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Occurrence of citrus viroids in Costa Rica

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Abstract: A survey for citrus viroids was conducted in the major citrus commercial growing areas in Costa Rica. Screening of 36 sweet orange and 12 lemon trees resulted in the detection of members of four of the five citrus viroid groups as determined by nucleic acid hybridization using specific RNA probes and polymerase chain reaction (PCR) using specific oligonucleotide primers. CEVd, CVd-IIa, CVD-IIb and CVd-III viroids were found to be widespread in the three main regions of commercial cituts production. CVd-Ib was only found in lemon in Nicoya.

Key words: viroids, detection, nucleic acid hybridization, PCR, dot blot assay.

Five distinct viroid groups are known to infect citrus trees around the world: the citrus exocortis viroid group (CEVd) includes the causal agent of the exocortis disease, the citrus viroid group I (CVd-I) consists of two viroids, CVd-Ia and CVd-Ib (Durán-Vila *et al.* 1988); the citrus viroid group II (CVd-II) includes CVd-IIa, the causal agent of mild exocortis disease, and CVd-IIb the causal agent of cachexia disease, both variants of hop stunt viroid (HSVd) (Semancik *et al.* 1988); the citrus viroid group III (CVd-III) consists of 4 viroids, CVd-IIIa, CVd-IIIb, CVd-IIIc and CVd-IIId (Semancik and Durán-Vila 1991, Rakowski *et al.* 1994). and the citrus viroid group IV (CVd-IV), consists of a single known member (Putcha *et al.* 1991). These groups are characterized by their biological properties in differential hosts, by their molecular size determined by electrophoretic mobility, and by their nucleic acid homology determined by nucleotide sequencing and by hybridization with specific probes (Levy and Hadidi 1995.)

Citrus is one of Costa Rica's most important nontraditional export crops. By 1995, approximately 20000 ha were dedicated to this crop. Citrus is grown predominantly in the northern part of the country. Sweet orange (*Citrus sinensis*) is the main cultivated species. Lemon (*C. limon*), grapefruit (*C. paradisi*) and mandarin (*C. deliciosa*) are also cultivated, but primarily in experimental areas or in small plantations or home gardens. Most of these species are grafted on trifoliate rootstocks (*Poncirus trifoliata*).

The presence of citrus viroid diseases in commercial fields and experimental plots in Costa Rica was first suspected about 5 years ago based on dwarfing, and bark scaling symptoms, however the viroid origin of these symptoms was not confirmed. In this paper we report for the first time the identification of citrus viroids in Costa Rica.

MATERIAL AND METHODS

Survey of citrus: A total of 48 bark samples were collected from sweet orange and lemon trees in 4 counties in Costa Rica. All of the lemon samples were collected in Nicoya, which is the only area of commercial lemon production. The sweet orange samples were collected in each of two commercial groves in La Cruz and San Carlos where most of the important commercial fields of sweet orange are located, and in Alajuela in the citrus germplasm collection of the University of Costa Rica. Samples were lyophilized and stored at room temperature in closed vials until analysis.

Extraction of nucleic acids: Total nucleic acids (TNA) were extracted from infected and healthy citrus samples using a previously described procedure (Diener 1979). Briefly 0.5 g of lyophilized bark tissue from each sample (2-3 g fresh tissue) was resuspended in 1.5 vol. of TES buffer (0.1M Tris-base, 0.01 M disodium ethylenediamine tetraacetic acid (EDTA), 0.1M NaCl, 1% sodium dodecyl sulfate (SDS), 0.005M dithiotreitol (DTT)). Each sample extract was mixed with phenol saturated with Tris-HCl buffer, pH 7.4 (Gibco-BRL Life Technologies, Inc. Gaithersburg, MD USA) and chloroform 1: 1 v/v. After centrifugation the aqueous phase was collected and the nucleic acid precipitated with 3 volumes ethanol (95-100%) and 0.5 volume 7.5M ammonium acetate at -20°C overnight. Following centrifugation, pellets were resuspended in sterile water and stored at -20°C. The nucleic acid concentration was determined spectrophotometrically at a wavelength of 260 nm.

