Chapter

A Current Overview of Two Viroids Prevailing in Citrus Orchards: Citrus Exocortis Viroid and Hop Stunt Viroid

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Abstract

Citrus exocortis viroid (CEVd) and hop stunt viroid (HSVd) are the main viroids circulating in all citrus-growing areas worldwide, and causing two well-known diseases on citrus trees; exocortis and cachexia, respectively. These viroids are small, covalently closed single-stranded RNA, allocated to the *Pospiviroidae* family. CEVd is the first viroid being described on citrus trees in 1948 in California. It is considered the largest citrus viroid at 371 nucleotides. It causes bark scaling disorder on the rootstock of citrus trees grafted on trifoliate orange and its hybrids and can cause dwarfing of trees grown on these rootstocks. HSVd was first observed in 1945 in Florida. It consists of 299 nucleotides. Stunting, chlorosis, bark gumming, stem pitting, decline, and depressions in the wood are the main symptoms of HSVd in mandarin and its hybrids. The introduction and propagation of infected budwoods are the main causes of viroids spread in citrus orchards. These agents are mechanically sap-transmissible and spread by contaminated tools. Neither seed transmission nor vectors have been reported for both viroids. Root transmission, though possible, would be overshadowed by mechanical transmission. Rapid and sensitive molecular-based detection methods specific to both viroids are available. Both diseases are controlled by using viroids-free budwoods for new plantations, launching budwood certification programs, and establishing a quarantine system for new citrus varieties introduction. The most important achievements in CEVd and HSVd researches are outlined in this chapter. This would help to provide a clearer understanding of the diseases they cause and contribute to the development of better control strategies.

Keywords: CEVd, HSVd, citrus, *Pospiviroidae*, transmission, diagnostic, interactions, synergy, antagonism

1. Introduction

Viroids are circular, highly structured, single-stranded RNA (ssRNA) phytopathogens. Although they do not code for any peptide, these enigmatic pathogens have evolved the capacity to replicate within cellular organella, the nucleus and chloroplast

for *Pospiviroidae* and *Avsunviroidae*, respectively [1–4]. Viroid replication is ensured through an RNA-based rolling-circle mechanism [1]. Intriguingly, viroids can induce severe diseases in susceptible host plants similar to those caused by numerous plant viruses [5–7]. From the seven known citrus viroids only, two, namely, CEVd and HSVd, have been reported to be associated with citrus diseases that can pose significant economic risks to global citrus production. These diseases are exocortis and cachexia, respectively [8]. Since their original description in 1948 and 1950, respectively, both diseases have been reported to be present in almost all citrus-growing areas of the world, as well as in early citrus budwood registration programs [9, 10]. Given the importance of and rapid research progress in citrus virology in recent years, this review emphasizes recent findings related to CEVd and HSVd, the most serious viroids associated with citrus. It comprises reviews and research articles covering broad research areas on the characterization of both viroids and their symptoms, the development of reliable and rapid diagnosis methods, and management strategies. A brief snapshot of the present situation of CEVd and HSVd in the Mediterranean region, with an emphasis on their spread in citrus-growing areas of Morocco, is included.

