

Sensitive Detection of Single and Mixed Viral Infections in Ornamental Plants at All Stages of the Propagation Cycle

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Introduction and Objectives

Detection of viruses in ornamental plants is particularly important at the propagation level since infected mother plants can be responsible for the dissemination of 1,000's of infected cuttings. In addition, viruses that cause mild or even no symptoms in their original ornamental hosts under greenhouse conditions, may cause severe symptoms in other varieties or species, including food crops, once plants become established in the landscape. New viruses are being found every year, most of which come into the U.S. through the movement of plant material from off shore locations. As new plant varieties are produced and distributed, their associated viruses come with them. These new viruses may escape detection since most routine screening relies on specific antibody or nucleic acid based tests for known viruses, and because symptoms may be absent or quite subtle, especially in young plantlets. Once discovered, new viruses can trigger state quarantines of plant products, disrupting the industry's ability to conduct business.

My program focuses on improving the detection of known plant viruses in single and mixed infections as well as characterizing new viruses in order to develop methods for their detection, assess their threat to agriculture, and to provide information to regulatory agencies to help avoid or reduce quarantines. Similar work with viroids is also being implemented.

Topic 2-Detection and Characterization of Angelonia flower break virus (AnFBV) in California

AnFBV is a recently discovered carmovirus of ornamental plants (1). It causes distinct disruptions of floral pigments, most notably in varieties of *Angelonia*, but is also visible in *Verbena* (Figure 4). In California all new pathogens are put on a Quarantine ("Q") list until the California Department of Food and Agriculture (CDFA) determines the distribution and threat to CA. This Q rating prohibits movement between counties within CA and other states may impose similar import restrictions based on the CA rating. Upon discovery of AnFBV in CA I conducted a survey of plants throughout the state to assess the current incidence and characterize the strains present. There have been 3 major sequence variants reported, from Florida, Maryland, and Israel.

Between June 2006 and July 2007, ornamental plant samples were collected from four counties in California (Riverside, Sacramento, San Diego, and Santa Barbara) and were tested for the presence of AnFBV using ELISA (Ardia, Inc., Elkhart, IN). Tissue samples were from propagation facilities or wholesale outlets except those from Riverside County, which were from retail stores. Sixteen positive samples (64%) were found in three varieties each of *Angelonia* and *Nemesia* spp. and seven varieties of *Verbena* spp., with at least one positive from each county (Table 3). Foliar symptoms ranged from asymptomatic to a mild mosaic with distinct flower breaking in the *Angelonia* spp. Results were confirmed by RT-PCR of the coat protein gene (1) and the 1,172-bp amplicons were sequenced. Viral isolates from the three varieties of *Angelonia* had 98 to 99% nucleotide similarity and 99 to 100% amino acid identity to the Maryland strain of AnFBV, with 91 to 92% nucleotide similarity and 96 to 97% amino acid identity to the Israel and Florida strains. All viral isolates from the *Nemesia* and *Verbena* spp. plants had nucleotide similarities of 96 to 98% and 98% amino acid identity to the Israel and Florida strains, with 91 to 92% nucleotide similarity to the Maryland strain.

These results indicate that multiple introductions of AnFBV have occurred in CA. This was the first report of AnFBV occurring in *Nemesia* plants and in CA in general (3). These results were given to CDFA to show that AnFBV was already established in CA and based on previously published data (1), it was not a threat to most agricultural crops, notably common food crop species. No quarantine has been implemented for this virus. This is the second case where my laboratory has been able to prevent or remove a Q rating on a new virus in CA, and I continue to work with the industry to address such regulatory actions.



Figure 4-Healthy (a) and infected (b and c) *Angelonia angustifolia* (Scrophulariaceae) and *Verbena x hybrida* (d) flowers showing flower break symptoms of *Angelonia flower break virus*.

Table 3. Incidence of *Angelonia flower break virus* in four California counties

County City	Species (# plants)	ELISA positive/total
San Diego Bonsall	<i>Angelonia</i> (5)	3/3
	<i>Verbena</i> (5)	5/5
	<i>Nemesia</i> (2)	2/2
Encinitas	<i>Verbena</i> (4)	3/4
Riverside Moreno Valley	<i>Angelonia</i> (2)	0/2
	<i>Verbena</i> (1)	1/1
Santa Barbara Carpinteria	<i>Verbena</i> (1)	1/1
	<i>Nemesia</i> (3)	0/3
Sacramento Sacramento	<i>Verbena</i> (4)	1/4

Summary 2

Introductions of new viruses can have major effects on the ability of plant producers to sell and distribute their products. This type of research can help avoid state or federal regulatory actions from impacting the industry. Myself and others continue to be vigilant in recognizing new viral pathogens and characterizing them to help minimize their role in agriculture.

Topic 1-Optimizing Virus Testing of Tissue Culture Plants

Tissue culture of shoot tip meristems is commonly used in order to eliminate viruses from plants. The uppermost cells of the meristem are generally virus free due to their rapid growth and lack of normal cell differentiation that allows virus movement and replication. This method for virus exclusion is not 100% effective however and appropriate screening of regenerated plants must be done to ensure selection of virus-free lines. Results presented at the last triennial FNRI meeting showed that many times virus titers are suppressed to below detectable levels through tissue culture, then as plants mature, virus levels increase allowing detection. If testing isn't continued throughout the life of the plant, propagation can occur from infected plants resulting in the distribution of thousands of infected cuttings. Research has been underway the last 2 years to improve the timing of, and methods used for, virus testing after tissue culture.

