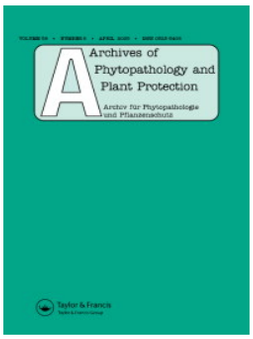

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RESEARCH ARTICLE

Molecular detection and identification of citrus bent leaf viroid (CBLVd) and hop stunt viroid (HSVd) in Palestine

Sabri M. Naser[a], Osama Alabdallah[b], Aswar Abualrob[a], Raied Abou Kubaa[c] and Raed Alkowni[a]

[a]Biology and Biotechnology Department, An-Najah National University, Nablus, Palestine; [b] National Agricultural Research Center (NARC), Biotechnology Department, Jenin, Palestine; [c]Department of Plant Pathology, University of California, Davis, California, USA

ABSTRACT

Citrus plants are natural hosts to several phytopathogens, including viroids, many of which have a significant economic impact on the crop. This research was to identify molecularly the Citrus bent leaf viroid (CBLVd) as well as Hop stunt viroid (HSVd) in citrus in Palestine. Field inspections were conducted during August 2020; where virus and virus-like symptoms were noted as epinasty; vein discoloration and leaf deformation were noticed in the citrus germplasm collection in the National Agriculture Research Center (NARC). Using a two-step RT-PCR detection method, HSVd was detected in 9.5% of samples and CBLVd in 7%. The amplified amplicons of both detected viroids were sequenced and deposited in gene bank as Palestinian isolates under the accession numbers PQ084650 and PQ084651, respectively. To our knowledge, this is the first molecular identification of CBLVd and HSVd in citrus in Palestine.

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KEYWORDS
Viroid; HSVd; CBLVd; citrus; Palestine; RT-PCR

Introduction

Citrus plants are natural host of several pathogens, including viroids, which have a significant economic impact. The importance of the viroids was reported in their influence on the quality and quantity of the citrus crop (Najar et al. 2017). These tiny, circular RNAs, with genomes ranging from 246 to 433 nucleotides (Flores et al. 2005; Serra et al. 2008) are known to be infectious and can cause remarkable disease symptoms (Ian et al., 2015). Viroids can replicate autonomously in plants; in spite of their genomes do not encode any proteins (Duran-Vila 2017).

Viroid pathogenicity is influenced by both the viroid and its host genomes. Viroids are unique in their structure and capability to replicate

CONTACT Sabri M. Naser sabri.mnaser@najah.edu Biology and Biotechnology Department, An-Najah National University, Nablus, Palestine.

within host cells; particularly in the nucleus and chloroplast (Murcia et al., 2015; Flores et al. 2005). Their noncoding nature allows them to redirect host machinery for infection. Viroids are causative agents of various diseases affecting herbaceous and woody plants, as well as agronomic and ornamental plants worldwide (Nemeth 1986; Roistacher 1992; Hammond and Owens 2006; Kaponi et al. 2024). They can cause symptoms such as chlorosis, leaf deformation, stunting, and even plant death in sensitive hosts (Flores et al. 2005). Prominent macroscopic manifestations of viroid infection encompass stunting, epinasty, vein discoloration, leaf distortion and mottling clearing, chlorotic or necrotic regions, cankers, bark scaling and bark cracking, as well as deformity of tubers, flowers, and fruits (Kovalskaya and Hammond 2014). In rare cases, plant death may also occur.

The viroids Citrus bent leaf viroid (CBLVd) and Hop stunt viroid (HSVd) are widely recognized in the citrus industry (Di Serio et al. 2017). Abualrob et al. (2024) have recently established the presence of three viroids, namely Citrus viroid-III (CVd-III), Citrus exocortis viroid (CEVd), and Citrus viroid-IV (CVd-IV), in the citrus germplasm collection at the National Agriculture Research Center (NARC) in Jenin, Palestine. In order to evaluate the sanitary status of citrus in the country from a viroid perspective, this study intends to expand the information on HSVd and CBLVd viroids from the Palestinian citrus plants.

