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DEVELOPMENT OF A SCREEN FOR RESISTANCE TO BLUEBERRY SHOESTRING VIRUS (BBSSV) AND ITS USE IN ASSAYING A BROAD RANGE OF GERMPLASM OF HIGHBUSH BLUEBERRY, VACCINIUM CORYMBOSUM

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DEVELOPMENT OF A SCREEN FOR RESISTANCE TO BLUEBERRY SHOESTRING VIRUS (BBSSV) AND ITS USE IN ASSAYING A BROAD RANGE OF GERMPLASM OF HIGHBUSH BLUEBERRY, *VACCINIUM CORYMBOSUM*

by

Nancy L. Schulte

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ABSTRACT

DEVELOPMENT OF A SCREEN FOR RESISTANCE TO BLUEBERRY SHOESTRING VIRUS (BBSSV) AND ITS USE IN ASSAYING A BROAD RANGE OF GERMPLASM OF HIGHBUSH BLUEBERRY, VACCINIUM CORYMBOSUM L.

By

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A screen for resistance to blueberry shoestring virus (BBSSV) was developed using rub inoculation and enzyme-linked, immunosorbent assay (ELISA) technique. It appears that high rates of infection can be obtained with a virus concentration of 0.25 mg/ml when it is applied to randomly selected leaves of container-grown blueberries of any age, during any portion of the growing season as long as healthy leaves are present. However, to accurately measure levels of infection with ELISA, plants must be sampled several times post-inoculation, preferably after a dormancy period has been satisfied.

Twenty-eight cultivars were tested for potential resistance to BBSSV. 'Concord', 'Elliot', 'June', and 'Pemberton' appeared to be resistant after one sampling, while the rest of the cultivars had infection percentages varying from 9 to 66%. It is hoped that the apparently resistant cultivars can be used to breed a new generation of resistant varieties.

This thesis is dedicated to my parents, Hal F. and Marian J. Schulte, for their support and encouragement along the way.

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Introduction

Blueberry shoestring has only recently been identified as a serious disease of the highbush blueberry, Vaccinium corymbosum L. The characteristic 'shoestring' symptoms were originally observed in New Jersey in the late 1940's on several cultivars and wild plants (Hutchinson, 1950). The symptoms were shown to be of virus origin in 1957 (Varney, 1957). The disease has been detected in Nova Scotia (Lockhart and Hall, 1962), Washington State, North Carolina, and Michigan (Ramsdell, Whallon, and Hancock, 1980).

Michigan's first cultivated highbush blueberry production began in the early 1930's and within ten to fifteen years, 'shoestring' symptoms were observed in these initial plantings. Since no symptoms have ever been reported in the natural populations indigenous to Michigan, the initial introduction of 'shoestring' virus into Michigan probably came from the east coast. 'Shoestring' disease has a four year latency period (Ramsdell, personal communication), and propagating wood could have unknowingly been taken from infected, but currently symptomless plants.

Blueberry Shoestring Virus (BBSSV) has caused serious economic losses in Michigan's blueberry industry. Approximately 16% of Michigan's total blueberry acreage was inspected for BBSSV in 1976 and 5724 diseased plants were found. This means that a total of 145,200 plants may have had BBSSV in Michigan in 1976, if the disease was evenly spread across the state and four times more plants were latent for BBSSV than those with visible symptoms. Diseased plants are generally

removed within 2-3 years of the onset of symptoms or as the yield and vigor of the bush declines (Nelson, personal communication). The time from diseased bush removal to full production of a new plant is typically ten years (allowing for site preparation and bush maturation). Therefore, with an average yield of 4000 lb/acre and a selling price of 40¢/lb, state wide losses in 1976 would have been $3,320,000 if all of the diseased plants had been removed.

Plant removal is particularly costly, since undiseased fields can produce high yields for long periods of time. New Jersey has several 80 year old fields still in production (Ramsdell, Whallon, and Hancock, 1980). Twenty-three percent of the producing acreage in Michigan is over 25 years in age. Due to economics and the longevity of the crop, whole plantings of an unfavorable or disease susceptible cultivar cannot always be arbitrarily removed and replanted with undiseased plants. In the case of BBSV, and most "woody" viruses, spread within a field takes years before the cost to maintain the planting is greater than its yield can support.

The spread of blueberry shoestring virus has not slowed since 1976 and in the last twenty-five years, it has been increasing as a logrithmic rate. It is estimated that 40,000 individuals will be infected by 1985 (Ramsdell, Whallon, and Hancock, 1980), and as the number of plants with 'shoestring' increases, the rate of spread will also increase due to a greater number of inoculum sources. Clearly, ways to prevent the spread of BBSSV must be found to avert serious economic disaster.

Characteristics of the Disease

Blueberry shoestring is primarily a disease of Vaccinium corymbosum L., although it has been reported in V. augustifolium (Lockhart and Hall, 1962). Symptoms are found on the stems, leaves, flowers and fruit. Symptom expression occurs randomly throughout the bush at any height where new growth has occurred.

Elongated reddish streaks and blotches of varying length develop early in the growing season on current and one year old stems. These are generally more numerous on the side of the stem exposed to the sun. The lesions on one year old stems, initiated the previous spring, fade throughout the season as the characteristic bark of older stems begins to form. Those of the current year's growth remain, being most apparent the following spring when the wood is greening up, but before bud break occurs.

The cane growth habit is also affected. Elongated, spindly canes develop with twists about their axes and multiple kinks and bends. These abnormal canes fail to produce blossom buds leading to a reduction in yield (MSU Ext. Bull. No. 370).

Leaf symptoms develop as the leaves emerge. Severely affected leaves are narrow and strap-like, being reduced in total leaf area by as much as ninety percent of their potential. Leaves on the same stem can also be crescent shaped and curled, where one side of the leaf is severely distorted, while the other half separated by the mid-rib is apparently normal. There may also be variations in leaf tissue thickness, with thin almost translucent areas of light green and

thickened areas of dark green. Another characteristic symptom is varying degrees of red coloration on the leaves. Strap-like leaves may be completely red, while other less distorted leaves can show red vein-banding, reddish streaking along the mid-rib and/or an oak leaf pattern emanating from the base of the petiole. Symptomed leaves can be found along the length of the stem, but symptoms are generally most severe at the base of a new cane, decreasing in severity towards the growing point. Older shoots often support new shoots of both diseased and symptomless tissue alike.

Pink longitudinal stripes occur occasionally on the blossoms, but this coloration can also be caused by environmental factors and is a poor disease indicator. Immature berries on diseased canes may develop a reddish-purple cast on the side exposed to the sun, while the rest of the berry crop is a dull green.

Under field conditions, visible symptoms take approximately four years to be expressed after the initial infection takes place. Due to the nature of the aphid vector Illinoia pepperi MacGillivray and the low virus concentration that it transmits (Morimoto, et al. 1982), multiple infections are probably necessary to get high enough levels of virus in the plant to induce symptom expression. Over time, the total percentage of diseased wood increases in an apparently random manner, resulting in progressively decreased yields and less total vigor.

