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Journal of Citrus Pathology

Brief Report

First Report of Citrus Bent Leaf Viroid and Citrus Dwarfing Viroid in Argentina

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Citation: Palacios, M., & Figueroa, J. (2022). First report of CBLVd and CDVd in Argentina. Journal of Citrus Pathology, 9. http://dx.doi.org/10.5070/C49151296 Retrieved from https://escholarship.org/uc/item/7212n0wp

Abstract

Samples collected from citrus trees with viroid-like symptoms in citrus orchards in Tucumán, Salta, and Jujuy provinces (northwestern Argentina) were initially indexed on citron (Citrus medica) and then analysed by s-PAGE. These samples were found to be infected with different viroid species, among them, CEVd and HSVd have been already identified. In order to determine the presence of other viroids, we performed a RT-PCR assay using specific primers for CBLVd, CDVd, Citrus bark cracking viroid (CBCVd) and Citrus viroid V (CVd-V).

Forty-two samples including 15 lemons, 15 oranges, 8 grapefruits, 2 citrumelos and 2 Cleopatra mandarins were analysed. On the basis of amplification of the appropriately sized DNA, CDVd was detected in thirty-eight samples and CBLVd in all grapefruit samples. CBCVd and CVd-V were not found in any samples to date. Analysis of the amplicon sequences revealed 96% and 97% identity with CBLVd GenBank reference sequences, and 96% to 98% with CDVd GenBank reference sequences.

This is the first report of CBLVd and CDVd in citrus trees in Argentina. The results indicate that CDVd is widely spread throughout the surveyed areas and it is even more prevalent than CEVd and HSVd. Moreover, a considerably high percentage of citrus species are affected by mixed viroid infection. It would be important to take it into consideration when developing citrus disease management strategies.

Keywords: CEVd, CBLVd, HSVd, CDVd, RT-PCR

Introduction

Citrus trees are natural hosts of several viroid species of the Pospiviroidae family: Citrus exocortis viroid (CEVd), Citrus bent leaf viroid (CBLVd), Hop stunt viroid (HSVd), Citrus dwarfing viroid (CDVd), Citrus bark cracking viroid (CBCVd), Citrus viroid V (CVd-V), Citrus viroid VI (CVd-VI) and the novel Citrus viroid VII (CVd-VII) (Duran-Vila, et. al., 1988; Ito et. al., 2001; Serra et. al., 2008; Chambers et. al., 2018). CEVd and HSVd are the causal agents of exocortis and cachexia diseases, respectively, causing economically important losses. The other viroids lead to minor effects with complex interactions when present in mixed infections (Ito et. al., 2002; Vernière et al., 2006). Although not inducing specific symptoms, CBLVd and CDVd have been reported to induce reduction in canopy volume and fruit production in infected citrus trees on trifoliate and trifoliate orange hybrid rootstocks. Moreover, citrus trees infected only with CBLVd or in combination with Citrus exocortis viroid (CEVd), Hop stunt viroid (HSVd) and CDVd have been associated with poor development of the root system. (Vernière et al., 2004; Murcia et. al., 2015).

Symptoms typical of exocortis and cachexia diseases were observed in northeast (NE) and northwest (NW) Argentina, the two producing areas of the country, on field-grown citrus trees (Fernández Valiela, 1961; Fernández Valiela et. al., 1965; Foguet and Oste, 1968). Trifoliata (Poncirus trifoliata (L.) Raf.) and citrange (Citrus sinensis x P. trifoliata) rootstocks have been found to be sensitive to infection from several viroids, exhibiting different symptoms like bark scaling, dwarfing and yield reduction (Roistacher et. al., 1993; Bani Hashemian et. al., 2009; Murcia et. al., 2015). The increased use of these rootstocks in Argentina has given symptomatic evidence of the presence of viroids in citrus groves. Local citrus production could be threatened given they are the most used rootstocks in the NW region, representing 86% of seed demand.

Previous studies carried out by biological indexing, using Etrog citron Arizona 861-S-1 grafted on rough lemon (Citrus jambhiri Lush.) seedlings as indicator plants, showed a wide range of symptoms including stunting, mild and severe epinasty, petiole wrinkle and petiole and midvein browning. In addition, inoculated citrons were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) indicating the presence of several viroids, in most cases present in mixed infections (Figueroa et. al., 2010).

The aim of this study was the detection and identification of citrus viroids present in commercial orchards of Northwestern Argentina by RT-PCR assays using a set of specific primers.

