Blueberry Red Ringspot Virus Detection in Crude Sap of Highbush Blueberry Plants

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ABSTRACT

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A two-animal ELISA (enzyme-linked immunosorbent assay) was developed to detect blueberry red ringspot virus (BBRRSV) in crude blueberry sap after ELISA with antiserum from one species proved unsatisfactory. Use of polyclonal mouse anti-BBRRSV globulin from ascites fluid as the detecting globulin after use of rabbit anti-BBRRSV as the trapping globulin permitted the routine detection of BBRRSV in crude sap from infected blueberry leaves during late summer and early autumn. This two-animal ELISA system also was useful for detecting BBRRSV in bark of fully dormant, infected blueberry shoots and provided a simple, reliable method of identifying latent infections of BBRRSV in dormant blueberry nursery stock.

Additional key words: *Vaccinium corymbosum*

Blueberry red ringspot virus (BBRRSV) is presumed to be a caulimovirus 42–46 nm in diameter that is widespread and sometimes latent in U.S. stocks of cultivated highbush blueberries (*Vaccinium corymbosum* L.) (8,10). BBRRSV has been characterized and a polyclonal antiserum prepared against it in rabbit (5,8). With this antiserum, the virus has been detected successfully by ELISA in partially purified preparations but not in crude sap extracts of infected blueberry leaves (5; R. F. Hepp and R. H. Converse, *unpublished*). This paper reports the development of an ELISA system that permits detection of BBRRSV in crude homogenates of highbush blueberry.

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MATERIALS AND METHODS

Virus purification. Purified BBRRSV was prepared from frozen infected leaves of blueberry cultivar Bluetta by the method of Gillett and Ramsdell (5). Virions were precipitated with polyethylene glycol and given one cycle of differential centrifugation. This was followed by fractionation from rate-zonal centrifugation in sucrose gradients made in 0.01 M sodium-potassium phosphate, pH 7.2. The gradients were prepared by two cycles of freezing and thawing of 25% (w/v) RNase-free sucrose (6) in a Beckman SW 40 Ti rotor at 180,000 *g* for 1 hr. After gradient fractionation, the virions were pelleted from sucrose at 100,000 *g* for 2 hr in a Beckman 65 rotor.

Serology. One BALB/c mouse (7) was immunized with 680 μg total ($E^{0.1\%}_{260nm} = 7$ [8]) of partially purified BBRRSV. Four intraperitoneal injections were given on days 1, 14, 49, and 59. The virus suspension was emulsified 1:1 (v/v) with Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant thereafter. The mouse developed an ascites from which fluid was collected on days 35, 63, and 70, yielding a total of 2.6 ml of ascitic fluid. From ascitic fluid, globulin (diluted to an A_{280nm} of 1.4 = 1 mg/ml) was obtained by ammonium sulfate precipitation. The globulin was made 0.2% with respect to sodium azide and stored at −20 C.

Polyclonal rabbit antiserum against BBRRSV was supplied by D. C. Ramsdell (Department of Botany and Plant Pathology, Michigan State University). Globulin was prepared from it by passing the whole serum through a CM-Affi-Gel Blue column (Bio-Rad Inc., Richmond, CA). Rabbit globulin concentration, adjustment, and storage were as described for mouse globulin.

Two ELISA procedures were used, a one-animal $F(ab')_2$ system described by Barbara and Clark (1) and a two-animal system (11). Two hundred microliters per well of each reactant was used, rinsing between steps (3). For the two-animal system, optimum concentrations of each globulin were determined in preliminary ELISA. Rabbit anti-BBRRSV globulin at 2.5 μg/ml was used to coat Immulon 2 ELISA plates (Dynatech Inc., Chantilly, VA). Antigen was prepared by grinding frozen infected and healthy blueberry leaves 1:10 (w/v) in modified ELISA virus buffer (described below) with a tissue homogenizer (Polytron, Brinkmann Instruments, Wartburg, NY) for 20 sec at setting 7. Homogenates were filtered

through Lintguard Poly Shield wipes (Kimberly-Clark Co., Roswell, GA), added to the wells, and incubated overnight at 5 C. Mouse anti-BBRRSV globulin at a concentration of 1 μg/ml was added after rinsing, incubated for 8 hr at 5 C, and rinsed. Because of high background in healthy controls, the diluted mouse globulin was incubated overnight at 5 C, mixed 50:1 (v/v) with 1:10 (w/v) healthy blueberry leaf sap obtained as described previously, and given a low-speed centrifugation before being used in the ELISA. Rabbit anti-mouse (heavy and light chain) globulin conjugated to alkaline phosphatase (Zymed Co., South San Francisco, CA) used at 1:1,000 (v/v) was added after rinsing, incubated overnight at 5 C, rinsed, and 1 mg/ml *p*-nitrophenyl phosphate was added (3). Absorbance readings (A_{405nm}) were made, usually within 60 min, in a through-the-plate ELISA reader (Biotek, Model EL 307 I, Burlington, VT).

