also failed to react with purified BBSSV. Mathematical analysis of field data indicated bush-to-bush spread of the disease and gave a compound interest rate "r" value of 0.27/unit/yr.

Additional key words: purification, electron microscopy, serology.

Blueberry shoestring disease, which originally was described in New Jersey (15), is found throughout the entire Michigan blueberry region and is the most important virus disease of blueberries in the state. In 1975 in Ottawa County, which produces some 39.5% of the state crop, a survey of 25% of the acreage by the Michigan State Department of Agriculture (about 750,000 plants) yielded a total count of 4,514 blueberry bushes showing symptoms of virus or mycoplasma-caused disease.

Of these, 3,688 or 81.7% were identified as having shoestring disease on the basis of symptomatology (about 0.5% of the total acreage surveyed). Shoestring-diseased bushes exhibit several characteristic symptoms. The most common symptom is the presence of elongated reddish streaks on stems. Other symptoms that may be present are a red to purple oak-leaf pattern on certain leaves and leaf deformities; e.g., crescent or "strap" shaped leaves. This latter symptom has given rise to the descriptive name "shoestring". Within a few years after infection the disease dramatically lowers berry yield. Virus spread occurs mostly along the rows in the field, but at present, the vector or mode of spread is unknown. Previous work indicated the graft-transmissibility of the disease (15) and the probable viral nature of its cause (6). This report presents proof of the viral nature of the disease, some properties of the causal agent, and evidence of bush-to-bush spread of the disease in the field. Preliminary accounts of this work have been reported (8, 9).

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MATERIALS AND METHODS

Purification techniques.—Two purification methods were used to obtain the virus for this study. The first was adapted from Gilmer et al. (5). Eighty to 100 gm of frozen, infected blossoms were ground, with 3 ml buffer/gm of tissue, in a Waring Blendor for 3 to 5 min in cold 0.2 M potassium phosphate buffer, pH 7.6 (PO_4 buffer) containing 0.001 M sodium diethyl dithiocarbamate (DIECA), and 0.1 M mercaptoacetic acid as virus stabilizers. The homogenate was squeezed through cheesecloth, centrifuged at low speed (12,100 *g* for 20 min) and the virus was then precipitated by the addition of 10% (w/v) polyethylene glycol (PEG), MW 6,000, to the supernatant liquid which contained 0.3 M NaCl. The precipitate was pelleted by a second low-speed cycle and the virus was resuspended in 0.2 M PO_4 buffer, pH 7.1, using 5 ml/30 ml initial supernatant. The virus was then pelleted in a Beckman No. 40 rotor at 137,600 *g* for 60 min, and resuspended in 0.02 M PO_4 buffer, pH 7.0, using 0.5 ml/30 ml initial supernatant, for use in further purification or inoculation attempts.

The second purification method, adapted from Tremaine et al. (12) involved the use of 0.05 M PO_4 extraction buffer, pH 8.0, containing 0.04 M DIECA and 0.04 M mercaptoacetic acid. Polyethylene glycol

precipitation was not used in this procedure. The virus was purified and concentrated by multiple cycles of differential centrifugation. Ultracentrifugation was done in a Beckman No. 30 rotor at 98,000 *g* for 90 min. The final pellet was resuspended in 0.02 M PO_4 buffer, pH 7.0.

Density gradient centrifugation.—Sucrose density gradient (SDG) tubes (Beckman SW 25.1 rotor) were layered with 4, 7, 7, and 7 ml of 10, 20, 30, and 40% (w/v) sucrose solutions respectively in 0.02 M PO_4 buffer, pH 7.0, and allowed to equilibrate for 12-16 hr at 4 C. One to two ml of virus at 1.3 A_{260} units/ml were layered onto the gradients which then were centrifuged at 23,000 rpm for 2.5 hr at 4 C. An identical preparation made from healthy blossoms was used as a control. The gradients were fractionated and scanned at 254 nm with an ISCO density gradient fractionator and UV-analyzer (Instrumentation Specialties Co., Lincoln, NE 68504).

Brakke's (2) method was used to estimate the sedimentation coefficient of BBSSV by comparing its sedimentation velocity in SDG with that of tobacco mosaic virus (TMV, U-1 strain) and southern bean mosaic virus (SBMV), supplied by J. L. White and F. S. Wu, respectively.

