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Relative Titer of Strawberry Vein Banding *Caulimovirus* and Comparison of Diagnostic Procedures

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Received: December 16, 2016; **Published:** February 01, 2017

Abstract

End-point PCR and gel analyses were used for detection and relative quantification of strawberry vein banding virus. In order to overcome the severe inhibitory effects of host derived components on *Taq* DNA polymerase activity, two different viral DNA isolation procedures were optimized to allow quick isolation of total nucleic acids prior to PCR. Different types of tissues obtained from infected strawberry plants including a virus indicator and three commonly growing cultivars were used to determine the most suitable tissues for virus testing and evaluate different diagnostic procedures. Nucleic acid preparations were diluted 10 - 10,000 folds in sterile water to determine the detection limits of PCR and the relative virus titer of samples based on dilution end point assay. The highest virus titer was found in old symptomatic leaves, whereas the lowest was in petioles. However, virus titer greatly varied from one host to another in similar tissues. To determine the PCR inhibition limits, concentrated preparations (up to 100x higher than those used in routine diagnosis) were used as templates. False negative results were interpreted as inhibition of *Taq* DNA polymerase activity by host derived contaminants with the highest levels in terminal roots and the lowest in young terminal leaves.

Keywords: *Limits of PCR inhibition/detection; Methods of Nucleic Acid Extraction; Systems of virus diagnosis*

Abbreviations

SVBV: Strawberry vein banding virus

Introduction

Diagnostic History of SVBV

SVBV is a member of the genus *Caulimovirus* which belongs to family *Caulimoviridae*. Several SVBV detection methodologies have been developed since its discovery by Frazier in 1955 [1]. SVBV diagnosis based upon symptomatology alone [2-8] has been shown to be inadequate to diagnose virus-infected plants in the field. This led to the development of indexing procedures for diagnosis or confirming field observation of SVBV infection [9,10].

With inability in purifying virions to raise specific antibodies, dot blot hybridization with a cloned-DNA probe was introduced as an alternative to ELISA [11]. However, this method required specialized laboratories for handling radioisotopes. Using a high-titer indicator plants (e.g., *Fragaria vesca* var. semperflorens) in a dot blot hybridization assay, Stenger and co-workers [11] reported that the virus could be detected from a minimum of 100 µg fresh tissue. However, they did not report SVBV detection from low titer cultivars. A non-radioactive probe and dot blot analysis was used for SVBV detection [12]. The sensitivity of this method (150 µg tissue from indicator strawberries) was not suitable for reliable detection of infected cultivars. This procedure was further modified by first using PCR for amplification of SVBV DNA and then blotting the PCR products on a membrane and detecting amplified viral DNA with a chemoluminescent dot blot analysis [12].

The objectives of this investigation were: 1) to develop PCR based methodologies for SVBV detection, 2) to develop a simple sample preparation procedure for testing strawberry plants without dependence to biotech kits, 3) to evaluate the sensitivity, efficiency, and reproducibility of the developed procedures, 4) to determine the highest and lowest concentrations of the templates prepared from various tissues and hosts where virus could be detected.

Methods and Materials

Virus Sources and Maintenance

UC-5 strawberry plants (*Fragaria vesca* L.) infected with a California isolate of SVBV were used as the main source of infected tissue in this study. These plants were originally inoculated by leaf-grafting and subsequently propagated vegetatively. F. x *ananassa* cvs. Carlsbad, Pacifica, and Seascape were inoculated by infiltrating with *Agrobacterium tumefaciens* LBA4404 cells carrying pCGN1547SVBV1.25mer construct [13]. Non-infected samples were obtained from virus free-plants that had been generated by heat-treatment and meristem-tip culture.

Sample Preparation

Method I

A sample preparation (Method I) procedure was developed by modifying the Method 4 of Rowhani and coworkers [14] and used in all the experiments to test the inhibition and detection limits [9,10,15]. Briefly, 250 mg of fresh strawberry tissue was homogenized in 5.0 ml grinding buffer (100 mM sodium or potassium phosphate, 2% [w:v] polyvinylpyrrolidone-40 [PVP-40, Sigma], 50 mM Benzoic Acid, 0.2% [v:v] β-mercaptoethanol, pH 7.0) using grinding bags and a mechanical homogenizer (Lenze, Homex 6, Bioreba Ag., Switzerland). A microfuge tube was filled with clear homogenate and tubes were centrifuged at 1,300 *g* for 3 min. One ml of the supernatant (tissue extract equivalent to 50 mg) was further centrifuged at 16,800 *g* for 30 min at 4°C, and the pellet was suspended in 0.5 ml of disruption buffer (50 mM Tris, 10 mM EDTA, 2.0% [w:v] SDS, 0.2% [v:v] β-ME, pH 8.0). The suspension was incubated at 65°C for 30 min, and then chilled at –10°C after adding 1/3 volume of 4.0 M potassium acetate (KAc), pH 5.5. The supernatant was recovered after centrifugation at 16,800 *g* for 10 min, and DNA was precipitated by adding an equal volume of isopropanol followed by chilling at –80°C for 15 min. The nucleic acids were pelleted by centrifugation at 16,800 *g* for 20 min at 4°C. The pellet was rinsed with 70% ethanol, air-dried, dissolved in 50 µl of sterile water, and stored at –20°C to be used in PCR.