Washing buffer (3) was modified by adding 0.2% (w/v) bovine serum albumin fraction V (PBS-Tween-BSA) (Sigma). ELISA virus buffer used for antigen and second and third globulins were modified by substituting 0.2% bovine serum albumin for ovalbumin (EVB-BSA). In each plate, aliquots were placed in each of three wells for each crude blueberry homogenate for known BBRRSV-infected extracts and for buffer alone. For healthy plant controls, six aliquot samples of crude sap from a healthy blueberry plant were used. The threshold positive reading for each plate was the mean healthy absorbance reading for that plate plus three standard deviations.

Immunosorbent electron microscopy. Grids were prepared using the method described by Chen et al (2). Rabbit anti-BBRRSV globulin at a concentration of 50 μg/ml was used to trap the virus particles from a partially purified preparation of BBRRSV (0.16 mg/ml) obtained from a field-grown infected Bluetta plant. This was followed by decoration with mouse anti-BBRRSV globulin at a concentration of 20 μg/ml. A partially purified sample of healthy Bluetta and normal rabbit globulin replacing rabbit anti-BBRRSV globulin were used for control grids. After staining the grids with 2% aqueous uranyl acetate, they were examined with a Philips EM 300 transmission electron microscope at 60 kV.

Blueberry plant sample preparation. The two-animal ELISA system was evaluated for the detection of BBRRSV in dormant tissue of a field-grown, BBRRSV-infected Bluetta plant collected 20 January 1986. As controls, tissues were used from a dormant, healthy Bluetta and a dormant, BBRRSV-infected Bluetta plant grown in the screenhouse at Oregon State University. Samples of roots, stem bark, and flower buds (0.5 g each) were ground to a powder with liquid nitrogen in mortar and pestle. After mixing with 5 ml of EVB-BSA, the samples were filtered and added to ELISA plates.

Because preliminary ELISA trials had demonstrated positive detection of BBRRSV in the stem bark of dormant, infected blueberry plants, the system was tested on 10 field-grown Bluetta plants that had shown moderate to severe red ringspot symptoms the previous growing season and on one continually symptomless Bluetta. Without symptom evaluation, four twigs 8–10 cm long were collected from different branches around each plant on 31 January, 14 February, and 28 March 1986. The bark was peeled off each twig with a razor blade, pooled (0.5 g/plant), and processed as described previously. As a negative control, bark from a bulk sample of healthy, dormant, screenhouse-grown Bluetta twigs was processed similarly.

RESULTS

ELISA detection of BBRRSV in crude extracts of frozen blueberry leaves without globulin absorption. Composite leaf samples from field-grown (Linn County, Oregon) highbush blueberry cultivar Bluetta plants showing leaf symptoms typical of BBRRSV infection were harvested from individual plants at intervals from April to October 1985, stored at −20 C, and evaluated for BBRRSV as crude sap by DAS-ELISA (5), by the F(ab′)$_2$ ELISA system, and by the two-animal ELISA system using mouse anti-BBRRSV globulin that had not been cross-absorbed (Table 1). BBRRSV was not detected by DAS-ELISA. BBRRSV was detected only in the October sample by the F(ab′)$_2$ system. The A_{405nm} value for this sample was 3.7 times the threshold value of 0.057. With the two-animal system (Table 1), BBRRSV was detected in three of seven samples. The A_{405nm} values for April, September, and October were, respectively, 1.2, 1.8, and 2.2 times the threshold value of 0.190. The A_{405nm} value obtained with the two-animal system (non-cross-absorbed) for frozen, crude infected leaf sap in October was twice as high as that obtained with the F(ab′)$_2$ system. This two-animal method did not detect BBRRSV in symptomatic leaf samples collected in May (twice), June, or August.

Detection of BBRRSV by the two-animal ELISA system using cross-absorbed mouse anti-BBRRSV globulin. Cross-absorbing mouse anti-BBRRSV globulin reduced healthy background to very low levels compared with non-absorbed globulin (Table 1). BBRRSV was readily detected in crude sap from frozen, infected blueberry leaves that had been collected in August, September, or October in two replicate tests but not from frozen, symptomatic leaf samples collected in April, May, or June (Table 1). For all subsequent tests, mouse globulin was routinely cross-absorbed.