BBSSV is an isometric, single component, single stranded RNA containing virus approximately 27 nm in diameter (Ramsdell, 1979). With the use of ultrathin sectioning and electron microscopy,

characteristic particles have been found in leaf epidermal, palisade and spongy mesophyll cells. Stacked crystalline arrays were present in root xylem cells and epidermal cells of diseased plants. Individual particles were also found in mature xylem, but not phloem tissue (Hartmann, Bath and Hooper, 1973).

BBSSV is unrelated serologically to any of the characterized spherical plant viruses, although it has similar physical and chemical characteristics to those viruses belonging to the proposed Southern Bean Mosaic Virus (SBMV) group described by Hull, 1977. Similar to other viruses in that group, BBSSV forms one band in CsCl and two or more bands in Cs_2SO_4 gradients. It is stabilized by divalent cations (Ramsdell, 1979) and forms crystalline arrays in the host's cytoplasm (Hartmann et al., 1973). Most viruses in the SBMV group are transmitted by beetles, but BBSSV is vectored by aphids (Ramsdell, 1979).

A reliable indicator plant has not been found for BBSSV. Upon rub inoculation with purified BBSSV and carborundum, 47 different herbaceous indicator plants have failed to show symptoms (Lesney et al., 1977), although Lockhart and Hall (1962) reported chlorotic spots within three days after inoculation of Phaseolus vulgaris (slender green) with crude sap from diseased lowbush tissue. These symptoms disappeared slowly after two weeks and this work has not been repeated.

The blueberry aphid, Illinoia pepperi MacGillivray has been shown to transmit BBSSV in a controlled greenhouse environment. I. pepperi fed on sachets containing sucrose and purified virus transmitted BBSSV to one year old rooted cuttings of cv. 'Jersey' after a prolonged

acquisition and inoculation feeding period (Morimoto, personal communication). Aphids fed radioactive I^{125} labeled virus have also been observed by scanning electron microscopy to have virus-like particles in all areas of their digestive systems. These results suggest that the virus maybe semi-persistent or persistent in the aphid vector (M. Petersen, personal communication).

The life cycle of I. pepperi begins late in the growing season. After fruit harvest, small succulent shoots arise at the base of the bush and oviparous females lay their eggs on these shoots (Elsner, 1981). Throughout the winter the eggs then fall into the leaf liter below the bush or into the branch crevices in the crown. In the spring, apterae (wingless) individuals emerge which multiply throughout the growing season, primarily colonizing the new shoots found throughout the bush (the severity and style of pruning determines the number and location of these shoots within the bush). As the shoots become crowded with aphids, either alatae (winged) individuals are formed which can fly to new bushes, or wingless aphids walk to new locations. This cycle repeats itself throughout the growing season until late summer when the egg laying stage again commences.

Illinoia pepperi MacGillivray has been observed to colonize red oak, black gum, red maple, winter berry holly, and Prunus sp., but only when food resources in the blueberry fields appeared to be limiting. Colonies survived a maximum of two weeks on these alternate hosts (Elsner, 1981).

Virus spread within a blueberry field occurs down the row from bush to bush, and not in a random manner (Lesney, 1976). Two reasons

have been suggested for this type of spread: 1) overcrowding causes apterate individuals to walk from one bush to another on overlapping branches, and 2) mechanical harvesters transport aphids to new bushes (Ramsdell, Hancock, and Whallon, 1982). A study using Rubidium-labeled aphids found live labeled aphids on the mechanical harvester at least 15 bushes from where they originated. Harvesters may be literally green with living aphids when environmental conditions have been favorable for colonization.

Control strategies

Plant viruses can be controlled by several genetic and non-genetic means, with the tightest control coming from a combination of overlapping strategies. Several different approaches have been undertaken to control shoestring disease, but most are in their infancy or have inherent problems.

The removal of symptom bearing plants has been an effective form of curbing disease spread, but many problems plague this means of control. The four year latency period for symptom expression keeps the virus one step ahead of the grower, since symptomless, infected plants are a constant source of inoculum. Also, a few diseased bushes may be missed in a grower's field or the producer may not be willing to commit the labor, time and money involved in the removal of a few bushes. BBSSV does not cause immediate vigor and yield decline, and it may be several years before a grower feels that yield is sufficiently reduced to justify the effort and economics of removal.

Planting virus free blueberry stock is an important control measure, but the state of Michigan does not have a vigorous testing procedure. The initiation of a certification program would slow virus spread, but such a system would take years to cause a major effect for two reasons: 1) fields are very slowly replanted, and 2) a good percentage of growers propagate their own plant material directly from production fields. If symptomless plants are used for this purpose, it is likely that the subsequent cuttings will possess BBSSV.

Chemical control of the vector, I. pepperi has been used to slow virus spread (Nelson, personal communication). An effective insecticide program can moderate aphid numbers because I. pepperi probably has a circulative and persistent relationship with BBSSV and the aphid requires a prolonged acquisition and inoculation time to transmit the virus. Unfortunately, several problems exist with this method of control. First, not all aphids are killed upon the application, with some virus transmission inevitably occurring between sprays. Secondly, natural predators of I. pepperi are killed which limits their potential as a control measure and finally, any use of chemicals presents a risk and potential hazard to the balance of our ecosystem.

The most effective protection against BBSSV would be to plant horticulturally acceptable plant varieties which are resistent to both the aphid and virus. Unfortunately, only a few varieties have been screened and most of those tested do not have resistance or are weak.

A field survey was conducted for aphid resistance in southwest Michigan on 16 cultivars over two growing seasons. The ability of the

cultivars to support aphid colonies separated into three groups (based on statistical significance) but the most resistant individuals still supported substantial aphid populations (Hancock et al., 1981). In an environmentall controlled chamber, plants from each of the significance groupings were again screened for aphid rearing potential and large aphid populations developed on all of the cultivars, resulting in no significant differences (Hancock, unpublished).

A limited greenhouse screen for BBSSV resistance was also conducted on six cultivars including 'Jersey', 'Rubel', 'Atlantic', 'Bluecrop', 'Blueray', and 'Elliot'. Roots and leaves of seedlings were rub inoculated with purified BBSSV and only Blueray showed complete resistance after six months. Bluecrop exhibits resistance to BBSSV in the field, but 10% of its seedlings became infected in this greenhouse screen (Ramsdell, 1979).