Method II

This alternative procedure, originally developed for dot blot hybridization analysis, was also used for PCR [9]. The tissue (250 mg) was homogenized in 5 ml of the above mentioned virus disruption buffer containing 2.0% PVP-40. Grinding bags containing the homogenates were immediately transferred to water bath (65°C) and incubated for 30 min. A volume of 1.0 ml from the extract (equivalent of 50 mg tissue) was transferred to a microfuge tube, and 600 µl KAc (4.0 M, pH 5.5) was added to the final concentration of 1.0 M. After brief chilling at –10°C, the extract was clarified by centrifugation at 16,800 *g* for 10 min, and precipitated with an equal volume of isopropanol. A colorless and viscous nucleic acid pellet was obtained. The pellet was suspended in 0.5 ml of sterile water, and the suspension was used in dot blot or PCR analyses.

DNAeasy Kit

The DNAeasy kit (Qiagen®, Valencia, CA) was used for isolating genomic DNA from plant tissues according to manufacturer's instruction.

PCR Detection and Analysis

PCR reactions were performed in final volumes of 20 µl containing 1-10 µl of template in a Perkin Elmer 9600 thermal cycler. The reaction conditions were 2 min of denaturation at 95°C followed by 35 cycles of 95°C for 30 sec (denaturation), 57°C for 30 sec (annealing) and 72°C for 1.0 min (elongation) followed by 7 min at 72°C (extension). The primer pair used in this study directed amplification

of a 944-bp fragment of the coat protein gene (sense 5'-ATGGTAAGCAGAAGAGAAAGA-3', position 1890-1910, and complementary sense 5'-GGACAACACATATTTCTACGTA-3', position 2833-2811).

The colorimetric PCR detection methodology described by Rowhani and coworkers [16] was used with representative samples to confirm the results obtained by visual detection of PCR-amplified DNA fragments in agarose gel (see next). A biotinylated primer (Bio-5'-GGTGGACTAACTCTAGAAGA-3', position 2969-2950) hybridizing with the 887-bp PCR products generated from CP gene was used in these analyses. These reactions were conducted in duplicate wells, and the average optical density (OD_{405nm}) readings were corrected by subtracting from background values generated in healthy control reactions.

Dot-blot Analysis

DNA suspensions prepared by Method II were used in the dot blot analysis. Equivalent amounts of DNA suspensions isolated from 1.0, 5.0, and 25 mg tissue were denatured with an equal volume of 1.0 M sodium hydroxide at room temperature for 10 min [17]. The denatured DNAs were further diluted to a final volume of 200 µl with 0.5 M NaOH, and then applied to High Bond membranes (Amersham) briefly presoaked in 0.5 M NaOH. A vacuum dot blot manifold (Bio Rad) was used to blot the samples onto the membrane. The membranes were immediately placed in a UV chamber (GS Gene Linker, Bio Rad, Foster City, CA), and DNA was cross-linked to the membrane at 150 milli-joules.

Dot blot hybridization analysis and colorimetric detection were done according to manufacturer's recommendations (Boehringer Mannheim®; Miltenburg., *et al.* [18]). The membrane was treated with standard hybridization buffer at 60 °C in a hybridization incubator (Model 310, Robbins Scientific) for 2.0 h and then hybridized overnight with digoxigenin-11-dUTP-labeled probe at the same temperature (the standard temperature of 68 °C was not used due to the low GC content of SVBV DNA). The DNA probe was synthesized by random priming of pSVBV-E3 DNA with Klenow fragment and 5% dig-11-dUTP/95% dTTP in 0.2 mM dNTP mix according to the manufacturer's recommendations. Washing, reaction with the anti-dig antibody (Fab fragment) conjugated with alkaline phosphatase, and colorimetric detection was conducted according to the standard protocols of Boehringer Mannheim [18]. Five percent non-fat dry milk in PBS containing 0.5% Tween-20 (PBST) was used for blocking membranes before reacting with antibody [19]. Excess antibody was washed away with PBST instead of using maleic acid washing buffer.

Data Collection and Analysis

A comparison of the detection and inhibition limits was performed for three DNA extraction procedures: Method I, Method II, and the DNAeasy kit. Symptomatic leaves from a SVBV-infected UC-5 plant and virus-free leaves from a non-infected UC-5 plant were used as controls. For each procedure, three DNA extracts from infected and one from uninfected leaves were prepared.

To evaluate the optimized detection procedure presented in this study, the inhibition/detection limits of PCR were determined for seven types of tissues: symptomatic lower (old) leaves, middle-aged leaves, young terminal leaves, petioles from middle-aged leaves, runner tips, flowers (clipped from peduncle), and terminal roots obtained from strawberry cultivars. Three UC-5 plants and three selected cultivars were examined. Three samples from each of the seven tissue types were tested.

Colorimetric PCR analysis was performed to confirm the visual evaluation of PCR-amplified DNA fragments in agarose gels (see next). Preparations from UC-5 samples (3 plants X 3 replicates) were pooled and representative samples were analyzed by standard and colorimetric PCR.

Dot blot hybridization was conducted to assess the final inhibition/detection data obtained by the PCR. Tissues containing the highest (old symptomatic leaves) and lowest virus titer (petioles) respectively from UC-5 and Seascape were used in this analysis. Each infected sample was tested three times. DNA was prepared by Method II. Sterile water and SVBV DNA (pCGN1547SVBV1.25mer) of known concentration were used as the negative and positive controls, respectively. The positive control was also used to estimate the virus titer in the infected samples. All of these preparations were also tested by PCR.