Dot blot and RNA hybridization: Forty ug of extracted TNA of each sample were denatured with 0.2ml sample denaturation buffer (SDB) (20X standard saline citrate buffer (SSC) containing 37% formaldehyde) (1X SCC= 150 mM NaCl, 15 mM Na-citrate, pH 7.0) and spotted onto positively charged nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, IN USA) using a Bio-Dot blotting manifold (Bio-Rad Laboratories, Richmond, CA USA). The TNAs were crosslinked to the membrane using a Stratalinker (Stratagene, La Jolla, CA USA). Membranes were incubated in prehybridization buffer (5x SSC, 50% formamide, 1x blocking stock (Boehringer Mannheim) and 3% SDS) for 1 h at 65°C followed by incubation with 10 µl (100 ng/µl) of the specific RNA probe diluted in 25 ml of hybridization buffer (same as prehybridization buffer but containing 0.02 % SDS) overnight at 65 °C. "CEVd" and "HSVd" RNA probes were used for detection of CEVd, and CVd-IIa/CVd-IIb, respectively, in all samples and were transcribed in the presence of digoxigenin (DIG) from plasmids containing the viroid inserts. Membranes were washed and developed according to the recommendations of the manufacturer (Boehringer Mannheim) using Fab anti-DIG and LumiPhos 530. The membranes were then exposed to Hyperfilm-MP (Amersham).

Viroid cDNA synthesis and PCR amplification: For the cDNA synthesis, 2 µl containing 2 to 10 µg of TNA were mixed with 1 µl pdN6 (random primers, 5 µg/µl, Pharmacia Biotec, Inc. Piscataway, NJ, USA), 2.5 µl 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin, Perkin-Elmer Cetus Corp, Norwalk, CT USA), 0.5 µl RNasin (20µ/µl, Promega Corp. Madison, WI USA) and 9.5 µl water. The mixture was incubated for 3 min at 97°C to denature the sample RNA followed by incubation at 68°C for 5 min for primer annealing. To the denatured RNA was added 2.5 µl 10 mM dNTPs, 2.5 µl 15 mM $MgCl_2$, 2.5 µl 100 mM DTT, 1 µl M-MLV RTase (200 µ/µl, Gibco-BRL Life Technologies, Inc.) and 1 µl water and the mixture was incubated at 37°C for 1-2 h for cDNA synthesis.

The primers used for the specific viroid amplifications are presented in Table 1. PCR reactions contained 5 µl of sample cDNA and 45 µl reaction mixture (5 µl 10x PCR buffer, 1 µl of the complementary specific primers (10

pmol/µl), 0.25 ul Ampli-Taq DNA polymerase (5µ/µl, Perkin-Elmer Cetus Corp.), 33.75 µl water and 4 ul 1 mM dNTPS). Cycling parameters were 1 min at 94 °C, 1min at 55°C, 1.5 min at 72°C for 35 cycles with the final extension at 72°C for 7 min in a DNA thermal cycler (Perkin-Elmer Cetus Corp.).

TABLE 1

Sequence of the primers used in PCR.

Primer	Sequence 5'→3'	Length	Viroid nucleotides*
CVd-Ib	(5'-AGAGCTCAACTTCGCCACCG-3')	20	141-160
CVd-Ib	(5'-GCTCTTCTAAGCTGTAACGG-3')	20	161-180
CVd-IIa	(5'-TTGCCCCGGGGCTCCTTTCTC-3')	21	67-87
CVd-IIa	(5'-CTCTTCTCAGAATCCAGCGG-3')	20	88-107
CVd-IIb	(5'-TTGCCCCGGGGCTCCTTTCTC-3')	21	66-86
CVd-IIb	(5'-CTCTTCTCAGAATCCAGCGA-3')	20	87-106
CVd-IIIb	(5'-ACTCTACCGTCTTTACTCCA-3')	20	120-138
CVd-IIIb	(5'-CTCCGCTAGTCGGAAAGACTCCGC-3')	24	139-162
CVd-IV-I	(5'-GACGATCGGATGTGGAGCCAGC-3')	22	160-181
CVd-IV-I	(5'-GCTTCTTCCTTCGCGACCTG-3')	20	182-201
CEVd	(5'-CTCCAGGTTTCCCCGGG-3')	17	94-110
CEVd	(5'-gcggatCCGGTGGAAACAACTGAAGC-3')	25	270-289

