

Detection of Blueberry Scorch Virus Strain NJ2 by Reverse Transcriptase-Polymerase Chain Reaction Amplification

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ABSTRACT

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A reverse transcription-polymerase chain reaction (RT-PCR) assay has been developed for the detection of strain NJ2 of blueberry scorch carlavirus (BBScV), which causes an economically important disease of blueberry. Four extraction methods were compared for their ability to yield nucleic acid from blueberry tissue that could be amplified by specific RT-PCR primers. Two extraction methods were found suitable when they were followed by an Elutip-r column purification step. These procedures were successful with blueberry blossoms and leaves. RT-PCR was shown to be considerably more sensitive than spot hybridization using a ³²P-labeled DNA probe. These purification systems enable use of nucleic acid-based RNA virus detection from blueberry.

Blueberry scorch carlavirus (BBScV) causes serious blighting diseases of blueberry (*Vaccinium corymbosum* L.) (9). A disease known locally as Sheep Pen Hill disease was first observed near Pemberton, New Jersey, in the 1970s (14). A similar disease observed on the West Coast near Puyallup, Washington, in 1982 was found to be caused by a different strain of the same virus (1,8). Spring symptoms of these diseases include necrosis of blossoms and new vegetation, and can be mistaken for frost injury or other blighting diseases. Resulting yield loss can be substantial. Autumn leaf symptoms may include a pronounced line or oak leaf pattern. Symptoms are variable among different cultivars (10; unpublished observations).

BBScV is a member of the carlavirus group. These are flexuous rod-shaped viruses with single-stranded, positive sense RNA genomes of 8.5 kb (1,8). The complete nucleotide sequence of one East Coast strain (NJ2) and the partial sequence of another (NJ1), and the sequences of two West Coast strains (WA1 and WA2) have been determined (1).

A rapid, sensitive detection system would be useful for diagnosis of BBScV. However, phenolic compounds and other inhibitory substances in blueberry plants and other woody plants including grape, apple, cherry, and peaches (3,11,13) interfere with nucleic acid extraction and virus detection. We examined four nucleic acid

extraction methods and compared their effectiveness in RNA spot hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR). The work presented in this report represents data obtained from the BBScV strain NJ2.

MATERIALS AND METHODS.

Source of plant material. Virus-infected blossom tissue and leaf tissue were obtained from Weymouth highbush blueberry plants in Pemberton. *Chenopodium quinoa* L., the herbaceous host plant used in these experiments, was grown in the greenhouse and mechanically inoculated with BBScV as described previously (1).

Selection of primers for PCR. Two oligonucleotides based on the BBScV sequence (1) were chosen as primers to amplify a 511-bp fragment from residues 6,025 to 6,535. This is in the second open reading frame of the BBScV genome, which encodes a 25-kDa putative helicase protein (1). Oligonucleotide 11, a 16-mer ([5'-AAAAGTCTGGCCGCGC-3']; $T_M = 51.3$), corresponds to residues 6,025 to 6,040 from the 5' end. Oligonucleotide 14, also a 16-mer ([5'-ATTTCGAGCGTCA-GTC-3'] $T_M = 53.3$), is complementary to residues 6,520 to 6,535.

Virus isolation and RNA purification. Virus was isolated from *C. quinoa* leaves essentially as described by Martin and Bristow (8). RNA was isolated from purified virus as described by Hillman et al. (5).

Sample preparation. Extractions were performed with 0.1 g of tissue, which is equivalent to approximately four blossoms or one leaf of plant tissue, unless otherwise noted. Four different nucleic acid extractions were performed on healthy and infected plant tissue. Samples were stored at -70°C after extraction.

Method 1. Direct proteinase K extraction (12): Tissue was ground with a pestle and small mortar in 1 ml 1× STE (0.1 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing 200 µg of proteinase K (GIBCO BRL Life Technologies, Gaithersburg, MD). Samples were transferred to 1.5-ml microfuge tubes, incubated at 42°C for 45 min, and placed in a boiling water bath for 10 min to inactivate the proteinase K; 5 µl was used in RT reactions.