2. Citrus exocortis viroid

2.1 Taxonomy

Citrus exocortis is a destructive disease infecting citrus species [9, 11]. The agent of this disease, citrus exocortis viroid [*Pospiviroidae*; *Pospiviroid*; CEVd], is a small, covalently closed ssRNA of about 371 nucleotides (nts) [12–14]. CEVd molecules can exist as either linear or circular [9]. As all viroids allocating to the *Pospiviroid* genus, CEVd lacks RNA self-cleavage activity and has a central conserved region (CCR), composed of two sets of conserved nucleotides in the upper and lower strands of its rod-like secondary structure, and a terminal conserved region (TCR) [15]. The rod-like secondary structure of CEVd takes the form of a model of five structural-functional domains. The latter is the Central (C), the Pathogenic (P), the Variable (V), the Terminal Left (TL), and the Terminal Right (TR) domains [16]. Based on their biological properties, CEVd sequences have been classified into two groups by using tomato as an experimental host: severe "Class A" and mild "Class B". Both classes of sequences differ by a minimum of 26 nts. These mutations affect two genomic regions, designated P_L and P_R, located respectively within the P and the V domains [17–19]. It is important to emphasize that CEVd strains of both classes cause distinct symptoms in gynura (*Gynura aurantiaca* (Blume) DC.) [20]. However, they induce only subtle differences in trifoliate orange (*Poncirus trifoliate* L. Raf.) used as a rootstock and a similar overall performance of the infected trees [21]. Sequencing of additional CEVd isolates revealed that further strains different from those of "Class A" and "Class B" existed. Furthermore, it seems that the sequence/pathogenicity relationship was more complex than originally anticipated [22]. Infectivity assays carried out with chimeric cDNA clones suggested that P_L is the pathogenicity-modulating domain. Although it remains to be explored how this domain modulates pathogenicity (i.e. stunting and epinasty). The role of the P_R domain is not known. However, infectivity assays suggested that it may influence the efficiency of viroid infection or replication in the plant [18]. Further infectivity assays of CEVd chimeras and another viroid of the *Pospiviroid* genus, tomato apical stunt viroid [TASVd], have been done to identify the role of individual structural domains. Firstly, it has been demonstrated that symptom severity is modulated by the TL and the P domains. Secondly, it has been shown that the V and the TR domains are involved in regulating viroid replication and/or accumulation [23].

2.2 Symptoms and economical impact

Citrus exocortis could affect a various part of the tree including the rootstock (at bark and wood levels), scion, leaves, and fruits, thus causing different types of damages such as bark scaling and cracking, bumps, severe stunting, low fruit-bearing, the poor appearance of the canopy [21, 24–27], and poor tree performance [28]. CEVd-infected trees in the orchard show typical symptoms. The most characteristic one is bark scaling on trifoliate orange rootstock, yellow stem blotch on trifoliate orange and its hybrids and Rangpur lime (*Citrus limonia* Osb.), and stunting on trifoliate orange or its hybrid rootstocks [9, 11]. It is important to note that classic exocortis symptoms are not always closely associated with all CEVd isolates. For instance, only transient flaking (Washington Navel orange L) or a fine reticulum of surface cracks (Washington Navel orange 3536) on the trifoliate orange rootstock have been observed on two CEVd-infected trees in Australia [28]. Additionally, no bark scaling symptoms have been observed in CEVd-infected Washington Navel orange trees grafted on Carrizo citrange although these trees presented lesions and blisters in the roots [26]. It is important to emphasize that bark scaling symptoms could be caused by viroids other than CEVd. Indeed, it has been proved that, in certain viroid combinations, synergistic effects occur and cause exocortis scaling symptoms in the absence of CEVd [24]. Furthermore, in Australia, a large number of trees showing exocortis-like symptoms including dwarfing and/or bud union abnormalities produced only mild epinasty when grafted on the indicator plant Etrog citron. The presence of viroids other than CEVd has been highlighted [28]. Bark scaling symptoms could be also the consequence of tree exposure to abiotic stresses such as sunburn [29]. A reduction in vegetative growth has been observed on commune clementine (*Citrus clementine* Hort. ex Tanaka) trees infected by CEVd as it has been determined by the height and rootstock and scion circumferences [21]. Similar but milder symptoms have been reported in CEVd-infected Washington Navel orange trees grafted on Carrizo citrange [26].

