Citrus Viroids in Colombia

N. Murcia[1,2], L. Bernad[1], A. Caicedo[2], and N. Duran-Vila[1]

[1]*Centro de Protección Vegetal y an. Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada (Valencia), Spain.*
[2]*Corporación Colombiana de Investigación Agropecuaria (CORPOICA).*

ABSTRACT. A sensitive Northern hybridization method, developed to detect viroids in commercial species and cultivars, was used to begin surveys for citrus viroids in Colombian orchards. Analysis of seven samples collected in the Tolima Region showed that five Tahiti lime sources were infected with HSVd and CVd-III, whereas two Mexican lime sources were viroid-free. Three additional Mexican lime sources collected in Magdalena municipality were also viroid-free, whereas two Tahiti lime sources were infected with HSVd and CVd-III or CEVd, HSVd and CVd-III. Cultivars of Valencia sweet orange, Mexican lime and Tahiti lime, maintained in the germplasm collection of Palmira, were all viroid-free. Of the three citron sources, also maintained in the germplasm collection, two were viroid-free and one was infected with CEVd and CVd-III. The base sequences of the viroids from the infected sources were determined and compared with the reference sequences. This is the first report on citrus viroids in Colombia.

Index words: exocortis, cachexia, CEVd, HSVd, CVd-III, sequence variants.

Colombia is a citrus-growing country with an estimated production of 1.1 million tonnes on a surface of 57,420 ha. Tahiti lime is the main export commodity, accounting for 12.6% of the overall citrus growing area (5), but with a productivity of only about 8 metric tonnes/ha in small orchards, much below the 15-25 metric tonnes/ha that can be reached in well managed orchards in Colombia and other countries of the region (18). *Citrus tristeza virus* (CTV), including stem-pitting isolates, is endemic in Colombia (7, 8) where it causes important economic losses. The presence of other graft-transmissible diseases has been reported on the basis of symptoms on sensitive rootstocks and cultivars. The need for high quality planting material has been acknowledged by ICA (Instituto Colombiano Agropecuario) and numerous international experts have recommended implementation of a "Plan Nacional de Certificación de Material de Propagación de Cítricos" as a way to maintain and improve the competitiveness of the citrus sector.

Grafted citrus trees may be infected with viroids without showing any signs of infection when both scions and rootstocks are tolerant to the viroids involved. Tahiti and Mexican limes on Volkamer lemon, widely grown in Colombia, are such tolerant scion/rootstock combinations. Here we report the results of a preliminary survey for citrus viroid identification in three municipalities of Colombia.

MATERIALS AND METHODS

Plant materials and nucleic acid extraction. Samples of Tahiti lime (10 samples), Mexican lime (six samples), Valencia sweet orange (one sample) and citron (three samples) were collected in Tolima, Magdalena and Palmira municipalities (Table 1).

TABLE 1
VIROIDS IN CITRUS SAMPLES COLLECTED IN COLOMBIAN MUNICIPALITIES

Municipality	Scion	Rootstock	Citrus viroids[1]					
			CEVd	HSVd	CBLVd	CVd-III	CVd-IV	CVd-V
Tolima	Tahiti lime 1	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 2	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 3	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 4	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 5	Volkamer lemon	-	+	-	+	-	-
Tolima	Mexican lime 1	Volkamer lemon	-	-	-	-	-	-
Tolima	Mexican lime 2	Volkamer lemon	-	-	-	-	-	-
Magdalena	Tahiti lime 6	Carrizo citrange	+	+	-	+	-	-
Magdalena	Tahiti lime 7	Volkamer lemon	-	+	-	+	-	-
Magdalena	Tahiti lime 8	Volkamer lemon	-	-	-	-	-	-
Magdalena	Tahiti lime 9	Cleopatra mandarin	-	-	-	-	-	-
Magdalena	Mexican lime 3	Volkamer lemon	-	-	-	-	-	-
Magdalena	Mexican lime 4	Volkamer lemon	-	-	-	-	-	-
Magdalena	Mexican lime 5	Volkamer lemon	-	-	-	-	-	-
Palmira	Tahiti lime 10	Unknown	-	-	-	-	-	-
Palmira	Mexican lime 6	Unknown	-	-	-	-	-	-
Palmira	Valencia sweet or.	Unknown	-	-	-	-	-	-
Palmira	Etrog citron 1	Unknown	+	-	-	+	-	-
Palmira	Etrog citron 2	Unknown	-	-	-	-	-	-
Palmira	Citron	Unknown	-	-	-	-	-	-

[1] +:Viroid detected; -:Viroid not detected

Bark (5 g) stripped from young shoots was reduced to powder in liquid nitrogen and homogenized in 5 ml of extraction buffer (0.4M Tris-HCl pH 8.9; 1% (w/v) SDS; 5mM EDTA pH 7.0; 4% (v/v) 2-mercaptoethanol) and 15 ml of water-saturated phenol (15). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1mM $MgCl_2$ pH 7.4). Aliquots of these preparations were used for Northern blot hybridization and RT-PCR analysis.