Materials and Methods

Forty-two samples from 5 different citrus species including Cleopatra mandarin (Citrus reshni), and distinct varieties of citrumelo (Poncirus trifoliata x Citrus paradisi), lemon (Citrus limon (L) Burm f.), grapefruit (Citrus x paradisi Macfad.) and sweet orange (Citrus sinensis) were collected from citrus orchards of Tucumán, Salta and Jujuy provinces (Table 1). Samples included symptomatic and asymptomatic trees from 3 to more than 60 years old. The main symptoms observed in sensitive varieties were bark scaling, dwarfing and loss of production of large fruit. Positive controls of CBCVd and CVd-V were provided by Instituto Valenciano de Investigaciones Agrarias (IVIA).

All samples were analysed by biological indexing and s-PAGE to corroborate the presence of viroids (data not shown).

Total RNA was extracted from 500 mg of a mix of leaf midrib and green bark tissue using the SDS-KAc method (Garnsey et. al., 2002).

Simplex two-step RT-PCR was performed using one specific primer pair for each different viroid as follows: CEVd (CEV-AM3 and CEV-AP3), HSVd (CV2-AM and CV2-AP), CBLVd (CBLV-CM2 and CBLV-AP2), CDVd (CV3-AM and CV3-AP), CBCVd (CV4-AM3 and CV4-AP4) (Ito. et. al., 2002) and CVd-V (CVV-P3 and CVV-P4) (Ito and Ohta, 2010).

The first strand of cDNA synthesis was accomplished using 2 µl of total RNA, 7.5 µM specific antisense primer, 10 mM dNTP, 25 mM MgCl2, 5X first-strand buffer, Ribolock RNase Inhibitor (Thermo Fisher Scientific Inc), and RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc) as per the manufacturer's protocol. The tubes were incubated at 55ºC for 1 h for CEVd and 42ºC for 1 h for the rest of the viroids.

The PCR reactions for the amplification consisted of 5 µl 10X Taq Buffer, 1 mM MgCl2, 0.2 mM dNTP, 1U Taq DNA Polymerase (Thermo Fisher Scientific Inc), 0.2 µM of each forward and reverse primer, 2 µl of cDNA product and molecular grade water to a total volume of 20 µl. PCR reaction conditions were; 1 cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 1 cycle of 72°C for 5 min. The final PCR products were visualised on a 1.5% agarose gel stained with GelRed 10000X. Some amplicons of positive samples of CBLVd and CDVd were purified using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany), sent to Centro de Referencia de Lactobasilus, CERELA (T4000 Tucumán, Argentina) and subjected to direct Sanger sequencing. Sequences obtained were deposited into GenBank. Taxonomic identities of each direct Sanger sequence was determined using the online version of the BLASTn algorithm (www.ncbi.nlm.nih.gov/BLAST) with the non-redundant nucleotide (nt) database.

Species	Variety	Plants Sampled	Location
Citrus reshni -	Cleopatra mandarin	2	Tucumán
Citrus paradisi x P. trifoliata	Swingle citrumelo	1	Tucumán
	75 AB citrumelo	1	Jujuy
Citrus limon	Adamo	1	Tucumán
	Frost Eureka	8	Tucumán, Jujuy
	Interdonato	1	Tucumán
	Frost Lisbon	2	Tucumán
	Limoneira 8A Lisbon	1	Tucumán
	Unknown	2	Tucumán
Citrus paradisi	Rouge La Toma	5	Tucumán, Salta
	Henninger´s Ruby	2	Jujuy
	Foster Seedless	1	Jujuy
Citrus sinensis	Valencia Late	4	Tucumán, Salta
	Ruby Blood	3	Tucumán
	Jaffa	2	Salta
	Marr's Early	1	Tucumán
	Pineapple	4	Tucumán
	Maltese	1	Tucumán

Table 1. Species, variety and sampling location (province) of the 42 citrus plants surveyed for viroid infection in NW Argentina.

Results

RT-PCR reactions were satisfactory for the six tested viroids as amplicons of each viroid corresponded to the expected size (Figure 1). CEVd, HSVd, CDVd and CBLVd were detected in the samples analysed. CBCVd and CVd-V were not found in any tested trees. These results agree with previous studies performed in our laboratory where four viroids were differentiated by sPAGE analysis (Escobar Ponce de León et. al., 2012).

CDVd was predominant, found in 38 out of 42 samples, which represent 90% of the samples analysed. Then, HSVd was detected in 79%, CEVd in 60% and the less frequent was CBLVd present in 19% of the samples (Table 2).