Materials and methods

Field inspections and sample collection

In August 2020, surveys were conducted to assess the overall sanitary status of citrus trees mainly from germplasm collection plot at (NARC), Jenin-Palestine (~10 years old). Samples were randomly collected from various citrus varieties including Lemon (*Citrus limon*), Clementine (Citrus × clementina), Pomelo (*Citrus maxima)*, Grapefruit (Citrus × paradisi), Kumquats (Citrus japonica (previously *Fortunella* spp), and Orange (Citrus × sinensis (Sweet Orange), as well as two of rootstocks: Trifoliate (*Poncirus trifoliata* (formerly *Citrus trifoliata*)) and Volkameriana (Citrus × volkamerian)). Forty-two samples of young citrus leaves were collected using a stratified random sampling methodology, which involves proportionally samples exhibiting virus-like symptoms. Samples were then labeled, and stored at 4 °C for molecular tests.

Total nucleic acid (TNA) extraction

The TNA extraction was conducted using the method described by Foissac et al. (2005). Citrous leaf tissue weighing 100–200 mg was

pulverized in a 3 ml grinding buffer containing 4 M Guanidine thiocyianate, 0.2 M NaOAc, pH 5.2, 25 mM EDTA, 1.0 M KOAc, 2.5% PVP-40, and 2% Sodium bisulphate. 500 microliters of the liquid after centrifugation were combined with 150 microliters of sodium sarkosyl and allowed to incubate at 70 degrees Celsius for 10 min. The mixture was then purified using a silica column (Sigma-Aldrich) that previously prepared in the Lab as described by Foissac et al. (2005). TNA was dissolved in water at a concentration of 1 µl/mg tissue and measured using a NanoDrop 2000/2000c UV-Vis spectrophotometer (JENWAY, Genova Nano, Fisher Scientific UK Ltd, England).

Reverse transcription and polymerase chain reactions

Two-step RT-PCR method was employed to detect any of Hop stunt viroid (HSVd) and Citrus bent leaf viroid (CBLVd) by using specific primers (Table 1). These primers were selected according to the available nucleotide sequences in GenBank.

The reverse transcription reaction was performed as mentioned in Abualrob et al. (2024), where SuperScript™ III RT (Life Technologies Corporation) was used in a final volume of 20 µl. The cDNA was then subjected to PCR mix using Taq polymerase (5 unit/µl) (Promega Corporation, USA). PCR parameters included 35 cycles of cDNA amplification. Each cycle included a 50-second denaturation period at 94 °C, followed by a 50-second annealing period at 55 °C, a 2-minute extension at 72 °C, and a final 10-minute extension at 72 °C. Visualization of the PCR products was achieved using a 2% TAE agarose gel and a UV light detector after stained with GelRed (Biotium, USA).

Sequence data analysis

The amplicons fragments generated from RT-PCR amplification of the two viroid's isolates (CBLVd and HSVd) were sent for Sanger sequencing facilities at Biotech for Medical supply (Mesk Bldg. P. O. Box 01756, Esack Michael St, Ramallah, Palestine). BLASTn analysis was conducted using the website service at the National Center for Biotechnology

Table 1. Sequences of primers employed for the RT-PCR identification of viroids.

Primer	Sequence 5→-3	Size bp	Position	Reference
CBLVd-R	TTCGTCGACGACGACCAGTC	234	86–104	Ashulin et al. (1991)
CBLVd-F	CCCTTCACCCGAGCGCTGCTT		188–208	
HSVd-R	CCGGGGCTCCTTTCTCAGGTAAG	302	59–82	(Sano et al., 1988)
HSVd-F	GGCAACTCTTCTCAGAATCCAGC		83–105	
18s[a]-R	TTCAGCCTTGCGACCATACT	844		Gambino and
18s[a]-F	CGCATCATTCAAATTTCTGC			Gribaudo (2006)

[a]referred to plant rRNA (as internal control); F is for sense primer; and R for antisense primer.

