A Unique, Infectious RNA Associated with Citron Showing Symptoms Typical of Citrus Exocortis Disease

A. Schlemmer, C. N. Roistacher, and J. S. Semancik

Department of Plant Pathology and Cell Interaction Group, University of California, Riverside 92521.
This reseach was supported in part by the Heinrich-Hertz Stiftung in Düsseldorf, FRG.
Accepted for publication 8 April 1985.

ABSTRACT

Schlemmer, A., Roistacher, C. N., and Semancik, J. S. 1985. A unique, infectious RNA associated with citron showing symptoms typical of citrus exocortis disease. Phytopathology 75:946-949.

A pathogenic RNA with a molecular weight of approximately 105,000 has been detected in extracts from citron (*Citrus medica*) by polyacrylamide gel electrophoresis. Under denaturing conditions, two bands can be observed that migrate faster than the respective circular and linear molecules of the citrus exocortis viroid (CEV). Leaf curling symptoms in citron are similar to those produced by CEV and are favored by high temperature and high light intensity. The isolate was readily transmitted from citron to citron by mechanical inoculation, but not to the known herbaceous hosts of CEV. These properties suggest that the isolate may be a new viroid. Since the most characteristic feature is the irregular or variable appearance of the leaf curling symptoms in citron, the name citron variable viroid (CVaV) is suggested. Eight isolates of what have been characterized as mild forms of the exocortis disease agent principally by their reaction on citron did not protect citron plants against infection by CVaV or severe CEV. Practical implication for the efficacy of citron and gynura (*Gynura aurantiaca*) as indicator plants for CEV are discussed. Furthermore, the question is raised whether CEV is the only causal agent of the syndrome known as citrus exocortis disease.

Additional key words: citrus disease, viroid protection.

Characteristic symptoms of exocortis disease of citrus are bark cracking of Rangpur lime (*Citrus limonia* Osb.) and bark scaling and shelling of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] accompanied by stunting of trees. Arizona 861-S-1 citron (*Citrus medica* L.) is the indicator plant commonly used for indexing purposes (12). Depending on the severity of the exocortis isolate, citron shows varying degrees of leaf curling, browning of veins, leaf tips and petioles, petiole wrinkle, yellowing of shoot tips, and twisting and stunting of plants (12).

The citrus exocortis viroid (CEV) has been described as the causal agent of the disease (15). CEV is a mechanically transmissible RNA consisting of 371 nucleotides in a known sequence, which can exist as either circular or linear molecules (4,17,18). The host range of CEV includes various plant species of Rutaceae, Solanaceae, and Compositae (15). Data presented in this article raises the question whether the specific infectious RNA known as CEV is the only causal agent of exocortis disease.

The pathogen described here was isolated in the mid-1950s from Eureka lemon [*C. limon* (L.) Burm.] on sweet orange [*C. sinensis* (L.) Osb.] growing in Ventura County, CA. The Eureka lemon showed severe bark shelling. When transmitted to calamondin (*C. madurensis* Lour.) on Morton citrange [*P. trifoliata* (L.) Raf. × *C. sinensis* (L.) Osb.] the rootstock expressed mild bark scaling symptoms at soil level. In citron, leaf curling, epinasty, necrosis of veins and stems, and moderate stunting were observed. From these data, the isolate was considered to be a moderately virulent strain of citrus exocortis virus (L. G. Weathers, *personal communication*).

The purpose of the research reported here was to compare the properties, symptomology, and host range of the isolate with those of CEV.

MATERIALS AND METHODS

Plants were grown in UC soil mix (7). Arizona 861-S1 citron, propagated either by rooted cuttings or by grafting on rough lemon (*C. jambhiri* Lush.) rootstock were used as the indicator plant. Host range studies were performed with *Gynura aurantiaca* DC., tomato (*Lycopersicon esculentum* Mill. 'Rutgers'), and petunia (*Petunia hybrida* Vilm. 'Burpee Blue'). Rooted cuttings of gynura and citron were inoculated by ten razor blade slashes, while tomato and petunia seedlings were inoculated by needle puncture when the first two leaves were well developed. In the protection experiment, the plants were graft inoculated with two buds per plant. Greenhouse temperatures ranged from 30–42 C daytime maximum to 18–20 C nighttime maximum in the protection experiment.

