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## Practical Field Detection of Citrus Viroids in Florida by RT-PCR

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**Abstract.** A practical approach to the detection of the three common citrus viroids in Florida citrus from field-collected tissue by RT-PCR was developed and tested. Reverse transcriptions were done with total nucleic acid extracts prepared by a SDS-potassium acetate extraction of small amounts of tissue pulverized in Tris buffer. PCR amplifications were done using previously described primer pairs specific for *Citrus exocortis viroid* (CEVd), Citrus viroid II (CVd-II) (*Hop stunt viroid*) and *Citrus viroid III* (CVd-III), and the products were analyzed by agarose gel electrophoresis. Effects of cultivar, tissue location and sampling time were investigated. CEVd, CVd-II, and CVd-III were all consistently detected in tissue from experimentally infected orange, lemon, and lime cultivars, but detection from grapefruit and mandarins was less consistent, especially for CEVd. CEVd and CVd-II were not detected in Meiwa kumquat and detection of CVd-III was rare. Bark tissue from woody, budwood-sized twigs was the best tissue source, and samples collected in warm weather yielded better results than those collected in winter. Field surveys of several hundred trees in commercial groves and test plots with varying viroid profiles were conducted. The correlation between results from RT-PCR and biological indexing exceeded 90%. RT-PCR was especially effective for detection of CVd-II, the citrus viroid most difficult to detect biologically or by sequential PAGE. Several isolates that caused moderate symptoms in Etrog citron were not amplified by our CEVd, CVd-II and CVd-III primers. One was identified as *Citrus viroid IV*, and this is the first report of this viroid in Florida. The others were identified as sequence variants of CVd-III. The methods developed have been used successfully by personnel in three different laboratories.

At least five distinct viroid species affect citrus (14) and there are multiple variants within some species (1). Exocortis, caused by *Citrus exocortis viroid* (CEVd), and citrus cachexia (xyloporosis), caused by Citrus viroid IIb (CVd-IIb), are economically important diseases (11). Other viroids, especially *Citrus viroid III* (CVd-III), can stunt trees grafted on trifoliolate orange and some of its hybrids (5, 15). CEVd, CVd-II and CVd-III are widespread in most citrus growing areas. They are readily spread by propagation of infected budwood and as contaminants on pruning tools (10). Citrus viroids are often present in low concentrations in citrus hosts, and, with the exception of CVd-IIb, are generally symptomless in commercial scion cultivars.

Control of viroid diseases is based on therapy of infected budwood sources, certification programs to prevent propagation of infected budwood, and decontamination to prevent movement via contaminated tools. Development of rapid and reli-

able indexing tests for viroids to expedite control has been a continuing process. Graft-inoculation of sensitive selections of Etrog citron allows detection of the four citrus viroids that produce good symptoms in this host, but this requires 3 to 9 mo plus good greenhouse facilities and experienced personnel (10). CVd-II does not produce easily recognized symptoms in Etrog, and while isolates of CVd-II that induce cachexia can be detected in 8-12 mo by graft-inoculation of Parson's Special mandarin, there are no good biological tests for CVd-IIa. Sequential gel electrophoresis (sPAGE) has been the most commonly used non-biological test for viroids (13) and is capable of detecting all citrus viroids. A drawback to sPAGE is that viroid concentration in commercial citrus cultivars is frequently too low to be detected reliably, and sPAGE normally must be conducted using extracts from graft-inoculated Etrog citron, a host in which viroids replicate well. Even with biological

amplification in citron, precise technique is required to detect CVd-II by sPAGE. Direct hybridization assays with labeled probes (6) offer another approach, but require preparation of probes with sufficient sensitivity to detect the low titers of viroids frequently encountered.

The advent of polymerase chain reaction (PCR) protocols for amplification of pathogen-specific nucleic acids offered a new approach to detection of citrus viroids. The cost and complexity of PCR testing limits its application for many pathogens, but its sensitivity, and a significant potential to reduce indexing time makes RT-PCR potentially attractive for detecting citrus viroids. Yang et al. (17) demonstrated that PCR could be used for detection of CEVd and CVd-II from extracts of infected citron and sweet orange plants under research conditions, and Levy et al. (8) demonstrated simultaneous detection of both viroids by use of multiplex primers. Although these studies were promising, the feasibility of using RT-PCR on a practical basis for detection of citrus viroids was not extensively tested. Limited results reported from Italy indicated some success in detecting viroids by PCR from field sources under summer conditions (16), but only two samples from sweet orange were tested.