The major susceptible citrus rootstocks, which show exocortis bark scaling symptoms, are trifoliate orange and its hybrids, Palestine sweet lime (*Citrus limettioedes* Tan.) or Rangpur lime [11]. Trees grown on trifoliate orange are the most severely affected, with symptoms of bark scaling and severe stunting usually developing when the trees are around 4 years old [29, 30]. Cracking and peeling of the bark below the bud union appear when bark scaling occurs on these rootstocks [30]. Symptoms of exocortis have been also reported on citrange and Swingle citrumelo rootstocks. However, unlike trifoliate orange, bark scaling symptom does not always occur on trees grown on citrange rootstocks. Trees grafted on these rootstocks exhibit symptoms somewhat late and the level of tree stunting is usually less severe than that on trifoliate orange. On another susceptible rootstock, CEVd-infected trees showed symptoms of stunting, yellowing of the canopy, and general tree decline, and occasional flaking of the rootstock bark. On these trees, fruit quality is not affected. However, tree yield is severely reduced since the viroid causes tree stunting [30]. The time required for disease expression by citron scions is believed to be directly associated with the inherent vigor of the rootstock, the environmental temperature, and cultural practices [31]. CEVd does not induce any symptoms in most sweet orange (*Citrus sinensis* (L.) Osbeck), mandarin (*Citrus reticulate* Blanco), and grapefruit (*Citrus paradisi* Macfad) scion cultivars. However, when CEVd-infected budwoods are grafted on one of the previous susceptible rootstocks, distinct symptoms may appear [11].

The type and severity of symptoms induced by citrus exocortis disease depend not just on the selected rootstock as described above, but also on the amount of viroid present in the scion and the infection with other citrus viroids. High

temperatures can also accelerate the development of symptoms [30]. The results of a long-term field trial carried out with clementine trees grafted on the trifoliate orange rootstock revealed that CEVd-induced effects might be both reduced or increased when CEVd-infected trees were exposed to mixed viroid infections. In other words, several interactions among viroids including CEVd have been revealed through comparative assays between symptoms developed on trees infected with CEVd alone or co-infected with other viroids. The most clear-cut interaction occurs between CEVd and Citrus viroid IV [*Pospiviroidae*; *Cocadviroid*; CVd-IV]. This interaction is manifested by the attenuation of bark-scaling or bark-cracking symptoms as a result of the occurrence of antagonism between both viroids. CVd-IV limits the negative effects of CEVd on tree performance. The reduction of tree size and fruit yield occurs mainly in trees infected with combinations containing CEVd or CVd-III and, to a lesser extent, those containing Citrus bent leaf viroid [*Pospiviroidae*; *Apscaviroid*; CBLVd] [24].

Numerous field trials have been conducted on different citrus species, varieties, and rootstocks under three different agroecosystems, to evaluate the effect of CEVd on vegetative growth and yield (**Table 1**). The first field trial has been conducted to assess the effect of CEVd infection on commune clementine trees grafted on Pomeroy trifoliate orange. CEVd-infected trees have been periodically monitored for a period of 12 years (from 1990 to 2002) for symptom expression, growth, and fruit yield. CEVd-infected trees showed a significant reduction of growth and yield, which became increasingly apparent over time with infection. Cumulative yield varied from 291,1 to 570,3 kg in 2001 for CEVd and the control, respectively. This equated to 50% cumulative yield lost. This yield attenuation was associated mainly with the loss of large fruit production. Indeed, it has been shown that CEVd reduced fruit production significantly for calibers 2 to 5. Cumulative weights were smaller than the control for caliber 0–1 and small calibers 6 and 7–8, with some significant difference [21]. The quality of fruits from CEVd-infected orange trees (Washington Navel) grafted on Carrizo citrange rootstock has been evaluated from 2004 to 2007. The results of this experiment showed that the quality of the fruit was not affected by CEVd infection [26].

2.3 Transmission and epidemiology

All citrus viroids are distributed primarily by the introduction and propagation of infected budwoods and subsequently by mechanical transmission, and CEVd is no exception [11]. Mechanical transmission of CEVd has been already reported. It took place on secateurs, tools, knives, and hedging equipment [9, 11, 27, 29] especially from lemon (*Citrus lemon* Bum. f.) to lemon [11]. Further, it has been shown that CEVd can survive for 8 days on steel knife blades. CEVd infectivity was not affected over a wide range of time intervals between knife contamination and transfer to citron or by 2 sequential transfers by this method. CEVd spread to susceptible hosts by contaminated tools was accomplished from numerous tested citrus species of great economic importance such as lemon, sweet orange, grapefruit, tangerine, and a trifoliate hybrid [31]. Another transmission assay carried out under greenhouse conditions showed that CEVd can be mechanically transmitted from citron to healthy citron [32, 33] and gynura herbaceous plant [33] by a single slash with a knife blade [32, 33]. CEVd-retransmission from infected gynura back to citron was successful [33]. Natural grafts of citrus roots seem to be associated with CEVd propagation. That is the case for example for a budwood multiplication block of an Australian nursery where the propagation of CEVd by natural grafts of roots induced the infection of some healthy lemon mother trees on which neither hedging nor pruning operations took place before

