Is Desert lime (*Eremocitrus glauca*) Resistant to Viroid Infection or Only a Poor Viroid Host?

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ABSTRACT. Desert lime (*Eremocitrus glauca*) has a genotype of interest for rootstock breeding purposes, but only limited information is available regarding its sensitivity to citrus pathogens. We evaluated its response to viroid infection using scions grafted on rough lemon as well as self-rooted plants. Analysis showed that the viroids could readily move from the inoculated *E. glauca* scion to the rootstock, in which they reached detectable titers. However, the inoculated viroids did not reach detectable titers in *E. glauca* tissues, and may not have multiplied at all. Ongoing studies aim to establish whether *E. glauca* is a truly resistant genotype or simply a poor viroid host.
Index words: CEVd, HSVd, CBLVd, CVd.III, CVd-IV

Desert lime (*Eremocitrus glauca*) is a small bushy tree confined to inland areas of Australia, usually on heavy, clay soils. Because of its habitat, it is a rootstock genotype of interest, as it may contribute to extend the areas of commercial citrus cultivation. Unfortunately, little is known about its field performance and its sensitivity to pathogens. Because of its potential use for rootstock breeding, studies were conducted to evaluate its sensitivity to viroid infection.

For the experiments, desert lime trees were propagated. The source of tissue for propagation was pathogen-free *E. gluaca* (IVIA-346) from the IVIA germplasm bank. Plants were graft propagated on rough lemon seedlings.

In the absence of seeds, self-rooted plants were obtained by *in vitro* micropropagation. Stem pieces (10 cm long) were stripped of leaves and disinfected in 70% (v/v) ethanol for 3 min and 1.5% (w/v) sodium hypochlorite for 10 min and rinsed in autoclaved water. Stem segments (1 cm long) with a single bud were split longitudinally and cultured with the longitudinal cut surface in contact with culture medium containing basic nutrient solution (BNS)(2) and 1 mg L^{-1} benzylaminopurine, pH 5.7. Four months later, shoots with a minimal length of 0.5 cm originated from the cultured explants were transferred to rooting medium containing BNS and 3 mg L^{-1} naphthalene acetic acid, pH 5.7. After six weeks, shoots showing root primordia were transferred to elongation medium containing BNS, pH 5.7 and transferred to soil 4 mo later.

The viroid sources used were CEVd (CEVd-117) (5), HSVd (X-707 and CVd-IIa-117) (7), CBLVd (CVd-Ia-117) (3), CVd-III (CVd-IIId) (3), and CVd-IV (CVd-IV-Ca) (4). CEVd-117 had been characterized as a severe strain (5) highly homologous to the CEVd sequences defined by Visvader and Symons (12, 13) as class A. HSVd isolates (X-707 and CVd-IIa-117) had been characterized as cachexia and non-cachexia inducing variants, respectively (7). Citron plants infected with these viroids were used as sources of tissue for graft-inoculation. An artificial mixture of these viroids had been previously obtained by graft-inoculating the viroids into a Fino lemon tree grafted on rough lemon rootstock; indexing confirmed the presence of the viroids in the tree.

E. glauca grafted on rough lemon and self-rooted *E. glauca* plants were

inoculated with viroids in September 2005. Three graft-propagated plants were graft-inoculated in the *E. glauca* scion using Fino lemon co-infected with the viroid mixture, and three uninoculated plants were kept as negative controls. The plants were trained to allow the growth of a branch from the Rough lemon rootstock for its further analysis. Self-rooted plants were graft-inoculated with one of 6 viroid sources that had been maintained in Etrog citron (two plants per viroid treatment). Two additional plants were graft-inoculated with the viroid mixture maintained in Fino lemon and two uninoculated plants were kept as negative controls.

For viroid analysis, samples (5 g) of bark tissue were powdered in liquid nitrogen and homogenized in 5 ml of extraction medium (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) β-mercaptoethanol) and 15 ml of water-saturated phenol (8). The total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 0.1 mM $MgCl_2$).