Northern blot hybridization. Aliquots (20 µl equivalent to 300 mg of fresh weight tissue) of the nucleic acid preparations were subjected to 5% PAGE (39:1) for 2 h at 60 mA under non-denaturing conditions (6). The RNAs were then electroblotted (400 mA for 2 h) from the gel to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) and immobilized by UV cross-linking. DIG-labeled viroid-specific probes were synthesized by PCR using cloned plasmids containing full-length viroid DNAs as described by Palacio-Bielsa et al. (10). Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and 6×SSC buffer (SSC: 150 mM NaCl; 15 mM sodium citrate, pH 7.0) containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking solution (Roche). After hybridization the membranes were washed twice in 2×SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1×SSC, 0.1% SDS at 60ºC for 60 min. DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and were visualized with the chemiluminiscence substrate disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Roche).

RT-PCR analysis.

Retro-transcription and PCR amplification 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®). Full-length viroid DNAs were recovered performing second strand synthesis and DNA amplification with sets of 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. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products.

Sequencing and sequence analysis. Whenever possible full-length uncloned viroid amplicons were sequenced. When the sequencing results were unclear, the amplicons were purified (GFX^{TM} PCR-DNA and Gel Band Purification Kit, Amersham Bioscience) and ligated to the pGEM-T vector (Promega). *Escherichia coli* cells (strain DH5α) were used for plasmid cloning. Uncloned amplicons and plasmid inserts were sequenced with the ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with Clustal W (17). Nucleotide distances were estimated considering alignment gaps and using the Jukes and Cantor method (3) for correction of superimposed substitutions with the MEGA 3.1 program (4). Phylogenetic tree was obtained with Neighbor-joining method (14) using the MEGA 3.1 program (4).

RESULTS

Eight of the 20 samples analyzed showed hybridization with some of the probes (Table 1). All the Mexican lime sources were viroid-free whereas seven out of ten Tahiti lime sources were infected with HSVd and CVd-III or with CEVd, HSVd and CVd-III. One out of three

citrons tested was infected with CEVd and CVd-III. Valencia sweet orange was viroid-free. Viroid infection was confirmed by RT-PCR. The consensus sequence of these viroid isolates was determined by sequence analysis of the uncloned RT-PCR amplicons or the inserts of recombinant plasmids.

Characterization of the CEVd isolates. The uncloned amplicons from the two CEVd sources gave ambiguous results and the consensus sequences were obtained from four clones of each isolate. The consensus sequences recovered from Tahiti lime and from Etrog citron presented the highest identity (94.9% and 96.5%) with the reference sequences of class A defined by Visvader and Symons (22, 23) (Table 2). Sequence alignment showed that CEVd from Tahiti lime differed in 14 nucleotide changes from the reference sequence of Class A but maintained the characteristic sequence of the P_L motif located in the Pathogenicity (P) domain. However a number of changes (four in the upper strand and three in the lower strand of the CEVd secondary structure) were identified within the P_R motif located in the Variable (V) domain. Three of the remaining changes located in the lower strand of the Central (C) and P domains had also been identified in a CEVd isolate previously described by Gandía et al. (2). CEVd from citron differed in eight nucleotide changes from the reference sequence of Class A that included a U316→A change in the lower strand of P_L motif located in the P domain. None of the changes affected the P_R motif located in the V domain. From the remaining changes four had been identified in a CEVd isolate previously described (2). As illustrated in the phylogenetic tree obtained with the Neighbor-Joining method (Fig. 1), the two CEVd sequences clustered with CEVd-A.