Study goals

BBSSV has been transmitted by three methods: 1) grafting, (Varney, 1957; Lockhard and Hull, 1962), 2) through the vector I. pepperi (Ramsdell, 1979, 1980; Morimoto, personal communication), and 3) mechanically by rub inoculation of purified virus (Ramsdell, 1978). There are disadvantages to using all three of these methods in resistance screens, but rub inoculation is generally more efficient and economically feasible than the others. Greenhouse space limitations necessitates the use of one year old rooted cuttings for stock wood, but these plants generally have shoots too small in diameter to graft efficiently. Also, several grafts per stock plant are needed to ensure

adequate virus adequate virus transmission and the process is extremely time consuming. Transmission through I. pepperi is an inadequate screening technique since the aphid is an inefficient vector of BBSSV (Morimoto, personal communication). A large group of aphids must be placed on one plant and they must feed there for an extended period of time to ensure adequate transmission. Rub inoculation is more rapid and it allows for equal virus distribution throughout the plant; however, it transmits a much higher concentration of virus than occurs through natural aphid transmission and for this reason, may miss lower levels of resistance. However, high inoculum levels do diminish the possibility that susceptible plants will escape detection.

The four year latency period exhibited by BBSSV and the absence of herbaceous indicator plants dictates that a biochemical assay must be used to detect BBSSV in symptomless plants. Enzyme linked immunosorbant assay (ELISA) is well adapted to this purpose as it is generally sensitive to small quantities of virus in plant material (Clark and Adams, 1977; Ramsdell, 1980), and it is less costly and time consuming than the other available techniques radioimmunoassay (RIA), and immunosorbant electron microscopy (ISEM).

The initial goal of this study was to perfect a technique for screening BBSSV resistance using rub inoculation and the ELISA technique. Several plant and environmental parameters were examined including: 1) the amount of purified virus used for inoculation (0.25, 0.5 and 1.0 mg/nl), 2) the plant tissue inoculated (light-green, unexpanded leaves vs. dark-green fully expanded ones), 3) the age of plants when

inoculated (1 or 5 months out of cold storage), 4) the season when plants were inoculated (spring vs. fall), 5) the length of time before plants were ELISA tested (2 week intervals up to 1 year), 6) the number of times an individual was tested (1-12), and 7) the plant tissue which was ELISA tested (light-green, unexpanded leaves vs. dark-green, fully expanded leaves or roots). After development of the screen, the secondary goal was to screen a broad range of highbush blueberry germplasm for resistance to BBSSV.

Materials and Methods

A. General Plant Culture:

Dormant 1 year old rooted cuttings of the cultivar 'Jersey' were used in all experiments. The plants were grown in a greenhouse at Michigan State University in 19 cm diameter plastic pots in a soil mix of 1:1 canadian sphagnum peat moss and agricultural grade coarse perlite. Average greenhouse temperatures during the course of the experiments were: summer (June, July, August) - 18-43°C day/18-27°C night; fall (September, October) - 18-30°C day/18-21°C night; winter (November, December, January, February) - 18-24°C day/18°C night; spring (March, April, May) - 18-32°C day/18-21°C night.

Plants were fertilized every two or three waterings during periods of active growth. The frequency of application was decreased to every 2 to 3 weeks when active growth ceased. The fertilizer mix was comprised of ammonium sulfate, potassium nitrate, magnesium sulfate and iron chelate (sequestrene 330 Fe, Ciba-Geigy Co., Greensboro, NC). The N-P-K-Mg-Fe concentrations were 200 ppm - 100 ppm - 100 ppm - 50 ppm - 100 ppm (W/W/W/W/W). Phosphoric acid was used to adjust the pH of the delivered water to 5.0. When any deficiency symptoms appeared, soluble trace elements were added for one to two waterings using "Peter's Soluble Trace Elements Mix (STEM)" at the half strength recommendation.

B. Virus Purification:

BBSSV was purified from frozen blueberry blossoms of naturally infected 'Jersey' using the procedure outlined by Ramsdell (1979): 1) blossoms were transported from the field on ice and were stored at -20°C until processed (1 month to 2 years later). 2) Frozen blossoms were homogenized for 3 to 5 minutes in a chilled Waring Blender in a 1:3 weight to volume (ml/gm) dilution of 0.1 M phosphate buffer, pH 7.0, containing 0.01 M 2-mercaptoethanol and 0.005 M thioglycolic acid. 3) Triton x 100 was then added to 8% concentration (V/W) and the homogenate was stirred on ice for 2 hr. 4) The resulting solution was strained through cheesecloth and both chloroform and n-butanol were each added to a 10% concentration (V/V) ratio and stirred for an additional 15 minutes. 5) The mixture was centrifuged for 10 minutes at 10,000 rpm using a Sorval RC-2-B centrifuge and a GSA 4354 rotor. 6) The upper aqueous phase was made 8% (W/V) with polyethylene glycol (PEG)(mol wt. 6000) and 0.1 M with NaCl. The mixture was stirred overnight at 5°C before being centrifuged for 10 minutes at 10,000 rpm. 7) The pellet was then resuspended in 0.05 M phosphate buffer containing 0.001 m dithiothreitol to at least 1/10 of the starting homogenate volume, and the solution was held overnight at 5°C. 8) After storage, the preparation was centrifuged for 20 minutes at 10,000 rpm, and then the supernatant was further centrifuged for 2 hours at 28,000 rpm using a Beckman LS-65 ultracentrifuge and a number 30 rotor. 9) The pellet was resuspended in 0.05 M phosphate buffer pH 7.0

containing 0.001 M dithiothreitol. The suspension was layered onto 5-30% linear-log sucrose gradients (0.3 ml each tube) which were centrifuged for 90 minutes at 38,000 rpm at 4°C using an SW 41 rotor. 10) The resulting band was collected, diluted 1:3 with 0.05 M phosphate buffer, pH 7.0 and centrifuged for 6 to 7 hours at 28,000 rpm to pellet the virus out of sucrose. 11) The final pellet was then diluted to 1 mg/ml virus concentration with 0.05 M phosphate buffer containing 0.001 M dithiothreitol, pH 7.0 and stored at 4°C for 1 to 2 months.

C. Experiments on Inoculation Techniques:

A randomized complete block was used to compare the effects of season, virus concentration and plant age on disease susceptibility. To evaluate inoculation season, plants were potted on either April 20 or September 10 of 1981 and they were each inoculated 30 days later with BBSSV at a concentration of 1 mg/ml. Plants were also planted on April 20, 1981, for the virus concentration experiment and they were inoculated on June 5, 1981 with several virus concentrations including 1.0, 0.5 or 0.25 mg/ml. To measure the influence of plant age or virus susceptibility, plants were potted both on September 20 and April 20, 1981. On October 20, 1981 they were inoculated with a virus concentration of 1.0 mg/ml. A total of 36 plants were used per treatment, with 12 plants in each block.

A completely randomized design was utilized to measure the effect of leaf maturity on BBSSV susceptiblity. Forty plants were potted on April 20, 1981, and they were inoculated on June 26, 1981

with 1.0 mg/ml of virus. Only light-green unexpanded leaves were inoculated on 20 of the individuals, while the other half of the plants received virus only on dark-green fully expanded leaves.