Here we report results of a multi-year effort to determine whether or not CEVd, CVd-II and CVd-III could be detected reliably directly from sweet orange and other citrus cultivars in Florida. Development of a practical testing method, its successful application to direct detection of viroids from field-grown trees and successful transfer of this technology to other testing laboratories are reported. A preliminary report of some results for CEVd and CVd-II has been previously published (4).

## METHODS AND MATERIALS

**Viroid sources.** Several previously described viroid isolates (7,

17) were used in developmental studies on extraction methods, host effects, seasonal conditions and tissue sources. E9 was used as a standard source of CEVd and X7 was used as a source of CVd-IIb. E11 contained a mixed infection of CVd-III and CVd-IIa. E36 was used as a pure source of CVd-III. It was originally obtained from a stunted pineapple sweet orange tree on a trifoliolate orange rootstock and subsequently passaged mechanically to Etrog citron and purified by sPAGE. A mixed source of citrus viroids (T68) was used in preliminary field tests and contained CEVd plus CVd-II and CVd-III. Isolates from a Florida viroid collection established by the senior author and currently maintained by the Florida Citrus Budwood Registration Program, plus isolates discovered while indexing budwood source trees for registration provided additional sources with predetermined biological properties. Field trees in several locations were selected for testing based on field symptoms observed.

To test host effects on viroid detection, multiple viroid-free propagations of Hamlin, Navel and Valencia sweet oranges, Redblush and Rio Red grapefruit, Satsuma mandarin, Sunburst and Ambersweet mandarin hybrids, Eureka lemon, Persian and Mexican limes and Meiwa kumquat were graft-inoculated with E9, X7 and E36, singly or in combination. Some replications of these plants were grown under glasshouse conditions and others were planted out doors for studies on seasonal effects on detection efficiency. Plants were grown in the field for at least 1 yr prior to testing.

**Biological indexing.** Indexing for CEVd or CVd-IIb on indicator plants was done in a greenhouse equipped with evaporative coolers and operated at a temperature range of 20-24°C night and 30-33°C day (Max). All plants were propagated from seed or virus-free source

plants in a commercial potting mix and fertilized via a fertigation system for vigorous growth. Insects were controlled by periodic application of suitable pesticides and all pruning equipment was dipped in 0.5% sodium hypochlorite prior to each use. Presence of CEVd and CVd-III was determined by graft-inoculation to Etrog Citron 861-S1 (10). Indicator plants were cut back after inoculation and each flush of growth was examined for at least 6 mo post-inoculation. Presence of CVd-IIb was determined by graft-inoculation to rough lemon seedlings pre-budded with Parson Special mandarin (10). Readings were made 10-12 mo post-inoculation by examining 6 cm long bark strips removed from across the budunion for gumming and pitting symptoms. Under our conditions CVd-IIa did not produce detectable symptoms in Etrog or in Parson Special, but mechanical inoculation of Bonnie Jean chrysanthemum with E11 produced typical CVd-II symptoms.

**Tissue sources.** For most tests, bark tissue was collected from recently matured stems approximately the size and age desirable for budwood. Composite samples were assembled by mixing diced tissue from several stems. For studies on the effect of tissue source on detection efficiency, tissues were also collected from the tender tips of new flushes of growth, tender young leaves (not fully expanded), bark peeled from young shoots, mature leaves, and bark patches from the trunk of test plants. Tissues collected were either extracted immediately after collection, or stored at  $-20^{\circ}\text{C}$  until extracted.

