Viroids in Gummy Bark Sources from the Sultanate of Oman

L. Bernad[1], P. Moreno[1], J. M. Bové[2], and N. Duran-Vila[1]

[1]Departamento de Protección y Biotecnología. Instituto Valenciano de Investigaciones Agrarias (IVIA). Apartado oficial. 46113 Moncada (Valencia), Spain;
[2]18, chemin Feyteau, 33650 La Brède, France

ABSTRACT. The gummy bark disease affects sweet oranges and rough lemon in some Mediterranean countries and most countries of the Near and Middle East regions. Diseased trees are usually stunted, and scraping the bark reveals gum deposits. Symptoms of gummy bark in sweet orange resemble those of cachexia on mandarin but the cachexia agent fails to induce symptoms in sweet orange. The causal agent of gummy bark is suspected to be a viroid, but previous studies conducted with sources from Turkey failed to identify a sequence variant of HSVd as the putative disease agent. In the present study, samples collected from Baladi, Valencia, Washington navel and Succari sweet orange trees showing the characteristic gummy bark symptoms were graft-inoculated on Etrog citron and analyzed by sPAGE and slot-blot hybridization using viroid-specific probes. In addition to HSVd, all samples also contained CEVd, CVd-III and CVd-IV. Sequence analysis of DNA amplicons generated by RT-PCR using specific primers for these viroids identified novel variants of CEVd (CEVd-gb) and CVd-III (CVd-III-gb) in all the gummy sources, whereas CVd-IV was identical to previously reported sequences. The relationship of these viroids to the putative agent(s) of gummy bark is discussed.

Index words. Exocortis, cachexia, CEVd, HSVd, CVd-III, CVd-IV.

Gummy bark is a disorder of sweet orange trees that was first observed in 1954 during a survey conducted in the Dahala and Kharga oases in Egypt where most Baladi sweet orange trees grafted on sour orange rootstock exhibited phloem discoloration (9). Further surveys showed the same disorder affecting other sweet orange cultivars (9, 10). Diseased trees are usually stunted, and scraping the bark of the trunk above the bud union reveals gum deposits. After bark removal, affected trees show stem pitting, symptoms resembling those of cachexia on cachexia-sensitive hosts. Nour-Eldin (11) reported that graft transmission of gummy bark sources to sweet orange grafted on Orlando tangelo resulted in the development of symptoms on the sweet orange scion in the absence of cachexia symptoms on Orlando tangelo. He, thus, concluded that the two diseases were not caused by the same agent. Conversely, transmission tests to sweet orange grafted on rough lemon induced a bud union constriction demonstrating that rough lemon is also sensitive to the putative gummy bark agent (11).

The disease has also been reported in a number of eastern Mediterranean, North African, Near East and Middle East countries including Egypt, Greece, Iran, Iraq, Pakistan, Syria, Lybia, Oman, Saudi Arabia, Yemen, Sudan and Turkey but never from North and South America (3).

Because of its graft-transmissibility, the disease was first considered to be caused by a virus. However, following the discovery that the cachexia disease was caused by a viroid (21), later characterized as a specific variant of HSVd (18), the hypothesis that the gummy bark disease might be also caused by a viroid gained popularity. This hypothesis was compatible with indirect evidences such as (i) symptoms that are virtually identical to those of cachexia disease on cachexia-sensitive hosts and (ii) the geographical distribution of the disease which has only been observed in countries

where high temperatures are known to favor viroid symptom expression.

The first attempt to associate the gummy bark disease with viroid infection was conducted with two gummy bark sources (20). This approach was continued by Önelge et al. (12) who conducted an extensive survey in the Çukorova region of Turkey. All the symptomatic trees were infected with CEVd, HSVd, CVd-III and CV-IV, whereas HSVd and CVd-IV were absent from non-symptomatic trees sampled in the same region. Following the finding that HSVd variants inducing cachexia differed from non-cachexia variants in only 5-6 nucleotides located in the V domain (18), the hypothesis that additional variants of HSVd might cause gummy bark was entertained. However, molecular characterization of HSVd variants present in gummy bark sources from Turkey did not allow identification of a distinct variant associated with gummy bark (13).