In the PCR analysis on the pooled samples, 50 mg of symptomatic young leaves from SVBV-infected UC-5 plants was mixed with 4.95 g of uninfected leaves. These samples were ground in liquid nitrogen followed by homogenization in 100 ml grinding buffer by a Polytron®. Three sub-samples of clarified supernatant including 1.0 ml, 1.0 ml, and 70 ml were used for DNA extraction by Method I. The procedure was scaled up for the 70-ml sample. The final products were suspended as equivalents of 1.0 mg tissue in 1.0 µl of sterile water. Further dilutions were made in sterile water and PCR was performed as described previously. Control samples (250 mg each) from uninfected plants were processed accordingly.

Relative Quantification and Data Analysis

DNA samples, prepared by Method I, were used in the PCR. Samples were diluted at 10, 100, 1,000 and 10,000 fold with sterile water. One, 3 and 10 µl of the original preparation and 1 and 3 µl of each of the diluted samples were used in a 20-µl PCR reaction. The quantity of DNA used in each reaction is presented as the equivalent amount of tissue used to isolate the template. Efforts were made to maintain the related variables as consistent as possible.

The entire 20-µl PCR product was analyzed by agarose gel electrophoresis in 1.0% agarose (Gibco BRL®) using Tris-acetate-EDTA buffer (TAE) for 15-20 min at 100 volts. Gels were stained with EtBr (1 µg/ml), and de-stained for 10 min in deionized water before photographing by a Kodak digital camera (model KDS 1D) at an exposure time of 2.0 sec. The brightness of the DNA fragments (bands) in the gels was rated from zero (no signal or band) to 6.0 (the highest brightness) as shown in Figure 1. The brightest band represented optimal levels of the DNA template and minimal concentration of PCR inhibitors (i.e., appropriate dilution) in strawberry tissues. Weak staining or no amplified DNA fragments with high concentrations of sample DNA was indicative of the inhibitors in the DNA samples. Weak staining or no amplified DNA fragments at low concentrations of sample DNA indicated the PCR detection limit.

All raw data from PCR analysis of inhibition/detection limits were used in a statistical analysis. Super ANOVA software (version 1.01, UC Berkeley) executed in a Macintosh computer was used for all computations, analysis of variance, and mean comparisons (Duncan Multiple Range Test, $P = 0.05$). All replicated experiments were analyzed as 2 (tissue type and replicate) or 3 factors (tissue type, host type and replicate) in the ANOVA. Interaction plots were generated by incorporating effects of two factors (i.e., tissue type X host type). Colorimetric PCR and dot blot hybridization assays were compared with the result from PCR analyses of quality confirmation. These experiments were not statistically analyzed due to low values of degrees of freedom for experimental error.

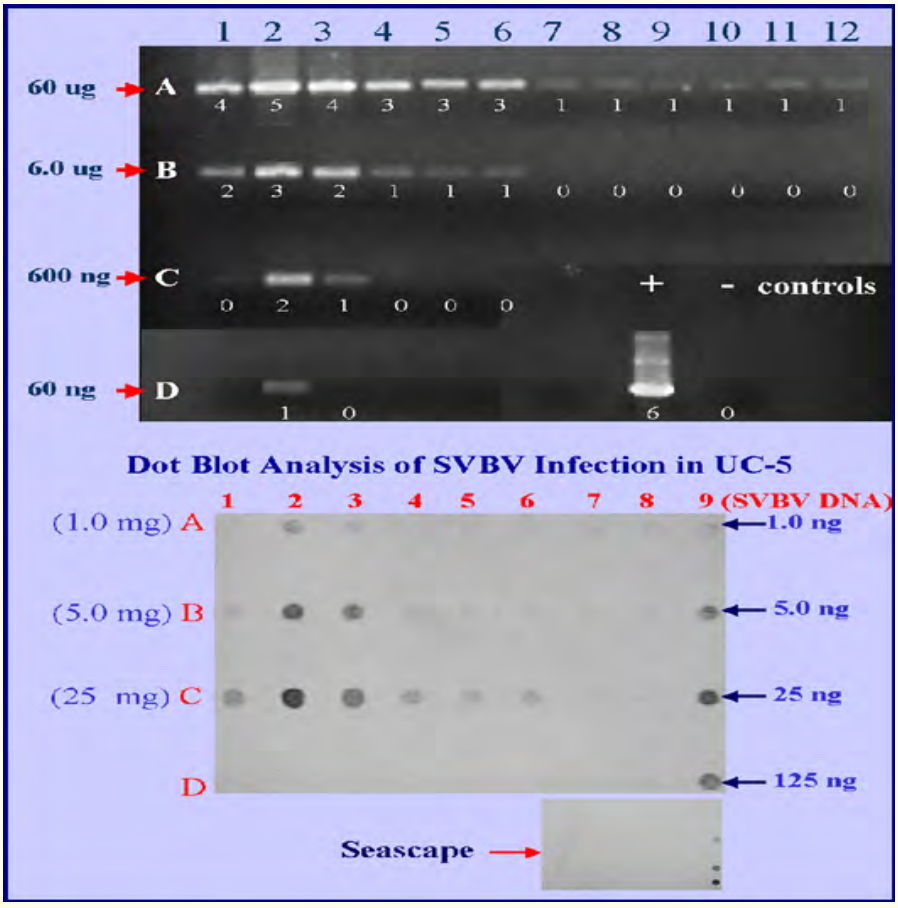


Figure 1 (above): *Visual rating system of PCR product: PCR-amplified SVBV DNA fragments in EtBr-stained agarose gels. Total nucleic acids were prepared from samples of symptomatic UC-5 leaves (lanes 1, 2, and 3) and petioles (lanes 4, 5, and 6), Seascape old asymptomatic leaves (lanes 7, 8, and 9) and petioles (lanes 10, 11, and 12) using Method II for DNA extraction. PCR signals from total DNA extracts equivalent to 60 µg, 6.0 µg, 600 ng, and 60 ng of fresh tissue are presented in rows A, B, C, and D. pSVBV-E3 DNA (1.0 ng) was used as a positive control and PCR reagents with no DNA template were used as a negative control. The numerical values under the bands represent the visual ratings. The most intensely stained DNA fragment (i.e., the positive control) was assigned the highest rating (6), whereas no amplified fragment (i.e., the negative control) was assigned the lowest rating (0).*