Young shoot tips were used for nucleic acid extraction as previously reported (9). The samples were enriched for viroidlike RNA by CF-11 cellulose (Whatman) chromatography (2) in highly purified preparations. The CF-11 cellulose step was omitted with the samples presented in Figs. 2 and 4. Samples were then analyzed on 5% polyacrylamide slab gels 13 × 16 cm, 2 mm thick as described in (10). Electrophoresis was at 60 mA for 2.5 hr at 4 C. The same size of slab gels was used for denaturing gels containing 8 M urea (14), with electrophoresis at 15 mA for 3–4 hr at room temperature. Gels were stained with ethidium bromide and observed with a UV transilluminator.

Nucleic acid bands were eluted in a buffer containing 500 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS by shaking overnight at room temperature (5). The nucleic acid was precipitated with ethanol and resuspended in TKM-buffer which is made of 0.01 M tris (tris[hydroxymethyl]aminomethane), 0.01 M KCl, 10^{-4} M $MgCl_2$ pH 7.4 (16).

RESULTS

The citron plant in which CVaV was maintained indexed negative for psorosis A, concave gum, tatterleaf, tristeza, and vein enation, and is currently under testing for xyloporosis (11). When CVaV was transmitted to citron by razor slashing or budding, leaf curling symptoms accompanied by necrosis of veins and stems occurred at irregular or variable intervals. Depending on light and temperature conditions the symptoms can either be very severe or not expressed at all (Fig. 1). High light intensity and high temperature appear to be necessary for symptom development; however, it was not possible to define the exact threshold conditions. The disease agent was readily transmissible from citron

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

to citron from symptomatic as well as asymptomatic tissue by bud-graft inoculation.

Mechanical inoculation of rooted Arizona 861-S-1 citron cuttings was possible by direct transmission with a cutting instrument contaminated from infected citron plants. The preparations from the following steps in the purification procedure were also infectious: the aqueous phase from the phenol extract, the 2 M LiCl-soluble nucleic acids, and the fraction eluted from the CF-11 cellulose column with STE-buffer (0.1 M NaCl, 0.05 M tris, 0.001 M EDTA, pH 7.2). Plants inoculated with 35 and 25% ethanol-buffer fraction did not show symptoms.

In nucleic acids extracted from infected plants, a unique RNA band with a molecular mass of about 105,000 daltons, approximately 20% smaller than CEV, can be seen migrating slightly slower than the 7S host component in PAGE (Fig. 2A). This band could not be detected in extracts from healthy plants, and when it was eluted from the gel and inoculated to healthy citrons, symptoms identical to those observed in the original plants were reproduced. When the RNA band was exposed to denaturing conditions, a fast- and a slow-migrating band could be detected, presumably corresponding to the linear and circular molecular forms of the viroid (Fig. 2B).

The intensity of the CVaV band from symptomatic and asymptomatic tissue was compared in three experiments. No significant difference in intensity could be observed in two experiments, while in one experiment the CVaV band from asymptomatic tissue was less intense.

Six citron, six gynura, 20 tomato, and 20 petunia plants were mechanically inoculated with nucleic acid extracts from CVaV-infected citrons to determine whether CVaV could infect the same herbaceous hosts as CEV. The experiment was performed under environmental conditions optimal for symptom expression of CVaV. No symptoms developed in any of the inoculated herbaceous plants, while all six citron plants inoculated in the same experiment became infected. Although the herbaceous plants did not show symptoms, nucleic acids were extracted from young shoot tissue to determine whether the viroid had replicated without expressing symptoms. No viroidlike band could be detected in extracts prepared from 9–10 g of tissue from herbaceous plants, while the CVaV band could be detected in extracts from 1.5–2.0 g of citron tissue.

Citron plants that had first been inoculated with CVaV and later challenged with a severe isolate of CEV no longer expressed variable symptoms but rather consistently displayed typical severe symptoms. Both the CEV and the CVaV band could be detected by PAGE (Fig. 2A and B [lane 3]).