Here, we report additional efforts to associate the gummy bark disease with either viroid infection or with the presence of dsRNAs of potential virus origin using samples collected from symptomatic sweet orange trees in the Sultanate of Oman.

MATERIALS AND METHODS

Plant materials. Samples were collected from sweet orange trees displaying the characteristic gummy bark symptoms and located in Tanuf and Sohar, two locations in the Sultanate of Oman (Table 1). They included the sweet orange cultivars Baladi and Succari introduced from Egypt, Valencia and Washington navel. Each of these sources was graft inoculated onto three Etrog citron plants and incubated for at least 6 mo before analysis for dsRNAs and viroids.

Double stranded RNA analysis. Total nucleic acid extracts from bark tissue of inoculated citrons were subjected to non-ionic cellulose column chromatography in the presence of 16.5% ethanol to recover dsRNA-rich preparations that were analyzed by PAGE (5% acrylamide) (8).

Viroid analysis. Samples (5 g) of sweet orange bark tissue, as well as young leaves and stems of citron 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 (22). 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 $MgCl_2$). Aliquots of these preparations were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) and slot blot hybridization.

TABLE 1
VIROIDS RECOVERED FROM SWEET ORANGE TREES SHOWING GUMMY BARK SYMPTOMS

		Viroids detected[z]				
Sweet orange cultivar	Location	CEVd	CBLVd	HSVd	CVd-III	CVd-IV
Baladi	Tanuf	+	–	+	+	+
Baladi	Tanuf	+	+	+	+	+
Baladi	Tanuf	+	+	+	+	+
Valencia	Tanuf	+	–	+	+	+
Washington navel	Sohar	+	+	+	+	+
Succari	Sohar	+	–	+	+	+
Succari	Sohar	+	–	+	+	+

[z]Viroids were identified by sPAGE, slot blot hybridization and RT-PCR analysis of graft inoculated citrons.

For sPAGE analysis, preparations (20 µl equivalent to 300 mg fresh weight) were subjected to a first analysis with polyacrylamide gel electrophoresis (PAGE) under non denaturing conditions at 60mA for 2.5 h. A segment of the gel between the CEVd and 7s RNAs was excised and subjected to a second PAGE (containing 8M urea) at 16mA for 4 h (19). The viroid bands were viewed by silver staining (6). For slot blot analysis, nucleic acids (10 µl equivalent to 300 mg fresh weight) were pretreated in 6× SSC and 8% formaldehyde for 15 min at 60°C, blotted onto positively charged Nylon membranes (Roche®) using an Hybri-slot filtration manifold (BRL®) and immobilized by UV cross-linking. Prehybridization and hybridization were carried out in 50% formamide and 5× SSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent using digoxigenin (DIG)-labeled DNA probes (14).

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, an extraction method that avoids the use of phenol was employed. Briefly, samples (0.5 g) of leaf and bark tissue were placed in a sealed plastic back containing 5 ml of TE buffer (0.1 M Tris-HCl pH 8.5; 50 mM EDTA; 0.5 M NaCl; 10 mM β-mercaptoethanol), and gently crushed with a hand homogenizer. The homogenate was subjected to alkaline denaturation (1, 15), and DNA was synthesized as described by Bernad and Duran-Vila (2). First-strand cDNA was synthesized at 60°C 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.

Sequencing and sequence analysis. DNAs synthesized by RT-PCR were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with Clustal W (5), and secondary structure analyses used MFOLD (circular version), from the RNA structure V.40 GCG package (26) and RNAviz (4).

RESULTS

Identification of dsRNAs and viroids in trees showing gummy bark symptoms. Attempts to identify dsRNAs in citrons that had been graft inoculated with gummy bark sources, were unsuccessful. sPAGE analysis of nucleic acids extracted from sweet orange bark tissue revealed the presence of low concentrations of viroid-like RNAs with the characteristic mobilities of HSVd and CVd-III (data not shown). These preliminary results were confirmed by the appearance of symptoms characteristic of viroid infection in the graft inoculated citrons. sPAGE analysis of inoculated citrons confirmed the presence of the viroid-like RNAs observed in sweet orange samples and additional viroid-like RNAs with the expected mobilities of CEVd, CBLVd and CVd-IV were also observed. The identity of these RNAs was further confirmed by slot blot hybridization using viroid specific probes and RT-PCR analysis. Results are summarized in Table 1.