**Preparation of nucleic acid extracts.** The extraction procedure used for most tests was an adaptation of a SDS-potassium acetate (SDS-KAc) protocol for extraction of dsRNAs from plant tissues (2). A 0.2 to 0.5 g sample of tissue was macerated in a Kleco Tissue Pulverizer (Kinetic Laboratory Equipment Co.,

Visalia, CA). The extraction buffer (1.5 to 3.0 ml) was 0.1 M Tris, pH 8.0, which contained 50 mM EDTA, 500 mM NaCl, and 10 mM 2-mercaptoethanol. In some cases, a Mini Bead Beater 8 tissue pulverizer (Bio Spec Products, Inc., Bartlesville, OK) was used or tissue was ground with a mortar and pestle in the presence of dry ice. After maceration, 100  $\mu\text{l}$  of 10% SDS was added per 750  $\mu\text{l}$  of extract and the resulting mixture was incubated for 20-30 min at  $65^{\circ}\text{C}$ . Next, 500  $\mu\text{l}$  of 5M KOAc was added and mixed thoroughly. After a 20 min incubation on ice, the mixture was centrifuged for 10-15 min in a microfuge and 400 to 500  $\mu\text{l}$  of the resulting supernatant was transferred to a fresh tube. NaOAc (0.1 vol.) and ethanol (3 vol.) were added and the mixture was incubated at least 2 hr at  $-20^{\circ}\text{C}$ . After centrifugation, the pellet was air dried, resuspended in distilled  $\text{H}_2\text{O}$  or Tris extraction buffer and stored at  $-20^{\circ}\text{C}$ . Reusable equipment was washed with water, soaked in 0.5% sodium hypochlorite and rinsed again to prevent possible cross contamination between samples

In some cases, a modification of the extraction procedures used for preparation of nucleic acid extracts for sPAGE (13) was used. In this case 1-5 g of infected tissue was extracted with a VirTis homogenizer using an extraction medium that contained 0.4 M Tris buffer, pH 8.9, 5 mM EDTA, 4% 2-mercaptoethanol, 1% SDS 1:1 with water-saturated phenol. Extraction was followed by centrifugation, ethanol precipitation of the aqueous phase in the presence of sodium acetate, dialysis, LiCl partitioning of the nucleic acids and ethanol precipitation of the lithium chloride soluble fraction. In some tests, tissue was powdered with a mortar and pestle in the presence of liquid nitrogen before extraction.

**cDNA synthesis.** For most tests 1  $\mu\text{l}$  of nucleic acid extract was added to 19  $\mu\text{l}$  of RT reaction mix which

consisted of RT buffer (10 mM Tris buffer, pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.75  $\mu$ M primers complementary to CEVd, CVd-II or CVd-III, 5 mM MgCl<sub>2</sub>, 1 mM each of dATP, dTTP, dCTP, and dGTP, 4 units of Rnasin (Promega) and 2.5 units of MMLV reverse transcriptase (Promega). The reactions were incubated in a thermocycler for 15 min at 42°C, 5 min at 9°C, and 5 min at 5°C. In some tests 2  $\mu$ l of nucleic acids, a primer concentration of 0.15 mM, and a thermocycler setting of 99°C were used. The primers used have been previously described (9, 17). The complementary primer for CEVd was the 20 mer 5'-CCCTGAAGGACTTCTTC-CCC-3' (17), for CVd-II the 19 mer 5'-GGCTCCTTTCTCAGGTAAG-3' (17), and for CVd-III the 20 mer 5'-ACTC-TACCGTCTTTACTCCA-3' (9).

**PCR Amplification.** For most amplifications, we used 20  $\mu$ l of the RT reaction product plus 80  $\mu$ l of a PCR reaction mix which contained PCR buffer (10 mM Tris, pH 8.3, and 50 mM MgCl<sub>2</sub>), 1.2 mM MgCl<sub>2</sub>, 1 unit of Taq DNA polymerase (Perkin Elmer), and 0.3  $\mu$ M of primers homologous to CEVd, CVd-II or CVd-III. Other variations in reactant concentrations were used for some tests. The homologous primer for CEVd was the 24 mer 5'-ATCCCCGGG-GAAACCTGGAGGAAG-3' (17), while the 25 mer 5'-CCGGGGCAACTCT-TCTCAGAATCCA-3' (17) was used for CVd-II and the 24 mer 5'-CTC-CGCTAGTCGGAAAGACTCCGC-3' (9) was used for CVd-III. Following initial denaturation at 95°C for 2 min, PCR was carried out for 35 cycles at 95°C (1 min), 60°C (1 min) and 72°C (1 min) followed by a final 5 min extension at 72°C.