Characterization of HSVd isolates. The consensus sequences of the seven HSVd isolates were obtained by sequencing the uncloned RT-PCR amplicons. The five HSVd isolates recovered from Tahiti lime trees (Tahiti lime 1 to 5) collected in Tolima municipality presented the highest sequence identities (99.3-99.6%) with the non-cachexia variants (CVd-IIa) of HSVd (Table 2), differing from the reference sequence in one to two changes. They all presented, within the Variable (V) domain, the six-nucleotide motif characteristic of non-cachexia sequence variants of this viroid (11, 12). One of the two HSVd isolates recovered from Tahiti lime (Tahiti lime 6) collected in Magdalena municipality, also presented the highest sequence identity (98.0%) with the non-cachexia variants (CVd-IIa) (Table 2). This sequence variant however, presented only four of the six nucleotides discriminating non-cachexia from cachexia-inducing sequence variants. It presented two compensatory deletions, –A(116) and –U (189), in the upper and lower strands of the V domain, which being characteristic of cachexia variants, were reported previously in a non-cachexia isolate from Cuba (19). The HSVd isolate recovered from Tahiti lime (Tahiti lime 7) also collected in Magdalena municipality presented the six-nucleotide motif characteristic of cachexia sequence variants with the highest sequence identity (99.6%) with CVd-IIc variants (13). As illustrated in the phylogenetic tree obtained with the Neighbor-Joining method (Fig. 1), all except one of the HSVd isolates clustered with the non-cachexia variant (CVd-IIa).

TABLE 2
SEQUENCE IDENTITIES OF VIROIDS IDENTIFIED IN COLOMBIA WITH THE TYPE MEMBERS OF CEVd, HSVd and CVd-III

Sample	Sequence identities (%)[1]							
	CEVd		HSVd			CVd-III		
	CEVd-A (M 30868)	CEVd-B (M 30870)	CVd-IIa (AF213503)	CVd-IIb (AF213501)	CVd-IIc (AF131250)	CVd-IIIa (S76452)	CVd-IIIb (AF184147)	CVd-IIIc (AF184149)
Tahiti lime 1	-	-	**99.6**	97.0	93.7	95.3	**99.3**	95.0
Tahiti lime 2	-	-	**99.3**	97.0	93.7	96.0	**100**	95.2
Tahiti lime 3	-	-	**99.3**	97.0	93.7	96.0	**100**	95.6
Tahiti lime 4	-	-	**99.6**	97.3	93.7	95.3	**99.3**	94.5
Tahiti lime 5	-	-	**99.3**	97.0	93.7	95.3	**99.3**	95.0
Tahiti lime 6	**94.9**	92.5	**98.0**	96.7	93.7	94.3	**99.0**	93.8
Tahiti lime 7	-	-	93.7	95.7	**99.6**	95.0	**99.3**	94.2
Etrog citron 1	**96.5**	93.0	-	-	-	95.6	**100**	95.2

[1]Figures in bold indicate highest sequence similarity among those reference variants to which they were compared.