Figure 1 (below): *Detection of SVBV in infected tissues by dot blot analysis: Total nucleic acid preparations obtained from PCR-positive UC-5 (enlarged image) and Seascape (reduced image) were tested in this analysis. Pooled preparations from UC-5 older symptomatic leaves obtained from 3 plants were blotted in lanes 1, 2, and 3, and DNA extracted from tissue equivalent to 1.0, 5.0, and 25 mg fresh tissue were blotted in rows A, B, and C. Preparations from 3 petiole samples were blotted in lanes 4, 5, and 6. The samples of healthy leaf and petiole DNA were blotted in lane 7. The diluting buffer alone (0.5 M NaOH) was applied in lane 8, and the known amounts (1.0, 5.0, 25, and 125 ng) of SVBV DNA (pCGN1547SVBV1.25) were blotted in lane 9. Similar preparations from Seascape (the cultivar with the lower virus titer) were applied to the second membrane shown as a reduced insert image). The blotted membranes were hybridized with a dig-labeled SVBV probe and were visualized by Boehringer Mannheim colorimetric detection (Miltenburg., et al. 1995).*

Results

Sample preparation procedures

Results from comparing three DNA extraction procedures including Method I [14] Method II [9], and the DNAeasy kit (Qiagen®) by relative quantification of the inhibition/detection limits in a PCR diagnostic system provided that the inhibition limits for Method I and its simplified version (Method II) were similar (Figure 2). DNA recovered with the DNAeasy kit had 10-fold higher PCR inhibitory for detection of SVBV DNA compared with the same concentration of DNA recovered by the other methods. Based on determination of the detection limit (dilution end point), Method II was slightly more sensitive than Method I, as brighter bands were obtained with template equivalent to 100 ng infected tissue. The detection limit of the DNAeasy preparation, a faster procedure, was 10-fold less sensitive than the other methods (Figure 2).

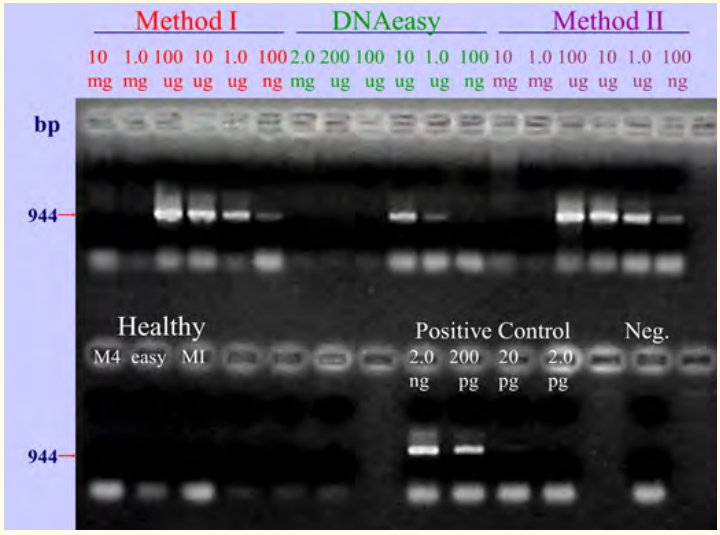


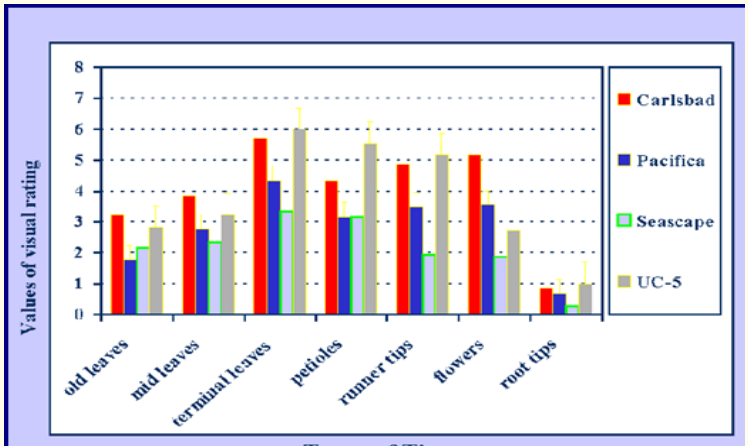
Figure 2: *Relative quantitative comparison of three sample preparation methods: Old symptomatic leaves of UC-5 strawberry were used in PCR analysis after DNA extraction by Method I, DNAeasy, and Method II. Different concentrations of DNA presented as equivalent fresh tissue weight were used in PCR to demonstrate levels of relative inhibition and detection as influenced by each procedure. No signal was detected in non-infected samples prepared by each procedure.*

