

**Optimization of Environmental Factors for  
Effective Detection of *Bean yellow mosaic virus*  
in *Verbena x hybrida***

by

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## AN ABSTRACT OF THE THESIS OF

Angela L. Obermeyer for the degree of Honors Baccalaureate of Science in Bioresource Research presented on May 21, 2004. Title: Optimization of Environmental Factors for Effective Detection of *Bean yellow mosaic virus* in *Verbena x hybrida*.

Abstract approved: \_\_\_\_\_  
Dr. Robert R. Martin

Horticultural and nursery crops are the leading agricultural commodity in Oregon. Among currently grown nursery plants, *Verbena x hybrida* is a staple ornamental. It is grown and shipped worldwide, thus requiring a high level of certainty concerning the plants' health and potential viability. *V. x hybrida* cultivars have been infected by a potyvirus that has been a sporadic but chronic problem in the floriculture industry for many years. Within the plant, detectable potyvirus titers fluctuate widely, making it difficult to detect in commercial nurseries. Virus detection can also be affected by changing environmental conditions, which also influence virus levels. The objectives of this study were to determine the environmental conditions that optimized detectable virus levels, to observe if the virus could be detected in tissue culture plantlets, to establish the earliest age at which plants could be accurately assayed, and to ascertain the most sensitive and reliable testing method for virus detection. The *V. x hybrida* cultivars in the study were infected 'Lavender Shades' and non-infected 'Twilight with Blue Eye.' Plants were tissue cultured or potted and placed in growth chambers at three temperatures with constant photoperiod and then at three photoperiods with constant temperature. The Enzyme-linked Immunosorbent Assay (ELISA) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) were used as the testing methods. Tests were performed at two week intervals for a duration of two months in each of the two growth chamber experiments. The resulting data support the conclusion that *V. x hybrida* plants should be grown in pots at 26°C and 16 or 20 hour photoperiod for at least 6 weeks before testing with RT-PCR for the most accurate detection of virus.

Honors Baccalaureate of Science in Bioresource Research thesis of  
Angela L. Obermeyer presented on May 21, 2004.

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I understand that my project will become part of the permanent collection of Oregon State University, the University Honors College, and the Bioresource Research Program. My signature below authorizes release of my project to any reader upon request.

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Angela L. Obermeyer, Author

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## **Optimization of Environmental Factors for Effective Detection of *Bean yellow mosaic virus* in *Verbena x hybrida***

### **INTRODUCTION**

The horticulture industry is an important component of the economies of many Western states, including Oregon. In 2002, nursery and greenhouse crops were the leading agricultural commodity in Oregon, with sales exceeding \$727 million (Oregon Agricultural Statistics Service). Among the currently grown nursery plants, *Verbena x hybrida* is a staple ornamental.

*V. x hybrida* is shipped locally and worldwide, thus requiring a high level of certainty concerning the plants' health and potential viability. In addition, reliable disease testing also contributes to the exporting of disease free plants to countries where strict quarantine conditions have been imposed (Katoch *et. al.*, 2002), which is of utmost importance for the export industry.

*V. x hybrida* belongs to the Verbenaceae family, which has over 250 genera. It is a perennial grown as an annual that has a long and vigorous flowering period, lasting from spring into early autumn. The plants grow in clumps that can reach up to one foot high, and can be of a mounding or trailing type. *V. x hybrida* flowers are found in clusters and can be white, red, pink, purple, or peach and often have white centers or eyes. The plant is used for edging, in pots or hanging baskets, or for general display (Guaragna *et. al.*, 2004).



Some cultivars of *V. x hybrida* are infected with a virus from the *Potyviridae* family that has been a sporadic but chronic problem in the floriculture industry for many years. The potyvirus found in *V. x hybrida* is related most closely to the Pea Mosaic Strain of *Bean yellow mosaic virus* (BYMV), having a 95-96% amino acid sequence identity (Guaragna *et. al.*, 2004). BYMV occurs worldwide and infects many legumes and non-legumes (Jones, 1997, Latham *et. al.*, 2001). The majority of the research dealing with BYMV in ornamental plants has been with gladiolus (Park *et. al.*, 1998, Katoch *et. al.*, 2002, Katoch *et. al.*, 2003), lily and tulip (Dekker *et. al.*, 1993). BYMV is transmitted by more than twenty aphid species in a non-persistent manner (Cheng *et. al.*, 1999). The virus is also transferred via contact between plants and/or contact between plants and contaminated objects.

The symptoms of BYMV vary greatly, and include chlorotic vein-banding, yellowing, mosaic mottling, necrosis, and distortion (Shukla *et. al.*, 1994, Hseu *et. al.*, 1987). Symptoms of BYMV in a *V. x hybrida* leaf are shown in Figure 1.



Figure 1. Potyvirus symptoms in *V. x hybrida* leaf tissue.