As previously described by Önelge et al. (12) in the case of gummy bark sources from Turkey, all the sources from the Oman sultanate were infected with CEVd, HSVd, CVd-III and CVd-IV. Since

the extensive work devoted to the characterization of HSVd isolates from Turkey did not yield any conclusive information (13), we focused on the characterization of the CEVd, CVd-III and CVd-IV isolates recovered from the gummy bark sources from Oman.

Characterization of CEVd isolated from gummy bark sources. Sequence analysis of the uncloned RT-PCR amplicons obtained using CEVd specific primers revealed that all the samples contained very closely related CEVd sequences. The seven consensus sequences were identical except for a single deletion (-U) in position 121 found in the Washington navel source. These sequences (CEVd-gb) were 92.5-93.0% and 94.6-95.0% identical with the class A and class B consensus sequences defined by Visvader and Symons (24, 25). Sequence alignment showed that most of the nucleotide substitutions characteristic of CEVd-gb were located in the P and V domains, but they were distinct from those distinguishing class A from class B (Fig. 1). From the 18 single or multiple adjacent nucleotide changes identified in CEVd-gb, seven were as in class B, one as in class A, and the rest were unique to CEVd-gb (Table 2). A survey of the CEVd sequences available in databases confirmed the uniqueness of CEVd-gb. Only a single CEVd sequence reported from Tunisia contained a similar collection of changes (Elleuch et al. NCBI accession AF540960).

Characterization of CVd-III isolates recovered from gummy bark sources. Sequence analysis of the amplicons obtained by RT-PCR using CVd-III specific primers confirmed that all the samples contained CVd-III. The seven consensus CVd-III sequences ranged in size from 287 to 292 nts. These sequences (CVd-III-gb) were 90.0-95.3% and 96.6-96.9% identical to CVd-IIIa and CVd-IIIb, respectively (17). In spite of differences in their nucleotide

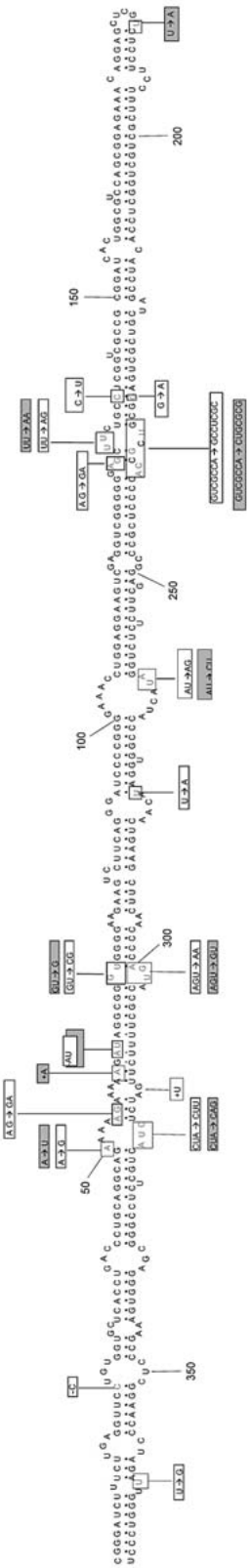

Fig. 1. Secondary structure of CEVd-30, type sequence of Class B (24, 25). Positions differing between the consensus sequences of Class A and Class B are boxed in the secondary structure of CEVd. Changes characteristic of Class A and CEVd-gb are shown as open boxes and shaded boxes, respectively.

