A Rapid Greenhouse Assay to Evaluate Viroid-induced Dwarfing

R. A. Owens[1], S. M. Thompson[1], and M. E. Hilf[2]

[1]*Molecular Plant Pathology Laboratory, USDA-ARS, Beltsville, MD 20705, USA*
[2] *Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL 34945, USA*

ABSTRACT. The potential of viroid infection to dwarf citrus growing in intensive plantings is well established. How viroids exert this dwarfing effect is not known, but one possibility involves limiting the size of the root system. As part of an ongoing effort to develop *Citrus viroid III* (CVd-III) for use with rootstocks other than trifoliate orange or its hybrids, we have studied the effects of viroid infection on root development under greenhouse conditions using three rootstock/scion combinations; i.e., rooted Etrog citron cuttings, trifoliate orange seedlings, and young Valencia orange/trifoliate orange grafted trees. Groups of 10 young Etrog cuttings growing under greenhouse conditions were slash-inoculated with CVd-IIIb RNA transcripts and then observed for up to 12 mo with periodic cutbacks. Three months post-inoculation, the viroid-infected Etrog plants were significantly shorter than the uninoculated controls. By 6 mo post-inoculation, the inhibitory effect of CVd-IIIb on root dry weight had also become statistically significant. Between 6 and 12 mo, effects on root weight and development continued to intensify. Graft inoculation of trifoliate orange seedlings or Valencia scions growing on trifoliate orange rootstocks with either CVd-IIIa or CVd-IIIb resulted in a similar (though not statistically significant) inhibition of root dry weight accumulation over an 18 mo period.

Interest in high density citrus plantings and tree size control is driven by several factors. As discussed by Hutton et al. (3), these include the need to improve early return on investment, make more efficient use of resource inputs, and respond more quickly to changing market demands. Smaller trees also offer the promise of reduced harvesting costs. Nearly 40 yr of research carried out in Australia, Israel, and California (e.g., 11) have demonstrated the feasibility of using viroid infection to achieve uniform tree size control, but the underlying physiological mechanism(s) is still unclear. The fact that deficit irrigation can be used to limit tree growth in high density plantings by reducing the size of the effective root zone (2) suggests that effects on the root system may play a key role in viroid-induced dwarfing.

Six different species of viroids, *Citrus viroids I - V*, and *Citrus exocortis viroid* (CEVd) have been isolated from citrus, and field trees often contain complex mixtures of these viroids (1, 12). Evidence for a causal relationship between viroid infection and dwarfing was provided by the consistent presence of *Citrus viroid III* (CVd-III) (9, 13) in graft-transmissible dwarfing "factors" originating from various citrus-growing regions around the world. Infection by other viroids (e.g., Citrus viroid-IIa) can also result in dwarfing of scions growing on trifoliate orange or citrange rootstocks, but the consistent absence of disease in field-grown trees infected with CVd-III (10) currently make this viroid the citrus dwarfing agent of choice.

We are interested in identifying variants of CVd-III that are suitable for use as dwarfing agents under sub-tropical conditions. In previous studies, we have characterized the sequence variability of CVd-III (5) and demonstrated the feasibility of isolating single sequence variants from mutagenized populations of CVd-IIIb (6). Here, we describe a bioassay using the indicator host Etrog citron that rapidly assesses the effect of viroid infection on root growth. The 6-12 mo required to complete this greenhouse assay is much shorter than the 3-4 yr required for dwarfing effects to become apparent under field conditions.

Actively growing Etrog 861S shoots were divided into 10-12 cm segments, each bearing two leaves, dipped in Rootone rooting hormone (Gardentech, Lexington, KY), and placed in 20 cm Ray Leach cone-tainers (Hummert International, Earth City, MO) containing a 1:1 mixture of perlite and Metro-Mix 510 (Griffin Greenhouse & Nursery Supplies, Morgantown, PA) on a mist bench. Approximately 2 mo later, 60 rooted cuttings were removed from the mist bench and transferred to 10 cm plastic pots containing the same potting mixture. After an additional 2 mo, 30 young trees were slash inoculated with precisely-full-length CVd-IIIb RNA transcripts as previously described (5). The remaining 30 young trees were used as healthy controls.