Most viruses of the *Potyviridae* family induce conspicuous symptoms either permanently or sporadically in their hosts (Shukla *et. al.*, 1994). Some *V.*

*x hybrida* plants infected with BYMV do not express visible symptoms of the virus (Katoch *et. al.*, 2002). The concentration of virus particles is distributed irregularly within plants, and although the site of greatest virus concentration within tissues has been determined for some plant/potyvirus systems (Stein *et. al.*, 1994), this information is unknown for the *V. x hybrida*/BYMV system.

Numerous studies on BYMV in different hosts have shown that the level or titer of the virus found in a plant can vary depending upon environmental conditions such as temperature and photoperiod (Tu, 1989, Merritt and Ting, 1995, Carpenter, 1963, Shukla *et. al.*, 1994). With such variations in titer, the virus can be difficult to detect in a plant. This has proven to be problematic in large-scale plant production, where thousands of infected plants may be produced due to unreliable testing for the virus in the starting material. This undermines the ability of the industry to produce disease free propagation material. It is imperative that symptomless plants be assayed for the presence of the virus before using them for healthy tissue culture propagation (Katoch *et. al.*, 2002). Development of disease-free stock plants and reliable testing protocols are essential for the production of virus-free nursery plants, and ultimately help to control the virus (Katoch *et. al.*, 2003).

Large-scale production of *V. x hybrida* typically is done through tissue culture propagation in greenhouses, through which plants can be produced at an extremely rapid pace. Nodal cuttings are the typical mode through which *V. x hybrida* is propagated (Hosoki and Katahira, 1994). Tissue culture plants are

usually tested for viruses using an Enzyme-Linked Immunosorbent Assay (ELISA), which can detect the presence of a specific virus recognized by an antibody (Kaufman, 1995). ELISA has been used to identify the presence of BYMV in plant tissue (Sasaya *et. al.*, 1998). Although ELISA is a means of virus detection that is available to producers, its utility is limited as the results are highly dependent upon the positive/negative threshold used to analyze the results (Slack *et. al.*, 1996).

Another test for virus detection, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), has also been utilized in the identification of BYMV. The RT-PCR test has been shown to be an improvement over ELISA for the detection of BYMV in gladiolus leaves (Vunsh *et. al.*, 1990). The RT-PCR method involves the amplification of certain virus genetic sequences using specific oligonucleotides as primers. The RT-PCR test is believed to be a more sensitive and accurate method of detection than ELISA. However, RT-PCR is not as readily available to producers due to the need for specialized facilities, equipment, and expertise.

The purpose of this project was to determine whether BYMV, carried along with propagation material of *V. x hybrida*, can be detected reliably in *V. x hybrida* tissue culture or stock plants. The earliest age at which propagation material can be reliably assayed was also determined, as an alternative to testing tissue culture plants. Growth chamber studies were carried out to determine the effect of temperature and photoperiod on virus titer and detection. The temperature and photoperiod that produce optimal detectable BYMV levels within

*V. x hybrida* were ascertained through this component. ELISA and RT-PCR were compared in terms of their sensitivity and accuracy in identification of the virus. The cultivars of *V. x hybrida* studied in this project were the infected 'Lavender Shades' and non-infected 'Twilight Blue with Eye.'

## **MATERIALS AND METHODS**

### **Collection and Propagation of Plant Material**

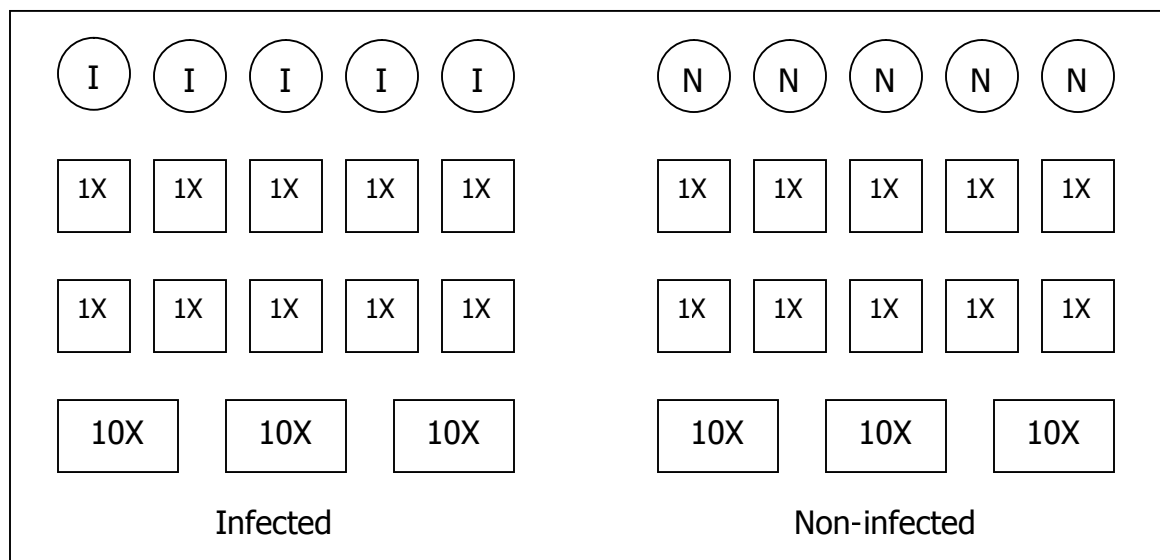
Both infected 'Lavender Shades' and non-infected 'Twilight Blue with Eye' *V. x hybrida* plant samples in tissue culture were provided by the California Florida Plant Company (P.O. Box 5310, Salinas, CA 93915). The samples were from symptomatic or non-symptomatic mother plants. Part of the plants were propagated using nodal cuttings (Hartmann, *et. al.*, 1997) and then tissue cultured in a simple MS medium with normal vitamins (Appendix 1) in magenta boxes. The remaining plants were potted in a commercial peat-based mix (Sun Gro Horticulture, Bellevue, Washington 98008) in four-inch square plastic pots. Plants for the temperature experiment were potted in the greenhouse on September 15, 2003, and tissue cultured on September 16, 2003. Plants for the photoperiod experiment were potted in the greenhouse on February 3, 2004. The plants were not fertilized and were watered when needed. The growth chambers used for the two experiments (Percival, Boone, Iowa 50036) were each lit with six 60 watt Phillips tube lights and four 52 watt Sylvania bulbs.