For Northern blot hybridization, aliquots (20 µl equivalent to 300 mg fresh weight) were subjected to 5% non-denaturing PAGE and the bands stained with ethidium bromide. The RNAs separated by 5% PAGE were electroblotted (400 mA for 2 h) to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), immobilized by UV cross-linking and hybridized with viroid-specific probes. Digoxigenin (DIG)-labeled DNA probes were synthesized by PCR using as template a cloned plasmid containing full-length viroid monomeric DNA, as described by Palacio-Bielsa et al. (6). Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and and 5XSSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent. After hybridization the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC, 0.1% SDS at 60ºC for 60 min, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminescence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

RT-PCR was performed as described by Bernad and Duran-Vila (1). First-strand cDNA was synthesized at 60ºC using 27-mer primers specific for each viroid and Thermoscript reverse transcriptase (Invitrogen®). In order to recover full-length viroid DNA, second strand synthesis and DNA amplification were performed using a set of two contiguous 18-mer forward and reverse primers specific for each viroid in 50 µl reactions containing 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of a 5 min denaturation at 94°C followed by 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (1 min) and finishing with a 5 min extension step at 72°C. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products.

Viroids were detected in the rough lemon rootstock of *E. glauca* plants 9 mo after these had been inoculated (data not shown). These plants were then decapitated below the graft-inoculation site and the remaining *E. glauca* scion was allowed to grow. After 2 mo all the viroids were detected in the rootstock by Northern blot hybridization and/or RT-PCR. As shown in Fig.1, CBLVd could only be detected by RT-PCR, indicating that this viroid was present at very low titers. None of the viroids could be detected in samples from the scion. The same results were obtained when the plants were tested again after four additional months.

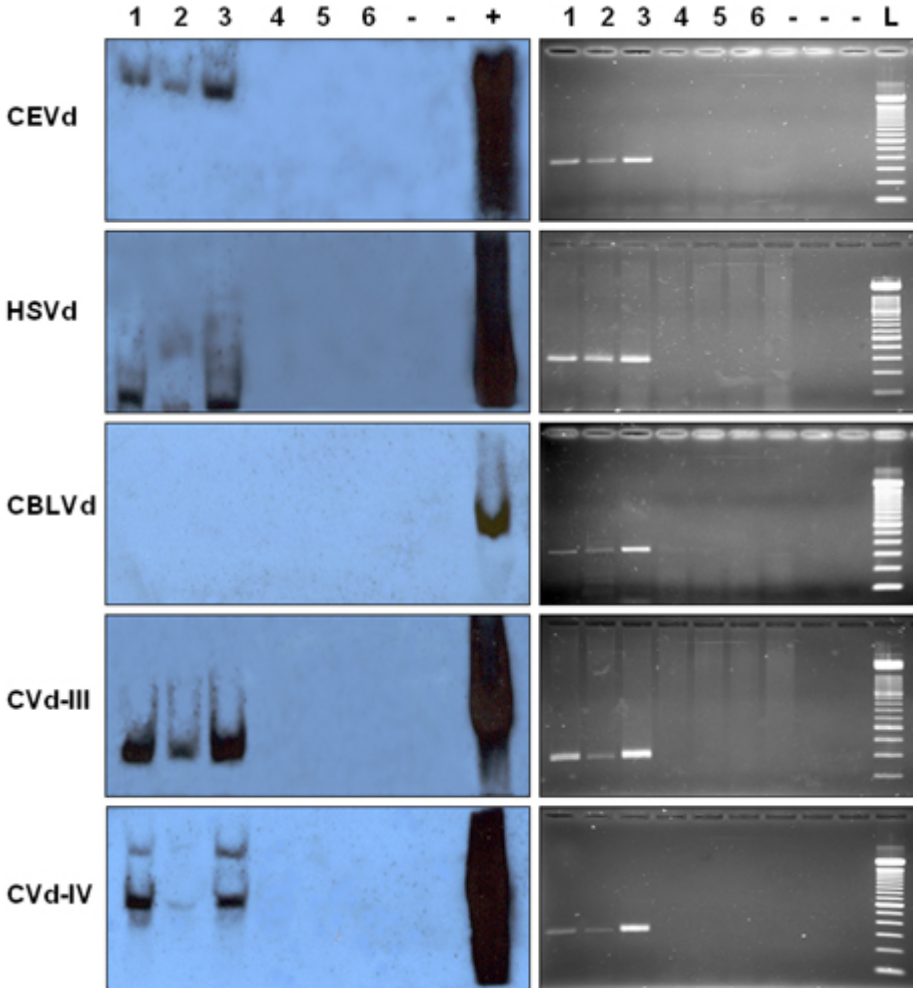

Fig. 1. Detection of inoculated viroids in *Eremocitrus glauca* plants graft-propagated on rough lemon rootstock. Viroids were detected by Northern blot hybridization (left) and RT-PCR (right) on samples from the rootstock (lanes 1, 2, 3) and from the scion (lanes 4, 5, 6). Samples from uninoculated (-) and viroid-infected (+) Etrog citron plants were included as negative and positive controls. L=100bp DNA ladder.