The ELISA technique was used to measure infection rates in the various treatments. The plants inoculated in different seasons (June vs. October) were evaluated twice; 1) September 15, 1982 and 2) November 3, 1982. The plants subjected to different virus concentrations were tested four times over a 15 month period: 1) August 4, 1981, 2) October 21, 1981, 3) June 15, 1982, and 4) September 15, 1982. The individuals inoculated at different ages were evaluated 11 months post inoculation on September 15, 1982. The plants used to test leaf maturity at time of inoculation were ELISA tested 3 times; 1) October 8, 1981, 2) January 12, 1982, 3) June 15, 1982.

D. Experiments on Evaluation Techniques:

To measure the effect of time and replication on disease detection with ELISA, 36 one month old 'Jersey' plants were inoculated with 1.0 mg/ml BBSSV on June 5, 1981 and these were tested for the presence of virus at two week intervals. The evaluations were begun on June 22, 1981 (2 weeks post inoculation) and continued every 2 weeks until November 11, 1981. Samples were also taken on December 15, 1981 and July 9, 1982.

Several experiments were also conducted to determine the best source material for ELISA testing: 1) 1 gm samples of roots and leaves were evaluated on December 15, 1981 using the 36 individuals

inoculated with 1 mg/ml BBSSV on June 5, 1982. 2) 1.0 gm samples of fully expanded leaves, unexpanded leaves and roots were compared on July 28, 1982 using 5 plants that had tested ELISA positive on at least 3 occasions, and 3) the five top and base leaves of 9 branches were evaluated from a single diseased plant on July 28, 1982.

All plants used in these and the previously described experiments were pretested using (ELISA) to confirm that they were free of BBSSV.

E. Inoculation Procedures:

In general, the plants were rub inoculated according to the procedures of Fulton (1966). Plants were completely shaded with a black-cloth for 36 hrs prior to inoculation. Ten, random leaf surfaces per plant were dusted with 320 mesh carborundum and a moderately coarse 2.5 cm^2 sterilized sponge was used to apply virus by making two separate strokes down the length of each leaf. Enough force was applied to slightly abraid the leaf surface, but not enough to gouge or tear the leaf in the process. Three to five minutes after inoculation, the plants were gently washed with tap water to remove excess carborundum and virus. Young, unexpanded leaves were preferentially chosen for transmission in all experiments except when age of leaf at time of inoculation was being tested.

F. ELISA Procedure:

Plant samples of 0.4 - 1.0 g were placed in 45 ml centrifuge tubes and diluted 1:10 (W/V) with a phosphate buffered saline solution, pH 7.4, containing 2% polyvinylpyrolidine, 0.2% ovalbumin

and 0.02% (W/V) sodium azide (extraction buffer). This was homogenized on ice for 30 to 120 seconds at approximately 60% of maximum speed (20,000 rpm) with a Tekmar Tissumizer (SDT-182EN Shaft, Cincinnati, OH 45237) and strained through cheesecloth. The filtrates were then stored at 5°C for no more than 48 hrs.

Microelisa ^{R} substrate plates (Immulon I, Flat bottom wells, Dynatech Lab Inc., Alexandria, VA 22305) with 96 wells per plate were used for the ELISA evaluations. The procedure consisted of 5 steps: 1) 200 µl of 1 µg/ml purified BBSSV α-globulin in sodium carbonate buffer, pH 9.6 was placed in each of the 96 wells and the plates were covered with plastic, sealed and incubated for 4 hrs at 37°C. 2) The plate(s) were then flipped over quickly to empty their contents and were rinsed with a wash bottle containing phosphate buffer saline solution, pH 7.4 with 0.5% (V/V) polyoxyethylene sorbitan monolaurate (tween 20). This washing procedure was repeated 3 times with at least 3 minute intervals between washes. 3) 200 µl samples of known healthy blueberry leaves in extraction buffer and samples to be tested for virus were then placed in wells of each plate. The control (healthy blueberry leaves, known diseased blueberry blossoms and straight buffer) were placed in a total of ten wells, 4 wells for the buffer and 3 each of the other two checks. The test samples were placed in the remaining 86 wells with replicates of each sample being run in the same microelisa ^{R} plate or a similar plate depending on the number of samples being analyzed. Each plate was kept on ice while samples were being

loaded and when a plate was full, it was covered with plastic, sealed and held at 5°C overnight. 4) After storage, the plant samples were removed from the wells using the previously described washing procedure, and 200 µl of a 1:800 dilution (in extraction buffer) of enzyme labeled BBSSV α-globulin (conjugate) was placed in each well. Plate(s) were again covered with plastic, sealed and incubated at 37°C for 3 to 6 hr. 5) The plate(s) were then washed and 200 µl aliquots of enzyme substrate (freshly prepared 1 mg/ml p-nitrophenol phosphate at 1 mg/ml in a substrate buffer of 10% diethanolamine) was added to each well. Plate(s) were left at room temperature for 30 minutes and then absorbance at 405 nm was read with a microelisa ^{R} spectrophotometer (Dynatech. Lab. Inc., Alexandria, VA 22305). Individuals were considered ELISA positive if their absorbance was equal to or greater than the mean A_{405} of the 3 healthy check wells plus 2 standard deviation units.

G. BBSSV Resistance Screen:

Container grown dormant one year old rooted cuttings of 28 cultivars were planted in a completely randomized design on May 5, 1981. The cultivars were: 'Atlantic', 'Berkeley', 'Bluecrop', 'Bluehaven', 'Bluejay', 'Bluetta', 'Burlington', 'Collins', 'Concord', 'Coville', 'Darrow', 'Dixie', 'Earliblue', 'Elizabeth', 'Elliot', 'GN-87', 'Herbert', 'Jersey', 'June', 'Lateblue', 'Northland', 'Patriot', 'Pemberton', 'Rancocus', 'Rubel', 'Spartan', 'Stanley', and '1316 A'. The plants were raised under the conditions

previously described until November 20, 1981, at which time they were moved to a greenhouse with a 15 hr daylength cycle provided by mercury vapor HID lamps. Their terminal flower buds were also removed to stimulate new growth. Ten to twelve plants of each cultivar were inoculated on April 14, 1982, with 1.0 mg/ml of purified BBSSV and the plants were evaluated with the ELISA technique between October 22 and November 11, 1982.

Results

Inoculation techniques

The total number of individuals which tested ELISA positive increased over time in all treatments (1.0, 0.5, and 0.25 mg/ml). Inoculum concentration did not appear to effect final rates of infection since there was no significant differences among treatments on the last sampling date (9/1/82) (Figure 1). There were significant differences ($P \leq 0.05$) in the percent of ELISA positive individuals on two other dates (8/4/81 and 9/15/82), but no discernible patterns were apparent across dates (Figure 2).