Approximately 3 weeks post inoculation (p.i.), all plants were cut back and allowed to grow out for 3 mo before initial symptom assessment. During this period, the young trees were transferred to 3.8 liter pots to allow the root system additional space to expand. Characteristic symptoms of CVd-III infection (leaf epinasty and veinal necrosis) began to appear in the foliage approximately 2 mo p.i. At 3 mo p.i., shoot heights and root dry weights were determined for randomly-selected groups of 10 healthy or viroid-infected trees, and the remaining 40 plants were cut back and allowed to re-grow. A second set of data was collected at 6 mo p.i., and the remaining 20 plants were transferred to 3.8 liter pots and allowed to grow for an additional 6 mo before collection of the final set of data.

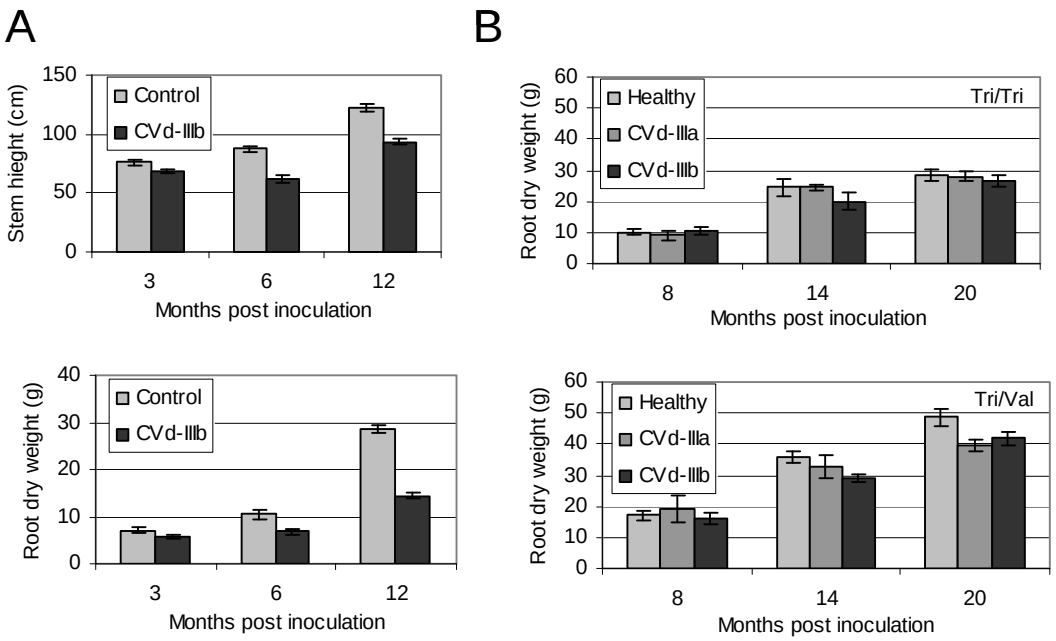

Fig. 1. Effects of CVd-III infection on Etrog citron and trifoliate orange. (A) Effect of CVd-IIIb on shoot height (upper panel) and root dry weight (lower panel) in Etrog citron. Values are means ± std error of the mean for 10 plants. (B) Effect of CVd-IIIa and CVd-IIIb on root dry weight accumulation by trifoliate orange growing on its own roots (upper panel) and Valencia sweet orange on trifoliate orange rootstock (lower panel). In all but one case, values are means ± std error of the mean for 7-9 plants. For CVd-IIIa infected Tri/Val trees sampled 8 mo p.i., only four trees were sampled. Differences between healthy and viroid-infected trees were not statistically significant in a Welch two sample t-test ($p \leq 0.05$).