We chose *Verbena* plants (Figure 1) for our model system because over the years it has been a host that is commonly infected with viruses, many times mixed infections of 3 or 4 viruses in the same plant. Extracts from *verbena* may inhibit certain detection systems, adding to the difficulty of virus detection. The first objective was to determine the optimal medium for tissue culture of *verbena* plants. Eight media were tested, 3 of which produced satisfactory shoot and root regeneration (Table 1). The 3 best media were retested and growth rates were compared (Table 2, Figure 2). One medium (M3) produced the fastest growth and the highest level of successful regeneration and is being used for all subsequent experiments. Typically regenerated plants can be tested starting at 3-6 weeks post meristem isolation when enough tissue is available (Figure 3).

The first virus being used for study is *Nemesia ring necrosis virus* (NeNRV) a recently characterized tymovirus. NeNRV infected plants have been used for regeneration of meristems through tissue culture with M3 and resulting plantlets are being tested by ELISA, RT-PCR and inoculation to *Nicotiana benthamiana* weekly. Initial results show that 32% of the regenerants contain detectable NeNRV at the first week of testing. Of those testing as negative in their first week, 7% tested positive the second week, and a single plant had 2 consecutive negative tests before testing positive the third week. Testing of the remaining 34 negative plants will continue for 3 months. ELISA and RT-PCR have been equally sensitive for the detection of NeNRV thus far. Having almost completed the initial study, we will repeat the NeNRV trial again, in addition to using *Angelonia flower break virus* (AnFBV) and mixed infections of NeNRV and AnFBV in future trials. Previous results indicate that mixed infections may affect the detection of one or both viruses



Figure 1. Typical flowers and leaves of *Verbena x hybrida*. Leaves have two major morphologies, oval (left) and pinnately compound (right).

Table 2. Survival and growth comparisons of three media used for *in vitro* isolation of healthy *verbena* shoot tip meristems. Shoot tips isolated were 0.3-0.5mm in length.

Tissue culture medium	Number of differentiated meristems/total	Average total length at 4 weeks	Average total length at 6 weeks	Average shoot length at 6 weeks	Average root length at 6 weeks
M1	8/16	1.7 cm	14.6 cm	8.8 cm	5.8 cm
M2	4/16	2.4 cm	18 cm	9 cm	9 cm
M3	11/16	2.5 cm	22 cm	10 cm	12 cm



Figure 2. Comparison of shoot and root lengths of healthy *verbena* plantlets regenerated from shoot tip meristems after 45 days of growth on three different tissue culture media.

Table 1. Optimization of media for tissue culture of *Verbena* meristems. Shoot tips isolated were 0.4-0.8mm in length. Media 1, 2 and 3 were selected for further study

Medium	Shoot tips planted	Shoots with Roots	Shoots, no Roots	Callus
1	10	7	3	0
2	10	8	2	0
3	10	7	3	0
4	10	6	4	0
5	10	1	9	0
6	10	0	10	0
7	10	0	1	9
8	10	0	0	0



Figure 3. Growth rate of shoot tip meristems isolated from *verbena* and grown in tissue culture after time periods noted above.

Summary 1

Viruses can escape detection during testing of plants regenerated via tissue culture. The ultimate goal of this project is to make recommendations to the industry on the proper timing and methods used for screening plants to increase the likelihood of reliable virus detection.

Topic 3. Characterization of Nemesia ring necrosis virus (NeNRV)

A new member of the tymovirus group, *Nemesia ring necrosis virus* (NeNRV), was first reported in Europe in 2004 (5) and the genome was characterized in 2005 (2). Our laboratory reported the first occurrence in North America in 2006 (4). The namesake necrotic rings are relatively rare in CA (Figure 5), although it isn't clear if that is due to the climate, plant varieties, or strains of the virus. We focused on the host range, sensitive detection and characterization of strains of the virus. Using European derived NeNRV primers for RT-PCR of plant samples that tested positive for NeNRV by ELISA, resulted in only 5 of 30 isolates producing an amplicon. The same samples were tested using generic tymovirus group primers (Ardia, Inc.), and all 30 samples were positive. After sequencing of these amplicons, major sequence changes were found in the second strand priming region for the NeNRV specific primers. These differences could result in false negatives if the European primers were used to screen for the presence of NeNRV in the U.S. After sequencing 95% of the entire genome of 4 isolates of NeNRV from California, it was determined that there were distinct strains of the virus present, but they were not divergent enough to be classified as new viruses. New primers have been designed that are expected to amplify all NeNRV strains. We continue to study this virus and use it as a model system for detection of ornamental viruses.

Figure 5. Necrotic ring symptoms of *Nemesia ring necrosis virus* on *Nemesia* leaves.



Summary 3

New viruses can go undetected when plants are screened for infections. It is important to use multiple methods when testing propagation stock to ensure that infected materials are not released.

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