

## SHORT COMMUNICATION

## CITRUS VIROIDS IN TUNISIA: PREVALENCE AND MOLECULAR CHARACTERIZATION

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## SUMMARY

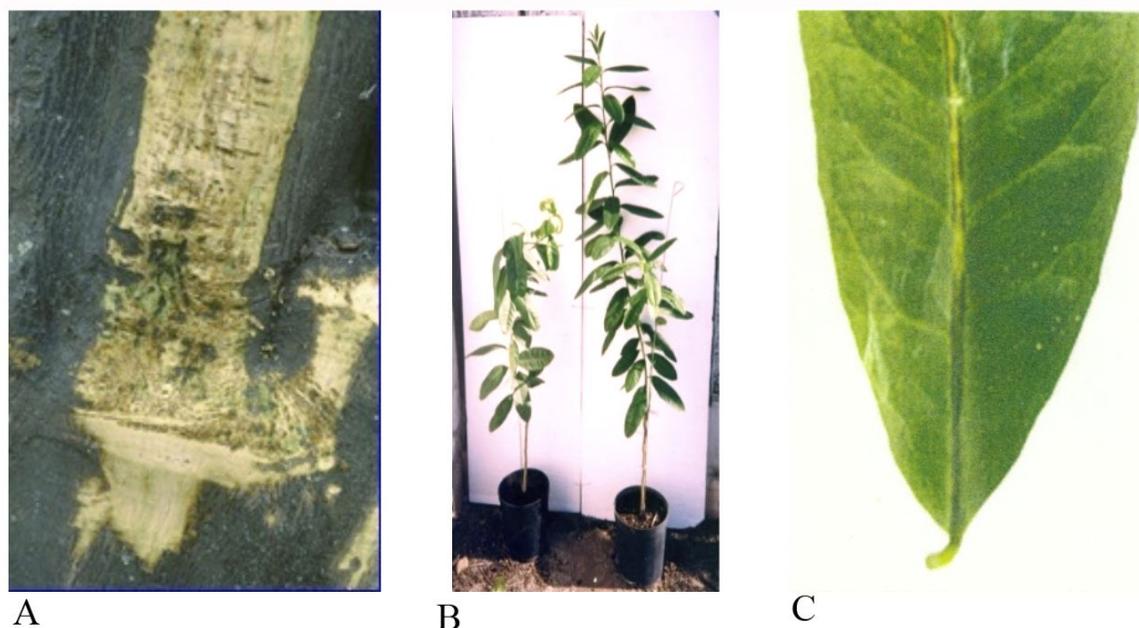
A field survey was conducted in commercial orchards of Cap Bon region and at the INRAT citrus collection to identify the prevalence of citrus viroids in Tunisia. Samples were collected from 202 trees grafted on sour orange (*Citrus aurantium* L.) including 35 common mandarin and 40 Cassar clementine trees showing cachexia symptoms. Sequential PAGE (sPAGE) analysis and molecular hybridization using viroid-specific probes revealed that all plants were infected with at least two viroids. Citrus exocortis viroid (CEVd), hop stunt viroid (HSVd) and citrus dwarfing viroid (CDVd) were found to be widespread and accounted for 70.3%, 72.3% and 78.2% of the tested trees, respectively. Citrus bent leaf viroid (CBLVd) and citrus bark cracking viroid (CBCVd) were found in 28.2% and 3.0% of trees, respectively. The most frequent viroid combinations found were CEVd+HSVd+CDVd (34.7%) and HSVd+CDVd (22.3%). Other combinations such as CBLVd+HSVd+CDVd (12.9%), CEVd+CBLVd+HSVd (11.9%) and CEVd+CDVd (10.8%) were less frequent. CEVd and CDVd isolates were RT-PCR amplified, cloned and sequenced, then compared with sequences from other parts of the world deposited in GenBank. The three CEVd clones sequenced had 99% similarity with each other and shared *ca.* 99% similarity with those from Iran, Greece, and Syria. CDVd isolates were 100% similar and shared >96% similarity with other isolates from Brazil, Cyprus, Greece, Uruguay, Israel and Spain.