Fig. 1A summarizes the data gathered from the six groups of Etrog plants. At 3 mo p.i., CVd-IIIb infection resulted in a modest, but statistically smaller increase in plant height. Over the succeeding 9 mo, the magnitude of this stunting increased, reaching a level of nearly 25% at 12 mo p.i. Evidence for an inhibition of root growth associated with CVd-IIIb infection could also be seen at 3 mo p.i., but this effect became statistically significant only after 6 mo. At 12 mo p.i., the mean root dry weight for young trees infected with CVd-IIIb was only half that of the uninfected controls; i.e., 14.39 g vs. 28.57 g. In addition to the well-known above-ground effects of CVd-IIIb infection on Etrog citron, these data indicate that development of the root system was also severely affected. Possible changes in root morphology associated with viroid infection (e.g., degree of branching or proportion of feeder roots) were not assessed.

Previous field studies have shown that trees growing on trifoliate orange (*Poncirus trifoliata*) rootstocks are highly susceptible to viroid-induced dwarfing (3), and our second bioassay was designed to determine how this rootstock would respond to CVd-III infection under greenhouse conditions. Both trifoliate orange seedlings on their own roots and Valencia orange scions grafted on trifoliate orange root stock were tested. Young (1-yr-old) bare-root trees obtained from Rucks Nursery (Frostproof, FL) were transferred to 11.4 liter pots containing the same 1:1 mixture of perlite and Metro-Mix 510 used for Etrog citron and, after a one month recovery period, groups of nine trees were graft-inoculated with buds collected from Etrog citron infected with either CVd-IIIa or IIIb. Control trees were not grafted.

The stem diameter of each *P. trifoliata* rootstocks was measured before inoculation, and each group of nine trees contained an equal number of trees randomly selected from the lower, middle, and upper third of the resulting distribution of stem diameters. Comparison of the stem diameters of surplus trees with the dry weights of their respective root systems allowed us to estimate the initial dry weight for both the budded and unbudded trees included in the trial, and effects of CVd-III infection on root dry weight accumulation were determined at 8, 14, and 20 mo post-inoculation. To eliminate position effects, the trees were systematically rotated on a weekly basis; i.e., trees were exchanged within their treatment groups, and the positions of all treatments on the bench top were changed. At each time point, the infection status of inoculated trees was assessed by RT-PCR (4), and only data for trees testing positive were included in our analysis.

Fig. 1B summarizes the data collected in this experiment. From left to right, the data in each panel indicate that although the root system continued to develop throughout the entire assay period, growth slowed noticeably after 14 mo. Comparing root dry weights for healthy and infected trees, viroid infection appears to have had little or no effect on *P. trifoliata* growing on its own roots (Fig. 1B, upper panel). In the case of Valencia orange growing on trifoliate rootstock, a modest (approx. 15%) inhibition of root growth was apparent 20 mo p.i (Fig. 1B, lower panel). Although consistent with the effects of CVd-IIIb infection seen in Etrog citron, these differences were not statistically significant. Taken together, our results suggest that i) the canopy size of the trifoliate orange trees growing on their own roots was too small to produce maximum rates of root growth under our conditions and ii) the more vigorous Valencia orange/trifoliate trees may have become pot-bound during 20 mo in the 11.4 liter containers used.