Gradient tubes were prepared using 3, 7, 7, 7, and 4-ml layers of 0, 10, 20, 30, and 40% (w/v) sucrose in 0.02 M PO_4 buffer solution, pH 7.0, respectively. Two tubes, each with 2 ml of BBSSV preparation, were compared with one tube layered with 2 ml of TMV or SBMV preparation. Centrifugation was at 24,000 rpm at 4 C for 60 min using a chilled Beckman SW 25.1 rotor, after which time migration of the sedimenting band was measured from the meniscus. The tubes then were centrifuged for an additional hour at which time band migration again was measured and a sedimentation rate curve was obtained which was used in calculation of the BBSSV sedimentation coefficient.

Ultraviolet spectrophotometry.—Equal quantities of healthy and diseased blossoms purified by the method of Gilmer et al. (5) were fractionated after SDG centrifugation, and equivalent fractions from the virus zone and from healthy preparations were dialyzed against 0.2 M PO_4 buffer, pH 7.1, overnight and pelleted at 137,600 *g* using a Beckman No. 40 rotor for 60 min. Pellets from healthy and diseased samples were resuspended in 1 ml of buffer and scanned on a Beckman DBG recording spectrophotometer. The virus was further purified by an additional cycle on SDG, and an $A_{260/280}$ ratio was obtained.

Electron microscopy.—Blueberry shoestring virus particles purified from frozen infected blossoms by means of PEG precipitation, followed by SDG centrifugation were examined for size determination on a Philips 300 transmission electron microscope, with purified TMV (U-1 strain) as an internal size standard. The two viruses were mixed and a drop of the mixture was placed on a Formvar-coated grid. The grid was blotted dry from its edge, dipped in glass-distilled water and blotted dry again. The viruses were stained using one drop of a saturated water solution of uranyl acetate which was applied to the grid for 2-5 min and then blotted dry. Fifty-six particles each of TMV and BBSSV were measured from photographic enlargements (× 100,000) and a relative size determination was made.

Serology.—A female New Zealand white rabbit was injected intramuscularly three times at 2-wk intervals, with one volume (0.75-1.0 ml) of purified virus (0.44, 0.51, and 0.16 A_{260} units/ml, respectively) emulsified with an equal volume of Freund's complete adjuvant. Serum was obtained at 1 wk, 12 days, and 25 days after the final injection. The antiserum was titered using the microprecipitation method and the gel diffusion method (1). All other serological tests were in agar double-gel diffusion plates (1) containing 15 ml of Ionagar No. 25 (Colab Laboratories Inc., Glenwood, IL 60425) in a 0.85% NaCl solution containing 0.05% sodium azide. Arabis mosaic, cherry rasp leaf, and raspberry ringspot virus antisera were obtained from R. Stace-Smith, Canada Department of Agriculture, Vancouver, B.C. Canada; grape fanleaf and grapevine false fanleaf virus antisera were obtained from J. K. Uyemoto, New York State Agricultural Experiment Station, Geneva, NY 14456; southern bean mosaic and turnip yellow mosaic antisera were obtained from H. H. Murakishi, Michigan State University, East Lansing, MI 48824; and Johannes-Seyve grape virus antiserum was obtained from H. F. Dias, Canada Agriculture, Vineland Station, Ontario, Canada. Peach rosette mosaic virus-grapevine strain antiserum was supplied by the authors. Cucumber mosaic-c strain, prune dwarf-b strain, prunus necrotic ringspot-g strain, rose mosaic, tobacco necrosis-tulip strain, tobacco ringspot-grapevine strain, and tomato ringspot-elderberry strain virus antisera were obtained from the American Type Culture Collection (ATCC), Rockville, MD 20852 (ATCC numbers are: PVAS 88, PVAS 19, PVAS 3, PVAS 72, PVAS 26, and PVAS 25, respectively). These heterologous antisera were tested against purified BBSSV at antisera dilutions of 1:1, 1:4, and 1:16. Because of size similarities with BBSSV, antisera to the following viruses were also tested: maize streak and cassava latent (3), from K. R. Bock, East African Agricultural and Forest Research Organization, P.O. Box 30148, Nairobi, Kenya; sugarbeet curly top (4, 10, 11) from J. Duffus, U.S. Department of Agriculture, Salinas, CA 93901; and potato leaf roll (7) from G. deZoeten, University of Wisconsin, Madison, WI 53706. These were tested against purified BBSSV at 1:1, 1:4, and 1:16 dilutions.