Information–NCBI web server to reveal sequence similarity, and the obtained sequences were then deposited at GenBank database. Pairwise Sequence Alignment-EMBOSS Water provided by European Molecular Biology Laboratory (EMBL) were used for finding the nucleotide sequence similarity. Phylogenetic trees were generated using MEGA-11 software (Tamura et al. 2021).

Results

Field inspections

Putative viroid-like symptoms were previously reported by Abualrob et al. (2024) from inspections made on citrus trees in germplasm collection at NARC, Jenin-Palestine. In fact; this was the motive to further investigate virus-like pathogens since these were virus tested one's. Symptoms were: cankers of bark, downward and upward leaf bending as well as leaf distortion and/or mottling (Figure 1).

RT-PCR detection and identification

These two tested viroids were confirmed positive by RT-PCR (Figure 2). Their incidence for HSVd was 9.5% of the surveyed samples, meanwhile CBLVd was reached 7% (Figure 2). HSVd and CBLVd were detected on Lemon and Clementine; where the HSVd was only detected in orange was the lowest incidence of the viroid. Mixed infection were the dominant in these viroids that reached up to 70% of tested samples.

Sequencing and genome analysis

The obtained PCR amplicons for each of these two detected viroids (CBLVd and HSVd) were sequenced to give a genomic size of 234 and 299 bases respectively. The obtained sequences were deposited at Gene Bank under the accession numbers: PQ084650 for CBLVd) and PQ084651 for HSVd.

Phylogenetic tree for CBLVd (Figure 3a) revealed that CBLVd clustering with those viroids isolated from Turkey. Meanwhile HSVd was found clustering with those from Laos and India.

The closest similar viroid of CBLVd-Ps isolate was from Citrus bent leaf viroid strain CBLVd5 isolated in Turkey (OQ366363.1) and revealed 97.1% identity, using EMBOSS Water algorithm to calculate the local alignment of these two sequences which provided by EMBL's European Bioinformatics Institute. Meanwhile the closest homology of HSVd-Ps isolate was found close to the Hop stunt viroid isolate L81, (MT917189.1) in Laos and revealed 95.4% identity.

Figure 1. On citrus, symptoms like those of a viroids were observed, including bark cracking (a), downward leaf bending in the (B to D); upward leaf bending (E) were noticed on young and/or old leaves, as well as leaf discoloration (F). Some plants exhibited these leaf symptoms on few branches.

Discussion

Viroids are gaining the attention of plant pathologists because of their detrimental impact on crop quality and yield (Gucek et al. 2017; Nazarov et al. 2020; Venkataraman et al. 2021). During field inspections of the citrus germplasm collection at NARC in Jenin, Palestine, potential viroid-like signs were initially noted, including bark cankers, downward and upward leaf curvature, together with leaf distortion and mottling. That seemed promising for the investigation of possible infections caused by viruses and virus-like organisms. The Citrus bent leaf viroid (CBLVd) and Hop stunt viroid (HSVd) were subsequently identified and molecularly sequenced.

It is worth to mention that Citrus bent leaf viroid (CBLVd) is recognized as one of the primary viroids prevalent in all citrus-growing regions globally (Ali et al. 2022), resulting in leaf bending or curling either downward or upward. Besides, Hop stunt viroid (HSVd) is recognized for infecting