Combination		Effect on			References
Scion	**Rootstock**	**Height**	**Canopy**	**Cumulative yield**	
CEVd					
Clementine	Orange Pomeroy trifoliate	**SE*** (Reduction of almost 14% for CEVd-117)	NSE	**SE** (Reduction of almost 50%)	[21]
Oranger Washington Navel	Carrizo citrange[c]	**SE** (Reduction of almost 10% and 15% for CEVd-129 and CEVd-117, respectively)	NSE (Reduction of almost 17% and 27% for CEVd-129 and CEVd-117, respectively)	NSE (Low reduction of about 2% and 10% for CEVd-129 and CEVd-117, respectively)	[26]
Orange Maltaise demi sanguine	Soor orange (*Citrus aurantium* L.)[a]	NSE	NSE	NSE (Reduction of almost 20%)	[46]
	Alemow *Citrus macrophylla* Webster [b]	NSE	NSE	NSE	
	Carrizo citrange[c]	NSE	NSE	NSE	
	Citrus volkameriana Ten. And Pasq.[c]	NSE	**SE** (Reduction of almost 33%)	NSE (Reduction of almost 20%)	
	Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.)[c]	NSE	NSE (Reduction of almost 25%)	NSE (Reduction of almost 20%)	
	Swingle citrumelo (Citru)[c]	NSE	NSE	NSE	
	Rangpur lime (*Citrus limonia* Osb.)[c]	NSE	NSE (Reduction of almost 28%)	NSE (Reduction of almost 20%)	
	Trifoliate orange[c]	**SE** (Reduction of almost 25%)	**SE** (Reduction of almost 49%)	NSE	
HSVd					
Clementine	Orange Pomeroy trifoliate[c]	Little or no real impact	NSE	**SE*** (Reduction of almost 34% for CVd-IIc)	[21]
Orange Washington Navel	Carrizo citrange[c]	NSE (Reduction of almost 15% for CVd-IIc)	NSE (Reduction of almost 8% for CVd-IIb)	No effect	[26]

Combination		Effect on			References
Scion	**Rootstock**	**Height**	**Canopy**	**Cumulative yield**	
Orange Maltaise demi sanguine	Soor orange (*C. aurantium*)[a]	NSE	NSE	NSE (Reduction of almost 20%)	[46]
	Alemow *C. macrophylla* Webster [b]	**SE** (Reduction of almost 33%)	**SE** (Reduction of almost 77%)	**SE** (Reduction of almost 76%)	
	Carrizo citrange[c]	NSE	NSE	NSE	
	C. volkameriana Ten. And Pasq.[c]	NSE	**SE** (Reduction of almost 30%)	NSE (Reduction of almost 20%)	
	Cleopatra mandarin (*C. reshni* Hort. ex Tan.)[c]	NSE	NSE	NSE (Reduction of almost 20%)	
	Swingle citrumelo (Citru)[c]	NSE	NSE	**SE** (Reduction of almost 36%)	
	Rangpur lime (*C. limonia* Osb.)[c]	NSE	NSE	NSE (Reduction of almost 20%)	
	Trifoliate orange[c]	**SE** (Reduction of almost 26%)	**SE** (Reduction of almost 45%)	**SE** (Reduction of almost 66%)	

SE: *Significant effect. NSE: No significant effect.*
[a]*Susceptible to citrus tristeza virus [Closteroviridae; Closterovirus; CTV] but viroids tolerant.*
[b]*Susceptible to CTV stem-pitting and cachexia.*
[c]*CTV tolerant.*
[*]*A function of the used viroid isolates.*

Table 1.
Results summary of the known field trials carried out in three citrus-growing countries of the Mediterranean area to evaluate the effect of CEVd and HSVd on vegetative growth and yield of different citrus scion and rootstock combinations.