Twenty other isometric viruses were tested against BBSSV antiserum: eighteen, including andean potato latent, belladonna mottle, bromegrass mosaic, carnation mottle, carnation ringspot, cocksfoot mild mosaic, cocksfoot mottle, dulcamara mottle, echtes ackerböhnenmosaic, Hungarian chrome mosaic, molinia streak, ononis yellow mosaic, pelargonium leaf curl, petunia asteroid mosaic, scrophularia mottle, sowbane mosaic, tomato bushy stunt type strain, and turnip yellow mosaic viruses were tested by H. Paul, Braunschweig, W. Germany. Blueberry shoestring virus antiserum was tested by R. W. Fulton, University of Wisconsin, WI 53706, against plum line pattern and tulare apple mosaic viruses.

Infectivity-assay.—The virus inoculum used came from the following sources: blossoms (virus purified via both methods previously described, before and after SDG), roots and leaves [purified via the method of Gilmer et al. (5)], and roots and leaves ground with mortar and pestle in 0.03 M PO_4 buffer, pH 8.0, containing 0.02 M 2-

mercaptoethanol and 3% w/v polyvinylpyrollidone (PVP).

Vaccinium corymbosum L. 'Jersey' plants were grown from seed from apparently healthy bushes and inoculated at the cotyledon or the one- to two-leaf stage. Roots, cotyledons, and leaves were dusted with 45-μm (320-mesh) Carborundum, a buffer-suspended virus preparation was rubbed on using small cubes of plastic sponge, and then the plants were rinsed with tap water. Control plants were dusted with Carborundum and inoculated with buffer. All inoculated and control plants were kept in a greenhouse environment supplemented with fluorescent lights to maintain a 14-hr day at 20 to 25 C.

Analysis of field data.—The mathematical model designed by van der Plank (13) for the estimation of random disease spread (outside vector or disease source) vs. non-random bush-to-bush spread (in-field vector) was used to analyze data from field maps made of a shoestring infected field during 1958 to 1959, near Holland, Michigan (courtesy of J. W. Nelson, Research Director, Michigan Blueberry Growers Association, Grand Junction, MI 49056) and from disease data obtained by the authors in 1976 from another field in the same vicinity. The compound interest rate "r" (14) for BBSSV spread was calculated using the 1958-59 data.

Test for seedborne virus.—Four hundred seeds each from healthy and diseased cultivar Jersey source bushes were planted in a peat-loam-perlite (2:1:1, v/v) mix in the greenhouse. Seedlings were counted upon germination and thereafter observed for symptom development for 1 yr. Seedlings were held under the same greenhouse conditions as previously described.

RESULTS

Purification techniques.—The PEG precipitation method usually produced the highest yield of virus. In many cases, the Tremaine et al.(12) method of differential centrifugation alone yielded no virus as determined by UV spectrophotometry.

Density gradient centrifugation and ultraviolet spectroscopy.—A visible, single band of virus sedimented in SDG 20 mm below the meniscus in preparations from infected tissue (Fig. 1) and it corresponded to a single UV-absorbing peak (Fig. 2). Preparations from healthy tissue yielded no visible band and no UV-absorbing zone. A UV-scan comparison of the corresponding SDG fractions from the diseased and healthy sample preparations is shown (Fig. 3-A, B). The virus, when re-run on SDG for added purification, gave an $A_{260/280}$ ratio of 1.47.

Comparison of the sedimentation rate of BBSSV to that of TMV (178.0S) and SBMV (110.3S) in SDG gave an estimated value of 78.5S ($\pm$ 3.1) for the sedimentation coefficient of BBSSV.

Electron microscopy.—Fifty-six particles each of both TMV (U-1 strain) and BBSSV were measured from photographic enlargement. Using 15 nm as the standard width value for TMV a comparison with BBSSV yielded a calculated average diameter of 24 nm $\pm$ 1.2 (Fig. 4). This varies somewhat from previous measurements made without an internal standard (6, 9).

