Citrus Viruses in Guatemala: Application of Laboratory-Based Assays

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ABSTRACT. In preparation for a citrus certification program in Guatemala, there was an urgent need to determine which graft transmissible citrus pathogens were present. Because of the lack of biological indicator plants, *Citrus tristeza virus* (CTV) and *Xylella fastidiosa* (causal agent for citrus variegated chlorosis) were tested for by ELISA, and we developed a protocol for the extraction of total nucleic acids from the samples to enable RT-PCR testing for CTV, *Citrus psorosis virus (CPsV)*, *Citrus exocortis viroid* (CEVd), Citrus viroid II (CVd-II, *Hop stunt viroid* variants), *Citrus viroid III (CVd-III)*, and PCR testing for *X. fastidiosa*. CTV was found in all citrus areas sampled, but was less prevalent in the central citrus area. The RT-PCR proved to be more sensitive than the ELISA for detecting CTV. *X. fastidiosa* was detected in one sample by ELISA, but this was not confirmed by PCR assay. Citrus viroids (CEVd, CVd-II, and *CVd-III*) were commonly found in samples collected from the three main citrus areas. CPsV was not detected by RT-PCR, and these results were confirmed later by biological indexing.

Index words. Citrus tristeza virus, *Citrus exocortis viroid*, Citrus viroid II, *Citrus cachexia*, *Citrus psorosis virus*, *Xylella fastidiosa*, RT-PCR, Guatemala.

Citrus is becoming increasingly important in Guatemala as a food crop mostly for many small farmers. New citrus plants are being established in the Santa Rosa area in the southern citrus growing region of Guatemala. Most of the older plantings of citrus are on sour orange rootstock while the more recent plants are on *Citrus tristeza virus* (CTV)-tolerant rootstocks (1).

A model certification program is being developed in Guatemala through a regional project sponsored by the InterAmerican Citrus Network and funded by the Food and Agricultural Organization of the United Nations (FAO). It is anticipated that this model program will eventually become a national certification program. In preparation for the implementation of a citrus certification program, there was a need to identify the prevalent graft transmissible diseases of citrus. Biological indexing using indicator plants is the commonly accepted method for determination of graft transmissible pathogens (7). However, due to the lack of both greenhouse facilities and available indicator plants, we developed an alternative method to enable testing for several common citrus pathogens based on PCR and RT-PCR using a single total nucleic acid extraction as the common basis for all PCR-based assays. We report here the laboratory based diagnostic method developed for this study as well as the results of the survey. We believe this protocol may be useful for other countries with similar situations.

MATERIALS AND METHODS

Survey areas. Three citrus areas in Guatemala were surveyed (Fig. 1). The South citrus area (Retalhuleu, Suchitepequez, Escuintla, and Santa Rosa) in the coastal plains near the Pacific Coast; the central citrus area which includes Quetzaltenango and Guatemala,

Fig. 1. Map of Guatemala showing the locations of the three main citrus growing areas (north, central, and south).

and the North citrus area which includes Chiquimula, El Progreso, Alto Verapaz, and Baja Verapaz. The North area is 800 m above mean sea level and has a more Mediterranean-like climate. This area has the older citrus plantings. Most of the new citrus plantations are in the southern area near Santa Rosa. Contact was made with citrus growers to help locate areas where citrus was not growing well, indicating that it may contain graft transmissible pathogens.

Collection of samples. Wherever possible, young flush tissue was selected from symptomatic parts of the field trees. The tissue was placed in plastic bags, labeled, and transported back to the laboratory on ice. All samples collected were screened by ELISA for CTV and *Xylella fastidiosa*. Samples from 45 trees suspected of having other pathogens were used for preparation of total nucleic acid extracts for later analyses.

ELISA assays. All of the samples collected (a total of 282) were analyzed by ELISA (4) to test for the presence of CTV using ELISA kits from Agdia (Elkhart, IN), which use a polyclonal antibody for trapping and a mixture of monoclonal antibodies for detection. A total of 267 of these same samples were tested for *X. fastidiosa*, the causal agent of citrus variegated chlorosis (CVC), using the Agdia ELISA kit for detection of *X. fastidiosa*. Positives from the ELISA tests were declared as recommended by the supplier (an optical density $(OD)_{405nm}$ value over 0.2 after subtracting the OD_{405nm} values obtained for the buffer only and healthy controls).