### **Evaluation of Temperature on Virus Titer**

On October 7, 2003, groups of the tissue culture and potted *V. x hybrida* plants were placed into three separate growth chambers to observe the effects of temperature on potyvirus infection over a period of two months. The chambers were set at a constant 16-hour photoperiod with temperatures of

18°C, 22°C, or 26°C. Both potted and tissue culture *V. x hybrida* were included in the chambers. Half of the plants were presumed infected with BYMV, the other half were presumed non-infected. The plants and their arrangement in each of the three chambers is depicted in Figure 2.

Figure 2. Contents and arrangement of each of three growth chambers in temperature experiment. "I" = potted infected *V. x hybrida*. "N" = potted non-infected, "1X" = magenta box with single tissue culture plantlet, "10X" = magenta box with 10 tissue culture plantlets.



The potted and tissue culture plants in the growth chambers were tested for BYMV by ELISA (Appendix 2, 3), using a monoclonal antibody specific for an epitope that is highly conserved among aphid-transmitted potyviruses, and a standard Goat anti-mouse alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO). The plants were assayed by ELISA at 0, 2, 4, 7, and 9 weeks after being put into the growth chambers.

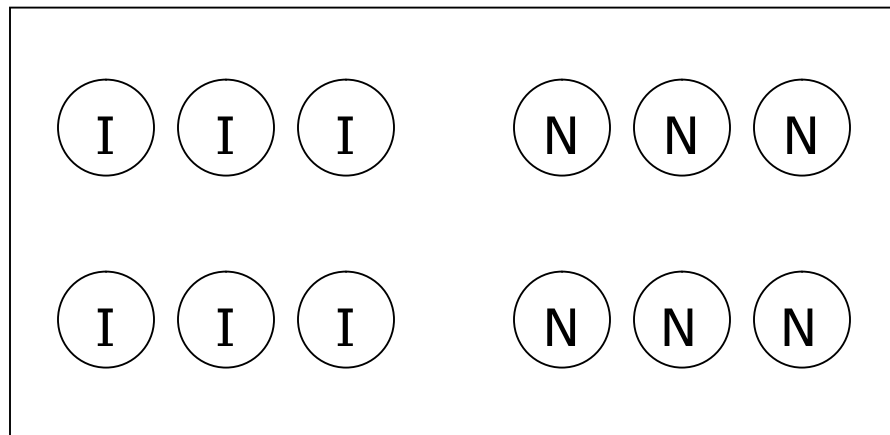
Once the virus titer was at a detectable level by ELISA in any of the chambers, the potted and tissue culture plants were then tested by extracting

single-stranded RNA from leaf tissue (Appendix 4) and implementing RT-PCR (Appendix 5, 6). All samples were tested with RT-PCR using two separate primer sets of VFA/VRA and VFB/VRB (Appendix 7). This was performed at week 6, 8, and 10.

### Evaluation of Photoperiod on Virus Titer

On March 10, 2004, potted *V. x hybrida* plants were placed into three growth chambers to observe the effect of varying photoperiod on BYMV expression. With the constant temperature of 26°C, as determined by the temperature experiment to be the optimal temperature for virus expression, photoperiods of 12, 16, and 20 hours were evaluated for their effect on virus titer. Each chamber contained six potted BYMV-infected and six potted non-infected *V. x hybrida* plants. No tissue culture plants were included in this experiment, since the tissue culture plants did not do well in the temperature experiment. The arrangement of the plants in the chambers is depicted in Figure 3.

Figure 3. Contents and arrangement of each of three growth chambers in photoperiod experiment. "I" = potted infected *V. x hybrida*, "N" = potted non-infected *V. x hybrida*.



The potted plants in the growth chambers were tested for BYMV with ELISA (Appendix 2, 3), using a monoclonal antibody and conjugate as described in the above experiment. The plants were assayed by ELISA at 0, 2, 4, 6, and 8 weeks after being put into the growth chambers.