TABLE 2
COMPARISON OF CEVD-GB VARIANTS ISOLATED FROM GUMMY BARK SOURCES WITH CLASS A AND CLASS B SEQUENCE VARIANTS[z]

Position (nt)	Class B	Class A	CEVd-gb	GB vs A and B	Location
21	C	-C	C	Class B	T_L
50	A	G	U	Unique	P (loop)
54-55	AG	GA	AG	Class B	P
60	–	–	+A	Unique	P
63-64	AU	-AU	-AU	Class A	P
71-72	GU	CG	G	Unique	P (loop)
131-132	AG	GA	AG	Class B	V (loop)
134-135	UU	AG	AA	Unique	V (loop)
140	C	U	C	Class B	V (comp.)[y]
230	G	A	G	Class B	V (comp.)[y]
233-239	GUCGCCA	GCCUCGC	CUGCGCG	Unique	V (loop)
264-265	AU	AG	CU	Unique	C (loop)
280	U	A	U	Class B	C
300-302	AGU	AA	GU	Unique	P (loop)
315-316	–	+U	–	Class B	P (loop)
317-318	–	–	+U	Unique	P
321-323	CUA	UCA	CAG	Unique	P (loop)
313	U	U	G	Unique	T_L(loop)

[z]The consensus sequence of CEVd-gb was compared to the sequences of CEVd-J and CEVd-30, the type sequences of Class A and Class B, respectively (24, 25).
[y]Compensatory change.

composition, all sequences contained three sets of characteristic changes located in the T_R domain of their secondary structure. These changes (GAGT→CGA, AAGA→G, T→C) (Fig. 2A) resulted in a unique reorganization of the base pairing of this domain (Fig. 2B).

Characterization of CVd-IV isolates recovered from gummy bark sources. Sequencing of the amplicons obtained by RT-PCR using CVd-IV specific primers confirmed that all the samples contained CVd-IV. All CVd-IV sequences contained 286 nucleotides and were identical to the CVd-IV sequences isolated from grapefruit (16), tangor, lemon and mandarin (7). Sequence alignment revealed three changes (+A, +U and U→C) in positions 134, 156 and 160 respectively that were absent in the CVd-IV reference sequence (16). However, these changes, all located in the T_R domain, were also present in the CVd-IV sources from lemon and mandarin reported by Ito et al. (7). Because none of the CVd-IV sequences reported by others were recovered from sweet orange, no conclusions could be drawn regarding the putative implication of CVd-IV as the causal agent of the gummy bark disease.

DISCUSSION

Previous studies conducted in Turkey showed that sweet orange trees affected with gummy bark disease were infected with several viroids (12). Similarly, our analysis of viroid content of sweet orange trees from the Sultanate of Oman displaying characteristic gummy bark symptoms showed that all these trees were also infected with CEVd, HSVd, CVd-III and CVd-IV. Because the symptoms of gummy bark in sweet orange resemble those of cachexia in cachexia-sensitive hosts, the hypothesis that other specific variants of HSVd may be responsible for gummy bark symptoms, was considered. Indeed, the

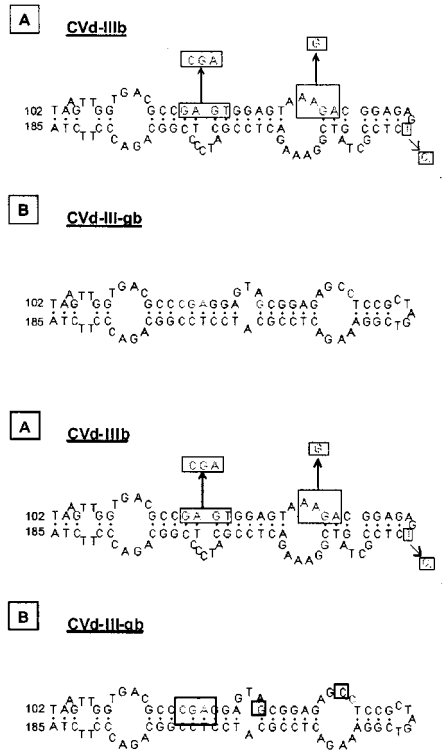

Fig. 2. Comparison of the T_R domains of CVd-IIIb and CVd-III-gb. A) Left part of the secondary structure of CVd-IIIb (17). Arrows indicate the nucleotide changes found in the consensus sequences of CVd-III-gb. B) Rearrangement of base pairing in CVd-III-gb.