**Analysis of PCR products.** Detection and analysis of PCR products was routinely done by electrophoresis on 1% agarose gels using a mini gel apparatus and standard protocols. Gels were stained with ethidium bromide. Identification of bands was determined by comparisons with products obtained with

healthy and appropriate viroid-infected controls. A 100 bp ladder (Promega) provided molecular weight markers. Further verification of band identity was accomplished in preliminary tests by southern transfer to positively charged nylon membranes (Boehringer Mannheim) and probing with DIG-labeled viroid-specific probes (Genius 4, Boehringer Mannheim) prepared per manufacturer's instructions.

**sPAGE.** In several instances, the presence and putative identity of viroids in various samples was confirmed by sPAGE (13) using extracts prepared from young flush tissue collected from S1 citron indicator plants inoculated at least 3 mo prior to assay.

## RESULTS

**Preliminary studies.** Initial experiments were conducted with known sources of viroids to gain experience and evaluate conditions that affect successful application of PCR. A number of variations in protocol were investigated to optimize reverse transcription and amplification conditions. We confirmed that the primers used for each viroid did not cross react with other viroids and that extracts obtained by the SDS-KAc method could be used successfully for reverse transcription and amplification. Typical results of the PCR products obtained using CVd-II primers are shown in Fig. 1. Although extracts obtained by the SDS-KAc method were less pure, and, apparently, had a lower viroid titer than those prepared by the protocol used for sPAGE, they yielded consistent results and allowed rapid processing of large numbers of samples. Relative concentrations of viroids and host nucleic acids in the SDS-KAc crude extracts were not determined, and testing several 10-fold dilutions of these extracts often helped optimize yield of the viroid product and minimize spurious host-associated products that were



Fig. 1. Gel electrophoresis of amplification products generated by RT-PCR from a panel of 28 citrus extracts using primers designed to amplify CVd-II. Extracts from plants infected singly with CVd-IIIb (lanes 13, 25), CVd-IIa plus CVd-III (lanes 5, 26), CVd-II in a mixture with CEVd and CVd-III from Valencia (lane 4), satsuma (lane 9), Redblush grapefruit (lane 21) and citron (lane 28) all yielded clearly detectable products along with five other sources with known or suspected CVd-II infection. Healthy plants of Redblush, Valencia, satsuma, and citron (lanes 8,12,18 and 24), as well as plants infected singly with CEVd (lane 17), CVd-III (lane 19) and CVd-IV (lane 1) did not yield detectable products. Lane 16 contains 100 BP ladder. Viroid bands appear near 300 BP marker.

sometimes observed. These spurious reaction products did not react with labeled viroid probes and could largely be eliminated by adjusting test parameters, especially for the RT step. In any case, they could be identified by comparison with healthy controls included in each test. Tests with samples stored frozen, or re-hydrated from lyophilized or dried tissue indicated that tissue samples could be stored for extended periods. Aliquots of lyophilized liquid nitrogen-powdered tissue provided a consistent reference source for studies conducted over extended periods.

**Effect of tissue source on viroid detection.** To investigate the source of tissue most suitable for detection of CEVd and CVd-II, sweet orange trees were inoculated with a source containing a mixture of viroids. After the plants had become systemically infected, shoot tips, tender leaves, tender bark, mature leaves, bark for mature stems (budwood) and bark from the trunk were collected. Extracts were prepared by

the SDS-KAc method from comparable amounts of tissue and tested by RT-PCR. Results are summarized in Table 1 and indicated that CVd-II was more readily detected in tissues of different ages than CEVd, but that very tender shoot tips or mature leaves were not good sources for either viroid. Mature stem bark gave good results with both viroids and because it was convenient for field sample collection, we used it routinely for subsequent tests.