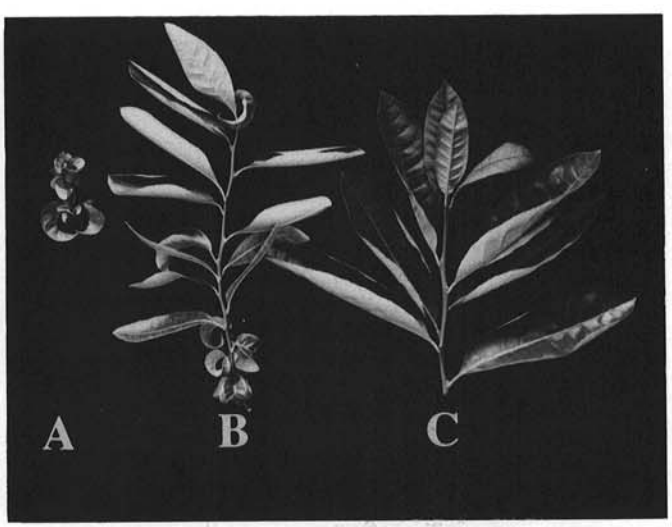

Fig. 1. Comparison of **C,** healthy citron with **B,** citron variable viroid (CVaV)-infected and with **A,** a severe strain of citrus exocortis viroid (CEV)-infected citron. Both symptomatic and asymptomatic phases of CVaV can be observed.

Since these data indicated an absence of protection by CVaV against CEV infection, eight isolates expressing very mild to moderate symptoms of the exocortis reaction in citron (Table 1) were tested in citron for protection from symptom expression of or infection with CVaV or CEV. These isolates had been selected over a period of 20 yr and had been maintained in the virus collection of the Citrus Clonal Protection Program at the University of California, Riverside (9). Isolates E803, E805, E806, and E818 had been isolated from trees on trifoliate orange showing bark cracking.

Six young citron seedlings were first graft inoculated with each of the mild strains. After a period of 10 mo when the mild symptoms were well established, the plants were challenge inoculated by bud graft with either severe CEV or with CVaV. Four months after challenge inoculation, plants challenged with CVaV showed typical CVaV symptoms, and plants challenged with severe CEV expressed typical severe CEV symptoms (Fig. 3 and Table 1). No evidence of protection was demonstrated in either challenged series.