Once the virus titer was at a detectable level by ELISA in any of the chambers, the plants were then tested by extracting single-stranded RNA from leaf tissue (Appendix 4) and implementing RT-PCR as described previously (Appendix 5, 6). This was performed at week 2, 4, 6, and 8 weeks.

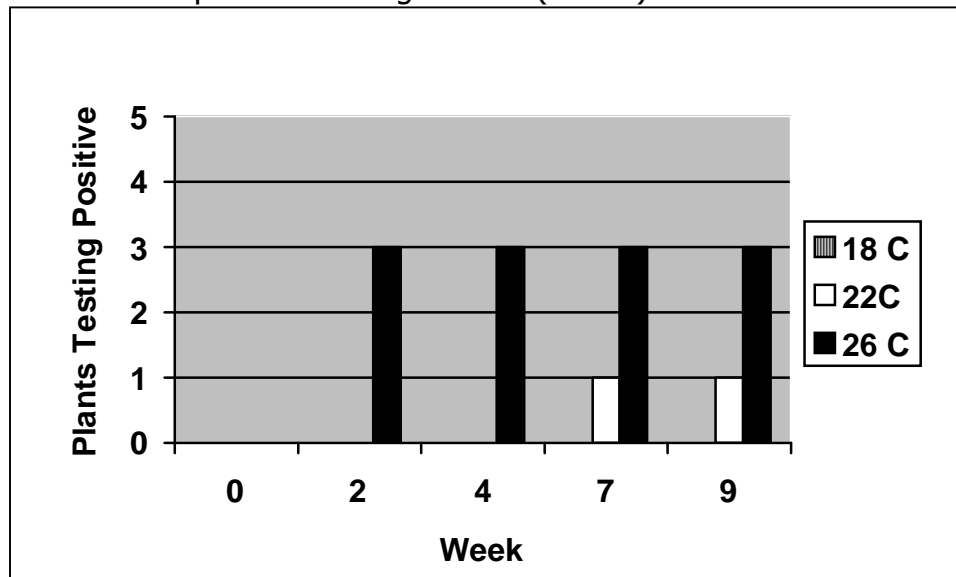


## RESULTS

### Temperature Experiment

The three growth chambers in the temperature experiment were set at temperatures of 18°C, 22°C, or 26°C, and at a constant photoperiod of 16 hours. Significant differences in the effect of temperature on BYMV expression in the growth chamber *V. x hybrida* were observed. The results of the ELISA tests performed at weeks 0, 2, 4, 7, and 9 are shown in Figure 4.

Figure 4. Number of plants that tested positive for BYMV at various temperatures using ELISA. (n = 5)



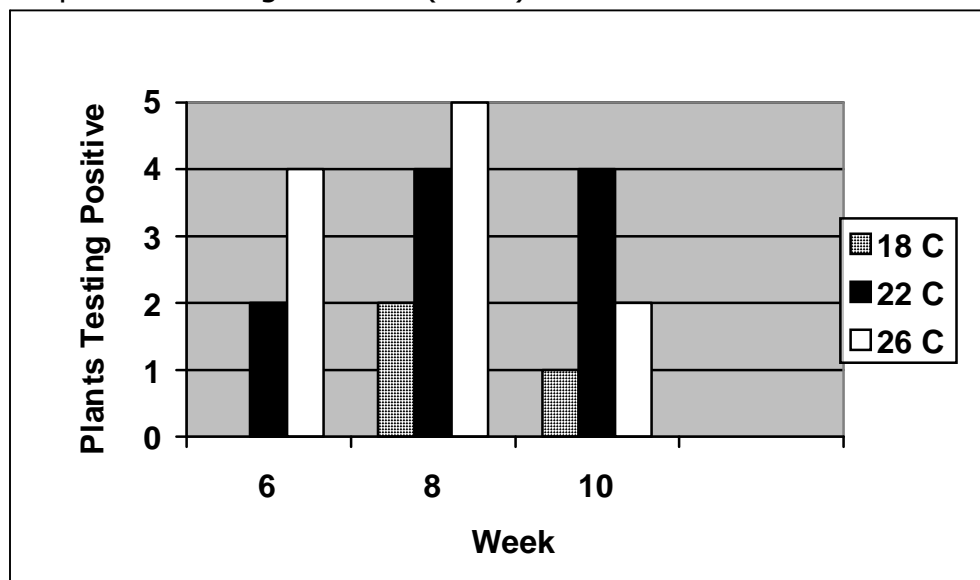
The ELISA tests gave positive results in weeks 2, 4, 7, and 9 of the temperature experiment. Three of six infected potted plants from the 26°C chamber tested positive at each of those testing intervals. Only one infected potted plant from the 22°C chamber tested positive at weeks 7 and 9. None of

the infected plants in the 18°C chamber tested positive at any time throughout the experiment. In addition, none of the infected tissue culture plants from any of the three chambers tested positive in any of the ELISA tests during this experiment.

More plants tested positive for BYMV using RT-PCR as compared to ELISA.