The inoculation of young expanded leaves vs. mature ones did not influence total infection rates, as 19 out of 20 plants in each treatment were ELISA positive after one year (Table 1). More of the individuals inoculated on young leaves were ELISA positive after 4 months, but this difference disappeared after one year.

The age of plants at inoculation did not significantly affect infection rate. Eleven months post inoculation, 54% of the plants inoculated at one month of age tested ELISA positive, while 46% of those inoculated after six months of growth were positive.

Significant differences were found between similarly aged individuals inoculated in different seasons (spring - 6/5/81 vs. fall - 10/20/81). Seventeen months after inoculation, 13.6% of the spring inoculated individuals were ELISA positive and 83.3% of the fall inoculated plants contained BBSSV.

EVALUATION TECHNIQUES

The highest percentage of times a single plant was ELISA positive over the 12 sampling dates was 50%. On an average, each plant was found ELISA positive only 18.3% of the time, with 61% having fewer than two positive readings.

On all but one sampling date, at least one new individual was found ELISA positive (Figure 3), and the cumulative number of diseased individuals increased over time until 91.7% of the inoculated plants tested positive. The greatest number of plants that were ELISA positive on any given sampling date was 61%, soon after dormancy was broken (7/9/82). Visible 'shoestring' symptoms were observed on only five out of 36 individuals 12 months after inoculation.

Roots appeared to contain slightly more BBSSV than other tissues. Among the five diseased plants broken into their component parts, 80% contained virus in their roots and only 20% had BBSSV in their leaves; no BBSSV was detected in the shoot tips. Of the 36 plants divided into roots and leaves, 8% had ELISA positive roots and 3% had ELISA positive leaves. In the plant whose nine shoots were divided into shoot tips and bases, 33% of the terminals had virus, while only 11% of the bases did.

Screen for BBSSV resistance

Six months after inoculation, 24 out of 28 cultivars tested ELISA positive for BBSSV. An apparently random pattern of infection rates was observed ranging from 0 to 66% (Table 5 and Figure 5). Four cultivars were not infected - 'Concord', 'June', 'Pemberton', and 'Elliot'. 'Bluecrop' had an infection rate of 42%, even though it appears to have field resistance.

Figure 1. Cummulative percentage of cv. 'Jersey' blueberry plants testing ELISA-positive on four dates (8/4/81, 10/21/81, 6/15/82 and 9/15/82). Three BBSSV inoculum concentrations (1.0, 0.5, and 0.25 mg/ml) were used on 6/5/81.

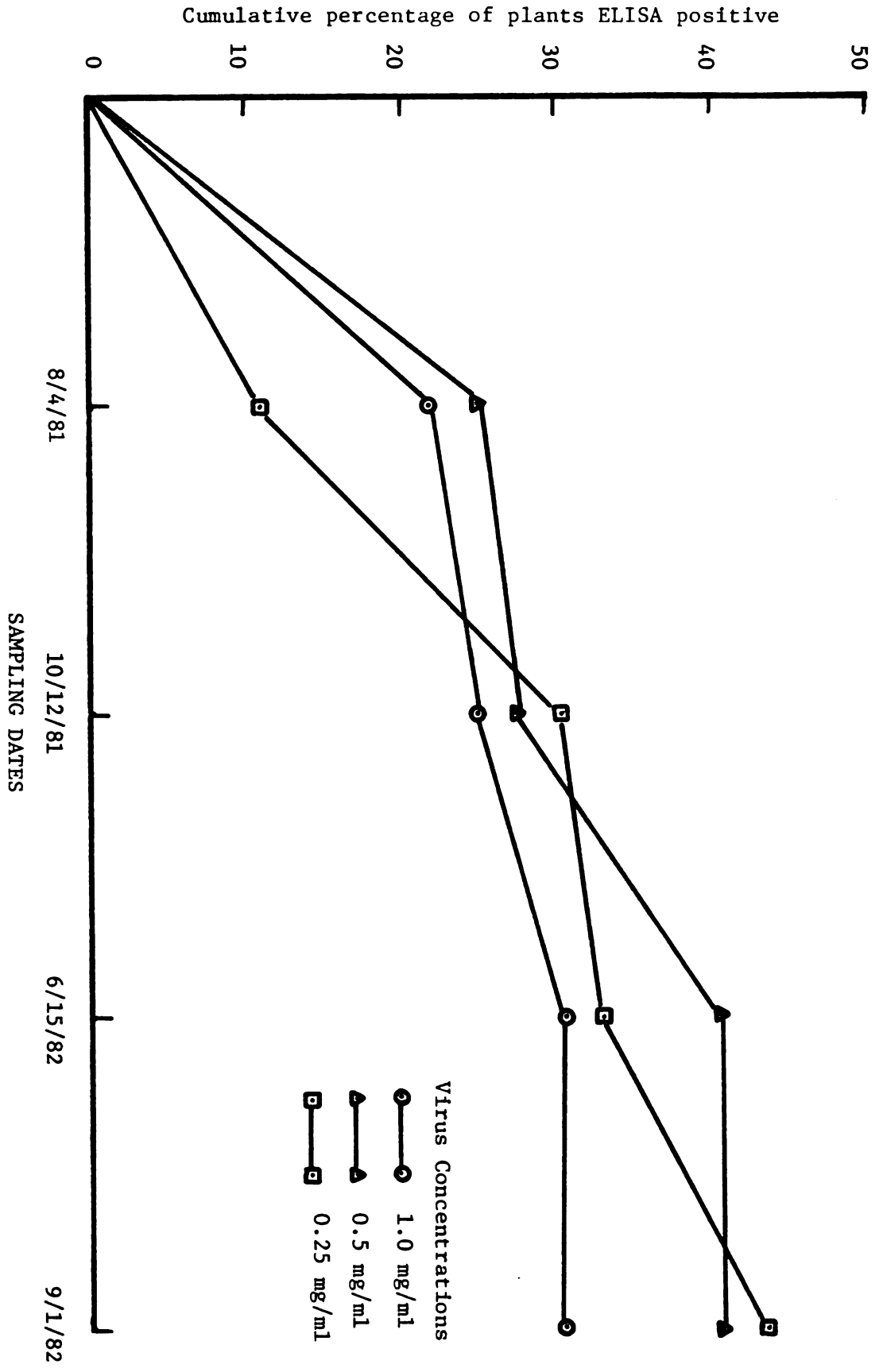
Cumulative percentage of plants ELISA positive
0
10
20
30
40
50
8/4/81
10/12/81
6/15/82
9/1/82
SAMPLING DATES
Virus Concentrations
1.0 mg/ml
0.5 mg/ml
0.25 mg/ml

Figure 2. Percentage of cv. 'Jersey' blueberry plants testing ELISA-positive on four dates (8/4/81, 10/21/81, 6/15/82, and 9/15/82).Three inoculum concentrations (1.0, 0.5, and 0.25 mg/ml) of BBSSV were used on 6/5/81.