Although suggestive, the ability of CVd-IIIb infection to reduce both root and shoot growth in Etrog citron does not prove a causal relationship between these two processes. To establish such a relationship requires that the bioassay be repeated, this time including two (or more) CVd-III sequence variants that differ in the severity of stunting induced. Unfortunately, the two most commonly recovered sequence variants (i.e., CVd-IIIa and IIIb) induce very similar symptoms in Etrog citron (unpublished observations). Studies with *Potato spindle tuber viroid* (PSTVd) have shown that sequence changes in a so-called "loop E motif" located in its conserved central region can have dramatic effects on both symptom expression (8) and host range (14). The central conserved region of CVd-III also contains a loop E motif, but its structural properties are quite different than those of PSTVd (7). Keeping in mind the possibility of significant host- and viroid-specific differences, systematic mutagenesis of the loop E motif of CVd-III may yield novel variants with altered host range and/or increased pathogenicity. Promising variants identified using the Etrog bioassay can then be further characterized by follow-up greenhouse studies on trifoliate orange or other dwarfing-sensitive rootstocks.

ACKNOWLEDGMENTS

We thank Cristina Paul and John Clark for assistance with plant propagation and bud inoculations and Matt Kramer for statistical analysis.

LITERATURE CITED

1. Duran-Vila, N. and J. S. Semancik
2003. Citrus viroids. In: *Viroids*. A. Hadidi, R. Flores, J. W. Randles, and J. S. Semancik (eds.), 178-194. CSIRO Publishing, Collingwood, Australia.
2. Golumb, A.
1988. High density plantings of intensive groves. A challenge and realization. In: *Proc. 6th Citrus Congr*., 921-930.
3. Hutton, R. J., P. Broadbent, and K. B. Bevington
2000. Viroid dwarfing for high density citrus plantings. Hort. Rev. 24: 277-317.
4. Owens, R. A., S. M. Thompson, P. A. Feldstein, and S. M. Garnsey
2000. Effects of sequence variation on symptom induction by citrus viroid III. In: *Proc. 14th Conf. IOCV*, 254-264. IOCV, Riverside, CA.
5. Owens, R. A., G. Yang, D. Gundersen-Rindal, R. W. Hammond, T. Candresse, and M. Bar-Joseph
2000. Both point mutation and RNA recombination contribute to the sequence diversity of citrus viroid III. Virus Genes 14: 243-251.
6. Owens, R. A., S. M. Thompson, P. J. Sieburth, and M. E. Hilf
2002. Limited sequence randomization: Testing a strategy to produce improved viroid dwarfing agents. In: *Proc. 15th Conf. IOCV*, 249-257. IOCV, Riverside, CA.
7. Owens, R. A. and T. Baumstark
2007. Structural variation within the loop E motif: Implications for the mechanism of viroid processing. RNA 13: 824-834.
8. Qi, Y. and B. Ding
2003. Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. Plant Cell 15: 1360–1374.
9. Rakowski, A.J., J. A. Szychowski, Z. S. Avena, and J. S. Semancik
1994. Nucleotide sequence and structural features of the group III citrus viroids. J. Gen. Virol. 75: 3581-3584.

10. Semancik, J. S.
2003. Consideration for the introduction of viroids for economic advantage. In: *Viroids*. A. Hadidi, R. Flores, J. W. Randles, and J. S. Semancik (eds.), 357-362. CSIRO Publishing, Collingwood, Australia.
11. Semancik, J. S., A. G. Rakowski, J. A. Bash, and D. J. Gumpf
1997. Applications of selected viroids for dwarfing and enhancement of production of Valencia orange. J. Hort. Sci. 72: 563-570.
12. Serra, P., C. J. Barbosa, J. A. Daròs, R. Flores and N. Duran-Vila
2007. A new citrus viroid: molecular characterization and synergistic interactions with other members of the genus *Apscaviroid*. Virology 370: 102-112.
13. Stasys, R. A., I. B. Dry, and M. A. Rezaian
1995. The termini of a new citrus viroid contains duplications of the central conserved regions from two viroid groups. FEBS Letters 358: 182-184.
14. Wassenegger, M., R. L. Spieker, S. Thalmeir, F. U. Gast, L. Riedel, and H. L. Sänger
1996. A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a noninfectious to an infectious RNA for *Nicotiana tabacum.* Virology 226: 191-197.