Results of RT-PCR from the temperature experiment are depicted in Figure 5.

Figure 5. Number of plants that tested positive for BYMV at various temperatures using RT-PCR. (n = 5)



Some of the infected potted plants in all three of the growth chambers tested positive for BYMV by RT-PCR at some time during the duration of the experiment. Plants at 18°C tested positive later and less frequently than plants at 22°C and 26°C. The 18°C chamber produced two plants with positive results at week 8 and one plant with positive results at week 10. The 22°C chamber produced two plants with positive results at week 6, four at week 8, and four at

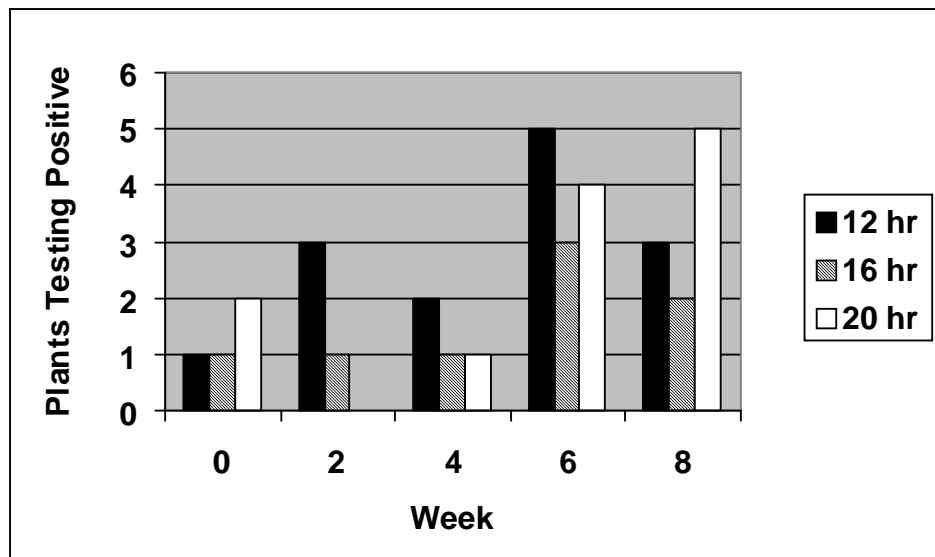
week 10. The 26°C chamber produced four plants with positive results at week 6, five at week 8, and two at week 10. The decrease in positive plants during week 10 at 26°C is due to the death of three infected potted plants. As before in the ELISA tests in the temperature experiment, none of the infected tissue culture plants tested positive by RT-PCR for BYMV at any time.

Because the highest percentage of potted infected plants that actually tested positive in both ELISA and RT-PCR were from the growth chamber set at 26°C, this was the temperature used for the photoperiod experiment.

## Photoperiod Experiment

*Verbena x hybrida* plants were grown at three different photoperiods of 12, 16, and 20 hours, at a constant temperature of 26°C. No tissue culture *V. x hybrida* plants were included in these chambers, since all tissue culture plants tested negative in the temperature experiment. Significant differences were observed in the effect of varying photoperiod on BYMV detection in the potted growth chamber *V. x hybrida*. The results of the ELISA tests performed at 0, 2, 4, 6, and 8 weeks are shown in Figure 6.

Figure 6. Number of plants that tested positive for BYMV at various photoperiods using ELISA. (n = 6)

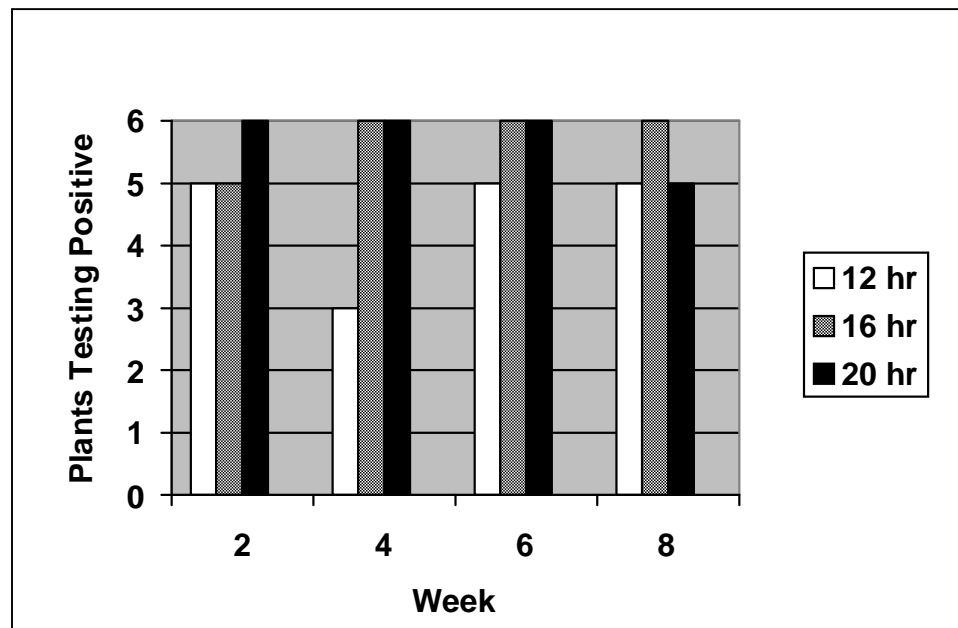


The ELISA tests gave positive results in weeks 0, 2, 4, 6, and 8. One of six infected potted *V. x hybrida* plants at the 12 hr photoperiod tested positive at week 0, three at week 2, two at week 4, five at week 6, and three at week 8. One of six infected potted plants at the 16 hr photoperiod tested positive at