Method 2. Glycine/phenol extraction (12): Tissue was ground to a fine powder under liquid nitrogen in a microcentrifuge tube. Eight hundred µl of extraction buffer (0.1 M glycine, pH 9.5, 0.1 M NaCl, 10 mM EDTA) was added, tissue was ground until emulsified, 800 µl of phenol was added, and the mixture was vortexed for 30 s. After centrifuging 15 min at 15,800 × g in a microcentrifuge, the aqueous phase was transferred to a clean tube and 1/20 volume 3 M NaOAc, pH 5.2, and 2.5 volume EtOH were added. Nucleic acid was collected by centrifuging 30 min at 15,800 × g. The resulting pellet was washed with 70% EtOH and centrifuged 10 min at 15,800 × g. The pellet was dried under vacuum and resuspended in 100 µl water; 2 µl was used in RT reactions.

Method 3. LiCl extraction (7): Tissue was ground to a fine powder under liquid nitrogen. Samples were homogenized with a microcentrifuge pestle in 400 µl of extraction buffer (1 M Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% SDS) and vortexed for 30 s with 400 µl of phenol. Samples were incubated for 5 min at 65°C, then centrifuged 5 min at 15,800 × g. The aqueous phase was transferred to a clean microfuge tube and extracted two times with phenol/chloroform (1:1). Nucleic acid was precipitated with 2.5 volume EtOH for 10 min at -70°C and centrifuged 8 min at 15,800 × g. Resulting pellets were washed and dried as above and resuspended in 20 µl of water; 2 µl was used in RT reactions.

Method 4. GT-phenol-chloroform extraction (2): Tissue was ground under liquid nitrogen. One ml of denaturing solution (4 M guanidine thiocyanate [GT], 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added, followed by 0.1 ml of 2 M NaOAc, pH 4.0, 1 ml of phenol, and 0.2 ml of chloroform-isoamyl alcohol (49:1). The mixture was shaken vigorously for 10 s, placed on ice for 15 min, and centrifuged at 14,000 × g for 20 min at 4°C. The aqueous phase was transferred to a clean tube, 1 ml of isopropanol

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was added, and the mixture was incubated at -20°C for 1 h. Samples were centrifuged at $14,000 \times g$ for 20 min at 4°C . Pellets were resuspended in 0.3 ml of denaturing solution and precipitated with 1 volume of isopropanol at -20°C for 1 h. Nucleic acid was collected by centrifuging at $14,000 \times g$ for 10 min at 4°C , and pellets were washed with 70% EtOH. Pellets were air-dried and resuspended in 50 μl water; 2 μl was used in RT reactions.

Elutip-r purification. Viral RNA was bound to Elutip-r minicolumns containing "low salt" buffer (0.4 M NaCl, 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA). Contaminants were washed off with low salt buffer, and the RNA was eluted from minicolumns with "high salt" buffer (1 M

NaCl, 1 M Tris-HCl, pH 7.4, and 1 M EDTA). Minicolumns were used according to the manufacturer's instruction manual (Schleicher and Schuell, Keene, NH). Samples were resuspended in 25 μl of water, and 2 μl was used in the RT step.

Reverse transcriptase reactions. Total nucleic acid (2 to 5 μl) samples were used in 20- μl reactions as described in *Promega Protocols and Application Guide* (Promega, Madison, WI).

Polymerase chain reactions. One hundred μl PCR reactions were performed using 5 μl of the RT products. Reactions contained 1.5 mM MgCl_2 , buffer provided by the enzyme manufacturer, and 1 U Taq polymerase (Perkin-Elmer, Norwalk, CT). Cycling incubations were performed on a Techne Model 2 thermocycler (Techne, Inc., Princeton, NJ) programmed for 35 cycles of 1.5 min at 92°C , 1.5 min at 52°C , and 1 min at 72°C , and a final extension cycle of 9.9 min at 72°C . Optimal steps were determined empirically.

Analysis of PCR products. PCR reaction products (10 μl) were analyzed by electrophoresis through a 1% agarose gel in TPE buffer (0.035 M Tris, 0.029 M NaH_2PO_4 , 0.8 mM EDTA) at 80 volts for 1.5 h. Gels were stained in ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 10 min, rinsed in water, and photographed on a UV-transilluminator. A 1-kb ladder (GIBCO BRL Life Technologies) was used to estimate sizes of PCR products.