**Keywords:** Biological indexing, sPAGE, molecular hybridization, sequencing.

Viroids are small, circular, single-stranded RNA, which can cause diseases of economic importance in several crops. Seven viroids reported to infect *Citrus* spp. belong to four genera of the family *Pospiviridae* (Serra *et al.*, 2008): 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) and citrus viroid VI (CVd-VI), with the latter having been reported only in Japan (Ito *et al.*, 2003).

CEVd and specific variants of HSVd (CVd-IIb and CVd-IIc) are the causal agents of two well-known citrus diseases, exocortis and cachexia, respectively (Semancik *et al.*, 1988; Duran-Vila and Semancik, 2003), which are present in almost all citrus-growing areas of the world. The characteristic field symptoms induced by CEVd are bark scaling and wood bumps on sensitive rootstock species like trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] and its citrange hybrids [*P. trifoliata* × *Citrus sinensis* (L.)]. Cachexia has been described as stem pitting and gum impregnation of the bark in sensitive species such as clementines (*Citrus clementina* Hort. ex Tan) and mandarins (*C. reticulata* Blanco). All these symptoms are associated with severe dwarfing and yield losses. Other viroids such as CBLVd and CDVd do not incite conspicuous symptoms, but they can reduce tree size and crop yield (Vernière *et al.*, 2006; Murcia *et al.*, 2009).

The citrus industry in Tunisia is based on several cultivars of sweet orange (*C. sinensis* L.), mandarin, clementine and lemon [*C. limon* (L.) Burm. f.]. The overall citrus growing area is about 24000 ha mainly located in the Cap Bon region (75%) with sour orange (*Citrus aurantium* L.) being the major rootstock. The prevalence of sour orange explains why most viroid symptoms are not observed in commercial orchards except in certain cultivars such as Cassar clementine and common mandarin on which cachexia symptoms have been observed. Actually, the substitution of sour orange by rootstocks resulting in symptomless rootstock/scion combinations is a desirable approach to prevent the damage associated with the citrus tristeza virus (CTV) in Tunisia. However, some promising citrus hybrid rootstocks such as Carrizo citrange and



**Fig. 1.** (A) Bark gumming on Cassar clementine. Symptoms of stunting, epinasty (B) and mid-vein necrosis (C) on Etrog citron.

citrumelo Swingle (*P. trifoliata* × *C. paradisi*) are sensitive to viroid infection (CEVd and HSVd). CEVd was originally reported in Tunisia by Elleuch *et al.* (2003) and further confirmed by Najar and Duran-Vila (2004) who in addition to CEVd, identified the presence of CBLVd, HSVd, CDVd and CBCVd.

As part of our continuing effort to assess the incidence of citrus viroids, a survey was conducted during 2015 and 2016 to identify the presence and prevalence of viroids in the available citrus sources. The genetic relationships of Tunisian viroid isolates with those reported from other parts of the world were also determined.

Samples were collected from twenty commercial orchards as well as from the INRAT citrus collection located in Cap Bon. A total of 202 samples were selected from sweet orange (Maltaise demi-sanguine, Washington navel and Valencia late), common mandarin, Cassar clementine and Eureka lemon trees and subjected to further analysis.

Biological indexing was performed using Etrog citron Arizona 861-S1 (*Citrus medica* L.) grafted on rough lemon (*C. jambhiri* Lush.) as the biological indicator species (Roistacher *et al.*, 1977). Each sample was graft-inoculated and maintained in a greenhouse under controlled temperature (28-32°C) and symptoms were observed 2-6 months after inoculation.

In order to determine if citrus viroid RNAs were present in the tested trees, nucleic acid preparations from the inoculated citrons were obtained as follows: five grams of bark tissues per tree, collected from shoots 30-50 cm long, were processed following the nucleic acid extraction protocol described by Semancik *et al.* (1975) with minor modifications as reported by Murcia *et al.* (2009). The nucleic acid preparations recovered from 5 g of bark tissue were resuspended in 300 µl TKM buffer (10 mM Tris, 10 mM

KCl, 0.1 mM MgCl<sub>2</sub>, pH 7.4) and 20 µl of the purified nucleic acids were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Duran-Vila *et al.*, 1993).