week 0, one at week 2, one at week 4, three at week 6, and two at week 8. Two of six infected potted plants at the 20 hr photoperiod tested positive for BYMV at week 0, none at week 2, one at week 4, four at week 6, and five at week 8.

BYMV detection by RT-PCR in provided a marked increase in the number of plants testing positive for the virus. Results of RT-PCR from the photoperiod experiment are depicted in Figure 7.

Figure 7. Number of plants that tested positive for BYMV at various photoperiods using RT-PCR. (n = 6)



The 12 hr photoperiod chamber produced five infected *V. x hybrida* plants testing positive for BYMV at week 2, three at week 4, five at week 6, and five at week 8. The 16 hr photoperiod chamber had five infected plants testing positive

for the virus at week 2, six at week 4, six at week 6, and six at week 8. The 20 hr photoperiod chamber had all six infected plants testing positive for BYMV at week 2, six at week 4, six at week 6, and five at week 8.

## **DISCUSSION**

In this project, environmental factors of temperature and photoperiod were optimized in terms of maximizing virus titer in *Verbena x hybrida* leaf tissue. In addition, the virus detection methods of ELISA and RT-PCR were evaluated in terms of their sensitivity in detecting the presence of BYMV in *V. x hybrida*.

In the experiment to optimize temperature, it was observed that tissue culture plantlets were not optimal for virus detection of BYMV in *V. x hybrida*. Throughout the entire experiment, there were no infected tissue culture plants that tested positive for the BYMV by either ELISA or RT-PCR assays. In addition, high contamination and mortality rates were observed in the tissue culture plants, especially at the higher temperatures, thus lowering their overall viability in the experiment. It was determined that virus detection in potted plants was much more reliable than in tissue culture plants.

Of the temperatures used in the first experiment, 26°C was the best for inducing detectable amounts of BYMV in *V. x hybrida*. However, since we did not test higher temperatures, it is not completely clear that 26°C is optimal. ELISA and RT-PCR results demonstrate that 26°C was superior to 18°C or 22°C for virus detection. More plants from the 26°C growth chamber tested positive in both tests than did plants from the 22°C or the 18°C growth chambers.

With the optimal temperature for virus titer established, the effect of photoperiod on virus expression was examined in a second experiment. The

optimal photoperiod was not as obvious as was the optimal temperature. It appears that temperature has a greater effect on virus titer than does photoperiod. However, the test results from the photoperiod experiment indicate that photoperiods of 16 and 20 hours provided the highest numbers of infected *V. x hybrida* plants testing positive by ELISA and RT-PCR.

The final objective of this project was to determine the most sensitive testing method for detecting BYMV in *V. x hybrida*. The test results from both experiments demonstrate that RT-PCR is more effective than ELISA for detection of BYMV in *V. x hybrida*. This is most likely due to the ability of RT-PCR to replicate any amount of virus present in the plant tissue, allowing even low initial virus levels to be highly present after PCR synthesis. However, ELISA can only detect the actual virus levels present, which can range from very high to very low.

The presence of visible BYMV symptoms in *V. x hybrida* plants played an important role in their detection result. The infected plants that were not visibly symptomatic did not test positive by ELISA. However, RT-PCR was able to detect BYMV in infected plants that did not exhibit visible symptoms. Once visible virus symptoms appeared in infected plants, they appeared to remain.

A recommendation to the nursery industry and producers would be that potted mother plants be grown at 26°C with a 16 or 20 hour photoperiod for 6 to 8 weeks before testing for BYMV. If the number of plants grown is large, an



initial ELISA test followed by RT-PCR on the negative plants is recommended. If the number of mother plants to assay is minimal, then RT-PCR is recommended.

Future experiments relating to BYMV detection in *V. x hybrida* would involve increasing uniformity and repetition among the growth chambers and plants. Because only three growth chambers were available for this experiment, the results are not entirely conclusive. The use of more growth chambers and more plants in those chambers would provide a more statistically significant number of results from which to make a recommendation to the nursery industry. Also, the growth chambers used could be monitored in order to ensure uniform and constant light levels, equal numbers of light bulbs used, and equal wattage of those light bulbs.

In addition, the electrophoresis gels on which the RT-PCR products were run often produced "ghost bands" in the wells of the negative *V. x hybrida* plants. These bands are most likely not indicators of virus in the negative plants, but rather background that could be reduced. The specificity of the gels could be increased by the use of chemicals or the alteration of the annealing temperature in the PCR program.