Extraction of the total nucleic acids. In the laboratory, the tender bark and leaf midrib tissue was chopped finely, mixed to form a composite sample and divided into 4 g aliquots. After freezing in liquid nitrogen, the tissue was pulverized using a mortar and pestle, then added to a 50 ml centrifuge tube containing 19 ml of 2× GPS buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, pH 9.6) with 0.5% (v/v) 2-mercaptoethanol, 5 mg bentonite powder, 1.0 ml 10% SDS, and 5.0 ml of a mixture of phenol/chloroform/pentanol (24/24/2). The tube was shaken vigorously for 1 min then put on ice and shaken vigorously several times for the next 30 min. The tube was then centrifuged for 10 min at 10,000 *g*. The upper aqueous phase was transferred to a sterile 50 ml tube, the volume adjusted to 20 ml with 2× GPS buffer, then divided equally into two 50 ml centrifuge tubes. Twenty five ml of 95% ethanol (2.5 volumes) and 1 ml of 3 M sodium acetate, pH 5.2 (0.1 volume) was added to each tube, mixed thoroughly and stored at -20°C. This preparation was referred to as a total nucleic acid (TNA) extract. The TNA extracts were then shipped on wet ice by express delivery services. Upon receipt, they were stored at -20°C until processed further.

Recovery of the double stranded RNA (dsRNA) fraction for analysis of viruses. Two milliliters of the shaken TNA extract was transferred to a sterile 2 ml

microcentrifuge tube and centrifuged at 13,000 *g* for 7 min at 4°C. The supernatant was discarded and another 2 ml of the TNA extract was added to the same microcentrifuge tube, and centrifuged again as above. The supernatant was discarded, and the pellet was air-dried. The pellet was then resuspended in 333 µl STE buffer (0.1 M Tris, 0.5 M NaCl, 1 mM EDTA, pH 8.0) then 50 mg CF-11 cellulose powder and 67 µl of 95% ethanol (for a final concentration of ethanol of 16%) were added, and the mixture was shaken on ice at about 120 cycles/min for 30 min. The sample was then centrifuged at 13,000 *g* for 3 min, the supernatant was discarded and the CF-11 pellet was resuspended in 400 µl of STE buffer containing 16% ethanol (wash buffer). This wash step was repeated three times. The dsRNA was then released from the CF-11 cellulose by mixing with 400 µl STE buffer without ethanol, centrifuging at 13,000 *g* for three min and transferring the supernatant into a sterile microcentrifuge tube. Next, 2.5 volumes of 95% ethanol and 0.1 volume 3 M sodium acetate, pH 5.2, were added. The mixture was stored at -20°C.

Recovery of RNA for analysis for viroids. A total of 4 ml of the TNA extract was collected in a single microcentrifuge tube as described above for dsRNA recovery. The air-dried pellet was resuspended in 333 µl STE buffer, mixed with 50 mg CF-11 cellulose powder and 147 µl of 95% ethanol (for a final concentration of ethanol of 35%). The mixture was shaken on ice at about 120 cycles/min for 30 min, then centrifuged at 13,000 *g* for 3 min. The supernatant was discarded and the CF-11 pellet was resuspended in 400 µl of STE buffer containing 35% ethanol (wash buffer). This wash step was repeated three times. The nucleic acid bound to the CF-11 cellulose was then released by adding 400 µl STE buffer without ethanol, mixing well, and centrifuging at 13,000 *g* for 3 min. The supernatant was transferred to a sterile microcentrifuge tube and 2.5 volumes of 95% ethanol and 0.1 volume 3 M sodium acetate, pH 5.2, were added. The mixture was stored at -20°C.