TABLE 1  
DETECTION OF CEVd AND CVd-II VIROIDS BY RT-PCR FROM SWEET ORANGE TISSUES OF DIFFERENT AGES

| Tissue        | CEVd             | CVd-II |
|---------------|------------------|--------|
| Tip flush     | 0/2 <sup>z</sup> | 0/2    |
| Tender leaves | 0/2              | 2/2    |
| Tender bark   | 0/2              | 2/2    |
| Mature leaves | 0/2              | 0/2    |
| Mature bark   | 2/2              | 2/2    |
| Trunk bark    | 2/2              | 2/2    |

<sup>z</sup>Number positive assays over total attempted from separate tissue samples.



**Seasonal and host effects on viroid detection.** To investigate the effect of seasonal conditions and cultivar on viroid detection, we inoculated 12 citrus cultivars with different viroids or viroid combinations. Initial tests were done with CEVd and CVd-II, the only viroids for which primers were available when the project was started. CVd-III was added when primers became available (9). Infected plants were maintained under glasshouse conditions and also planted outdoors in an experimental plot. Samples were collected periodically over several years during warm weather (May–September) with daily maximum temperatures averaging 32°C or higher and daily minimums near 21 to 23°C, and during periods of cool weather (December and January) when maximum temperatures were 8 to 21°C and minimums were 0 to 12°C. The results of these tests, summarized in Table 2, indicate that CVd-II and

CVd-III could be detected regularly from all hosts except meiwa kumquat in warm weather. Detection of CEVd from sweet oranges, Ambersweet, Eureka lemon and limes was also consistent during warm weather, while detection from grapefruit, Satsuma and Sunburst was erratic, and no detection was possible from Meiwa kumquat. In general, detection rates were reduced somewhat in samples collected in cool weather, but detection levels for CVd-II and CVd-III still remained high in most hosts. The greatest reduction occurred with CEVd.

While none of the three viroids could be detected from Meiwa kumquat (with the exception of one positive test for CVd-III), all three viroids were detected in bark samples collected from the rough lemon rootstock of the same plants. Viroids could be detected sporadically from Meiwa by graft-inoculation to Etrog citron, but the low levels of detection confirmed

TABLE 2  
SEASONAL AND CULTIVAR EFFECTS ON DETECTION OF CITRUS VIROIDS BY RT-PCR

| Cultivar                    | CEVd              |                   | CVd-II |       | CVd-III |       |
|-----------------------------|-------------------|-------------------|--------|-------|---------|-------|
|                             | warm <sup>z</sup> | cool <sup>y</sup> | warm   | cool  | warm    | cool  |
| Oranges                     |                   |                   |        |       |         |       |
| Hamlin                      | 6/6*              | 3/8               | 6/6    | 8/8   | 4/4     | 8/8   |
| Navel                       | 6/6               | 3/8               | 6/6    | 8/8   | 4/4     | 8/8   |
| Valencia                    | 6/6               | 3/8               | 6/6    | 8/8   | 4/4     | 7/8   |
| Grapefruit                  |                   |                   |        |       |         |       |
| Redblush                    | 5/6               | 3/8               | 6/6    | 8/8   | 4/4     | 6/8   |
| Rio Red                     | 1/6               | 3/8               | 4/6    | 7/8   | 3/4     | 8/8   |
| Mandarin/hybrid             |                   |                   |        |       |         |       |
| Satsuma                     | 3/6               | 1/8               | 6/6    | 8/8   | 3/4     | 5/7   |
| Sunburst                    | 1/6               | 1/8               | 6/6    | 7/8   | 4/4     | 3/8   |
| Ambersweet                  | 5/5               | 6/7               | 6/6    | 8/8   | 4/4     | 8/8   |
| Eureka lemon                | 6/6               | 6/8               | 6/6    | 8/8   | 4/4     | 8/8   |
| Mexican Lime                | 6/6               | 1/8               | 6/6    | 8/8   | 4/4     | 7/8   |
| Persian lime                | 6/6               | 7/8               | 6/6    | 8/8   | 4/4     | 8/8   |
| Meiwa kumquat               | 0/6               | 0/8               | 0/6    | 0/8   | 0/4     | 1/8   |
| Adjusted total <sup>w</sup> | 51/65             | 37/87             | 64/66  | 86/88 | 42/44   | 76/87 |

Data expressed as samples positive/total samples.