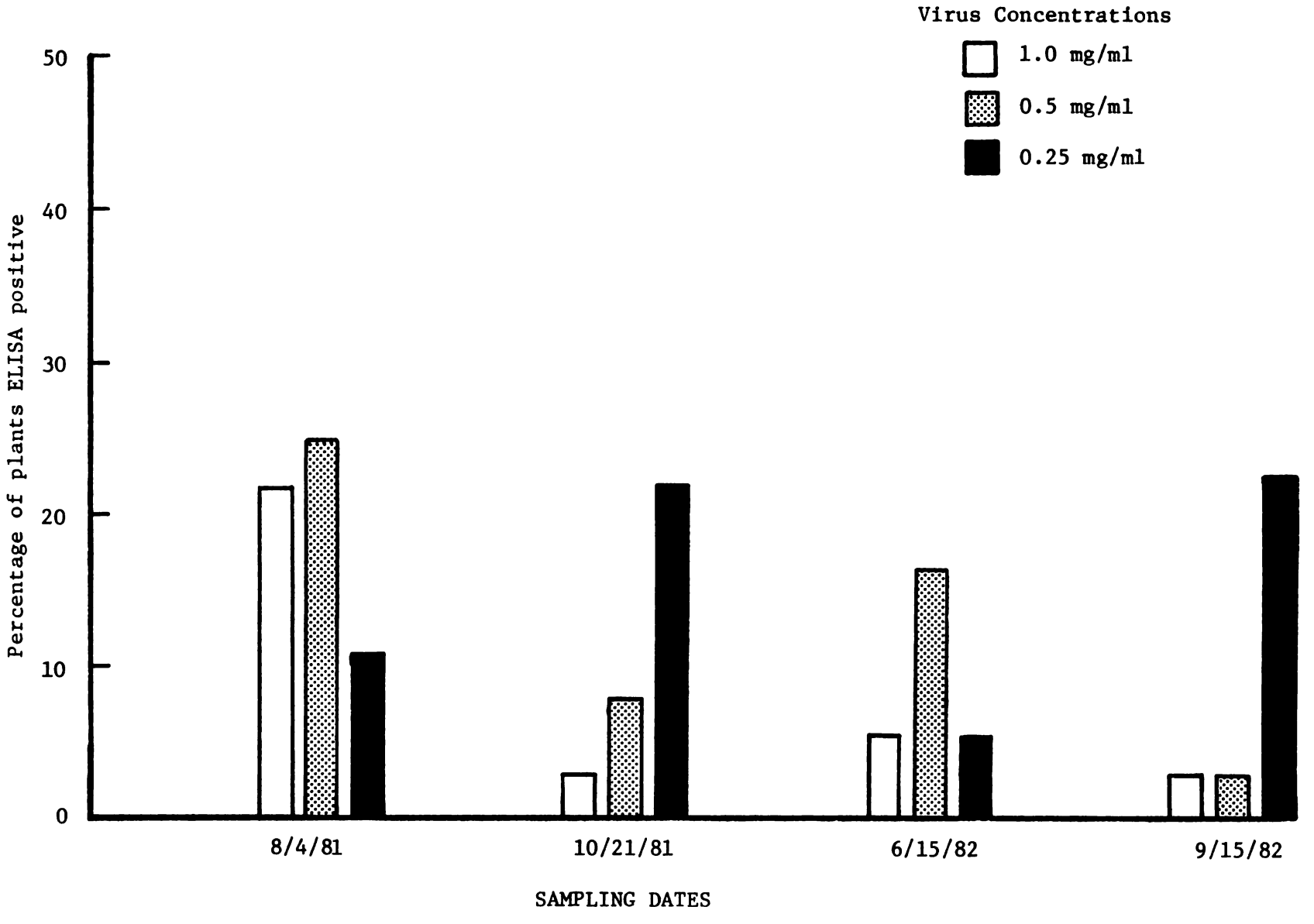
Virus Concentrations
1.0 mg/ml
0.5 mg/ml
0.25 mg/ml
Percentage of plants ELISA positive
50
40
30
20
10
0
8/4/81
10/21/81
6/15/82
9/15/82
SAMPLING DATES

Figure 3. Cummulative percentages of cv. 'Jersey' blueberry plants testing ELISA-positive on 12 sample dates. Plants were inoculated with 1.0 mg/ml BBSSV on 6/5/81.

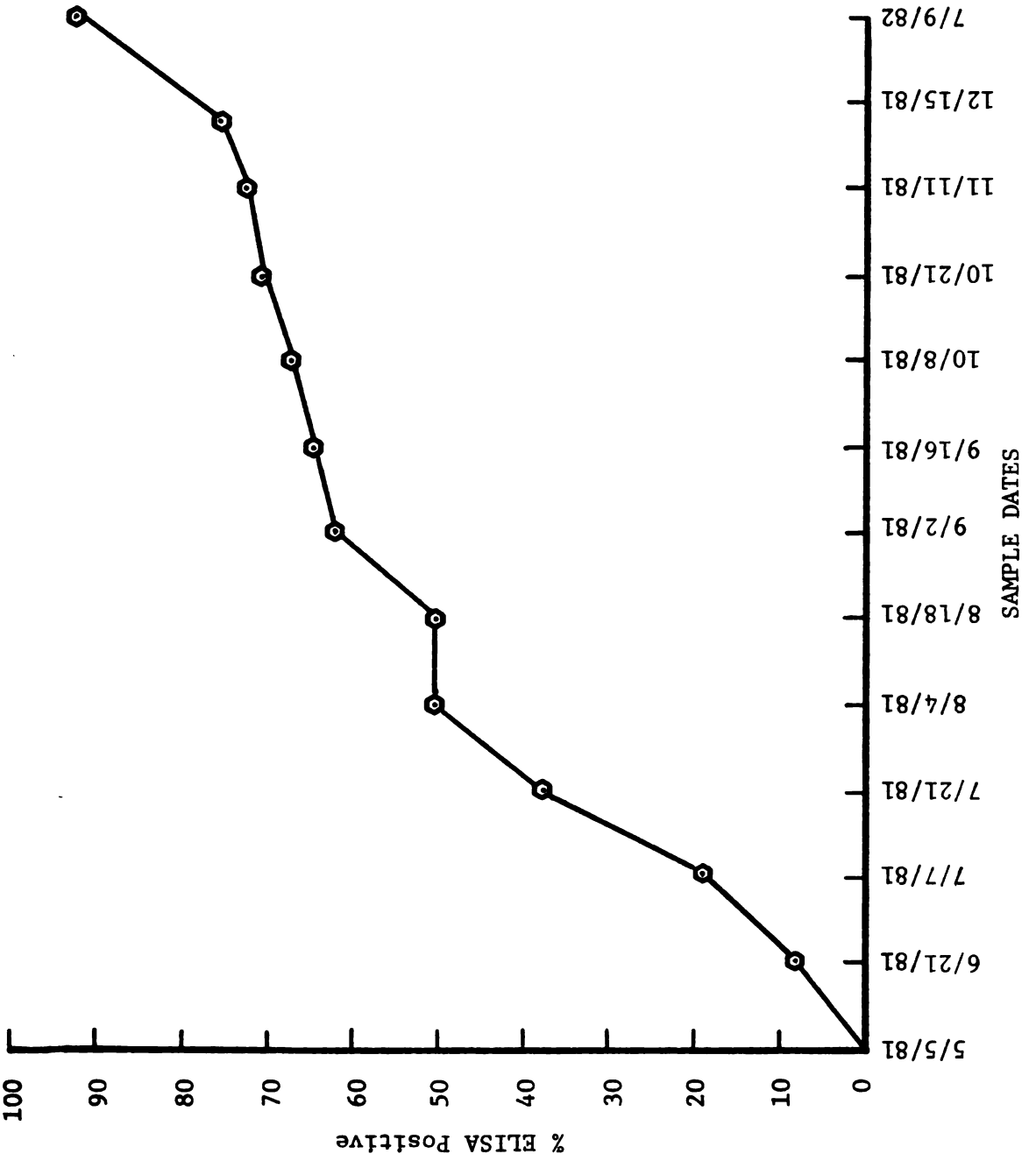
% ELISA Positive
100
90
80
70
60
50
40
30
20
10
0
5/5/81
6/21/81
7/7/81
7/21/81
8/4/81
8/18/81
9/2/81
9/16/81
10/8/81
10/21/81
11/11/81
12/15/81
7/9/82
SAMPLE DATES