their removal. This may be mainly linked to the fact that citrus trees were planted close to each other (within 2 m). The role of root grafting in CEVd transmission was assessed by excavating root systems [29]. CEVd root transmission, though possible, would be overshadowed by mechanical transmission. CEVd is not known to be a vector- or seed-transmitted [9, 11]. The role of gots as possible vectors of viroids, including CEVd, has been investigated. The experiment was carried out by rubbing healthy citrus plants with goat horns previously rubbed for 24 h on infected Etrog stems. Results highlighted the detection of CEVd in the tested plants. Therefore, transmission through gots could have facilitated the long-range spread of CEVd among both cultivated and wild plants and *vice versa* and also among graft-incompatible plants [34].

3. Hop stunt viroid

3.1 Taxonomy

Cachexia is a destructive disease infecting citrus species [11]. The agent of this disease, hop stunt viroid [*Pospiviroidae*; *Hostuviroid*; HSVd], is a small covalently closed ssRNA of about 300 nts [3, 35]. HSVd is a single member of the genus *Hostuviroid* [36]. HSVd molecules can exist as either circular or linear [35]. HSVd isolates are divided into five groups: three major and two minor groups. The first groups, composed of "plum-type", "hop-type" and "citrus-type", are composed of isolates from a limited number of isolation hosts. As to the second group, it has been suggested that they are the results of the occurrence of recombination events between members of the main groups [37]. Like CEVd, HSVd takes the form of a model of five structural-functional domains within the rod-like secondary structure: C, P, V, TR, and TL [15]. However, HSVd has a genus-specific CCR and a terminal conserved hairpin (TCH) and lacks a TCR [10]. It is worth mentioning that two HSVd-related Group II citrus viroids that differ by a "cachexia expression motif" have been described. It includes a cachexia disease non-pathogenic variant (CVd-IIa) and two pathogenic variants (CVd-IIb and CVd-IIc) [38–40]. Electrophoretic profiles obtained with single-stranded polymorphism (SSCP) allowed deciphering the variability among and within cachexia-inducing sources of citrus isolates of HSVd. SSCP allowed discrimination between non-cachexia and cachexia sources of HSVd. Sequence analysis showed that the V domain was extremely conserved among all the cachexia variants. Indeed, 5 nts differences, affecting both the upper (3 nts) and the lower (2 nts) strands of the V domain, were identified as the most characteristic motif allowing the discrimination between cachexia and non-cachexia sequences. It has been suggested, therefore, that the 5 nts affect the organization of a short helical region and two flanking loops of the V domain, thus modifying the three-dimensional geometry of the molecule [41]. Subsequently, it has been shown that only a single change in HSVd modulates citrus cachexia symptoms [38].

3.2 Symptoms and economical impact

Cachexia could affect a various part of the tree including the trunk, bark, twigs, branches, leaves, and fruits, thus causing different types of damages such as bark and trunk gumming with a rough and rugose appearance, bark-cracking, moderate and severe tree stunting, chlorosis, decline and death of severely affected trees, brown stipple spotting on the underside of the leaves, and the appearance of small pits on the wood [9, 21, 24]. Cachexia disease mainly affects some mandarins and their hybrids such as tangelos, and *Citrus macrophylla* Wester. Most other citrus species seem to be symptomless unless grafted on susceptible rootstocks [10]. Cachexia-inducing variants were proven to cause gummy bark disease of sweet orange [42, 43] and split bark disorder of sweet lime (*Citrus limetta* Risso) [44]. HSVd variants have been reported to induce yellow corky vein disease of Kagzi lime (*Citrus aurantifolia* (Christm.) Swingle) [45] and sweet orange [44] in India and Iran, respectively. It was subsequently shown that cachexia and a similar disorder previously described in Palestine sweet lime, known as xyloporosis, are caused by the same type of HSVd variants [40].

As mentioned before, for CEVd, the type and severity of citrus cachexia symptoms depend also on the presence of other citrus viroids in the tree. The results of a long-term field trial carried out with clementine trees grafted on the trifoliate

orange rootstock revealed that HSVd-induced effects might be both reduced or increased when HSVd-infected trees were exposed to mixed viroid infections. The most clear-cut interaction occurs between HSVd and CVd-IV. This interaction is manifested by a slight increase in fruit yield and reduction of scion circumferences [24].