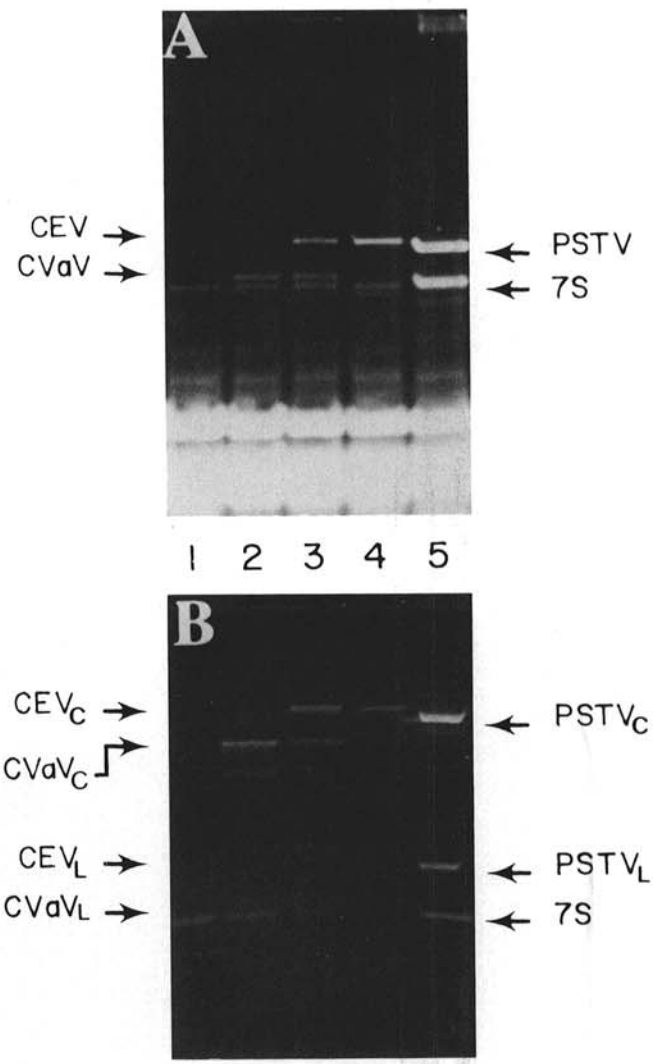

Fig. 2. Electrophoresis of citrus exocortis viroid (CEV) and citron variable viroid (CVaV). **A,** Analysis of nucleic acids on a 5% polyacrylamide gel under nondenaturing conditions. Electrophoresis was for 2.5 hr at 60 mA. **B,** The area between 7S RNA band and CEV band from the nondenaturing band was excised and a second electrophoresis was performed under denaturing conditions (5% polyacrylamide, 8 M urea, and tris-borate buffer). Electrophoresis was for 3 hr at 15 mA. Migration of circular (c) and linear molecules (l) is indicated. Lane 1, healthy citron; lane 2, CVaV-infected citron; lane 3, mixed infection of CEV and CVaV; lane 4, CEV-infected citron; lane 5, potato spindle tuber viroid (PSTV) infected tomato. Each lane represents 15 μg of total nucleic acid (based on $A_{260\ nm}$).

The isolates E818 (which shows very mild symptoms in citron) and E807 (a moderate isolate) were used as examples for analysis of the protection experiment by PAGE. The isolates by themselves had no CEV or CVaV-like bands; however, when challenged with CVaV or CEV the bands typical for those viroids could be detected (Fig. 4).

DISCUSSION

The pathogen described in this paper has characteristics similar to CEV. It is readily mechanically transmissible and under certain environmental conditions shows identical symptoms in citron. The infectious, low-molecular-weight RNA can be resolved during electrophoresis under denaturing conditions into two bands which presumably correspond to the circular and linear forms of the viroid.

Several isolates of CEV have been sequenced, all of them had the same number of nucleotides and only slight differences in nucleotide sequence (4,18). However, all had been maintained in an herbaceous host prior to purification and sequencing which introduces the possibility of screening by a selective host.

CVaV is significantly smaller than CEV and potato spindle tuber viroid (PSTV). While severe CEV reaches higher concentrations in its herbaceous hosts than in citron, CVaV did not produce symptoms in herbaceous hosts of severe CEV and could not be detected by PAGE in extracts from these plants in our experiments. In contrast to severe CEV, which consistently causes severe leaf curling and stunting in citron, symptom expression in citron plants infected with CVaV is variable. These properties would appear sufficiently different to propose a new and unique viroid of citrus. However, determination of the nucleotide sequence of CVaV will be necessary to confirm this assumption. A comparison of the sequence between CEV and CVaV would also confirm if CVaV might represent a variant of CEV characterized by a deletion of approximately 20%. Whether the missing sequences are responsible for the restricted host range also remains an intriguing question.

The practical implication of this work for viroid indexing suggests that in addition to CEV at least one other viroid which is not transmissible to gynura infects citron. This introduces the question whether citron can be considered to be a specific indicator plant for CEV. For indexing purposes of citrus viroids, gynura will have to be considered as the less sensitive, but more specific, rapid indicator, while citron the more sensitive; however, not specific for CEV. The trifoliate orange or Rangpur lime as a rootstock under a vigorous scion when grown under warm conditions for 3–6 yr still remains the ultimate indicator for the exocortis disease.

Cross protection among different strains of PSTV in tomato was first reported by Fernow (1); however, the protection was usually only partial and temporary. It was also shown that although severe symptoms are delayed if tomato plants had been previously inoculated with a mild strain, the severe strain still could replicate in doubly infected plants (1). A similar cross protection reaction among different viroids has been reported (8). Mild strains of PSTV could protect against symptom expression of CEV and severe strains of PSTV, and both viroids could replicate in the doubly infected host. Different responses of different hosts were

TABLE 1. Symptom expression in Arizona 861-S-1 citron (*Citrus medica*) of mild exocortis isolates alone and subsequently challenge-inoculated with CVaV or severe CEV

		Symptom expression[a]		
			Challenge inoculation	
Isolate[b]	Origin[c]	Control inoculation	CVaV	severe CEV
E 803	Frost navel orange	6.0	6.5	10.0
E 805	Bearss lime	4.0	6.0	10.0
E 806	Frost Eureka lemon	2.8	6.0	10.0
E 807	Cleopatra mandarin	4.5	7.0	10.0
E 813	Buddha's Hand citron	4.8	6.3	10.0
E 818	Atwood navel orange	0.8	5.3	9.0
E 819	Washington navel orange	0.8	5.5	9.3
CVaV		5.8	...	...
CEV (severe)		9.8	...	...