Figure 4. Percentage of cv. 'Jersey' blueberry plants testing ELISA-positive on 12 sampling dates. Plants were inoculated with 1.0 mg/ml BBSSV on 6/5/81.

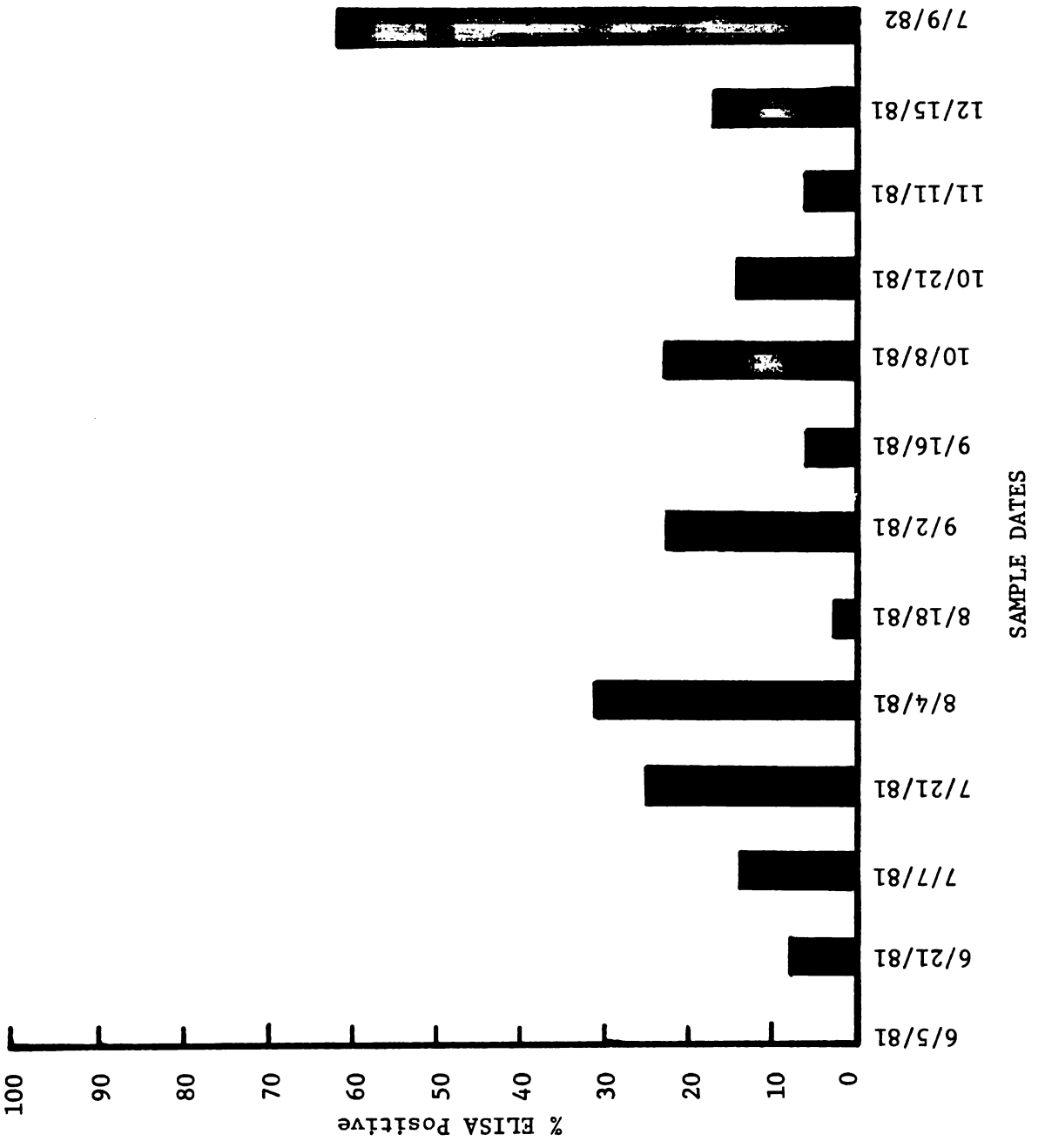
% ELISA Positive
0
10
20
30
40
50
60
70
80
90
100
SAMPLE DATES
6/5/81
6/21/81
7/7/81
7/21/81
8/4/81
8/18/81
9/2/81
9/16/81
10/8/81
10/21/81
11/11/81
12/15/81
7/9/82

Table 1. Percentage of cv. 'Jersey' blueberry plants testing ELISA-positive after inoculation[a] of young, unexpanded leaves vs mature, expanded ones.