Spot hybridization. Spot hybridizations were performed with a BRL apparatus and Zeta-Probe (Bio-Rad, Richmond, CA) nylon membranes following the suggested Bio-Rad protocol. Complementary DNA plasmid pBB-44 (1), corresponding to nucleotides 3,528 to 7,000 of the BBScV genome, was ^{32}P -labeled by random priming as previously described (5).

RESULTS

Detection of BBScV from *C. quinoa*.

Four extraction procedures were evaluated for their ability to yield nucleic acid for amplification by RT-PCR and ability of the nucleic acid to be detected by hybridization. Figure 1A shows an agarose gel of cDNA amplified from healthy and infected *C. quinoa*. Nucleic acid extracts obtained from methods 2 (glycine/phenol) and 3 (LiCl) yielded products that were successfully amplified to the expected 511-bp fragment (lanes 4 and 6). These methods were used for blueberry tissue experiments. Methods 1 (proteinase K) and 4 (GT-phenol-chloroform) did not consistently yield nucleic acid that allowed amplification of the expected PCR products. Inclusion of a final Elutip-r minicolumn step did not improve results for methods 1 and 4, and they were not examined further. Samples extracted from healthy tissues by all methods were consistently negative. Method 3 (LiCl) resulted in the strongest hybridization signal in spot hybridization experiments (Fig. 1B).

Detection of BBScV from blueberry tissue. RT-PCR was inhibited by blueberry tissue, but inhibition was overcome when an Elutip-r minicolumn purification procedure was performed before the RT step (Fig. 2). This was necessary for adequate amplification (lane 7). As shown in lane 2, when extraction method 2 or 3 was used on infected blueberry blossom tissue, no amplification product was detected. Amplification was achieved using infected *C. quinoa* (lane 3), but addition of healthy blueberry tissue to an infected *C. quinoa* sample during the RT step (lane 4) or the PCR step (lane 6) inhibited amplification.

BBScV was detected consistently from blueberry blossoms using the glycine/phenol or LiCl extraction protocols and Elutip-r minicolumns (Fig. 3). BBScV was also detected from infected blueberry leaf tissue by RT-PCR when LiCl nucleic acid extraction and Elutip-r minicolumns were employed (results not shown). Consistent amplification was achieved when the amount of leaf tissue was increased to 0.4 g.

Using purified nucleic acid as template, the lower limit of detection by RT-PCR was 10 fg of vRNA and 100 fg of plasmid DNA. The lower limit of detection for both vRNA and plasmid DNA was 100 pg by spot hybridization using the ^{32}P -labeled plasmid probe (data not shown).

Detection of BBScV from field samples. To examine the consistency of the

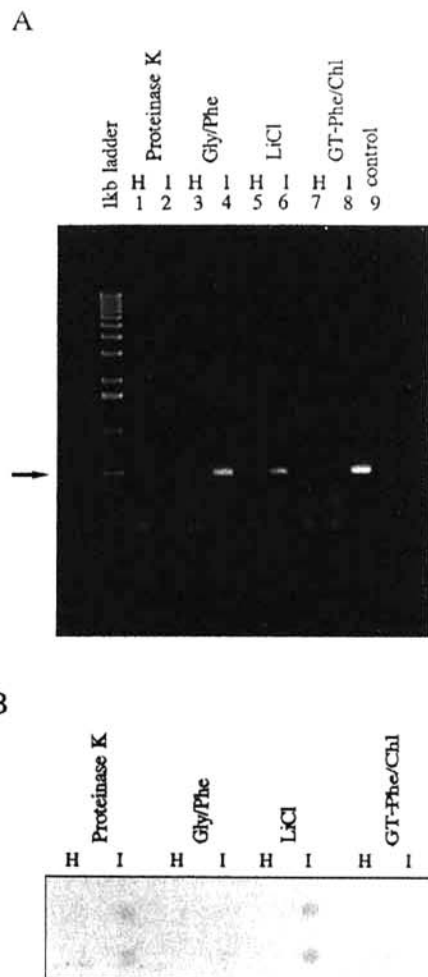


Fig. 1. Detection of blueberry scorch virus (BBScV) from healthy (H) and infected (I) *Chenopodium quinoa* leaves comparing the four extraction methods described in Materials and Methods using reverse transcription-polymerase chain reaction (RT-PCR) and spot hybridization. (A) Ethidium bromide-stained agarose gel of reverse transcription-polymerase chain reaction (RT-PCR) amplified products. Arrow indicates predicted 511-bp product. Lanes 1, 3, 5, and 7, healthy leaves; lanes 2, 4, 6, and 8, infected leaves; lane 9, plasmid control. (B) Autoradiograph of spot hybridization with ^{32}P -labeled cDNA plasmid representing BBScV sequences.