Detection Methodology

PCR analysis indicated that the highest and lowest SVBV titer was in the older symptomatic leaves and petioles of UC-5 and Seascape respectively. To confirm these results, the tissues and hosts were tested by dot blot analysis (Figure 1). The DNA preparation used for dot blot analysis was also tested by PCR analysis (Figure 1). In dot blot analysis, the entire 25 mg samples did not pass through the membrane due to the high concentration of host contaminants that plugged the pores in the membrane. In different experiments, it was shown that preparations from 10 mg tissue were the maximum amounts of DNA extract that could be applied onto the membranes (data not shown). The signals from total nucleic acids from SVBV-infected plants obtained in this analysis were compared to those in lane 9 (known amounts of SVBV cloned DNA) to quantify the virus titer. The amount of signals in lanes 1-3 in row B are roughly equal to signals from 1.0, 15, and 5.0 ng DNA per 5 mg tissue, respectively. Petiole samples of UC-5 that did not produce any signal would contain less than 1.0 ng DNA per 5 mg tissue. (Samples in row C could not be quantified accurately, because they were not entirely blotted onto the membrane).

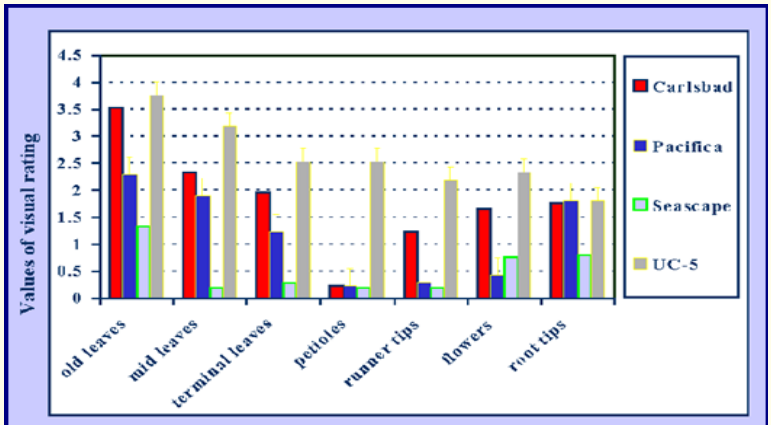
Tissue specific detection and inhibition

Using Method I procedure, consistent or similar data were obtained in all experimental replicates in studying relative tissue distribution of the virus and range of tissue-specific PCR inhibition. Each experiment was repeated 3 - 10 times by sampling tissues from different plants. No significant variations were observed among these replicates ($P = 0.05$). The highest rate of virus detection was observed in old symptomatic leaves and the lowest in leaf petioles. The inhibition was strongly observed in root samples, which tittered intermediate followed by old leaves. The minimal inhibition was observed on young leaves and petioles using template concentration of 10-100x of what used in routine detection. The consistency of these data was confirmed by extending the survey to three commonly growing strawberry cultivars. Tissue-specific inhibition (using concentrated DNA templates in PCR) from one host to another is shown in Figure 3. Due to consistency of the data, mean numerical values from 4 different hosts for each tissue type are compared in accompanied table (Figure 3). Similar analyses were conducted to survey detection limits or relative virus titer by using diluted DNA templates in PCR. Consistent trend of tissue-specific virus titer with considerable variation among the hosts is shown in Figure 3. Inhibition and detection ranges of terminal leaves in different hosts, as averaged from 3 replicates, are illustrated in Figure 4.



Tissue Type	PCR Signal	Group Letter
Terminal roots	0.694	a
Old symptomatic leaves	2.500	b
Mid leaves	3.042	b c
Floral Structures	3.333	c d
Runner Tips	3.875	d e
Petioles	4.056	e
Young terminal leaves	4.847	f

Figure 3: *Interaction plot (type of tissue X type of host) of PCR inhibition range: This assay was determined in different tissues/hosts by visual rating of PCR-amplified SVBV DNA fragments in EtBr-stained agarose gels. Values in Y axis represent mean for 3 replicates at 6 concentrations (equivalents of 10 mg, 3.0 mg, 1.0 mg, 300 µg, and 100 µg fresh tissue weights). The most intensely stained DNA fragment (i.e., the positive control) was assigned the highest rating (6), whereas no amplified fragment (i.e., the negative control) was assigned the lowest rating (0). No signals were detected in negative controls. In attached table, each value represents mean for 3 replicates at above concentrations using all four different hosts (UC-5, Carlsbad, Pacifica, and Seascape). Mean values were ranked by Duncan Multiple Range Test (P = 0.05).*



Tissue Type	PCR Signal	Group Letter
Old symptomatic leaves	2.726	a
Mid leaves	1.917	b
Young terminal leaves	1.488	b c
Root tips	1.452	b c
Floral Structures	1.143	c d
Runner tips	0.988	c d
Petioles	0.774	d

Figure 4: *Interaction plot (type of tissue X type of host) of PCR detection limits: This assay was determined in different tissues/hosts based on PCR-amplified SVBV DNA fragments in EtBr-stained agarose gels. Each value represents mean of 3 replicates at 7 concentrations (equivalents of 30 µg, 10 µg, 3.0 µg, 1.0 µg, 300 ng, 100 ng, and 30 ng fresh tissue weights). The most intensely stained DNA fragment (i.e., the positive control) was assigned the highest rating (6), whereas no amplified fragment (i.e., the negative control) was assigned the lowest rating (0). In overall detection limit (relative virus titer) UC-5, Carlsbad, Pacifica, and Seascape were grouped from the highest to the lowest respectively. No signals were detected in negative controls. In attached table, each value was obtained from averaging 3 replicates at above concentrations using all four hosts (UC-5, Carlsbad, Pacifica, and Seascape). Mean values were grouped by Duncan Multiple Range Test (P = 0.05).*

Quality control of visual ratings

To confirm the accuracy of visual ratings in PCR analyses, the results were compared with optical density (OD) measurements of the same samples analyzed by colorimetric PCR (Figure 5). Representative samples of these replicates (made by pooling DNA preparations) were used in colorimetric PCR to compare the results from the visual ratings. The signal strength obtained with the colorimetric PCR was comparable to the signals obtained for the visually-rated PCR analysis.