To confirm the identity of the viroid bands observed by sPAGE analysis, the nucleic acid preparations were further subjected to either dot-blot or slot-blot hybridization using viroid-specific probes for CEVd, CBLVd, HSVd, CDVd and CBCVd. Ten µl of nucleic acid preparations as well as the corresponding controls were denatured with 7.4% formaldehyde in 6×SPPE (3 M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.02 M EDTA) at 60°C for 15 min and loaded on positively-charged nylon membranes, which were further hybridized with digoxigenin (DIG)-labeled specific probes generated by PCR. The DIG-labelled hybrids were detected with an anti-DIG-alkaline phosphatase antibody and visualized the CSPD chemiluminescent substrate (Roche Diagnostics, Switzerland) (Palacio *et al.*, 2000).

For sequence analysis, the present study focused on CEVd and CDVd because they are among the most widespread viroids. Three clones from each viroid were sequenced. First, two-step RT-PCR procedures were performed using specific CEVd and CDVd primers (Semancik *et al.*, 1993; Kofalvi *et al.*, 1997) to amplify the corresponding sequences (around 371 and 294 bp). The amplicons were cloned into the pGEM-T vector (Promega, USA) and sequenced using an ABI PRISM DNA sequencer 377 (Perkin Elmer, USA).

For nucleotide sequence comparisons, CEVd and CDVd sequences were downloaded from GenBank and aligned with those obtained in this study using MUSCLE (Edgar, 2004). The aligned sequences were used to infer a Neighbor-Joining phylogenetic tree with the Juke-Cantor nucleotide substitution matrix and 1000 bootstrap

iterations. Branches with less than 50% bootstrap support were collapsed using TreeGraph (Stover and Muller, 2010). Viroid sequence pairwise identities were calculated using SDT v1.2 (Muhire *et al.*, 2014).

Most surveyed trees showed no visible symptoms because of the prevalence of sour orange as the main rootstock. However, 35 common mandarin and 40 Cassar clementine trees showed pronounced cachexia symptoms and heavy gum impregnation (bark gumming) (Fig. 1A).

A total of 184 field samples from the 202 that were graft inoculated onto Etrog citron Arizona 861-S1, induced characteristic viroid-like symptoms. Citron plants with severe symptoms were very stunted and presented severe leaf epinasty (Fig. 1B) and vein necrosis. Moderate symptoms were characterized by mild stunting, mild leaf epinasty and petiole necrosis, which sometimes were also observed along the mid-vein (Fig. 1C). A few plants presented very mild leaf epinasty affecting only few leaves.

The five viroid species described by Duran-Vila *et al.* (1988) were identified in Tunisian citrus by sPAGE and molecular hybridization (Table 1). All the samples that induced a severe reaction on Etrog citron contained CEVd, whereas those showing moderate and mild symptoms contained different combinations of CBLVd, HSVd, CDVd and CBCVd. CEVd, HSVd and CDVd were highly widespread accounting respectively for 70.3, 72.3 and 78.2% of the sources tested. CBLVd and CBCVd were only found in 28.2% and 3.0% of the tested trees (Table 1). CEVd, HSVd and CDVd were found in almost all the cultivars analyzed, whereas CBCVd was only detected in a total of six trees of Maltaise demi-sanguine sweet orange (2 out of 33), Common mandarin (1 out of 35) and Eureka lemon (3 out of 20) (Table 1).