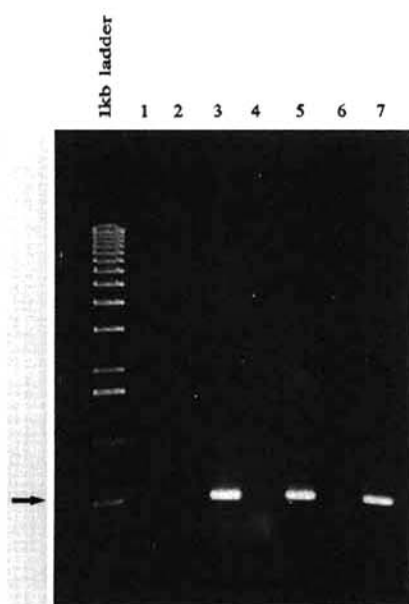


Fig. 2. Inhibition of reverse transcription (RT) and polymerase chain reaction (PCR) by blueberry blossom extracts. Lane 1, healthy blueberry blossom extract; lane 2, infected blueberry blossom extract; lane 3, infected *Chenopodium quinoa* leaf extract; lane 4, infected *C. quinoa* extract and healthy blueberry blossom extract; lane 5, plasmid control (1 ng); lane 6, PCR plasmid control with blueberry blossom extract; lane 7, infected blueberry blossom extract with Elutip-r column purification step.

RT-PCR assay method for routine detection of BBScV, field samples were collected for 5 years and tested. Figure 4A shows results from some field samples tested during spring 1993. Blossoms from six different plants consistently tested positive for BBScV (lanes 1 to 4, 8, and 9). PCR products of the expected size were amplified from 1:2 and 1:4 dilutions of 0.05 g of infected blossom tissue (lanes 13 and 14). Specificity of these primers to BBScV was demonstrated by their failure to amplify from samples infected by blueberry red ringspot caulimovirus (6) (lanes 15 and 16). Results from field samples using spot hybridization are shown on Figure 4B. Blossoms from the same six plants (lanes 1 to 4, 8, and 9) hybridized to 32 P-labeled plasmid DNA probe.

DISCUSSION

In this study, we showed that RT-PCR is sensitive and effective for detecting strain NJ2 of BBScV from blueberry tissue with proper sample preparation. A single infected blossom in a pooled sample could be detected by RT-PCR.

We evaluated four total nucleic acid extraction methods. We were unable to amplify products from *C. quinoa* and blueberry blossom tissue extracted using method 1 (proteinase K extraction), even when Elutip-r column purification was included. Method 4 (GT-phenol-chloroform extraction) resulted in a faint PCR band from infected *C. quinoa* tissue, but

results did not improve to a satisfactory level with additional purification steps. Method 3 (LiCl extraction) produced consistent results with infected *C. quinoa* tissue, but was inconsistent when used with blueberry tissue. Inclusion of an Elutip-r column purification in the LiCl extraction protocol resulted in consistent amplification. Finally, method 2 (glycine/phenol extraction) produced nucleic acid from *C. quinoa* that was amplified by RT-PCR, but gave consistently negative results from blueberry tissue when used alone. Inclusion of an Elutip-r column purification step in the glycine/phenol procedure resulted in amplification similar to that obtained by the LiCl/Elutip-r column method. Therefore, the LiCl and glycine/phenol methods are sensitive and reliable for detecting BBScV from blueberry blossoms when used with Elutip-r purification. Hadidi et al. (4) also reported improved detection of RNA viruses by RT-PCR following Elutip-r column purification. We typically use the LiCl/Elutip-r column procedure to prepare RNA from blueberry plants for RT-PCR.

We found RT-PCR to be more sensitive than spot hybridization with a 32 P-labeled

probe. Both indirect ELISA and direct tissue blotting using alkaline phosphatase-based detection systems gave accurate and consistent results with *C. quinoa* tissue but unacceptably high backgrounds with blueberry tissue (results not shown). Sandwich ELISA was reported to be useful for BBScV detection from mature leaves, but not from immature leaves or leaves beginning to senesce (7).