Detection of BBRRSV in dormant blueberry plant tissues. In dormant, field-grown Bluetta, BBRRSV was detected only in the pooled stem bark (Table 2). For each plant sampled, several stems were collected and their bark removed and pooled without noting presence or absence of stem symptoms of BBRRSV infection. The A_{405nm} value obtained from this infected stem bark was 10.6 times the threshold value of 0.032. No BBRRSV was detected in field samples of roots or flower buds. In the screenhouse-grown infected plant, BBRRSV was detected in the stem bark and flower buds; the A_{405nm} values were 8.0 and 4.9 times the threshold values of 0.032 and 0.025, respectively. The average A_{405nm} reading for the healthy

Table 1. Detection of blueberry red ringspot virus (BBRRSV) in crude extracts of symptomatic, frozen blueberry cultivar Bluetta leaves by a nonabsorbed rabbit F(ab′)$_2$ ELISA system and by a two-animal ELISA system, using non-absorbed and cross-absorbed mouse globulin

Date tissue collected (1985)	Mean absorbance at 405 nm (three wells per mean)			
	Rabbit F(ab′)$_2$ nonabsorbed	Two-animal nonabsorbed	Two-animal cross-absorbed[a]	
			Test 1	Test 2
23 April	0.032	0.225*[b]	0.012	0.002
14 May	0.014	0.063	0.014	0.006
31 May	0.022	0.062	0.002	0.001
24 June	0.026	0.064	0.015	0.000
22 August	0.035	0.144	0.862*	0.433*
23 September	0.046	0.339*	0.240*	0.289*
15 October	0.210*	0.426*	1.088*	0.373*
15 October (HBB)[c]	0.037	0.134	0.011	0.003
Threshold[d]	0.057	0.190	0.031	0.007

[a] First globulin (rabbit) at 2.5 μg/ml; second globulin (mouse) at 1 μg/ml. Diluted mouse globulin cross-absorbed 50:1 (v/v) with 1:10 (w/v) healthy blueberry leaf sap. Substrate incubation time = 45 min.
[b] * = Positive for BBRRSV by ELISA (i.e., exceeds threshold positive values).
[c] HBB = healthy Bluetta blueberry control (mean of six wells).
[d] Threshold positive values = healthy blueberry mean (six wells) + three standard deviations.

Table 2. Detection of blueberry red ringspot virus (BBRRSV) in crude extracts of tissues of dormant blueberry cultivar Bluetta by a two-animal ELISA system[a]

Plant source[b]	Mean absorbance at 405 nm		
	Roots	Flower buds	Stem bark
Field, infected	0.104	0.022	0.339*[c]
Screenhouse, infected	NT[d]	0.122*	0.256*
Screenhouse, healthy	0.199	0.016	0.019
Threshold[e]	0.232	0.025	0.032

[a] First globulin (rabbit) at 2.5 μg/ml; second globulin (mouse) at 1 μg/ml. Diluted mouse globulin cross-absorbed 50:1 (v/v) with 1:10 (w/v) healthy blueberry leaf sap. Substrate incubation time = 60 min.
[b] Tissue samples collected and tested on 20 January 1986 without symptom evaluation.
[c] * = Positive for BBRRSV by ELISA (i.e., exceeds threshold positive values).
[d] NT = not tested.
[e] Threshold positive values = healthy Bluetta blueberry mean (six aliquot wells from a healthy plant) + three standard deviations.

Table 3. Detection of blueberry red ringspot virus (BBRRSV) by a two-animal ELISA system[a] in crude extracts of bark[b] from dormant blueberry cultivar Bluetta plants collected on different dates in 1986 from a field in Linn County, Oregon

Plant number[c]	Mean absorbance at 405 nm		
	31 Jan.	14 Feb.	28 Mar.
1	0.208*[d]	0.227*	0.468*
2	0.378*	0.156*	0.158*
3	0.050*	0.159*	0.124*
4	0.038*	0.451*	0.090*
5	0.293*	0.564*	0.132*
6	0.076*	0.028	0.025
7	0.430*	0.141*	0.052*
8	0.064*	0.421*	0.096*
9	0.046*	0.028	0.029
10	0.041*	0.047	0.044*
11	0.028	0.020	0.016
HBB[e]	0.034	0.030	0.022
Threshold[f]	0.037	0.052	0.032