*Nucleotide number in the viroid sequence for CVd-Ib (Ashulin *et al.* 1991), CVd-IIa (Levy and Hadidi 1993), CVd-IIb (Levy and Hadidi 1993, Francis *et al.* 1995), CVd-IIIb (Rakowski *et al.* 1994), CVd-IV (Punchta *et al.* 1991), CEVd (Visvader *et al.* 1982). Lowercase letters are non-viroid sequences present in the primer.

TABLE 2

Survey for citrus viroids in Costa Rica 1995.

Viroids	Number of trees infected/number of trees indexed				
	Nicoya	La Cruz	San Carlos	Alajuela	Total
CEVd	2/12	5/11	7/17	0/8	14/48
CVd-Ib	7/12	0/11	0/17	0/8	7/48
CVd-IIa	9/12	1/11	7/17	2/8	19/48
CVd-IIb	3/12	6/11	11/17	2/8	22/48
CVd-IIIb	9/12	1/11	7/17	0/8	17/48
CVd-IV	0/12	0/11	0/17	0/8	0/48
Healthy	0/12	5/11	3/17	6/8	14/48

Analysis of PCR products: Five µl aliquots of the PCR products were analyzed by electrophoresis on 5% native polyacrylamide gels at 200 V for 45-60 min in 1X TBE (89 mM Tris, 89 mM borate, and 2.5 mM EDTA, pH 8.3) and stained with ethidium bromide. The molecular DNA marker *Hae*III-digested pST B14 with fragment sizes (bp) of 587, 458, 434, 359, 320, 288, 275, 267, 174, 119 and 80 was used.

RESULTS

A total of 68.75% of the samples analyzed were positive for at least one of the 5 viroids reported here. Citrus viroids CEVd, CVd-IIa, and CVd-IIb were detected by dot-blot hybridization using nonradioactive probes for CEVd and HSVd (Fig. 1 a, b). Specific primers for citrus viroids CEVd, CVd-Ib, CVd-IIa, CVd-IIb, and CVIIIb.were also used for viroid