<sup>z</sup>Samples collected during summer months with warm weather.

<sup>y</sup>Samples collected during cool weather in December and January.

<sup>\*</sup>Results from multiple assays over several years.

<sup>w</sup>Totals for all hosts with kumquat results excluded.

that neither CEVd nor CVd-III replicated well in this cultivar.

**Detection of viroids from field trees.** Budwood-sized twigs were collected from field trees in different locations to test the ability to detect CEVd, CVd-II and CVd-III directly from the field. Samples were collected during warm weather. Viroid-infected sources were selected based on presence of field symptoms and/or prior knowledge. Samples from trees of the same cultivars that were thought to be viroid-free were collected simultaneously, but not necessarily from the same plantings. Samples collected for testing for CEVd and CVd-III were also graft-inoculated to Etrog citron indicators. In the case of CVd-II, limited numbers of samples were also tested by graft-inoculation of Parson Special mandarin, but in most cases the biological data was based on prior testing of source plants on Orlando tangelo rootstocks. In each test known sources of viroids were included along with healthy controls.

The results are summarized in Tables 3-5 and indicate high levels of detection of all three viroids from trees suspected of being infected. In several cases, where positive tests were obtained from supposedly viroid-free plants, the initial RT-PCR data was confirmed by subsequent biological testing. Companion

testing for CEVd by RT-PCR while analyzing samples for CVd-III (Table 5) confirmed a close parallel between field and or biological indexing results and RT-PCR data. In several tests, aliquots of tissue from the same set of samples were blind coded and shared with other laboratories for independent processing. The results obtained matched closely, and where differences arose, they were usually associated with samples that appeared to have a low titer.

**Testing of viroid isolate collections.** A collection of 55 viroid isolates derived from 44 different sources representing various locations and hosts within Florida was tested by RT-PCR. Prior citron indexing data was available for most of these isolates. Twenty-two sources tested positively for CEVd and included all 20 isolates that had produced typical CEVd symptoms in citron, plus two isolates that produced more moderate citron symptoms. Forty-five isolates tested positively for CVd-III and all of these produced mild to moderate leaf epinasty in citron (in the absence of co-infection with CEVd). Most produced petiole browning symptoms typical of CVd-III infection. Based on analysis with selective probes, these isolates included CVd-IIIa and CVd-IIIb (both singly and as co-infections), plus several

TABLE 3  
FIELD TESTING SWEET ORANGE TREES FOR CITRUS EXOCORTIS VIROID (CEVd) INFECTION BY RT-PCR

| Cultivar | Trees tested | Reported viroid content <sup>a</sup> | CEVd Citron reaction <sup>b</sup> | RT PCR assay <sup>c</sup> |
|----------|--------------|--------------------------------------|-----------------------------------|---------------------------|
| Navel    | 12           | CEVd                                 | 11                                | 11                        |
| Hamlin   | 9            | CEVd                                 | 6 <sup>d</sup>                    | 6                         |
| Valencia | 16           | CEVd                                 | 16                                | 16                        |
| Navel    | 13           | None                                 | 1                                 | 1                         |
| Hamlin   | 10           | None                                 | 0                                 | 0                         |
| Valencia | 15           | None                                 | 0                                 | 0                         |

<sup>a</sup>Suspected viroid content based on field symptoms or prior biological test results.

<sup>b</sup>Severe leaf epinasty symptoms observed in graft-inoculated S1 citron indicators.

<sup>c</sup>Results from two separate PCR tests.

<sup>d</sup>Three isolates produced moderate leaf epinasty symptoms in citron typical of CVd-III.



TABLE 4  
FIELD TESTING SWEET ORANGE TREES FOR CITRUS VIROID II (CVd-II) BY RT-PCR

| Cultivar     | Trees tested | Reported viroid content | RT-PCR assay results <sup>a</sup> |
|--------------|--------------|-------------------------|-----------------------------------|
| Navel        | 12           | cachexia <sup>a</sup>   | 12                                |
| Parson Brown | 4            | cachexia                | 3                                 |
| Sweet sdlg   | 9            | cachexia                | 9                                 |
| Navel        | 13           | None                    | 0                                 |
| Parson Brown | 9            | None                    | 0                                 |
| Sweet sdlg   | 3            | None                    | 0                                 |

<sup>a</sup>Samples collected from trees that had previously indexed positively for CVd-IIIb by propagation on Orlando tangelo rootstocks in budwood registration indexing tests.