characterization of HSVd variants collected from trees showing gummy bark symptoms in Turkey, showed that they were identical, except for single nucleotide insertions in the T_R domain, to CVd-IIc, a variant of HSVd known to induce cachexia (13). Additionally, as in the case of CVd-IIc, the variants associated with gummy bark in Turkey contain the 5-6 nucleotide motif characteristic of cachexia inducing variants and indexed positive for cachexia in the Parson's Special bioassay (13). Since earlier transmission assays (11) showed that grafting certain gummy bark sources onto Orlando tangelo resulted in the development of symptoms on the sweet orange scion but the absence of cachexia symptoms on the Orlando tangelo rootstock, it seems unlikely that the variants of HSVd identified in gummy bark sources would cause gummy bark in sweet orange. Field assays being conducted in Turkey (13) will provide a definitive answer.

Since CEVd and CVd-III have been identified in many sweet orange sources from different citrus growing areas in which the gummy bark disease has never been reported, their role as causal agents of the disease has been considered unlikely. However, in the present study, unusual variants of CEVd (CEVd-gb) and CVd-III (CVd-III-gb) have been identified in the gummy bark sources from Oman. The specific changes identified in the P and V domains of CEVd-gb have been reported from only a single CEVd source from Tunisia (Elleuch et al. NCBI accession AF540960), and are quite distinct from the Class A and Class B changes considered responsible for differences in the severity of CEVd in tomato (25). Similarly, the changes identified in the T_R domain of CVd-III-gb result in a unique reorganization of the base pairing of this domain not found in other CVd-III sequences reported as CVd-IIIa and CVd-IIIb (17). Therefore, CEVd-gb and CVd-III-gb may represent new variants that deserve further characterization.

CVd-IV is the least widespread of all citrus viroids. Its presence in all the gummy bark sources from Turkey and Oman make it an interesting candidate as the putative causal agent. Unfortunately, all previously reported CVd-IV isolates were recovered from species other that sweet orange and rough lemon, the only citrus species known to be sensitive to the disease; thus, their role in gummy bark remains to be determined. Moreover, the effect of CVd-IV on tree performance has only been established in Clementine trees grafted on trifoliate orange (23), a rootstock/scion combination that does not exhibit gummy bark.

At present, we do not have strong evidence of the involvement of a virus or viroid as the causal agent of gummy bark disease. Previous results and the data presented here rule out CBLVd as the causal agent, but we have no clues to entertain or reject the possibility that CVd-IV or the unusual variants of CEVd and CVd-III identified in the present study may be involved. Additionally, the finding that certain viroid combinations cause exocortis-like symptoms in the absence of CEVd (6) shows that multiple viroid infections may result in the expression of unexpected symptoms. Therefore, since all gummy bark sources analyzed are infected by several viroids, the hypothesis that the disease may be caused by specific viroid combinations should also be entertained.

ACKNOWLEDGMENTS

This work was partially supported by RTA01-119 from the Ministerio de Ciencia y Tecnología (MCyT). The first author is recipient of pre-doctoral fellowship of the Generalitat Valenciana. The authors wish to thank Rosario Carbó for technical assistance. We like to thank very much our colleagues from the Sultanate of Oman, Dr. Yousuf M. Al-Raeesy (Crop Protection Research Center, Seeb) and Dr. Herbert Dietz (Directorate General of Agricultural and Veterinary Services, Royal Court Affairs, Sohar) for their help in collecting the gummy bark isolates.