[a] First globulin (rabbit) at 2.5 μg/ml; second globulin (mouse) at 1 μg/ml. Diluted mouse globulin cross-absorbed 50:1 (v/v) with 1:10 (w/v) healthy blueberry sap. Substrate incubation time = 60 min.
[b] One-half gram of bark per plant pooled from four stems selected without symptom evaluation per 5 ml of buffer (NaCl, 8.0 g; KH_2PO_4, 0.2 g; $Na_2HPO_4 \cdot 12\ H_2O$, 2.9 g; KCl, 0.2 g; NaN_3, 0.2 g; Tween 20, 0.5 ml; polyvinylpyrrolidone, mol wt 10,000, 20 g; and bovine serum albumin, 0.2 g in 1 L of H_2O, pH 7.4).
[c] Plants 1–10 showed typical symptoms of BBRRSV infection in September 1985. Plant 11 was symptomless in September 1985. Not all the symptoms were of the same degree of severity.
[d] * = Positive for BBRRSV by ELISA (i.e., exceeds threshold positive values).
[e] HBB = healthy blueberry Bluetta control.
[f] Threshold positive values = healthy blueberry mean (six wells) + three standard deviations.

root sample was higher than that for the field-grown, infected plant, indicating that nonspecific binding had probably occurred, because the mouse globulin used was only cross-absorbed against healthy blueberry leaf sap before use.

In January 1986, BBRRSV was detected by cross-absorbed, two-animal ELISA in dormant twig bark in 10 of 10 Bluetta plants that had previously shown typical red ringspot symptoms on stems and leaves (100% reliability). The A_{405nm} values obtained ranged from 1.0 to 11.6 times the threshold positive value of 0.037 (Table 3). In February, BBRRSV was detected in seven of 10 of these plants (70% reliability), with A_{405nm} readings ranging from 2.7 to 10.8 times the threshold value of 0.052. In March, BBRRSV was detected in eight of 10 of these plants (80% reliability), and the A_{405nm} values were 1.4 to 14.6 times the threshold value of 0.032. Overall, seven of these plants (70% reliability) tested positive for BBRRSV throughout this experiment (plants 1, 2, 3, 4, 5, 7 and 8, Table 3). Plants 6 and 9 were positive for BBRRSV in the January sampling only, and plant 10 was positive in the January and March samplings. Plant 11, which had not shown any red ringspot symptoms the previous season, was negative on all three sampling dates.

Immunosorbent electron miroscopy. After decorating the immunospecific electron microscope grids with mouse anti-BBRRSV globulin and staining with uranyl acetate, a globulin halo was observed on and around the trapped virus particles from plant 8 (Table 3). The individual virion-globulin complexes were about 96 nm, about twice the size of BBRRSV virions. No viruslike particles were found on the control grids from plant HBB (Table 3) or when normal rabbit globulin was used.

DISCUSSION

Highbush blueberry plants (cultivar Bluetta) infected with BBRRSV can be identified by a two-animal ELISA system. In our laboratory, a two-animal system is more sensitive than a one-animal system [$F(ab')_2$] in detecting BBRRSV in crude homogenates of frozen, symptomatic leaves. Although the one-animal system has been able to detect the virus only in crude leaf homogenates taken at the end of the growing season (October), the two-animal system has proven effective in early spring (April) as well as in late summer (August through October). We believe that the data in Table 1 indicate that the titer of BBRRSV in leaves of infected blueberry plants remains low from the time of spring foliation until late August and then increases when the leaves develop their strongest symptoms as they mature (10). More important, the two-animal system can accurately detect BBRRSV in crude extracts of bark of infected Bluetta regardless of bark symptoms during the dormant season. This is particularly valuable for nursery workers, who for the first time will be able to use this technique to screen for BBRRSV when preparing to dig their dormant blueberry nursery stock plants. Our data suggest that such testing be done when plants are fully dormant rather than when buds are breaking and that bark be tested from a pool of twigs gathered around a plant because of uneven distribution of BBRRSV in infected plants. The reliability of the test seems to drop as the season progresses and budbreak begins.

The immunospecific electron microscopy work shows that both the rabbit immunoglobulin and the mouse ascitic polyclonal immunoglobulin used were able to bind to viruslike particles that were of the proper size to be caulimovirus-like virions. The positive results using immunospecific electron microscopy with blueberry leaf samples that were ELISA-positive but not with those that were ELISA-negative reinforce the confidence that can be placed in both methods for detecting BBRRSV.

Mouse ascitic fluid has been widely used in monoclonal antibody production as a technique for increasing immunospecific antibodies for indirect ELISA applications (7). The advantages of using mouse polyclonal ascitic fluid for detecting plant viruses, however, were mentioned by Scott et al in 1969 (9). Recently, Davis (4) has reported the production and use of a high-titer polyclonal ascites fluid in mice immunized with zucchini yellow mosaic virus in indirect ELISA. As far as we are aware, the results obtained in this investigation constitute the first report of the successful use of mouse polyclonal ascitic fluid as part of a two-animal ELISA system for detecting a plant virus.

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