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## **APPENDIX 1—TISSUE CULTURE MEDIA RECIPE**

### **Simple Murashige Skoog (MS) medium with normal vitamins and 3% sucrose**

- no growth regulators
- solidified with 6.5g/1 agar

#### **Recipe:**

- 1 pack MS salts (M-5524 Sigma)
- 30 g sucrose (table sugar)

Bring to 1L with distilled water.

Adjust pH to 5.7-5.8.

Add 6.5 g agar.

Autoclave 10 minutes to melt agar.

Dispense 50 ml per magenta box.

Autoclave on metal tray for 30 minutes.

Let cool under hood.

\*\*Makes media for approximately 25 magenta boxes.

## **APPENDIX 2—ELISA PROTOCOL**

### **Indirect ELISA, Agdia protocol**

#### **Grind and dilute samples**

Grind tissue in indirect sample extraction buffer (Appendix 2) at a 1:100 ratio (tissue weight: buffer volume).

#### **Dispense samples**

Dispense 100 µl of prepared sample into sample wells. Dispense 100 µl of positive control into positive control wells, and dispense 100 µl of indirect sample extraction buffer into buffer wells.

#### **Incubate plate**

Set the plate inside the humid box and incubate for 1 hour, or incubate overnight at 4° C.

#### **Prepare antibody**

A few minutes before the incubation is complete, prepare the antibody, using 1X ECI buffer (Appendix 2) and the antibody vial. First dispense 1X ECI buffer, then add antibody according to the dilution on the label (1:200). The volume of 1X ECI buffer required depends on the number of test wells used, with 100 µl needed per test well. After adding the antibody, mix thoroughly. Set the prepared antibody aside. You will need it after washing the plate.

#### **Wash Plate**

When the sample incubation is complete, wash the plate. Fill all the wells to overflowing with 1X PBST (Appendix 2), then quickly empty them again. Repeat 4 to 8 times. After washing, hold the frame upside down and tap firmly on a folded paper towel to dry the wells.

#### **Add Antibody**

Dispense 100 µl of prepared antibody per well.

#### **Incubate Plate**

Set the plate inside the humid box and incubate for 2 hours at room temperature or overnight in the refrigerator (4° C).

#### **Prepare Enzyme Conjugate**

A few minutes before the incubation is complete, prepare the enzyme conjugate, using 1X ECI buffer and the enzyme conjugate vial. First dispense the ECI buffer, then add the enzyme conjugate according to the dilution given on the label (1:200). Prepare the same volume of enzyme conjugate as antibody. After adding the enzyme conjugate, mix thoroughly. It is important to mix the enzyme conjugate well. Set the prepared enzyme conjugate aside. You will need it after

washing the plate. (Note: Always prepare enzyme conjugate within 10 minutes before use.)

**Wash Plate**

As before, wash the plate 4 to 8 times with 1X PBST.

**Add Enzyme Conjugate**

Dispense 100  $\mu$ l of prepared enzyme conjugate per well.

**Incubate Plate**

Incubate the plate in the humid box for 1.5 hours at room temperature.

**Prepare PNP Solution**

Each PNP tablet will make 5 ml of PNP solution, at a concentration of 1 mg/ml, about enough for five 8-well strips. About 15 minutes before the end of the above incubation step, measure 5 ml of room-temperature 1x PNP buffer (Appendix 2) for each tablet you will be using. Then, without touching the tablets, add the PNP tablets to the buffer.

**Wash Plate**

As before, wash the plate 4 to 8 times with 1X PBST.

**Add PNP Solution**

Dispense 100  $\mu$ l of PNP solution per well.

**Incubate Plate**

Incubate the plate for 30 to 60 minutes in a humid box.

**Evaluate Results**

Examine the wells by eye, or measure on Molecular Devices microtiter plate reader at 405 nm. Wells in which color develops indicate positive results. Wells in which there is no significant color development indicate negative results. Threshold for positive results was an optical density reading of 1.0 or higher at 405 nm.

## **APPENDIX 3—ELISA BUFFERS**

### **Indirect sample extraction buffer**

Dissolve in distilled water to 1000 ml:

Sodium carbonate (anhydrous)	1.59 g
Sodium bicarbonate	2.93 g
Sodium azide	0.2 g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g

Adjust pH to 9.6. Store at 4° C.

### **PBST Buffer (Phosphate buffered sodium containing .05% Tween 20)**

Dissolve in distilled water to 1000 ml:

Sodium chloride	8.0 g
Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic(anhydrous)	0.2 g
Potassium chloride	0.2 g
Tween-20	0.5 g

Adjust pH to 7.4

### **ECI Buffer**

Add to 1000 ml 1X PBST:

Bovine serum albumin (BSA)	2.0 g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g
Sodium azide	0.2 g

Adjust pH to 7.4. Store at 4° C.

### **PNP Buffer**

Dissolve in 800 ml distilled water:

Magnesium chloride hexahydrate	0.1 g
Sodium azide	0.2 g
Diethanolamine	97.0 ml

Adjust pH to 9.8 with hydrochloric acid. Adjust final volume to 1000 ml with distilled water. Store at 4° C.