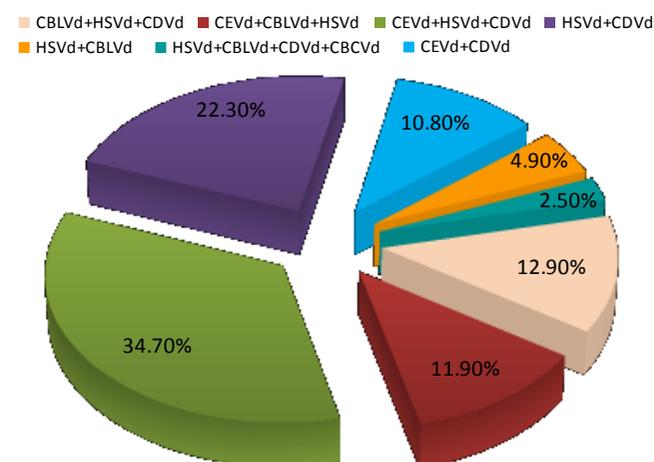
Most samples either from commercial orchards or from the citrus INRAT collection were infected with at least two viroids. The most frequent viroid combinations were CEVd+HSVd+CDVd (34.6%) and HSVd+CDVd (22.3%). Other combinations such as CBLVd+HSVd+CDVd (12.9%), CEVd+CBLVd+HSVd (11.9%) and CEVd+CDVd (10.9%) were less frequent, whereas HSVd+CBLVd (4.9%) and HSVd+CBLVd+CDVd+CBCVd (2.5%) were rather infrequent (Fig. 2).

The three CEVd clones sequenced had 99% similarity to each other and shared *ca.* 99% similarity with other viroid sequences from the GenBank accessions (GU825980, GU825981, FJ626864, FJ626863, FJ626866, KJ538556, KJ538554, KJ538555, LN681196, JX259392, AF540963, JX885866, U21126, EF126047, EF186991, EF186989, GQ260199, AF540960, JX885867 and KF265340) from Iran, Greece and Syria with 88% branch support (Fig. 3). The most similar variant was CEVd Class B from Greece (JX259392).

The CDVd isolates were similar with each other and showed 96% similarity with other isolates (AB054621, AB054622, AB054623, AB054632, AF123863, AF123866, AF123870, AF540966, EU564176, EU872279, EU934025,

**Table 1.** Detection of CEVd, CBLVd, HSVd, CDVd and CBCVd in different citrus cultivars from the Cap Bon region in Tunisia by sPAGE and molecular hybridization

Cultivar	Samples	CEVd	CBLVd	HSVd	CDVd	CBCVd
Maltaise demi-sanguine	33	22	10	23	24	2
Maltaise sanguine	23	13	0	15	18	0
Orange double fine	10	7	3	6	9	0
Washington navel	26	21	8	18	24	0
Valencia late	15	10	0	6	5	0
Common mandarin	35	23	8	30	28	1
Cassar clementin	40	28	16	36	33	0
Eureka lemon	20	18	12	12	17	3
Total	202	142	57	146	158	6
% infection		70.3	28.2	72.3	78.2	3.0



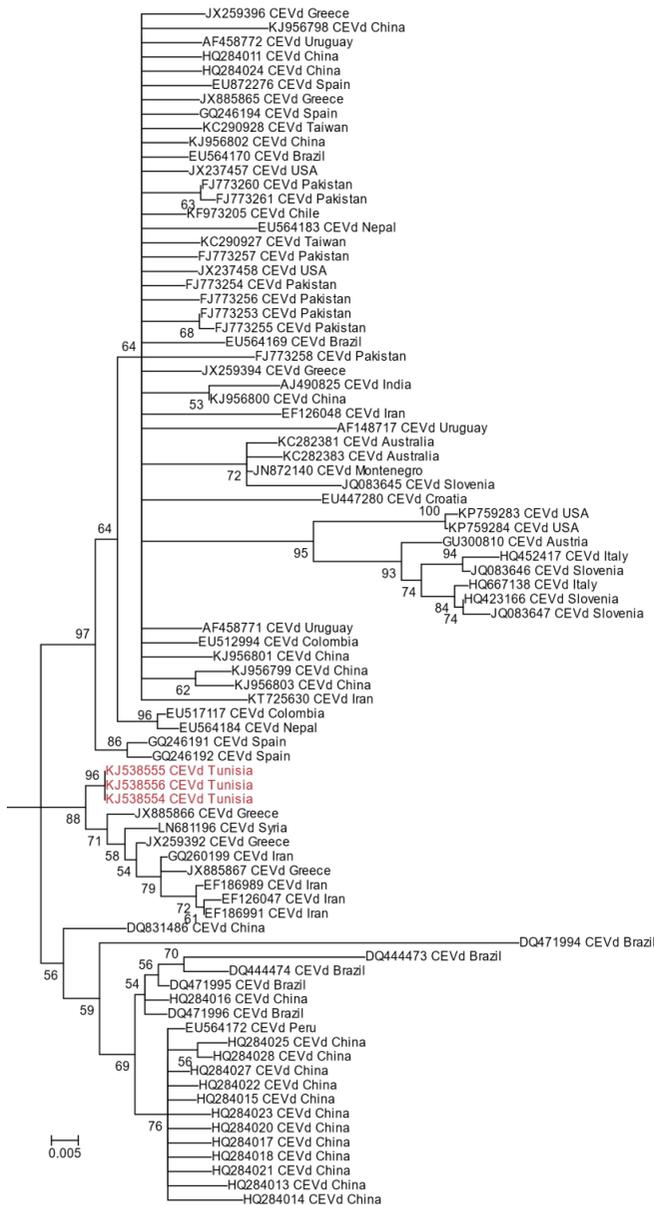
**Fig. 2.** Frequency of citrus viroid combinations found in Tunisian citrus orchards.