The same field trials described before to evaluate the effect of CEVd on vegetative growth and yield (**Table 1**) were used for the same purpose for HSVd. Little or no effect in vegetative growth has been observed on commune clementine trees infected by HSVd as it has been determined by the measure of height and rootstock and scion circumferences [21]. Cumulative yield varied from 377,6 to 570,3 kg in 2001 for HSVd (CVd-IIc isolate) and the control, respectively. This equated to 34% cumulative yield lost [21]. The negative impact of HSVd infection on cumulative yield has been reported in another study carried out on Orange Maltaise demi sanguine grafted on Alemow (*C. macrophylla*). HSVd-infected trees have been periodically monitored for a period of 12 years (from 2005 to 2017) for growth and fruit yield. HSVd-infected trees showed a significant reduction of yield of about 76% compared to healthy control [46]. As for CEVd, the effect of HSVd infection on the quality of fruit from Washington Navel orange trees grafted on Carrizo citrange rootstock has been evaluated from 2004 to 2007. The results of this experiment showed that the quality of the fruit was not affected by HSVd infection. However, a reduction occurred in the diameter of the harvested fruits [26].

3.3 Transmission and epidemiology

As pointed out before, for CEVd, propagation of infected budwoods and mechanical inoculations with contaminating tools were reported as the principal causes for the omnipresence of multiple viroid species, including HSVd, among citrus orchards [34]. Mechanical transmission of HSVd has been already reported. Indeed, the results of a transmission assay carried out under greenhouse conditions revealed that all HSVd strains are mechanically transmitted from citron to healthy citron by a single slash with a knife blade [32]. As for CEVd, the potential involvement of gots in HSVd spread has been shown under controlled conditions [34]. Top working, a common practice in Mediterranean countries, seems to have largely contributed to HSVd spread in Mediterranean citrus orchards [9]. HSVd is not known to be seed-borne [47] in citrus or to have natural vectors [11, 48].

4. Signaling pathways in citrus exocortis and cachexia pathogenesis

It is usually accepted that although the mechanisms through which viroids interact with their hosts are beginning to be dissected, the key triggering events and molecular mechanisms underlying viroid pathogenesis remain unclear [49, 50], and CEVd and HSVd are no exception. As demonstrated by various types of citrus pathogens [51, 52], further investigation of the molecular basis of viroid-host interactions is crucial to better understand the pathogenesis of viroids, and thus help to develop effective strategies to combat viroid diseases [50, 53]. Important changes occur in the chloroplast, cell wall, peroxidase, and symporter activities upon infection of Etrog citron with CEVd [54]. The CEVd-infected citron system has been subsequently used for studying the feedback regulation mechanism using transcriptomic analysis. The analysis of the woody host response to CEVd revealed the activation of basic defense and RNA-silencing mechanisms following CEVd infection. In other words, a large number of genes (about 1530) encoding key proteins involved in the RNA silencing pathway, and proteins related to basic defense

responses are expressed following CEVd infection [53]. Furthermore, a recent study elucidates the role of phytohormone pathways, particularly those linked to ethylene, in disease development and ribosomal stress caused by CEVd infection by using tomato as an experimental host [55]. For HSVd, a small RNA-mediated gene silencing response has been highlighted upon the infection of lemon by HSVd. The large amounts of HSVd-small interfering RNA (siRNA) from both central and variant domains have been suggested to be involved in interference with host gene and symptom development [56].

5. Detection methods

5.1 Biological indexing and cross protection

Generally, biological assays based on indicator host plants expressing typical symptoms of infection and able to withstand higher levels of viroid replication played an essential role in both viroid detection and characterization [57]. CEVd and HSVd are viroid diseases present in citrus orchards around the world [11]. The biological diagnosis through indexing method is considered as an efficient tool to test the health status of a plant, regarding a disease by inoculation with the grafting of the budwood or any other infected tissue in indicator plants that allow viroid replication, symptoms expression [11, 58], and the enhancement of viroid concentration [59]. However, bioassays for CEVd and HSVd detection and identification may require a panel of indicator host plants [60]. Certain considerations need to be respected for the proper indexing of citrus viroids. These include the use of excellent plants, the work under warm temperatures, and the use of citron index plants grown one per container. As mentioned previously, citrus viroids are highly mechanically transmissible and tools must be disinfected to avoid their spread [9].