LITERATURE CITED

1. Astruc, N., J. F. Marcos, G. Macquaire, T. Candresse, and V. Pallás
1996. Studies of diagnosis of hop stunt viroid in fruit trees: detection by molecular hybridisation and relationship with specific maladies affecting peach and pear trees. Eur. J. Plant Pathol. 102: 837-846.
2. Bernad, L. and N. Duran-Vila
2005. An improved RT-PCR strategy for detection of citrus viroids. In: *Proc. 16th Conf. IOCV*, 452-455. IOCV, Riverside, CA.
3. Bové, J. M.
1995. *Virus and Virus-like Diseases of Citrus in the Near East Region*. FAO, Rome. 518 pp.
4. De Rijk, P. and R. De Wachter
1997. RnaViz, a program for the visualization of RNA secondary structure. Nucleic Acids Res. 25: 4679-4684.
5. Higgins P. G. and P. M. Sharp
1989. Fast and sensitive multiple sequence alignment on a microcomputer. Comput. Appl. Biosci. 5: 151-153.
6. Igloi, G. L.
1983. Silver staining for the detection of nanogram amounts of tRNA following two-dimensional electrophoresis. Analyt. Biochem. 134: 184-188.
7. Ito, T., K. Ieki, K. Ozaki, T. Iwanami, K. Nakahara, T. Hataya, T. Ito, M. Isaka, and T. Kano
2002. Multiple citrus viroids in citrus from Japan and their ability to produce exocortis-like symptoms in citrus. Phytopathology 92: 542-547.
8. Moreno, P., J. Guerri, and N. Muñoz
1990. Identification of Spanish strains of citrus triteza virus by analysis of double stranded RNAs. Phytopathology 80: 477-482.
9. Nour-Eldin, F.
1956. Phloem discoloration of sweet orange. Phytopathology 46: 238-239.
10. Nour-Eldin, F.
1959. Citrus virus research in Egypt. In: *Citrus Virus Diseases*, J. M. Wallace (ed.), 219-227. Berkeley, Div. Agric. Sci. Univ. Calif. Berkeley, CA.
11. Nour-Eldin, F.
1968. Gummy bark of sweet orange. In: *Indexing Procedures for 15 virus Diseases of Citrus Trees*, 50-53. Agricultural Handbook No. 33., Washington, D.C., Agr. Res. Service, USDA.
12. Önelge, N., A. Çinar, U. Kersting, and J. S. Semancik
1996. Viroids associated with Citrus Gummy bark disease of sweet orange in Turkey. In: *Proc. 13th Conf. IOCV*, 245-251. IOCV, Riverside, CA.

13. Önelge, N., A. Çinar, J. S. Szychowski, G. Vidalakis, and J. S. Semancik
2004. Citrus viroid II variants associated with 'Gummy Bark' disease. Eur. J. Plant Pathol. 110: 1047-1052.
14. Palacio-Bielsa, A., X. Foissac, X., and N. Duran-Vila
2000. Indexing of citrus viroids by imprint hybridization. Eur. J. Plant Pathol. 105: 897-903.
15. Pallás, V., J. A. Sánchez-Navarro, P. Más, M. C. Cañizares, F. Aparicio and J. F. Marcos
1998. Molecular diagnostic techniques and their possible future use in stone fruit certification schemes. Options Méditerranées Ser. B. 19: 191-207.
16. Puchta, H., K. Ramm, R. Luckinger, R. Hadas, M. Bar-Joseph, and H. L. Sänger
1991. Primary and secondary structure of citrus viroid IV (CVd IV), a new chimeric viroid present in dwarfed grapefruit in Israel. Nucleic Acids Res. 19: 6640.
17. 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.
18. Reanwarakorn, K. and J. S. Semancik
1998. Regulation of pathogenicity in hop stunt viroid-related group II. J. Gen. Virol. 79: 3163-3171.
19. Rivera-Bustamante R. F., R. Gin, and J. S. Semancik
1986. Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system. Analyt. Biochem 156: 91-95.
20. Semancik, J. S. and N. Duran-Vila
1991. The grouping of citrus viroids: additional physical and biological determinants and relationships with diseases of citrus. In: *Proc. 11th Conf. IOCV*, 178-188. IOCV Riverside, CA.
21. Semancik, J. S., C. N. Roistacher, R. Rivera-Bustamante, and N. Duran-Vila
1988. Citrus cachexia viroid, a new disease of citrus: Relationship to viroids of the exocortis disease complex. J. Gen. Virol. 69: 3059-3068.
22. 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.
23. 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 on Clementine trees grafted on trifoliate orange. Plant Dis. 88: 1189-1197.
24. Visvader, J. E. and R. H. Symons
1983. Comparative sequence and structure of different isolates of citrus exocortis viroid. Virology 130: 232-237.
25. 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.
26. Zuker, M.
1989. On finding all suboptimal foldings of an RNA molecule. Science 244: 48-52.