EU934032, JX259427, JX259428, KJ538558, KT725632, KU861550 and S76452) from Brazil, Cyprus, Greece, Uruguay, Israel and Spain with a branch support of 55% (Fig. 4).

The phylogenetic analysis showed that all new Tunisian CEVd and CDVd variants cluster in the same group (Fig. 3, Fig. 4). These results suggest that these two viroids may have a common origin and have been introduced in Tunisia with the same propagation material.

The results of this survey confirm that citrus viroids are infecting the main citrus cultivars grown in Tunisia. Their high incidence was probably enhanced by the traditional inadequate phytosanitary grafting, harvesting and pruning procedures for which contaminated grafting and cutting tools are used. In fact, early studies demonstrated the mechanical transmission of cachexia and exocortis agents by stem slashing with a knife blade (Roistacher, 1983). A more recent greenhouse transmission study showed that CBLVd, HSVd and CDVd can mechanically be transmitted by a single slash (Barbosa *et al.*, 2005).

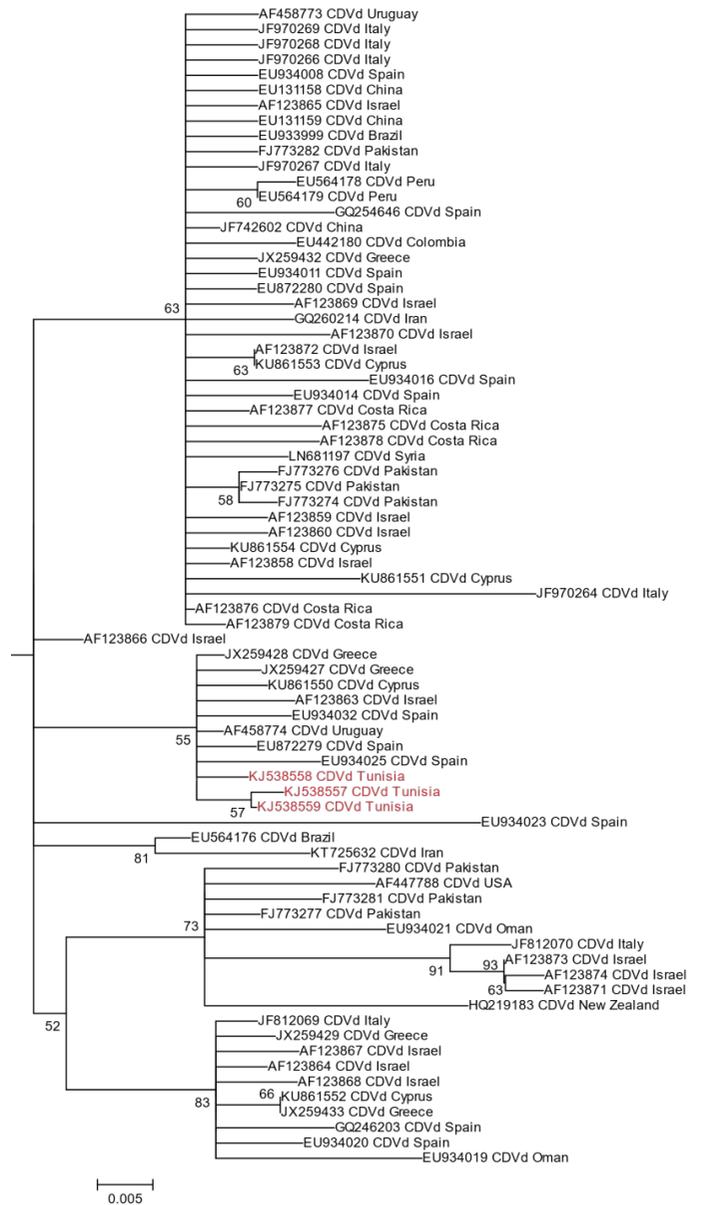
CEVd, HSVd and CDVd were found to be the most widespread viroids in the Cap Bon region being detected in almost all citrus cultivars (Table 1). They were also