Leaf type	% testing ELISA-positive		
	10/8/81	1/12/82	6/15/82
Young, unexpanded	20	0	95
Mature, expanded	0	0	95

[a]Plants were inoculated with 1.0 mg/ml BBSSV on 6/26/81

Table 2. Percentage of blueberry plants in 28 cultivars testing ELISA positive between 10/22/82 and 11/11/82.[a]

Cultivar	Total plants ELISA tested	Number ELISA positive	Percentage ELISA positive
Atlantic	3	2	66
Berkeley	12	2	17
Bluecrop	12	5	42
Bluehaven	7	1	14
Bluejay	9	2	22
Bluetta	11	7	64
Burlington	11	3	27
Collins	5	2	40
Concord	6	0	0
Coville	10	5	50
Darrow	11	1	9
Dixie	12	4	33
Earliblue	11	5	45
Elizabeth	11	2	17
Elliot	12	0	0
GN-87	12	2	17
Herbert	11	3	27
Jersey	10	6	54
June	12	0	0
Lateblue	12	5	42
Northland	8	2	25
Patriot	11	4	36
Pemberton	11	0	0
Rancocus	12	1	8
Rubel	10	4	40
Spartan	12	5	42
Stanley	10	3	30

Table 2. Continued

Cultivar	Total plants ELISA tested	Number ELISA positive	Percentage ELISA positive
1316A	9	3	33

[a]An inoculum level of 1.0 mg/ml BBSSV was applied on 4/14/82.

Figure 5. Percentage of blueberry plants in 28 cultivars testing ELISA-positive between 10/22/82 and 11/11/82. An inoculum concentration of 1.0 mg/ml BBSSV was applied on 4/14/82.

% ELISA Positive
0
10
20
30
40
50
60
70
80
Atlantic
Berkeley
Bluecrop
Bluehaven
Bluejay
Bluetta
Burlington
Collins
Concord
Coville
Darrow
Dixie
Earliblue
Elizabeth
Elliot
GN-87
Herbert
Jersey
June
Lateblue
Northland
Patriot
Pemberton
Rancocus
Rubel
Spartan
Stanley
1316A

Discussion

Inoculation techniques

The basic objective of this study was to develop a screen that effectively tests breeding stock for resistance to BBSSV and gives a reliable prediction of a cultivar's susceptibility or resistance with the least amount of effort, and cost.

Inoculation practices implemented by Ramsdell in 1979 in his preliminary cultivar screen (use of purified BBSSV, carbonundum and washing off virus 3 to 5 minutes after inoculation) were further modified in accordance with Fulton (1964) (preinoculation dark period, 36 hours, 2 passes of sponge on leaf surface and consistent application time-early to mid-morning to avoid diurnal effects). The variables inoculum concentration, age of leaf tissue, age of plant, and season of inoculation were tested in hopes of increasing the accuracy and flexibility of the BBSSV screening technique.

After assaying test plants on four successive dates, an inoculum concentration of 0.25 mg/ml produced 45% infectivity, which was not significantly different than that caused by 1.0 mg/ml. Thus, the lower rate can be used in screens without significantly effecting infection rates. This means that the potential number of plants that can be inoculated with purified BBSSV can be increased at least 4 fold with no decrease in infection rate.

Previous studies suggest that seasonal variations can influence infectivity in woody plants due to variations in light intensity (low light intensity favors rate of infection) (Fulton, 1964, Bawden and

Roberts, 1947). In this study, the fall inoculation (October) showed a significantly higher rate of infection than the summer one (June). However, these differences may be spurious since the other summer inoculated plants had an infection rate of 91.7% (Figure 3). Further comparisons need to be made to correctly evaluate the interrelationship between season and infectivity.

Several studies (Yardwood, 1962; Costa and Bennett, 1955; Bawden and Roberts, 1947) suggest that infectivity decreases with increasing leaf age on herbaceous plants. In this study, different leaf and plant ages at time of inoculation were compared and no significant differences were observed. It can be concluded from these data that young succulent growth is not mandatory for infection to occur in highbush blueberries. This suggests that a large range of plant growth stages can be inoculated in a resistance screen without prejudicing the results.

EVALUATION TECHNIQUES

To increase the accuracy of ELISA detection of BBSSV in blueberries several variables were tested including sample number, sample time and source material. These factors were chosen because they have been shown to be important in virus detection (Clark, 1981).

After twelve sampling dates, 91.7% of the 'Jersey' plants tested ELISA positive at least once, a two-fold increase over that obtained by Ramsdell, 1980. After two samplings, only 20% of the plants were ELISA positive and after five samples just 50% of the plants tested positive for BBSSV. These data suggest that several samples must be taken to accurately recognize susceptible genotypes, unless several replicates

of each genotypes can be used. However, the final sample taken soon after the plants had broken dormancy, produced 61% infected individuals which was at least twice the value of any other single sample. This indicates that BBSSV may be most accurately detected in expanding shoots just after dormancy requirements are met.

BBSSV was detected several times in new shoots, random leaf samples and roots of blueberries during the course of the growing season. Bennett (1956) suggested that virus movement through a plant can be correlated with the flow of carbohydrates, implying that the virus may accumulate in areas with the greatest sink potential. The limited sample size of their study prohibited testing this hypothesis, but further research should examine all plant parts including expanding flower blossom buds, expanding shoot tips and roots over all seasons. In this way, the optimum source and time of plant growth could be determined that results in the least sampling error.

BBSSV Screen

Twenty-eight cultivars, representing most of the genes present in blueberry cultivars were sampled once after inoculation with 1.0 mg/ml BBSSV. Both of the cultivars 'Bluecrop' and 'Atlantic', observed to be field resistant were found ELISA positive, while four cultivars, 'Concord', 'Elliot', 'June', and 'Pemberton' had 0% infection after one test date. It appears that there may be resistance to BBSSV in several of the available cultivars, but as the previous data indicate, more samples must be taken to substantiate this.

'Bluecrop' and 'Atlantic' may have become infected in the screen because the inoculum level used (1.0 mg/ml) was much higher than that typically transmitted under field conditions by the vector I. pepperi. It has been reported that in controlled tests, I. pepperi accumulates only from 3 to 80 ng virus (K. Morimoto, personal communication). It is possible that 'Bluecrop' and 'Atlantic' are resistant to BBSSV, but at levels much lower than those used in the rub inoculation. The use of high inoculum concentrations in screens reduces the possibility of rating a susceptible cultivar as resistant although it increases the possibility that low levels of resistance will be missed.

If the four cultivars 'Concord', 'Elliot', 'June', and 'Pemberton' are still deemed resistant after several additional ELISA evaluations, they can be used to breed new cultivars. There is a need for additional later fruiting cultivars with BBSSV resistance to extend the fruiting season.

Summary

A screen for resistance to blueberry shoestring virus (BBSSV) was developed using rub inoculation and the ELISA technique for assaying resulting infections. It appears that high rates of infection can be obtained with a virus concentration of 0.25 mg/ml when it is applied to randomly selected leaves of container grown blueberries of any age, during any portion of the growing season as long as healthy leaves are present. However, to accurately measure infection rate with ELISA, plants must be sampled several times post inoculation, preferably after a dormancy period has been experienced.

Twenty-eight varieties were tested for potential resistance to BBSSV. 'Concord', 'Elliot', 'June', and 'Pemberton' appeared to be resistant after one sampling, while the rest of the cultivars had infection rates varying from 9 to 66%. It is hoped that the apparently resistant cultivars can be used to breed a new generation of resistant varieties.

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