[a] Rating 0 = no symptoms, 2 = mild symptoms, 4 = moderate symptoms, 6 = moderate to severe, 8 = severe, 10 = very severe. Six plants were used per treatment.
[b] Isolates from the Citrus Clonal Protection Program at the University of California, Riverside.
[c] Cultivar and kind of plant.

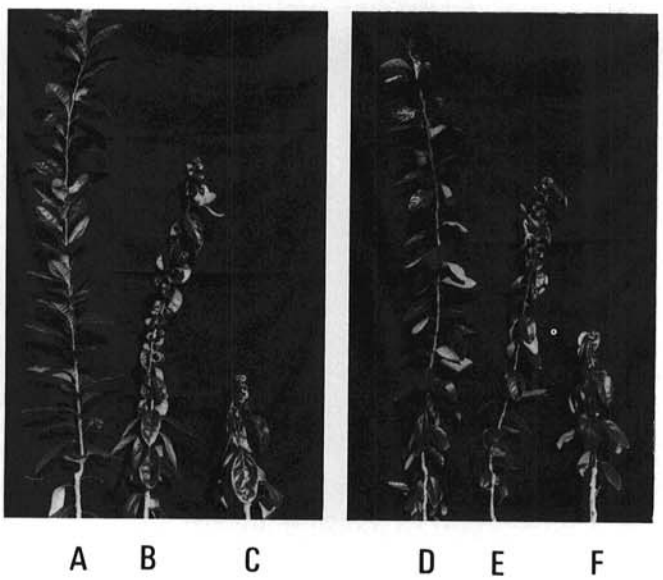

Fig. 3. Results of the protection experiment with citrus exocortis viroid (CEV) mild and severe isolates and citron variable viroid (CVaV) in citron seedlings: **A,** healthy plant; **B,** plant inoculated with CVaV; **C,** plant inoculated with severe CEV; **D,** plant inoculated with isolate E818 of CEV; **E,** plant inoculated with E818 and challenged with CVaV, and **F,** plant inoculated with E818 and challenged with severe CEV.

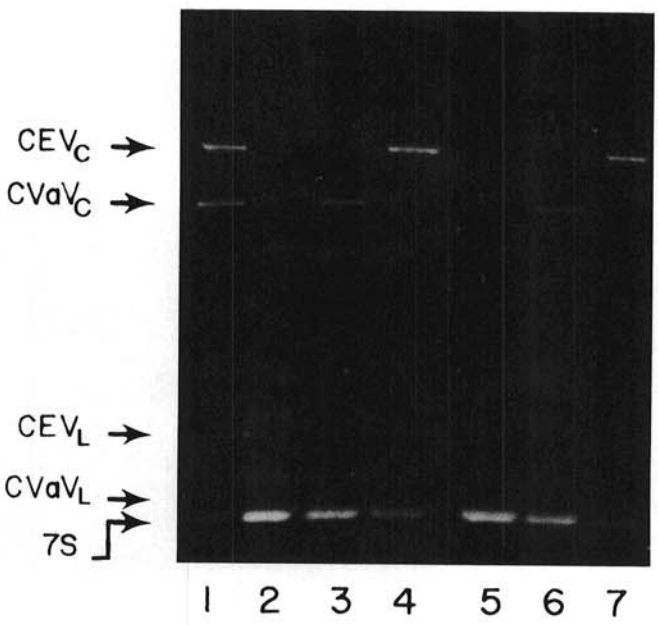

Fig. 4. Denaturing gel of the protection experiment involving citrus exocortis viroid (CEV) mild and severe isolates and citron variable viroid (CVaV). Electrophoresis conditions as described in legend to Fig. 2. Lane 1, standard (double inoculation with severe CEV and CVaV); lane 2, E807 nonchallenged; lane 3, E807 challenged with CVaV; lane 4, E807 challenged with severe CEV; lane 5, E818 nonchallenged; lane 6, E818 challenged with CVaV; and lane 7, E818 challenged with CEV. Each lane represents 20 μg of total nucleic acid (based on $A_{260\ nm}$).

observed. In doubly infected tomato plants, severe symptoms were not expressed at all, while in chrysanthemum the expression of severe symptoms was only delayed (8).