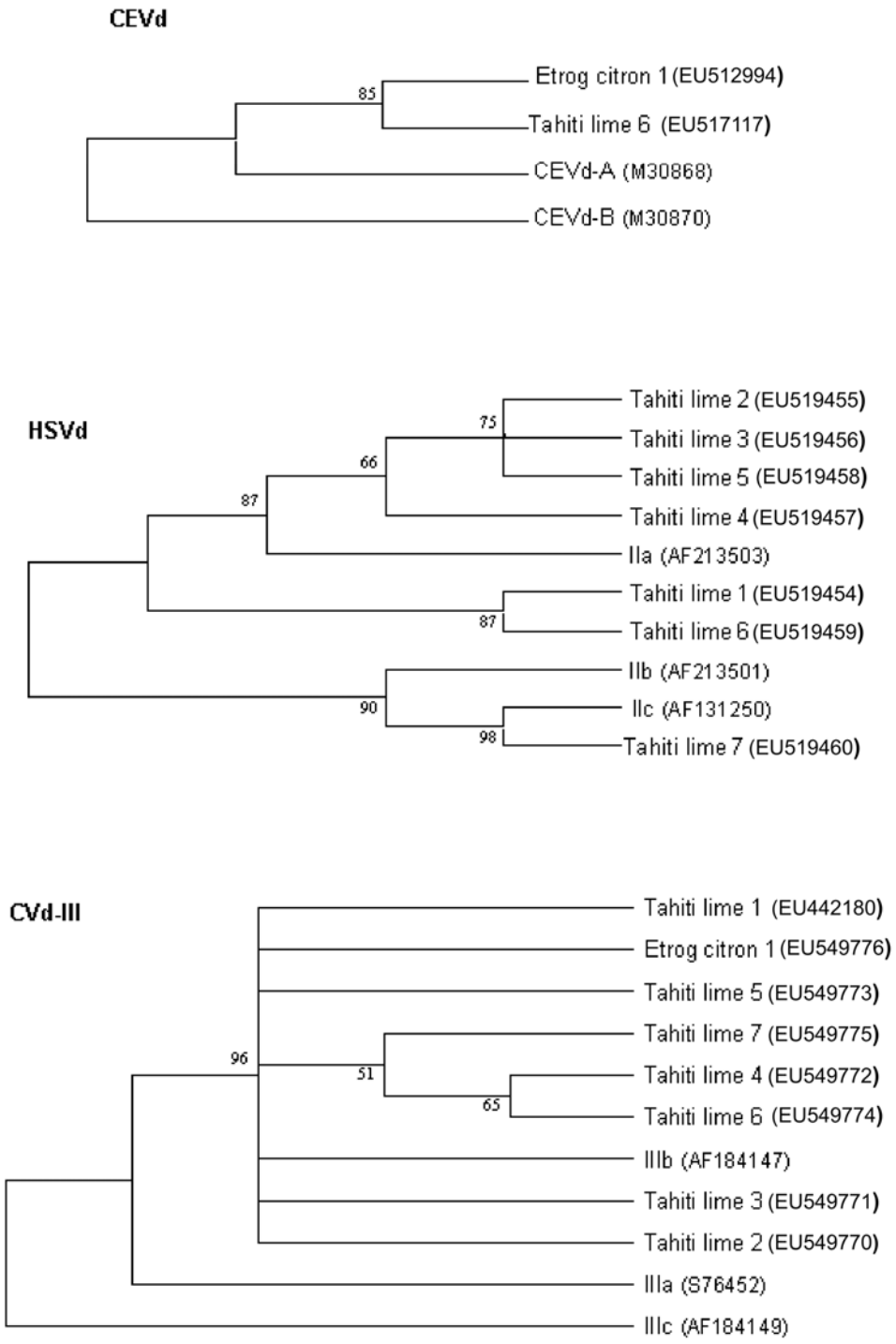

Fig. 1. Neighbor-joining phylogenetic tree obtained with the CEVd, HSVd and CVd-III sequences recovered from Tahiti lime and Etrog citron with 10,000 replicates. Nucleotide distances were estimated using the Jukes and Cantor method (3). Condensed branches have bootstrap values lower than 50%. Reference sequences were included in each case: CEVd the reference sequences of Class A and Class B (22, 23); HSVd reference sequences of CVd-IIa, CVd-IIb and CVd-IIc (13); CVd-III reference sequences CVd-IIIa, CVd-IIIb and CVd-IIIc (12, 16).

Characterization of CVd-III isolates. The consensus sequences of four CVd-III isolates (Tahiti lime 3, 5, 6, 7) were obtained by sequencing the uncloned RT-PCR amplicons whereas the remaining (Tahiti lime 1, 2, 4 and Etrog citron 1) were obtained from the sequences of three to four clones from each isolate. All the sequences presented the highest sequence identities (ranging from 99.0 to 100%) with CVd-IIIb (12) (Table 2). The consensus sequences of three isolates (Tahiti lime 2, 3 and Etrog citron 1) were identical to the reference sequence of CVd-IIIb defined by Rakowski et al. (12). The reference sequences of the other five isolates differed from two to seven changes from the reference sequence of CVd-IIIb (12) and the changes were found to be located in different regions of the secondary structure of the viroid molecule: V domain (Tahiti lime 1), T_R domain (Tahiti lime 4), P domain (Tahiti lime 5) and T_L (Tahiti lime 6, 7). As illustrated in the phylogenetic tree obtained with the Neighbour-Joining method (Fig. 1), all the CVd-III isolates clustered with the variant CVd-IIIb described by Rakowski et al. (12).

DISCUSSION

The results of the survey show that CEVd, HSVd and CVd-III are present in Colombia, affecting at least some commercial groves of Tahiti lime. All but one of the HSVd sources, were found to be non-cachexia strains. The CVd-III sources, which seem to be widespread in Tahiti lime groves, present high identities with CVd-IIIb (12) but contain changes affecting the regions that correspond to four domains of the viroid secondary structure. Even though HSVd and CVd-III are the most widespread viroids in Tahiti lime, CEVd is also present at least in one of the sources. Since bark-cracking symptoms in Tahiti lime are not infrequent in Colombia, viroid infections could be the cause (9). In addition, CEVd infection of trees also co-infected with HSVd and CVd-III may produce undesirable effects, such as those reported by Vernière et al. (20, 21) in the case of trees grafted on the viroid-sensitive trifoliate orange. One of the viroid-infected Tahiti lime sources from Magdalena municipality (Tahiti lime 6) was obtained from a tree grafted on Carrizo citrange, which is also viroid-sensitive, and therefore such trees may suffer from the sensitivity of both scion and rootstock.