Serology.—Purified BBSSV reacted at a homologous titer of 1:256 in agar gel double diffusion tests and 1:1,024 in microprecipitin tests. The antiserum did not react with purified healthy preparations, nor with 0.85% saline. Purified BBSSV did not react with normal serum.

None of the 23 heterologous antisera reacted with purified BBSSV in gel double diffusion tests at the antisera dilutions tested, although BBSSV did react with its own antiserum. No reaction occurred between the BBSSV antiserum and any of the 20 heterologous viruses tested.

Infectivity assay.—Blueberry seedlings from apparently healthy source plants were inoculated with BBSSV and expressed severe, typical disease symptoms 5 to 6 mo after inoculation with the following virus sources: (i) virus purified from infected blossoms via differential

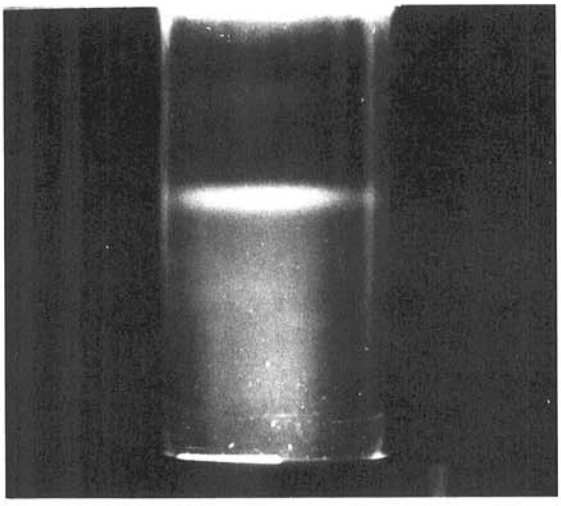

Fig. 1. Blueberry shoestring virus in a single band 20 mm below the meniscus of a 10-40% linear sucrose gradient following centrifugation at 23,000 rpm for 2.5 hr in a Beckman SW 25.1 rotor.

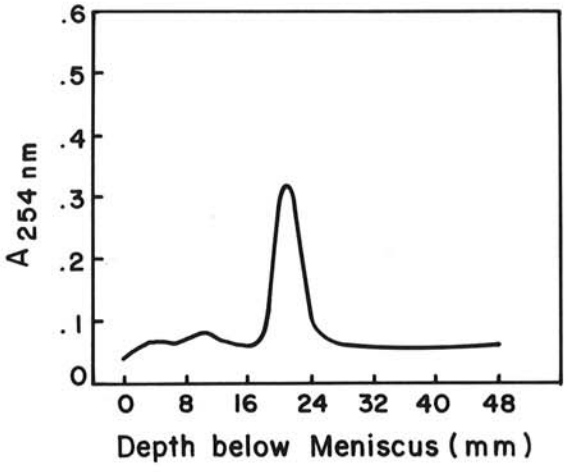

Fig. 2. Ultraviolet absorption profile of blueberry shoestring virus sedimented in a linear 10-40% sucrose density gradient. Direction of sedimentation is from left to right.

centrifugation alone (Fig. 5-A, B); four of five inoculated seedlings developed typical shoestring symptoms; (ii) virus purified from infected blossoms using PEG precipitation, followed by two cycles of SDG; a;; four inoculated seedlings were badly stunted. Three of these died and the remaining plant developed typical shoestring symptoms; (iii) roots from infected plants were ground in PO_4 buffer with 2-mercaptoethanol and PVP; one of three inoculated plants developed typical shoestring disease symptoms. Inoculum from roots and leaves subjected to the purification schemes and leaves ground in the PO_4 buffer containing 2-mercaptoethanol and PVP was not infectious to a total of five plants tested with each treatment. In all cases buffer inoculated controls remained symptomless (Fig. 5-C).