Our experiments indicated an absence of protection in citron. The eight mild isolates of exocortis could not prevent symptom expression when challenged with CVaV or CEV. It was also shown that CVaV and CEV both could replicate in plants preinfected with a mild isolate. Similar results have been reported in the literature (3,6,13). Whether these data can be used to establish the unique nature of CVaV as unrelated to CEV isolates or raise questions of the occurrence of cross protection among viroids in citrus or woody hosts in general remains to be resolved.

It has not yet been conclusively demonstrated, that either CEV, CVaV, or any of the mild isolates by themselves cause bark scaling, the typical exocortis symptom, when inoculated into viroid-free Rangpur lime or trifoliate orange rootstock. Further, the observation that different viroids can replicate simultaneously in citron introduces the possibility that the disease syndrome known as exocortis in the field may be caused by a complex of different viroids.

LITERATURE CITED

1. Fernow, K. H. 1967. Tomato as test plant for detecting mild strains of potato spindle tuber virus. Phytopathology 57:1347-1352.
2. Franklin, R. M. 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Nat. Acad. Sci. 55:1504-1511.
3. Garnsey, S. M., and Cohen, M. 1965. Response of various citron selections to exocortis infection in Florida. Proc. Fla. State Hortic. Soc. 78:41-48.
4. Gross, H. J., Krupp, G., Domdey, H., Raba, M., Jank, P., Lossow, C., Alberty, H., Ramm, K., and Sänger, H. L. 1982. Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur. J. Biochem. 121:249-257.
5. Haseloff, J., and Symons, R. H. 1981. Chrysanthemum stunt viroid: primary sequence and secondary structure. Nucleic Acids Res. 9:271-275.
6. Kapur, S. P., Weathers, L. G., and Calavan, E. C. 1974. Studies on strains of exocortis virus in citrus and *Gynura aurantiaca*. Pages 105-109 in: Proc. 6th Conf. Int. Organiz. Citrus Virol. L. G. Weathers and M. Cohen, eds. IOCV, Riverside, CA.
7. Nauer, E. M., Roistacher, C. N., and Labanauskas, C. K. 1968. Growing citrus in modified UC potting mixtures. Calif. Citrogr. 53:456-461.
8. Niblett, C. L., Dickson, E., Fernow, K. H., Horst, R. K., and Zaitlin, M. 1978. Cross protection among four viroids. Virology 91:198-203.
9. Martón, L., Duran-Vila, N., Lin, J. J., and Semancik, J. S. 1982. Properties of cell cultures containing the citrus exocortis viroid. Virology 122:229-238.
10. Morris, T. J., and Wright, N. S. 1975. Detection on polyacrylamide gel of a diagnostic nucleic acid from tissue infected with potato spindle tuber viroid. Am. Potato J. 52:57-63.
11. Roistacher, C. N. 1976. Detection of citrus viruses by graft transmission: a review. Pages 175-184 in: Proc. 7th Conf. Int. Organiz. Citrus Virol. E. C. Calavan, ed. IOCV, Riverside, CA.
12. Roistacher, C. N., Calavan, E. C., Blue, R. L., Navarro, L., and Gonzales, R. 1977. A new more sensitive citron indicator for detection of mild isolates of citrus exocortis viroid (CEV). Plant Dis. Rep. 61:135-139.
13. Salibe, A. A., and Moreira, S. 1965. Strains of exocortis virus. Pages 108-112 in: Proc. 3rd Conf. Int. Organiz. Citrus Virol. W. C. Price, ed. University of Florida Press, Gainsville.
14. Sänger, H. L., Ramm, K., Domdey, H., Gross, H., Henco, K., and Riesner, D. 1979. Conversion of circular viroid molecules to linear strands. Fed. Eur. Biochem. Soc. Lett. 99:117-122.
15. Semancik, J. S. 1980. Citrus Exocortis Viroid. No. 226 in: Description of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
16. Semancik, J. S., and Weathers, L. G. 1972. Exocortis virus: An infectious, free-nucleic acid plant virus with unusual properties. Virology 47:456-466.
17. Visvader, J. E., Gould, A., Bruening, G., and Symons, R. H. 1982. Citrus exocortis viroid: Nucleotide sequence and secondary structure of an Australian isolate. Fed. Eur. Biochem. Soc. Lett. 137:288-292.
18. Visvader, J. E., and Symons, R. H. 1983. Comparative sequence and structure of different isolates of citrus exocortis viroid. Virology 130:232-237.