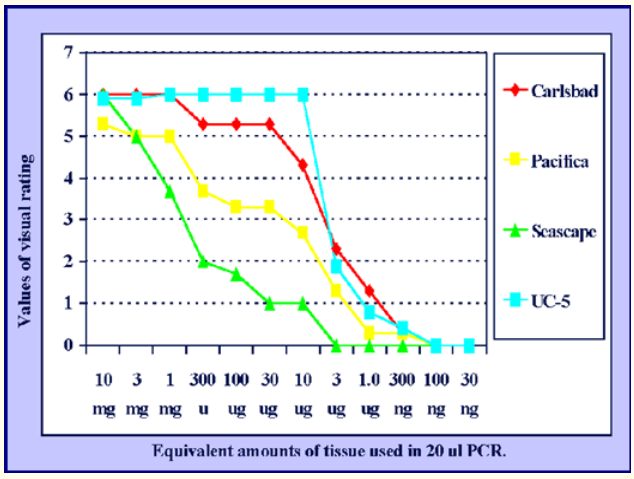


Figure 5: *PCR inhibition range and detection limits: These limits were determined in terminal leaves from 4 different hosts by visual rating of PCR products in EtBr-stained agarose gel. Each value was obtained from averaging 3 replicates. The most intensely stained DNA fragment (i.e., the positive control) was assigned the highest rating (6), whereas no amplified fragment (i.e., the negative control) was assigned the lowest rating (0). No signals were detected in negative controls.*

PCR Analysis of Pooled Samples

Detection of the PCR-amplified SVBV DNA fragment in pooled samples is shown in Figure 6. The PCR-amplified virus DNA was detected after pooling samples from infected UC-5 with 100-folds uninfected tissue and diluting the preparation up to 300 fold.

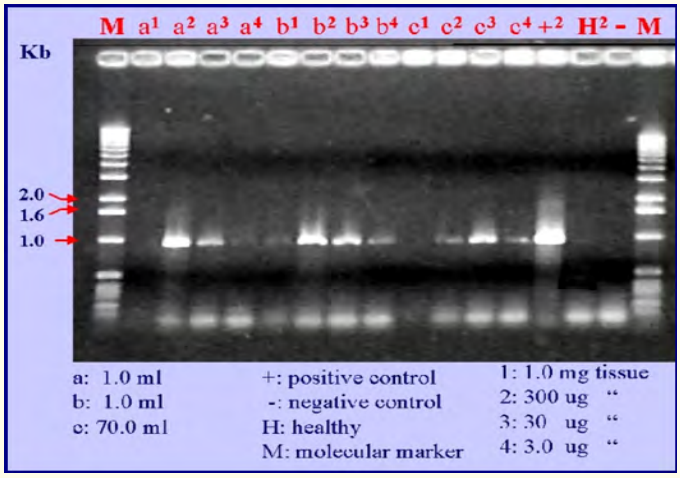


Figure 6: *PCR analysis of pooled samples: DNA was isolated from pooled samples (50 mg of UC-5 symptomatic young leaf pooled with 100 folds non-infected tissue) which were subsequently used in PCR after serial dilution. Templates are represented as equivalent tissue weight used for isolation of DNA. Sample preparation was repeated 3 times, and each template was analyzed in 4 concentrations. A healthy sample was prepared in parallel and applied only in an optimal concentration as negative control. The target DNA fragment is 944 bp.*

Discussion

Strawberry virus indexing [20,21] has been the standard method of virus diagnosis for several decades. Grafting infected strawberries onto several indicators can theoretically allow for diagnosis of all virus and virus-like infections of strawberry. Leaf grafting is a tedious task and requires skilled and experienced technicians. The procedure is also time-consuming and costly. It takes 8 - 12 weeks to obtain the results, and several indicators have to be maintained in the glasshouse. Although false positives are less likely with these tests, false negatives can occur e.g., due to inefficient grafting. Differentiation of virus symptoms from those of nutritional and abiotic symptoms on indicators also can be misleading. Because comprehensive detection procedures are lacking, indexing is still the main diagnostic method used to certify that mother plants are virus-free before mass propagation.

PCR was developed as a tool to detect SVBV in strawberry plants independently from Mráz and co-workers [12,22]. These authors published procedures for SVBV detection by a chemoluminescent dot blot assay (1996) and a PCR/chemoluminescent dot blot assay (1997). Isolation of DNA by the conventional methods (e. g., phenol extraction) produced extracts that contained plant-derived PCR inhibitors. Dilution of these preparations to reduce the concentration of the PCR inhibitors resulted in a reduction of the virus titer so that the virus was not detected by PCR [9]. The use of other DNA extraction methods such as the DNAeasy Kit and Method 4 [14] resulted in positive detection of SVBV by PCR.