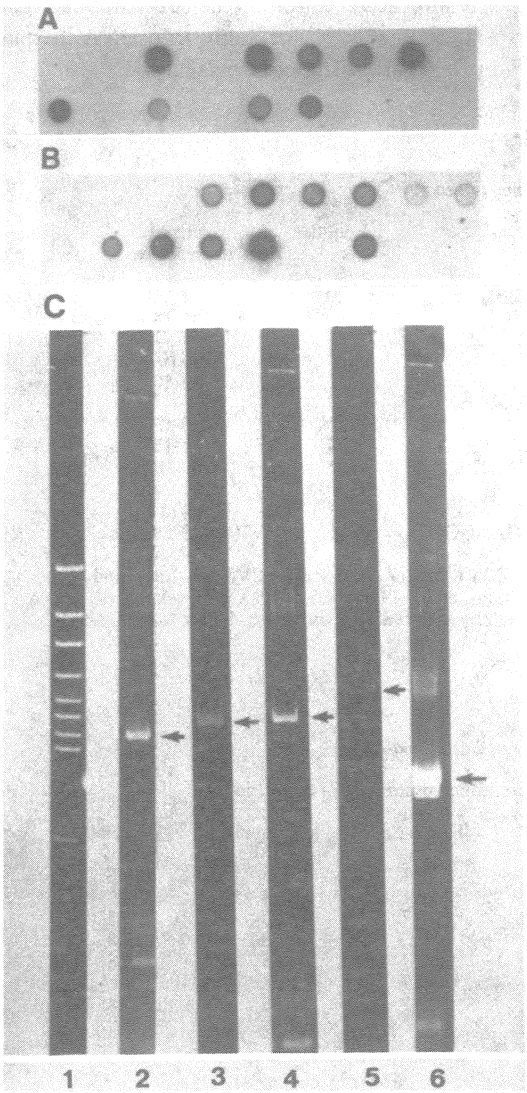

Fig.1. Detection of citrus viroids by dot blot hybridization and polymerase chain reaction (PCR). Dot blot hybridization using non-radioactive CEVd-RNA probe (A) and HSVd-RNA probe (B).The spots correspond to positive field samples. Polyacrylamide gel electrophoretic analysis of polymerase chain reaction (PCR) products of citrus viroids (C). Molecular DNA marker HaeIII-digested pST B14 with fragment sizes (bp) of 587, 458, 434, 359, 320, 288, 275, 267, 174, 119, and 80 (lane 1), PCR product (approx. 290 bp) of CVd-IIIb (lane 2), PCR product (approx. 275 bp) of CVd-IIa (lane 3), PCR product (approx. 275 bp) of CVd-IIb (lane 4), PCR products (approx. 320 bp) of CVd-Ib (lane 5) , and PCR products (approx. 210 bp) of CEVd. Arrows indicate the major PCR products.

detection and individual viroids were identified based on the molecular size of the amplified fragments (Fig. 1 c).

CEVd, CVd-IIa, CVd-IIb and CVd-IIIb were found to be widespread in the three principal regions of commercial citrus production in Costa Rica (Table 2). CVd-IIa and CVd-IIb were found in all 4 surveyed locations. CEVd was detected in all areas of commercial production included in this study, but was not detected in the germplasm collection in Alajuela. CVd-Ib was found only in lemon in Nicoya. CVd-IV was not detected in any of the samples. Mixed infections of two, three and four viroids were frequent. Approximately 85% of the infected trees were infected with more than one viroid, whereas only 15% were infected with a single viroid (CVd-IIb, CEVd or CVd-IIIb).

DISCUSSION

The viroids in this work were identified on the basis of their homology to CEVd and HSVd probes by dot blot hybridization and on the basis of the size of PCR amplification products using specific primers to CEVd, CVd-Ib, CVd-IIa, CVd-IIb, CVd-IIIb and CVd-IV. Using these criteria, viroids corresponding to four of the five known citrus viroid groups were detected in Costa Rica (Table 2).

Other viroids reported in Spain, Australia and the USA, including CVd-Ia, CVd-IIc, CVd-IIIa, CVd-IIIc, CVd-IIId and CVd-IV (Duran-Vila *et a.* 1988, Gillings *et al.* 1991) have not yet been detected in citrus in Costa Rica. Due to the extensive exchange of citrus material between Costa Rica and other countries, and the previous lack of viroid diagnosis capabilities by our quarantine facility, a more extensive survey for the presence of viroids in our country may reveal their widespread occurrence.

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RESUMEN

Se realizó un estudio sobre los viroides que infectan los cítricos en las principales regiones de Costa Rica donde se siembran comercialmente estos frutales. Se analizaron 36 muestras de árboles de naranja dulce y 12 de limón mediante las técnicas de hibridación de ácidos nucleicos con sondas ARN específicas y de la reacción en cadena de la polimerasa (PCR) usando imprimadores espécificos. Se detectaron viroides pertenecientes a cuatro de los cinco grupos de viroides conocidos en el mundo. Los viroides CEVd, CVd-IIa, CVd-IIb y CVd-IIIb se encontraron ampliamente distribuidos en las tres regiones principales de producción comercial de cítricos del país. CVd-Ib se encontró solamente en árboles de limón en Nicoya.

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