None of the following 47 herbaceous indicators test-inoculated with the virus from purified blossom preparations developed symptoms: *Antirrhinum majus* 'Red crimson giant', *Beta vulgaris*, *Capsicum annuum*, *Chenopodium amaranticolor*, *C. quinoa*, *Citrullus vulgaris*, *Coleus blumei*, *Crotolaria juncea*, *Cucumis maxima*, *C. melo*, *C. pepo*, *C. sativus*, *Cyamopsis occidentalis*, *Datura stramonium*, *Dolichos biflorus*, *Gomphrena globosa*, *Helianthus annuus*, *Hordeum vulgare*, *Lycopersicon esculentum* 'Marglobe', *Momordica balsamina*, *Nicotiana clevelandii*, *N.*

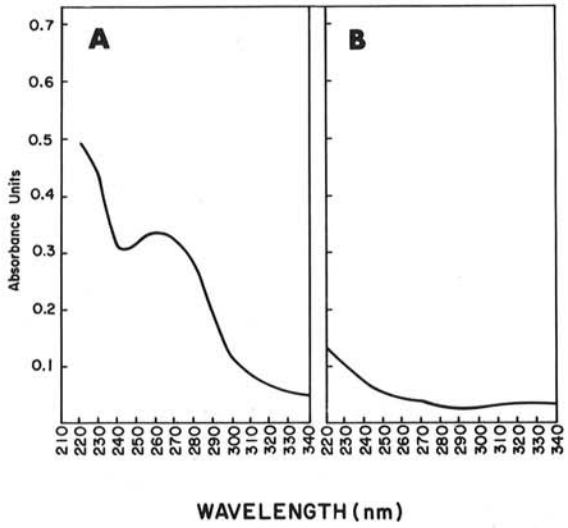

Fig. 3-**A**,**B**. Ultraviolet absorption spectrum of **A**) blueberry shoestring virus purified from diseased blueberry (cultivar Jersey) blossoms **B**) purified sap from healthy blossoms. The spectrum of both preparations was measured following separation from a sucrose density gradient and dialysis.

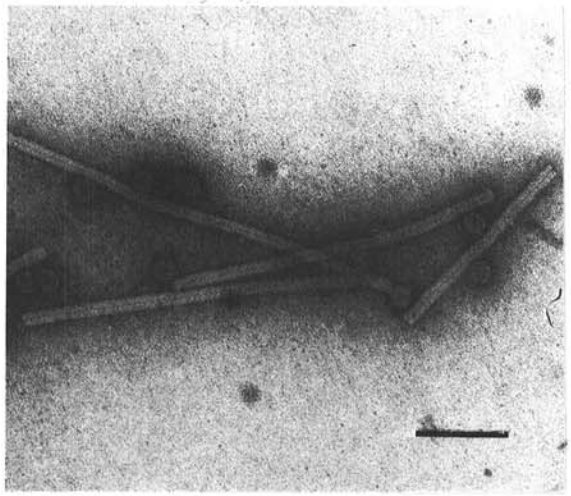

Fig. 4. Blueberry shoestring virions stained with uranyl acetate following purification from diseased (cultivar Jersey) blueberry blossoms. Tobacco mosaic virus (U-1 strain) particles were used as a size comparison standard. Bar = 100 nm.