Mixed viroid infections were detected in 86% of the samples. A total of 5 samples were individually infected with CDVd and only one with CEVd. CBLVd was always found with CDVd, and it was interestingly detected only in grapefruit, and in all grapefruit varieties analysed from

the three provinces. Since most samples were co-infected with several viroids, it is difficult to make a correlation between field symptoms and viroid infection with

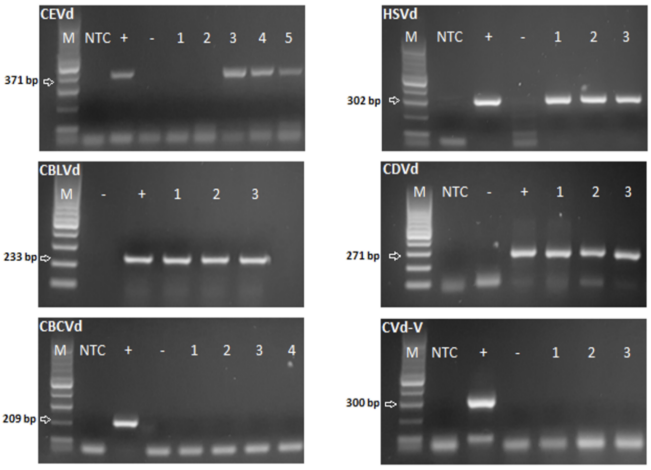

Figure 1. Detection of citrus viroids using RT-PCR assay followed by electrophoresis analysis. Positive results were indicated by appearance of bands at expected size position. Lane M, 100 bp DNA ladder; NTC, no template control; +, positive control; -, healthy control. Numbered lanes indicate different samples collected from several commercial orchards in Argentina. Specific amplified fragments are indicated by an arrow.

Species	Nº isolates	CEVd	HSVd	CBLVd	CDVd	CBCVd	CVd V
Citrus limon	15	12	11	0	12	0	0
Citrus sinensis	15	8	12	0	14	0	0
Citrus paradisi	8	5	7	8	8	0	0
Citrus paradisi x P. trifoliata	2	0	1	0	2	0	0
Citrus reshni	2	0	2	0	2	0	0
Total	42	25	33	8	38	0	0
	Total %	60	79	19	90	0	0

Table 2. Detection of viroid infection in different citrus species by simplex RT-PCR reactions indicating the total number of isolates tested and the number of plants with positive results for each viroid.

assurance. It is notable that all plants showing bark scaling in sensitive rootstock were found to be infected with CEVd. Other remarkable associations can be done based on symptoms of a Eureka lemon tree infected only with CDVd. This sample was collected from a field trial of lemon cultivars recovered by shoot-tip grafting (STG) compared with their original mother clones. The STG line of Frost Eureka (viroid free) developed a larger canopy volume and yielded 80% more fruit than the source tree (CDVd infected), while no statistical differences were found for the other tested cultivars, which were free of any viroid (Foguet et. al., 2013). Regarding sequences obtained, there was 96-98% similarity between CDVd (Accession No. OK181215, OK181216 and OK181217) and 99.5% for CBLVd sequences (Accession No. OK181218 and OK181219). Additionally, BLASTn analysis revealed 96-98% identity to the corresponding GenBank reference sequences.

Discussion

This is the first report of CDVd and CBLVd in Argentina. CDVd was the most frequently detected viroid, present in the greatest number of samples and in all varieties sampled. CBLVd was only present in grapefruit samples and all of them were infected with this viroid. Mixed viroid infection occurs very frequently. The correlation between viroid composition and sample species was observed only in grapefruit with the presence of CBLVd, but no other correlation was found regarding geographical location or citrus variety.

Similar results were obtained in Uruguay (Pagliano et. al. 2013), Costa Rica (Villalobos et al., 1997) and Australia (Gillings et al., 1991) where they reported the presence of CEVd, HSVd, CDVd and CBLVd in commercial orchards, with a high frequency of mixed viroid infections.

The presence and distribution of viroids in citrus groves in NW Argentina reinforces the need for accurate diagnostics to support certification programs, particularly for pathogens that represent a significant threat to the industry and consequently the regional economy.

The methods used in this study are available for use in quarantine and sanitation programs to test the health status of propagation sources in Argentina. We will continue working on the survey of citrus viroids in more samples from the region. Further studies will be performed on the characterization of the isolates found.

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