**Fig. 3.** Neighbor-joining phylogenetic tree (1000 bootstrap replicates) of selected CEVd isolates (whose country of isolation is available in GenBank records). Branches with less than 50% bootstrap support have been collapsed.

frequently found as mixed infections (Fig. 2). These results are in agreement with those of previous studies conducted in many parts of the world showing that these three viroids are the most prevalent in citrus orchards (Duran-Vila and Semancik, 2003; Onelge *et al.*, 2004; Malfitano *et al.*, 2005; Wang *et al.*, 2013).

The high incidence of HSVd as revealed by sPAGE and hybridization agrees with the high number of trees showing cachexia symptoms in the field-grown common mandarin and Cassar clementine. Despite the relatively high incidence of CEVd, exocortis scaly bark symptoms have never been observed because of the exclusive use of the sour orange which is an exocortis tolerant rootstock (Duran-Vila and Semancik, 2003).



**Fig. 4.** Neighbor-joining phylogenetic tree (1000 bootstrap replicates) of selected CDVd isolates (whose country of isolation is available in GenBank records). Branches with less than 50% bootstrap support have been collapsed.

CBCVd was only detected in two Maltese demisanguine sweet orange, one common mandarin and two Eureka lemon trees. The incidence of this viroid is significantly lower as compared to what reported from other citrus-growing countries like Turkey (Onelge *et al.*, 2004), Italy (Malfitano *et al.*, 2005), Sudan (Mohamed *et al.*, 2009) and Greece (Wang *et al.*, 2013). The occurrence of mixed infections found in the Cap Bon region can be considered as very high (Fig. 2), since all tested trees were infected by more than one viroid. We did not find trees infected with a single viroid, probably because the surveys were conducted in very old orchards (>40 years old). Pagliano *et al.* (2013) reported that HSVd was found more frequently in single than in mixed infections and it was detected not

only in older but in young (three-year-old) symptomless trees.

The results of the present study are in agreement with those reported from Southern Italy and Greece (Malfitano *et al.*, 2005; Wang *et al.*, 2013) that probably share similar nursery and agronomic practices. In our surveys, the most frequent viroids found under field conditions were CEVd, HSVd and CDVd. In fact, it has been suggested that the low frequency distribution of CBCVd may be due to a relatively recent introduction from an unknown host into citrus plants (Malfitano *et al.*, 2005). This viroid has been recently identified as a highly aggressive pathogen affecting hops in Slovenia (Jakse *et al.*, 2015).

The CDVd clones sequenced presented 99% of nucleotide similarity with each other and showed high sequence conservation (*ca.* 99% similarity) with other CDVd deposited in GenBank suggesting that CDVd presents a low genetic variability, as also observed by Murcia *et al.* (2009) and Eiras *et al.* (2013).

Furthermore, it is important to mention that the high viroid infection/contamination rate should be taken into consideration when new rootstocks will be chosen to manage tristeza disease. In this case, the control of foundation blocks and nurseries through a rigorous certification of citrus budwood should be implemented.

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