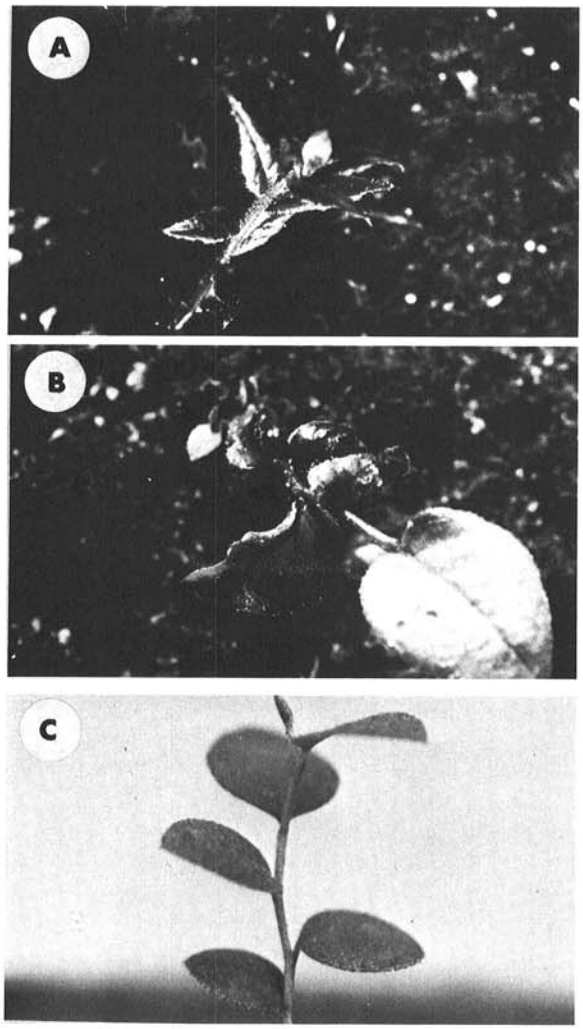

Fig. 5-(**A to C**) **A**,**B**) Blueberry (cultivar Jersey) seedlings showing shoestring symptoms 5 mo after inoculation with purified blueberry shoestring virus. **C**) Buffer-inoculated control seedling.

glutinosa, *N. megalosiphon*, *N. rustica*, *N. tabacum* 'Havana 423', *N. tabacum* 'KY16 Burley', *N. tabacum* '425 Tobacco', *N. tabacum* 'Xanthi NC', *Petunia hybrida*, *Phaseolus limensis*, *P. vulgaris* *'Prince Bean'*, *P. vulgaris* var. *humilis*, *Pisum sativum*, *Raphanus sativus*, *Sesbania exaltata*, *Solanum melongena*, *Spinacea oleracea*, *Taraxacum officianale*, *Tetragonia expansa*, *Tithonia* speciosa, *Triticum aestivum* 'Ionia', *Vicia faba*, *Vigna* cylindrica, *Vigna sinensis*, *Vinca rosea*, *Zea mays*, and *Zinnia elegans*.

Field analyses of disease spread.—The following virus spread information was determined from the Holland, Michigan field map data obtained during 1958-1959. Using the van der Plank (13) formula:

$$d = \frac{\mu(\mu\text{-}1)}{h}$$

in which

d = total number of doublets expected
h = total number of plants = 3,525
μ = total number of diseased plants = 383
$J\bar{d}$ = range of error 6.4
T = h-1 = total number of doublets = 3,524
d is calculated as = 41.3 ± 3.2
The observed number of diseased doublets = 192

Chi-square (χ^2) analysis was performed (using H_o: observed results = expected results) (i.e., for amount of random disease) by comparing healthy doublets (observed vs. expected). The χ^2 value was 556.42 (df = 1); therefore H_o is rejected, since the probability of $\chi^2 = 556.42 >> P = 0.001$.

Similar analysis of data from a field map made by the authors in March 1976, where h = 340, μ = 75, d = 16.3 and the observed number of doublets = 31, resulted in a χ^2 value of 13.96, causing H_o to be rejected, since 13.96 > 7.88 ($P = 0.005$). Virus spread in the field definitely occurs from bush-to-bush and is not the result of continuous, random introduction of the agent from outside sources.

The compound interest rate (r) for observable field spread of the virus over a 1-yr period (March 1958 to March 1959) for a population of 3,525 bushes was calculated from the formula:

$$r = \frac{2.3}{t_2 - t_1} \log_{10} \frac{X_2}{1\text{-}X_2} - \log_{10} \frac{X_1}{1\text{-}X_1}$$

where

$t_2 - t_1$ = time period between the second observation of disease incidence (at t_2) and the first observation (at t_1).
X_1 = proportion of diseased plants at t_1.
X_2 = proportion of diseased plants at t_2.
The resulting r-value was 0.269/unit/year (14).

Determination if BBSSV is seedborne in blueberry.—Of 400 seeds from BBSSV-infected bushes, 65 germinated after a 2-mo period. Of 400 seeds from healthy bushes, 67 germinated in the same period. In 10 mo of subsequent observations, none of the seedlings from infected bushes have shown virus symptoms.

DISCUSSION

The isometric particles purified from blossoms of naturally infected blueberry plants have been demonstrated to be the causal agent of the shoestring disease. Isolation of the particles from diseased, but not from healthy plants, and the occurrence of disease symptoms on seedlings mechanically inoculated with the purified virus, offer proof of pathogenicity.