Recovery of DNA for analysis for *X. fastidiosa*. Four milliliters of the TNA extract was concentrated in a single 2 ml microcentrifuge tube as described above. The air dried pellet was resuspended in 400 µl STE buffer, mixed with 2.5 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2. The mixture was stored at -20°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The recovered RNA fraction (for viruses and viroids, described above) was centrifuged at 13,000 g for 10 min. The pellet was washed by adding 400 µl cold 70% ethanol and centrifuged at 13,000 *g* for 10 min. The resultant pellet was air dried and resuspended in 40 µl sterile water. For RT-PCR, 10 µl of RNA was mixed with 0.5 µl of a mix of 10 µM each of sense and antisense primers (Table 1), incubated at 85°C for 10 min and immediately cooled on ice. A mixture of 4 µl of 5× first strand buffer (250 mM Tris, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 2 µl of 0.1 M DTT and 1 µl of 10 mM dNTPs, (Invitrogen) was prepared, incubated for 3 min at 42°C, then transferred to room temperature. One microliter each of Superscript II reverse transcriptase and RNase Out (Invitrogen) was added. The enzyme mixture (9 µl) was added to the heat denatured RNA template mixed with primers. The tube was gently mixed and incubated at 45°C for 1 hr followed by enzyme inactivation at 72°C for 15 min.

One microliter of cDNA from the reverse transcription reaction was used as template and added to a reaction mixture containing 5 µl of 10× PCR buffer, 5 µl 25 mM $MgCl_2$, 1 µl of the 10 mM dNTPs (Invitrogen), 2.5 U Taq polymerase

TABLE 1
SUMMARY OF PRIMERS USED FOR DETECTION OF DIFFERENT PATHOGENS, THE EXPECTED PRODUCT SIZE, AND ANNEALING TEMPERATURE USED IN PCR

Primer	Target pathogen	Sequence	Expected product size	Annealing temperature
CN119[6] z	Citrus tristeza virus	5'AGATCTACCATGGACGACGAAACAAAG3' (sense)	672 bp	50°C
CN120[6]	Citrus tristeza virus	5'GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC3'		
CEVd-AP3[5]	Citrus exocortis viroid	5'GGAAACCTGGAGGAAGTCGAG3' (sense)	371 bp	60°C
CEVd-AM3[5]	Citrus exocortis viroid	5'CCGGGGATCCCTGAAGGACTT3'		
CV2-AP[5]	Hop stunt viroid	5'GGCAACTCTTCTCAGAATCCAGC3' (sense)	302 bp	60°C
CV2-AM[5]	Hop stunt viroid	5'CCGGGGCTCCTTTCTCAGGTAAGT3'		
CV3-AP[5]	Citrus viroid III	5'CTCCGCTAGTCGGAAAGACTCCGC3' (sense)	271 bp	60°C
CV3-AM[5]	Citrus viroid III	5'TCACCAACTTAGCTGCCTTCGTC3'		
CPV1[2]	Citrus psorosis virus	5'GCTTCCTGGAAAAGCTGATG3' (sense)	654 bp	50°C
CPV2[2]	Citrus psorosis virus	5'TCTGTTTTTGTCAACACAACTCC3'		
RST31[3]	Xylella fastidiosa	5'GCGTTAATTTTCGAAGTGATTCGATTGC3'	391 bp	50°
RST33[3]	Xylella fastidiosa	5'CACCATTCGTATCCCGGTG3'		

[z]The number in the parenthesis indicates the reference source in LITERATURE CITED.

(Promega), 100 pmoles of each primer, and water for a volume of 50 µl for each reaction. The PCR reaction was conducted with the following conditions: one cycle of denaturation at 94°C for 2 min; then 30 cycles of denaturation at 94°C for 30 s, followed by annealing for 30 s at indicated temperatures (Table 1) for the specific primers, extension at 72°C for 1 min; followed by a final cycle of 72°C 10 min. The PCR amplicons were analyzed by electrophoresis of a 5-10 µl sample of the product on a 0.8% agarose gel in 0.5× TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8.0) at 100V for 40 min. After ethidium bromide staining, the gels were viewed over UV light and photographed.

PCR detection of *X. fastidiosa*. Ten microliters of the resuspended TNA as described above was used as a template for PCR as described above. Prior to PCR, the template and primer mixture (Table 1) was denatured at 85°C for 5 min followed by quick cooling on ice.