The DNAeasy method was not routinely used in this study because of its significant cost per sample. Method 4 [14] which was introduced as Method I in this study after modification, originally designed to isolate DNA from phytoplasma-infected plants, was simplified for extracting DNA from strawberry tissues. This simplified protocol (Method II) reduced the processing time and eliminated the risks of cross-contamination in preparing numerous samples for PCR analysis by reducing the scale and modifying the procedure. Using extracts prepared with Method I did not alter the results of PCR analysis, and results obtained were accurate and consistent (Figure 2). The DNAeasy protocol could not be used for accurate quantification due to losses that occurred in grinding a 50-mg sample in a mortar. It was also shown that limits of inhibition and detection in samples prepared by this kit were lower than Method I (Figure 2).

Quantification of PCR analysis was designed to allow for determination of the limits of confidence in SVBV detection. Interpretation of positive results was straight forward (i.e., the presence of the PCR-amplified SVBV fragments). Negative results were more difficult to interpret, because false negative results could be due to PCR inhibition, low virus titer, or non-infected samples. Therefore, it is essential to incorporate multiple controls to assure the reliability of the procedure. This research has established the proper concentration of DNA template to allow for reliable detection of SVBV from optimal tissues. A standard sample preparation method in this research involves the final suspension in a 10-fold excess volume of original tissue weight, i.e., product of 50 mg tissue would be suspended in 500 µl of sterile water. Multiple PCR analyses were successfully performed with lower amounts of the template (e.g., 1 and 3 µl from series of 10-fold dilutions prepared by adding 20 µl of preparation to 180 µl of sterile water).

Defining the limits of PCR detection in various tissues and hosts was another objective of this study. What is the confidence limit obtaining a positive signal in diluted preparations? What is the influence of each tissue type in this assessment? What is the relative titer of SVBV in a given tissue or host? Are there any significant variations in virus titer across the hosts and the tissues? To answer these questions detection limits of PCR templates were determined. The inhibition ranges were established in various tissues and the host genotypes. The maximum levels of PCR inhibitors were found in preparations from root samples followed by those from older leaves. Floral structures with small fruiting bodies (containing significant quantities of pectin) were shown to be different from the samples lacking the inflated receptacle. Variations in samples of floral structures were observed in replicates. Consistency of tissue-dependent inhibition was demonstrated across different host genotypes. Young leaves followed with petioles and runner tips had the lowest amounts of PCR inhibitors, whereas middle-aged leaves were intermediate.

However, preparations obtained from old symptomatic leaves provided the best source of DNA for detection of SVBV, and were significantly different from other types of tissue. Furthermore, PCR detection of SVBV was correlated with symptoms, i.e., symptoms were usu-

ally observed in older and lower leaves, which also produced higher signals at the lower dilutions compared to other tissues. These results indicated that a virus indicator (*Fragaria vesca* L. var. UC-5) and a newly released cultivar (*F. x ananassa* cv. Carlsbad), which expressed strong vein banding symptoms, provided higher levels of PCR-amplified SVBV DNA in any given tissue compared to the same tissue in the other tested host genotypes. In contrast, symptomless older leaves produced low PCR signals. In symptomatic leaves, the symptoms are associated with accumulation of inclusion bodies, which are the sites for virus replication and encapsidation [23,24]. However, old symptomless leaves from hosts with latent infection had lower PCR-signal compared to young terminal leaves from the same plant.

The lowest PCR signals were observed in petiole samples. A significant portion of petioles consists of stele (vascular tissue), which is not homogenized efficiently using a mechanical mortar like other tissue samples. Therefore, the virus yield per biomass may be reduced in these samples. It has been reported that long distance movement of *Caulimoviruses* as free virus particles occur in vascular system in the same direction used by photoassimilates from source to the sink [24,25]. SVBV inclusion bodies have also detected in parenchyma cells of vascular system [26]. Therefore, low virus titer in petioles or runners (without leaves) may reflect the absence of a significant amount of the virus in these tissues.

Colorimetric PCR was used as an alternative assessment to confirm the data generated by visual rating. The limited results obtained with this procedure confirmed the reliability of visual rating in PCR analysis and showed that the method would also be used to detect SVBV.

Dot blot hybridization analysis, assayed by colorimetric detection, was optimized by using preparations of total nucleic acids from SVBV infected tissues. This analysis was conducted as an alternative to confirm the accuracy of data generated by PCR analysis (Figure 1). In dot blot analysis, a different sample preparation procedure was designed to recover the total nucleic acids from different strawberry tissues. The sensitivity of the dot blot hybridization method, even under optimal conditions, was not enough to detect low titers of SVBV samples [12]. Therefore, to overcome this problem, higher volumes of the plant extracts were applied into the membrane. Unfortunately, this method did not work properly, because the high viscosity of the homogenate or clarified extract plugged the membrane pores and only small amounts of extract were applied to the membrane. These DNA applied to the membrane either was too small to be detected, or the concentration of plant-derived contaminants was too high so that competed with the DNA binding to the membrane. It was presumed that non-bound DNA would be lost during initial pre-hybridization step. This competition effect of strawberry extracts or homogenates on the blotting of DNA to the membranes was demonstrated by internal controls, where cloned DNA added to the plant extract could not be detected either, but strong signals were detected when the DNA was applied in the absence of plant extract (data not presented).

Other investigators have attempted to optimize dot blot analysis for SVBV detection, and this has involved development of long and tedious procedures for sample preparation [11,22]. With these procedures, higher sensitivities of detection were reported compared with dot blot data presented in this study.