The CEVd identified in a symptomless Etrog citron tree from the germplasm bank of Palmira municipality contains a single mutation in the P_L motif with respect to the reference variant. Since this motif is responsible for modulating symptom expression (22, 23), further characterization of this peculiar CEVd strain should reveal interesting information on the pathogenicity of CEVd.

ADDENDUM

Since this information was presented at the 17th IOCV Conference held in 2007, the International Committee on Taxonomy of Viruses (ICTV) has accepted some changes in the viroid nomenclature. The new names for CVd-III and CVd-IV are *Citrus dwarfing viroid* and *Citrus bark cracking viroid*, respectively. Additional information regarding the northern blot hybridization method used in now available (N. Murcia, P. Serra, A. Olmos, and N. Duran-Vila. 2009. A novel hybridization approach for detection of citrus viroids. Mol. Cell. Probes 23:95-102).

ACKNOWLEDGMENTS

This research was supported by grant AGL2005-01468 from the Ministerio de Educación y Ciencia. N. Murcia and L.

Bernad are recipients of fellowships from CORPOICA and IVIA, respectively. The authors would like to acknowledge J. J. Rivera and A.E. Diaz (CORPOICA) for supplying the plant material, R. Carbó for technical assistance and J. M. Bové for critical reading of the manuscript.

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. 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.
3. Jukes, T. H., and C. R. Cantor
1969. Evolution of protein molecules. In: *Mammalian protein metabolism*. H.N. Munro (ed.), 21-132. Academic Press, New York.
4. Kumar, S., K. Tamura, and M. Nei
2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5: 150-163.
5. Ministerio de Agricultura y Desarrollo Rural (MADR) Observatorio Agrocadenas Colombia.
2005. La Cadena de cítricos en Colombia. Una Mirada global de su estructura y dinámica 1991-2005. Documento de trabajo No.107. C. F. Espinal, H. J. Martínez C. and Y. Peña (eds.) Marín. 54 p.
6. Morris, T. J., and N. S. Wright
1975. Detection on polyacrylamide gel of a diagnostic nucleic acid from tissue infected with potato spindle tuber viroid. Amer. Potato J.: 52: 57-63.
7. Murcia, N., J. A. Osorio, F. Morales, and L. Calvert
2002 Distribución y caracterización serológica de aislamientos del virus de la tristeza de los cítricos en Colombia. Fitopatol. Colomb. 26: 21-16.
8. Murcia, N., A. Caicedo, L. Calvert, M. Sánchez, G. Davila, A. Domínguez, and H. Martinez.
2005. Caracterización de diez aislamientos colombianos del virus de la tristeza de los cítricos. Fitopatol. Colomb.28: 31-36.
9. Murcia, N., S. M. Bani Hashemian, K. Bederski, N. A. Wulff, C. J. Barbosa, J. M. Bové, and N. Duran-Vila
2010. Viroids in Tahiti lime scions showing bark cracking symptoms. In: *Proc. 17th Conf. IOCV,* 167-175. IOCV, Riverside, CA.
10. Palacio-Bielsa, A., X. Foissac, and N. Duran-Vila
2000. Indexing of citrus viroids by imprint hybridization. Eur. J. Plant Pathol. 105: 897-903
11. Palacio-Bielsa, A., J. Romero-Durbán, and N. Duran-Vila
2004. Characterization of citrus HSVd isolates. Arch. Virol. 149: 537-552.
12. Rakowski, A G., 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.
13. Reanwarakorn, K. and J. S. Semancik
1998. Regulation of pathogenicity in hop stunt viroid-related group II. J. Gen. Virol. 79: 3163-3171.
14. Saitou, N. and M. Nei
1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.
15. 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.
16. 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.
17. Thompson, J. D., D. G. Higgins, and T. J. Gibson
1994. CLUSTAL W. Improving the sensitivity of progressive multiple sequences alignment

through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680

18. Toro, J. C., R. García, and H. Rodríguez
2002. Especialización del sector frutícola del Valle del Cauca. SAG, Sociedad de Agricultores y Ganaderos del Valle del Cauca. 54pp.
19. Velázquez, K., M. Soto, R. Pérez, J. M. Pérez, D. Rodríguez, and N. Duran-Vila.
2002. Biological and molecular characterization of two isolates of citrus viroids recovered from Cuban plantations. In: *Proc. 15th Conf. IOCV*, 258-263. IOCV, Riverside, CA.
20. 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.
21. Vernière, C., X. Perrier, C. Dubois, A. Dubois, L. Botella, C. Chabrier, J. M. Bové, and N. Duran-Vila
2006. Interactions between citrus viroids affect symptom expression and field performance of clementine trees grafted on trifoliate orange. Phytopathology 96: 356-368.
22. 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
23. 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