## **APPENDIX 4—RNA EXTRACTION PROTOCOL**

Extract RNA from ~100 mg of tissue in 1 ml extraction buffer using a roller press. (Extraction buffer consists of: 200 mM Tris pH 8.5, 1.5% LDS (lithium dodecyl sulfate), 300 mM lithium chloride, 10 mM EDTA, 1% deoxycholic acid, 1% NP 40 or Nonidet P 40 substitute, and 1%  $\beta$ -mercaptoethanol—add just before use.)

Add 500  $\mu$ l of the extract to 500  $\mu$ l of 6 M KoAc (potassium acetate).

Centrifuge for 10 minutes at max speed.

Collect 750  $\mu$ l of the supernatant and add equal volume of isopropanol. Invert several times.

Centrifuge for 20 minutes at max speed.

Discard supernatant and wash RNA pellet with 1 ml of ice cold 70% ethanol. Centrifuge for 2+ minutes at max speed.

Repeat wash. Dry pellet briefly.

Resuspend RNA pellet in 40  $\mu$ l of RNase-free water.



## **APPENDIX 5—REVERSE TRANSCRIPTASE MIX/PROTOCOL**

**(NOTE:** The following master mix was prepared for 24 reactions of 25  $\mu$ l each.)

5X RT Buffer	150 $\mu$ l
100 mM DTT	75 $\mu$ l
dNTPs 10 mM each	30 $\mu$ l
Sterile distilled water	405 $\mu$ l
Superscript III	4.5 $\mu$ l
RNAse Out	1.5 $\mu$ l
Reverse Primer	7.5 $\mu$ l

Add 20  $\mu$ l mix to 5  $\mu$ l RNA template for 25  $\mu$ l reaction.

Incubate at 50° C for 50-60 minutes.

Inactivation for 15 minutes at 80° C.

**APPENDIX 6—PCR MIX AND PROTOCOL**

**(NOTE:** This PCR mix was prepared for 24 reactions of 25 µl for each of primer sets A and B. Primer information in Appendix 7.)

Primer set A:

10X PCR Buffer	75 µl
MgCl (2 mM)	45 µl
dNTPs (10 mM)	15 µl
Forward Primer A	15 µl
Reverse Primer B	15 µl
Sterile water	510 µl
Taq	4.5 µl

Add 20 µl mix to 5 µl RT template for 25 µl reaction.

Primer set B:

10X PCR Buffer	75 µl
MgCl (2 mM)	45 µl
dNTPs (10 mM)	15 µl
Forward Primer B	15 µl
Reverse Primer B	15 µl
Sterile water	510 µl
Taq	4.5 µl

Add 20 µl mix to 5 µl RT template for 25 µl reaction.

PCR Machine Program:

<b>Cycles</b>	<b>94° C</b>	<b>55° C</b>	<b>72° C</b>
<b>1</b>	4 min	1 min	1 min
<b>40</b>	45 sec	45 sec	1 min
<b>1</b>	45 sec	45 sec	5 min

PCR product run out on 2% Tris-phosphate-EDTA (TPE) electrophoresis gel (as adapted from Sambrook and Russell, 2001):

	<b><u>Working solution</u></b>	<b><u>Stock solution/Liter</u></b>
TPE	1X 90 mM Tris-phosphate 2 mM EDTA	10X 108 g of Tris base 15.5 ml of phosphoric acid (85%, 1.679 g/ml) 40 ml of 0.5 M EDTA (pH 8.0)

2% TPE Gel: 200 ml 1X TPE + 4 g agar

Add 1 µl Ethidium Bromide/100 ml TPE immediately before pouring gel.

Visualize and photograph results using UV light.

## **APPENDIX 7—RT-PCR PRIMERS**

A set of primers specific to BYMV was used in a preliminary *Verbena x hybrida* RT-PCR that confirmed the virus' relation to BYMV and its potential for sequencing. The PCR product was cloned and sequenced. Various computer programs were then used to determine appropriate primers specific to this virus strain.

### **Primers ordered from Invitrogen:**

#### **Verbena Forward Primer A (VFA):**

Sequence: TGC AGA TGA AGG CAG C  
5' Position: 54      3' Position: 69      Length: 16  
Invitrogen Primer Number: 64511C02

#### **Verbena Reverse Primer A (VRA):**

Sequence: CTA CAA AGA TCA GGC CCA  
5' Position: 367      3' Position: 350      Length: 18  
Invitrogen Primer Number: 64511C03

#### **Verbena Forward Primer B (VFB):**

Sequence: TGC AGA TGA AGG CAG C  
5' Position: 54      3' Position: 69      Length: 16  
Invitrogen Primer Number: 64511C04

#### **Verbena Reverse Primer B (VRB):**

Sequence: CAA AGA TCA GGC CCA CTC  
5' Position: 364      3' Position: 347      Length: 18  
Invitrogen Primer Number: 64511C05