As described in Methods and Materials, colorimetric substrates were used for virus detection in dot blot hybridization. The objective of comparison was to generate quantitative data. The procedures applied in the above-mentioned reports [11,22] or the templates prepared by Method I (in this study) could not be used for quantification of total virus titer. In Method II, total viral DNA was extracted during partial purification and applied onto the membrane. The final signal represented virus in the inclusion bodies, free virions, and naked DNA in the nucleus. The genomic and sub-genomic RNAs probably did not contribute to signal production due to their likely degradation in high-pH denaturation step. Attempt was made to estimate the virus titer in UC-5 tissues (Figure 1).

Dot blot analysis performed in this study provided an alternative assessment of virus titer in old symptomatic leaves of UC-5. Figure 1 clearly demonstrates the two extreme levels of virus titer in old symptomatic leaves vs. leaf petioles as shown by dot blot analysis. This data was also supported by PCR analysis. Figure 1 also illustrates the inability of dot blot analysis to detect SVBV in Seascape. This result agrees with PCR analyses in measuring relative virus titer in different hosts, in which Seascape was found to have the lowest viral titer. However, there are other cultivars with lower SVBV titers than the Seascape [13], and infections in these cultivars probably would not be

detected by dot blot analysis either. High-titer cultivars may be detected by dot blot analysis. That is why Mráz and coworkers [12] used a PCR amplification step before proceeding with dot blot hybridization to test field samples for SVBV infection. While it is true that the sensitivity of colorimetric detection is somewhat lower than ^{32}P and chemoluminescent methods used by Stenger., *et al*. [11] and Mráz., et al. [22], these investigators were also unable to detect the SVBV in any strawberry cultivars. In contrast, SVBV was detected in DNA preparations, from petiole and asymptomatic leaf samples from 3 Seascape plants by PCR.

The DNA extraction method (Method II) used for dot blot analysis was developed late in this study, after completion of the research, based on extracts prepared with Method I. However, due to the simplicity of the method and better recovery of total nucleic acid, Method II is recommended for use in sample preparations for PCR and dot blot hybridization. It should be noted that samples prepared by Method II have 10 - 100 fold higher rates of inhibition compared with those prepared by Method I. Higher quantities of host derived DNA, and possibly polysaccharides are isolated by this procedure. The PCR inhibition could be overcome with 10-100 fold dilution effect.

Findings in respect to inhibition and detection limits established a significant confidence in sampling (choice of tissue) and designing the experiments involved with virus diagnosis. This information will result in the proper sampling and testing of the large number of plants in the field and nursery. Additionally, the information could be considered more generally when preparing samples for PCR testing of other viruses in strawberry plants. Regardless of SVBV, this data established the maximum number of samples from a given tissue that can be pooled in a single PCR without observing inhibition. Of course, this would depend on the virus titer in the given tissue as well. The virus titer also varies in different cultivars [9,10], so designing a pooled sample experiment would depend on the type of host, and the sampling methodology could be readily re-designed with a dilution end-point assay. It is safer to design the mass virus screening in such a way that signals occur in the lower level of saturation point. This would vary from 200 ng to 20 µg of infected tissue per reaction in respect with different tissues and hosts. The maximum number of samples that can be pooled depends on degree of inhibition observed in particular type of tissue, which was shown to be consistent in different types of hosts.

As demonstrated in Figure 6, SVBV DNA from a 50-mg-leaflet of *Fragaria vesca* var. UC-5 could still be detected after diluting 100-fold with extract from uninfected tissue and after further dilution to 300-fold with sterile water. As shown in this analysis (Figure 6), the level of inhibition increased at least 30 fold in pooled samples compared to sample DNA not diluted with healthy DNA as shown in Figure. 3. This increased level of inhibition is due to an increased ratio of inhibitory molecules to viral template or to diluting the virus DNA with 100x inhibitors during sample preparation. On the other hand, the detection limit of pooled samples (3.0 µg tissue diluted 100-fold with non-infected tissue) was similar to those observed for symptomatic leaves (30 ng) as seen in Figure 4. This data showed that samples could be pooled from number of strawberry plants and SVBV could still be detected by PCR.

For PCR detection of field samples, the dilution end point should be determined for any given host not examined in this study. The range of inhibition level should be tested in pooled samples. Root samples are not recommended for virus detection analyses. These samples showed the highest levels of inhibition and relatively lower levels of virus titer. Older leaves are preferred, but only if they are symptomatic. In some cultivars, young terminal leaves have slightly lower titer than the old or middle-aged symptomatic leaves, but they provide good sampling source for virus detection as they have low levels of inhibitors.

In addition to both Method I and Method II, Method 4 [14] and DNAeasy kit also could be used for detection of SVBV. However, according to data presented in this study, young terminal leaves are the best choice for virus testing. The method of choice depends on the matter of PCR inhibition, which varies in respect with number of samples in the pool. To test individual samples, the Method II is the method of choice. The DNA extract should be suspended in excess of 10-100 fold tissue weight to avoid inhibition. For testing large numbers of samples in the pool, Method I is the safest method to use in respect with PCR inhibition. To assure the virus detection, it is necessary to use at least 200 ng of equivalent fresh tissue of each sample or sub-sample in a 20- µl PCR reaction mixture.

Acknowledgements

This study was accomplished at the Department of Plant Pathology and Foundation Plant Service (FPS), University of California, Davis. Hereby, I express my deepest gratitude to Dr. Adib Rowhani for kindly sponsoring the project and Dr. Stephan D. Daubert for being my mentor throughout conducting my project.

Conflict of Interest

I declare that there is no financial interest or any conflict of interest exists.

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