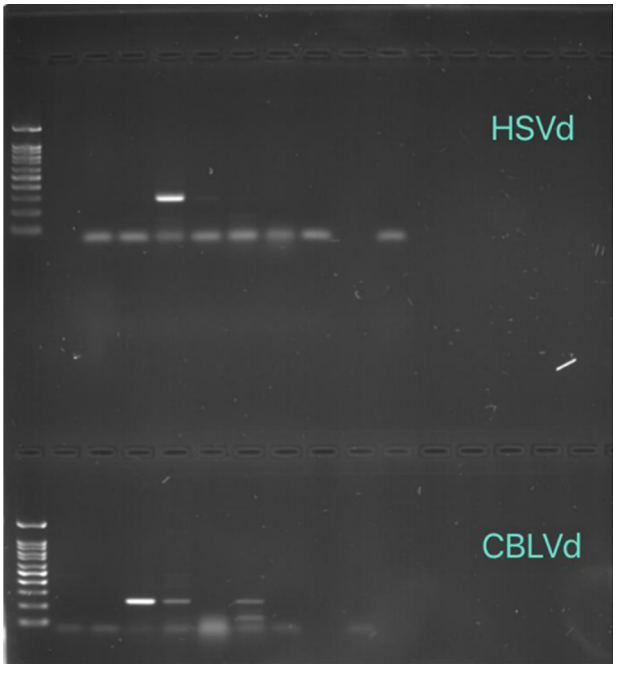

Figure 2. Specific primer sets were capable of detecting HSVd (up) and CBLVd (down); where (M) represent 100 bp ladder marker.

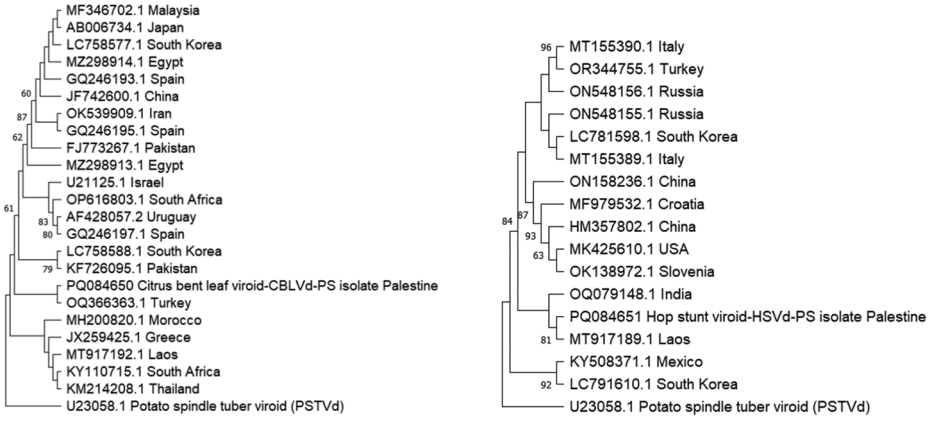

Figure 3. Phylogenetic analysis on the sequences of citrus bent leaf viroid (CBLVd) [accession No.: PQ084650] and hop stunt viroid (HSVd) [accession No.: PQ084651] sequences with their corresponding viroids accessions from GenBank. Potato spindle tuber viroid (PSTVd) was used as an outgroup for both trees. The MEGA-11 program was used to construct the phylogenetic trees, by inferring the evolutionary history using the Neighbor-Joining method in the bootstrap clustering test with 1,000 repetitions. The proportion of replicated trees below 50% was not shown on the tree.

multiple hosts, particularly affecting citrus trees by causing "cachexia" disease, characterized by wood pitting, gumming, bark cracking, and reduced tree vigor (Hadidi et al. 2017). The viroids were confirmed positive through

RT-PCR analysis with incidence rate 9.5% and 7% for HSVd and CBLVd, respectively. Despite this low rate, their notable ease of transmissibility, extensive host range, and significant genetic heterogeneity due to their RNA composition, which lacks a genetic correction mechanism (Flores et al. 2022; Ortolá and Daròs 2023), render them a source of dissemination.

Conclusions and recommendations

This research significantly advances the knowledge on the existence of HSVd and CBLVd citrus viroids in the West Bank of Palestine, representing the inaugural documentation of these viroids in the region. The two-step RT-PCR efficiently detected the presence of these two citrus viroids, underscoring the urgent necessity to establish a certification method that incorporates the use of viroid-free propagation plants.

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Author's contribution statement

A.A.; O.A.; R.A; S.N, and R.A. wrote the main manuscript text. A.A, O.A. and R.A. did the molecular detection work. O.A. and R.A. designed the experiment work. All authors shared data analysis and result interpretation including figures and tables.

Disclosure statement

No potential conflict of interest was reported by the author(s).

ORCID

Sabri M. Naser http://orcid.org/0009-0008-9138-9918

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