BBScV RNA can be amplified and detected from infected blueberry tissue in a relatively short time with proper sample preparation. The relative expense of the Elutip-r columns would preclude their use on a large-scale basis, such as for epidemiological studies. However, for identification of virus from a newly infected field or propagation material, RT-PCR is a useful alternative detection method.

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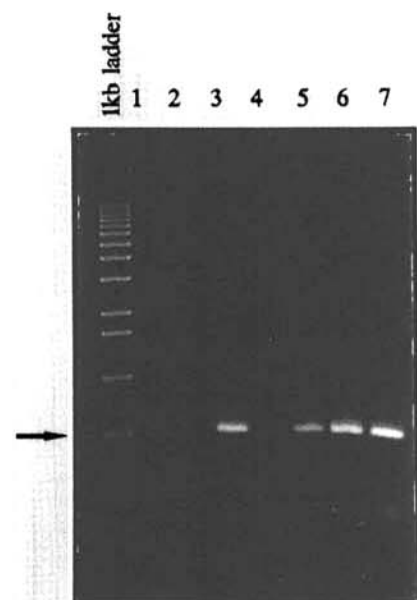


Fig. 3. Detection by reverse transcription-polymerase chain reaction (RT-PCR) of blueberry scorch virus (BBScV) from blueberry blossoms. Lanes 1 to 3, glycine/phenol extraction (method 2) applied to healthy (lane 1) and infected (lanes 2 and 3) blueberry blossoms. The extract in lane 3 was purified through an Elutip-r minicolumn before RT-PCR. Lanes 4 to 6, same as 1 to 3, but using LiCl extraction (method 3). Lane 7, 1 ng of purified viral RNA subjected to RT-PCR.

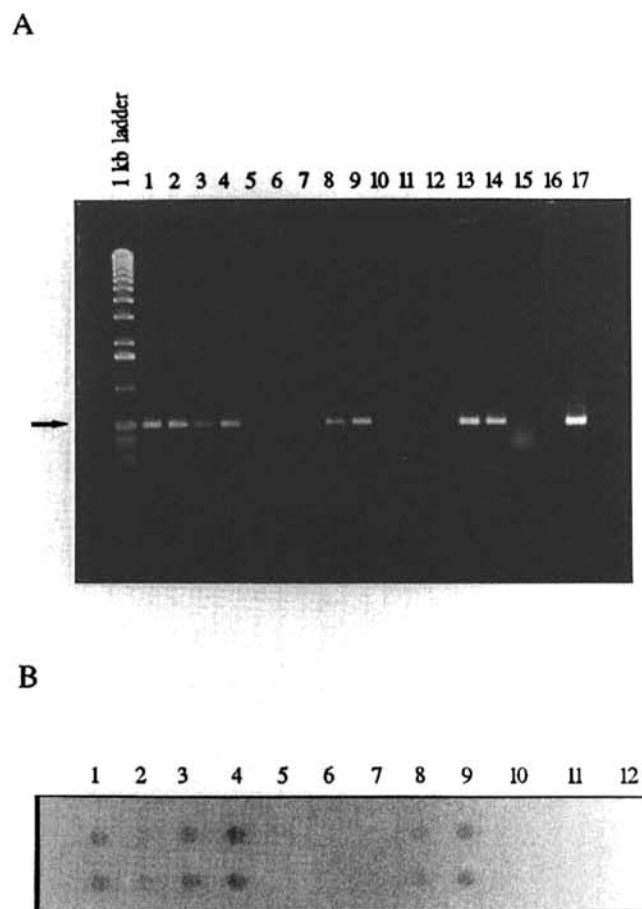


Fig. 4. Consistency and specificity of reverse transcription-polymerase chain reaction (RT-PCR) for detection of blueberry scorch virus (BBScV). (A) Arrow indicates expected 511-bp fragment. Lanes 1 to 12, blueberry blossom extracts from field samples; lanes 13 and 14, two- and fourfold dilutions of blossom sample; lanes 15 and 16, BBScV primers used on blueberry tissue samples infected with red ringspot virus; lane 17, PCR plasmid control. (B) Autoradiograph of spot hybridization performed as in Figure 1. Lanes 1 to 12, blueberry blossom extracts from the field samples used in panel A.

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