Self-rooted *E. glauca* plants were analyzed for presence of viroids 18 mo after inoculation using tissue collected above and below the inoculation site. The results of Northern blot hybridization and RT-PCR analysis were negative for all the inoculated viroids, whether the plants had been inoculated with single viroid sources or co-inoculated with the viroid mixture.

Citrus and citrus relatives are hosts of several viroids, two of which (CEVd and HSVd) induce disease symptoms in sensitive species; other viroids produce only subtle effects that may result in small trees with reduced crops (9, 10, 11). These effects are only perceived in a few sensitive species whereas in most commercial cultivars viroids replicate without inducing symptoms. Such symptomless carrier trees are responsible for viroid spread. Only when buds from these trees are graft propagated on sensitive rootstocks, will symptoms be expressed.

The results reported here indicate that *E. glauca* is either a very poor host or a viroid resistant genotype. Indeed the inoculated viroids did not reach detectable

titers in the *E. glauca* scion of graft-propagated plants nor in the self-rooted *E. glauca* plants. However, in the case of graft-propagated *E. glauca* plants, the viroids were present in detectable titers in the rough lemon rootstock, even though they were undetected in the *E. glauca* scion. This shows that the *E. glauca* scion allows long distance movement of viroids.

The results presented here are only preliminary and must be extended. The decapitated *E. glauca* plants have now been top-worked with Etrog citron. Analyses of the citron tissue for presence of viroids will provide additional evidence on whether *E. glauca* is a true resistant genotype or only a very poor host.

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LITERATURE CITED

1. Bernad, L., and N. Duran-Vila
2006. A novel RT-PCR approach for detection and characterization of citrus viroids. Mol. Cell. Probes 20: 105-113.
2. Duran-Vila, N., V. Ortega, and L. Navarro.
1989. Morphogenesis and tissue cultures of three citrus species. Plant Cell Tiss. Organ Cult. 16: 123-133.
3. Foissac, X., and N. Duran-Vila
2000. Characterization of two citrus apscaviroids isolated in Spain. Arch. Virol. 145: 1975-1983.
4. Francis, M., J. A. Szychowski, and J. S. Semancik
1995. Structural sites specific to citrus viroid groups. J. Gen. Virol. 76: 1081-1089.
5. Gandía, M., L. Rubio, A. Palacio, and N. Duran-Vila
2005. Genetic variation and population structure of an isolate of citrus exocortis viroid (CEVd) and of the progenies of two infectious sequences variants. Arch. Virol. 150: 1945-1957.
6. Palacio-Bielsa, A., X. Foissac, and N. Duran-Vila
2000. Indexing of citrus viroids by imprint hybridization. Eur. J. Plant Pathol. 105: 897-903
7. Palacio-Bielsa, A., J. Romero-Durbán, and N. Duran-Vila
2004. Characterization of citrus HSVd isolates. Arch. Virol. 149: 537-552
8. Semancik, J. S., T. J. Morris, L. G. Weathers, G. F. Rodorf, and D. R. Kearns
1975. Physical properties of a minimal infectious RNA (viroid) associated with the exocortis disease. Virology 63: 160-167.
9. Semancik, J. S., A. G. Rakowski, J. A. Bash, and D. J. Gumpf.
1997. Application of selected viroids for dwarfing and enhancement of production of "Valencia" orange. J. Hort. Sci. 72:563-570.
10. Vernière, C., X. Perrier, C. Dubois, A. Dubois, L. Botella, C. Chabrier, J. M. Bové, and N. Duran-Vila
2004. Citrus viroids: symptom expression and effect on vegetative growth and yield of clementine trees grafted on trifoliate orange. Plant Dis. 88:1189-1197.
11. Vidalakis, G., D. J. Gumpf, J. A. Bash, and J. S. Semancik
2004. Finger imprint of *Poncirus trifoliata*: A specific interaction of a viroid, a host, and irrigation. Plant Dis. 88: 709-713.
12. Visvader J. E., and R. H. Symons
1985. Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. Nucleic Acids Res 13: 2907-2920
13. Visvader J. E., and R. H. Symons
1986. Replication of in vitro constructed viroid mutants: location of the pathogenicity modulating domain of citrus exocortis viroid. EMBO J 5: 2051-2055.