<sup>b</sup>Results from two separate RT-PCR assays.

other sequence variants (12), Two isolates that produced mild to moderate leaf epinasty symptoms on Etrog citron did not yield a CVd-III amplification product in repeated tests. sPAGE analysis indicated that one of these isolates was a *Citrus viroid IV* (CVd-IV), and the other was a group III variant in which sequence divergence prevent amplification with the primers used. Both isolates were obtained from citron plants that had been used to index a collection of old varieties, and, therefore, may have different origins than isolates commonly distributed in commercial Florida plantings. Thirty-four isolates tested positively for CVd-II. In many cases no biological indexing information was available to confirm these results, but all sources known to cause cachexia or to contain CVd-IIa (based on S-PAGE)

tested positively. Comparative testing of 22 isolates by another laboratory yielded identical results.

## DISCUSSION

The results presented here indicate that CEVd, CVd-II and CVd-III can be detected from field samples of sweet orange growing under Florida conditions by RT-PCR procedures suitable for practical large scale applications. Seasonal effects on testing were noted, especially for CEVd, but CVd-II and CVd-III were successfully detected under the mild winter conditions experienced in Florida. When using bark samples from budwood-sized stems for testing, cambial activity may be a more reliable guide for sampling than temperature, and sampling should probably be avoided when bark can-

TABLE 5  
FIELD TESTING SWEET ORANGE TREES FOR CITRUS VIROID III (CVd-III) BY RT-PCR

| Cultivar             | Trees tested | Canopy symptoms | Bark symptom <sup>a</sup> | Citron reaction <sup>b</sup> | CVd-III RT-PCR | CEVd RT-PCR |
|----------------------|--------------|-----------------|---------------------------|------------------------------|----------------|-------------|
| Val/TFO <sup>c</sup> | 13           | stunted         | none                      | 13M                          | 13             | 0           |
| Val/TFO              | 8            | stunted         | scaling-M                 | 8 M                          | 8              | 1           |
| Val/TFO              | 5            | stunted         | scaling-S                 | 4 S,1 M                      | 5              | 4           |
| Val/Carr             | 11           | none            | none                      | 3 M                          | 3              | 0           |
| Val/Carr             | 2            | blight          | none                      | 2 M                          | 2              | 1           |
| Val/SFS              | 11           | none            | none                      | 0                            | 0              | 0           |

<sup>a</sup>Val/TFO were 30 yr old Valencia orange grafted on trifoliolate orange rootstock, Val/ Carr were 23 yr old Valencia orange grafted on Carrizo citrange rootstock and Val/SFS were 12 yr old Valencia orange grafted on Smooth Flat Seville orange rootstock.

<sup>b</sup>Visual symptoms on rootstock. Scaling M = mild scaling, scaling S = strong scaling.

<sup>c</sup>Symptoms observed on Etrog citron. M = moderate leaf epinasty plus petiole browning and S = strong leaf epinasty plus petiole browning.

not be easily peeled from the stem. Appropriate positive control samples collected from the same hosts and under the same conditions remain the best means to verify suitability of sampling conditions. A detailed comparison of different tissues was not done for CVd-III, but the mature stem bark favorable for detection of CEVd and CVd-II also worked well for CVd-III.

Some cultivar-specific influences on viroid detection by RT-PCR were observed. While detection from sweet orange, Eureka lemon, limes and Ambersweet was highly consistent for all three viroids tested, detection of CEVd from grapefruit, Satsuma and the Sunburst mandarin hybrid was less consistent than that of CVd-II and CVd-III. This may reflect a lower titer or more erratic distribution in these hosts. The most notable failure of RT-PCR was with Meiwa kumquat where only one of 40 samples yielded a positive test. This is apparently due to the resistance of Meiwa to viroid infection rather than a problem associated with the RT-PCR methodology. While CEVd and CVd-III were sometimes detected from our experimentally inoculated Meiwa plants by biological indexing on Etrog, most attempts to graft-transmit these viroids from Meiwa failed. This indicates that there is either a very low overall titer or a very irregular distribution of these viroids in Meiwa. All three viroids were detected by PCR from the bark of the rough lemon rootstocks, indicating that the original inoculations had been successful.