Hartmann et al. (6) apparently failed to obtain infectious preparations of virus. It is to be noted, however, that he used only partially purified preparations, his material was from leaves and not blossoms, he inoculated Jersey blueberry seedlings at an age at least several weeks beyond the cotyledon stage, and observed the inoculated plants for no longer than 1-2 mo. Our work indicates the apparent necessity of inoculating very young seedlings and of waiting the requisite 4-6 mo for symptom development to occur.

The apparent differences in size between the earlier particle measurements (28-31 nm) as determined by Hartmann et al. (6) from partially purified preparations, the 21 nm determined by the authors in a previous report (9), and those determined in the present study can be accounted for by the fact that an internal standard had not been hitherto used. Calibration grid comparisons used for previous measurements must have introduced a large error factor.

The presence of a single major UV-absorbing zone in SDG indicates that BBSSV is a single-component virus. The $A_{260/280}$ ratio of 1.47 obtained after two cycles of SDG is somewhat lower than that of most isometric plant viruses.

The lack of serological reaction between BBSSV and antisera to 23 other isometric plant viruses and between BBSSV antiserum and 20 other isometric plant viruses has been demonstrated. This, coupled with its apparently limited host range, indicates that BBSSV is unrelated to those previously described viruses.

The analysis of field map data indicates the existence of bush-to-bush spread in the field which is prominently observed in the spread of the disease "along the rows" in blueberry plantings. Isolated diseased bushes, although appearing fairly infrequently, do occur. Nematode surveys of several fields in which the disease is spreading have failed to reveal the presence of known potential vector species. Experiments are in progress to determine the mode of spread of the virus.

LITERATURE CITED

1. BALL, E. M. 1974. Serological tests for the identification of plant viruses. Am. Phytopathol. Soc., Plant Virology Committee. 31 p.
2. BRAKKE, M. K. 1960. Density gradient centrifugation and its application to plant viruses. Adv. Virus Res. 7:193-224.
3. BOCK, K. R. 1975. Maize streak, casava latent and similar viruses. Page 93 *in* Abstr. 3rd Intl. Congress for Virology. Madrid, Spain.
4. DUFFUS, J. E., and A. H. GOLD. 1973. Infectivity neutralization used in serological tests with purified beet curly top virus. Phytopathology 63:1107-1110.
5. GILMER, R. M., J. K. UYEMOTO, and L. J. KELTS. 1970. A new grapevine disease induced by tobacco ringspot virus. Phytopathology 60:619-627.
6. HARTMANN, J. X., J. E. BATH, and G. R. HOOPER, 1973. Electron microscopy of virus-like particles from shoestring-diseased high bush blueberry.

Phytopathology 63:432-436.
7. HEPP, R. F., and G. A. DE ZOETEN, 1976. Studies on potato leaf roll virus. Proc. Am. Phytopathol. Soc. 3:252.
8. LESNEY, M. S., and D. C. RAMSDELL. 1976. Purification and some properties of blueberry shoestring virus. Proc. Am. Phytopathol. Soc. 3:252 (Abstr.).
9. LESNEY, M. S., and D. C. RAMSDELL. 1976. Purification and some properties of blueberry shoestring virus. Acta Horticulturae 66:105-109.
10. MINK, G. I., and P. E. THOMAS. 1974. Purification of curly top virus. Phytopathology 64:140-142.
11. MUMFORD, D. L. 1974. Purification of curly top virus. Phytopathology 64:136-139.
12. TREMAINE, J. H., W. R. ALLEN, and R. S. WILLISON. 1964. The extraction of viruses from fruit tree petals. Plant Dis. Rep. 48:82-85.
13. VAN DER PLANK, J. E. 1944. A method for estimating the number of adjacent diseased plants in a homogeneous field. Trans. Roy. Soc. S. Afr. 31:269-278.
14. VAN DER PLANK, J. E. 1963. Plant diseases: epidemics and control. Academic Press, New York. 349 p.
15. VARNEY, E. H. 1957. Mosaic and shoestring, virus diseases of cultivated blueberry. Phytopathology 47:307-309.