RESULTS

Of the 282 samples collected from field trees and assayed by ELISA for CTV, an average incidence of 13.5% was found (Table 2). The southern citrus area showed the highest incidence of CTV (about 19%). From a total of 267 samples were assayed by ELISA for *X. fastidiosa*; one ELISA positive was found, but this could not be confirmed by PCR analysis. Forty-five samples were selected for analyses using RT-PCR based on presence of field symptoms when the samples were collected. Of these 45 samples which were used for TNA extractions and subsequently analyzed by RT-PCR, 11% were positive for CEVd, 29% were positive for Citrus viroid II (CVd-II), 7% were positive for *Citrus viroid III* (CVd-III), and 27% were positive for CTV (Table 2). The RT-PCR assay for CTV proved to be much more sensitive as compared to the ELISA method. Of the 45 samples tested by RT-PCR for CTV (all were negative by ELISA), 12 were positive for CTV by RT-PCR. Some of the RT-PCR amplicons for CEVd, CVd-II, CVd-III, and CTV were cloned and sequenced to verify the identity of the products (data not shown).

The RT-PCR tests for psorosis gave variable results, and no definite positives could be determined using the psorosis-specific primers (Table 1). Subsequent biological indexing on sweet orange seedlings did not reveal any symptoms associated with psorosis.

DISCUSSION

The ideal way to index for graft transmissible pathogens is the use of indicator plants. However, there are occasionally situations where a graft transmissible pathogen is suspected

TABLE 2
SURVEY RESULTS FROM THE DIFFERENT CITRUS AREAS SAMPLED IN GUATEMALA

Citrus Area	*Xylella fastidiosa* ELISA	CTV ELISA	CTV[z]	CEVd[y]	CVd II[x]	*Citrus viroid III*[w]
North	0/84[v]	6/87	4/16	3/16[u]	5/16	0/16
Central	0/19	0/24	3/8	0/8	1/8	0/8
South	1/164	32/171	5/21	3/21	7/21	3/21
Total	1/267	38/282	12/45	6/45	13/45	3/45

[z]*Citrus tristeza virus* (CTV) tested by RT-PCR as described in the Materials and Methods.
[y]*Citrus exocortis viroid* (CEVd) tested by RT-PCR as described in the Materials and Methods.
[x]Citrus viroid II (CVd II) tested by RT-PCR as described in the Materials and Methods.
[w]*Citrus viroid III* tested by RT-PCR as described in the Materials and Methods.
[v]Number found positive by the indicated test/number of samples tested.

based on symptoms in the field, but it is not possible to do biological indexing because of the lack of indicator plants and/or adequate facilities in which to conduct the biological indexing. There also are occasions when a rapid diagnosis is required. The protocol described here has proven to be useful for preparing extractions of total nucleic acids from citrus samples for later analyses using RT-PCR for diagnosis of viruses and viroids and for PCR to diagnose *X. fastidiosa*. The extractions are non-infectious and are stable enough as alcohol precipitated preparations (at least for a brief period) for shipment and/or short term storage at ambient temperatures.

The lack of a definitive detection of psorosis was not totally unexpected (7). The unreliable detection from the suspect field samples could be due to several factors. The field symptoms caused by psorosis and concave gum are similar, but the primers used here are capable of detecting only psorosis. Psorosis also is known to have irregular distribution in infected trees, especially under warmer conditions (8). Biological indexing performed later was used to confirm the negative results for diagnosis of psorosis.

The results of the survey indicate that CTV, CEVd, CVd-II and CVd-III are common in Guatemala. The significance of these findings is great when considering rootstock usage. CTV limits the use of sour orange and alemow rootstocks. CEVd limits the use of trifoliate-type rootstocks including citranges and citrumelos. In addition, the detection of CVd-II indicates the potential presence of citrus cachexia caused by CVd-IIb and IIc variants. Cachexia greatly limits the use of mandarin type rootstocks including Cleopatra mandarin. The detection of CTV, CEVd and the potential presence of cachexia highlights the need for a citrus certification program for Guatemala whereby clean budwood materials should be produced on clean rootstocks.

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