The detection of viroids from field-collected lemon, orange, grapefruit and tangerine and tangelo by PCR has been previously reported (16), but most samples were from lemon, and details on results for specific viroid-cultivar combinations were not given. The data presented here provides much additional information about the reliability of RT-PCR for testing

field samples. However, caution will be needed in extending use of RT-PCR detection other cultivars until known positive controls from those specific cultivars are tested.

The generally more robust detection of CVd-II and CVd-III by RT-PCR as compared to CEVd is in contrast to experience with other methods such as sPAGE (13). Since similar results were obtained even with Etrog citron, an excellent host for CEVd, the primers used for CEVd may be less efficient than those used for CVd-II, and CVd-III. We also observed the presence of spurious amplification products more frequently with the CEVd primers than those for CVd-II and CVd-III. These could be recognized with appropriate controls but are still undesirable for routine diagnostic work. Subsequent development of other primer pairs for CEVd is reported in a companion paper (12). Although the PCR-based detection of CEVd needs further optimization, we still were able, with the few exceptions noted above, to detect CEVd with reasonable ease from samples collected in warm weather. Fortunately, RT-PCR worked very well for detection of CVd-II, the viroid that has been the most difficult to detect biologically or by sPAGE.

An important objective of this study was to develop methodologies that could be readily adopted by other laboratories. The results obtained initially in one research laboratory were successfully duplicated in two other small moderately well equipped laboratories used primarily for diagnostic purposes. Personnel in all three labs had backgrounds in micro and molecular biology, but little prior experience in PCR methodology. Each lab was also able to modify the general protocol to further adapt it to their specific conditions and equipment. Although the collection of samples from mature stems and the SDS-KAc extraction method are both rapid and convenient, RT-PCR for viroids is still technically demanding and it

is subject to errors that must be recognized and controlled. In contrast to sPAGE and various hybridization assays, the sensitivity achieved through amplification of viroids present in the initial extract also means that contamination or careless handling can easily yield false positive test results. Appropriate controls are needed for collection and processing of samples, and these should be collected under the same conditions and from the same hosts. In some cases, as when a previously untested cultivar is evaluated, it may be necessary to deliberately inoculate plants to create a known infected control. Appropriate controls are also essential during the RT and amplification steps to be sure that all reagents are functioning appropriately.

Our results indicate that RT-PCR can be a useful option for detection of citrus viroids. It offers some particular advantages for detection of CVd-II which is often difficult to detect by other methods. It is also advantageous for situations where rapid detection of viroid infections in field sources is necessary or no glasshouse facilities suitable for biological indexing are available.

There are obvious limitations to RT-PCR. Analysis of amplification products by gel electrophoresis requires only minimal equipment and reagents, but processing large numbers of samples is time consuming and interpretation of band identity requires some experience and appropriate controls. Access to labeled viroid-specific probes allows analysis of the PCR products by blot hybridization and may be a useful option for some applications. More importantly, only those viroids with sufficient homology to the primers will be detected. In situations where

a broad spectrum test is needed, biological testing and sPAGE retain advantages. Advance knowledge of the diversity and relative distribution of different viroid genotypes to be encountered may be required to accurately define the accuracy that can be predicted for RT-PCR testing. For example, further studies will be required in Florida to determine the distribution of CVd-IV and the CVd-III variants that were not detected in the current study. While the detection of CVd-II by RT-PCR appears robust, the potential for missing sequence variants is difficult to verify by comparative biological testing. RT-PCR is also relatively expensive in terms of reagents and labor costs. These costs, however, are still likely to be competitive with other methods available and can be reduced by testing composite samples, especially where incidence of positive trees is expected to be low (12). Further studies relative to the incorporation of RT-PCR methods into a Florida Citrus Budwood testing program are presented in a companion paper (12).

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