The citron test is a very sensitive and diagnostic index for determining the presence of CEVd [9]. However, indexing, *in vivo* for CEVd diagnosis is time-consuming, labor-intensive, and requires technician greenhouses [59]. It can take 90 days after inoculation or grafting onto indicator plants [61–63]. Symptoms of slight to severe epinasty leaves wrinkled and twisted to the reverse with light to dark brown cracks in petiole and branches, blisters in the petiole, corking of the midrib, and reduced growth are the main symptoms observed on Etrog citron Arizona 861-S indicator plants graft-inoculated with CEVd-infected budwoods [28, 29, 59, 63]. An *in vitro* indexing procedure has been developed to minimize the risks of epidemics caused by viroids including CEVd. It has been proved that the *in vitro* indexing of CEVd is efficient as well as the *in vivo* diagnosis, and requires between 20 and 40 days less to reach the maximum incidence after inoculation. Epinasty, growth reduction, and rugged leaves with dry tips, and reduced size are the main symptoms observed on the sprouts planted *in vitro* and grafted with CEVd-infected callus [59]. The same symptoms have been reported for sprouts grafted with CEVd infected cortex [62]. Cuban Shaddock (*Citrus maxima* (Burm.) Merr.) has been proved to be the best rootstock, compared to rough lemon (*Citrus jambhiri* Lush.) or Volkamer lemon (*Citrus volkameriana* Ten.), for symptom expression on Arizona 861 S-1 citron indicator plants for indexing exocortis [64]. Gynura is also considered as an excellent indicator for CEVd. This latter reacts strongly in this host plant [9].

As to cachexia, Parson's special mandarin budded on vigorous root-stock such as rough lemon or Volkamer lemon is reported as an excellent indicator for the disease [9, 65]. The biological indexing may take up to one year before symptoms are seen. The reaction of Parson's Special mandarin may differ depending on HSVd isolates. In other words, some isolates are very mild reacting, whereas others are quite severe

in their reaction to the indicator plant. Indeed, a mild strain reaction consists of just a slight browning at the bud union or cut back region of the Parson's Special mandarin while a severe reaction consists of the appearance of gum in the wood that may extend via the entire plant [9]. Cuban Shaddock has been proved to be the best rootstock, compared to rough lemon or Volkamer lemon, for symptom expression on Clemeline 11–20 indicator plants for indexing cachexia. Furthermore, the application of 0,5% foliar urea sprays, alone or in combination with 20 ppm gibberellic acid showed to produce more intense expression of cachexia symptoms in the indicator Clemeline 11–20 than the unsprayed control [64].

Cross protection is a biological assay, in which the infection of a plant with a viroid strain ensures protection from infection with another strain of the same viroid. This bioassay can be used for indirect viroid biological indexing. It has been applied in the diagnosis of several viroids including CEVd and HSVd. Typically, the principle of this method is based on the infection of the plant with a mild strain of a viroid, followed by its inoculation with inoculum from a plant suspected to be infected with a severe strain of the same viroid. Positive indexing of the viroid is revealed by the non-expression of symptoms in the tested plants [60]. It has been shown in a cross-protection assay, performed with CEVd-129 as a "protecting" strain against the severe type strain of CEVd that a mild strain of CEVd could lead to apparent "protection" against challenge inoculation with the severe strain. However, it is important to highlight that variability has been shown in the induced protection effect. The latter varied from only a brief delay to almost total impairment of symptom expression. The level of protection depends on the length of the interval between the inoculations with the mild and severe strains [66].

5.2 Nucleic acid-based methods

Since viroids lack a protein capsid, serological techniques used routinely in plant viruses' detection are not applicable [67]. Nucleic acid-based methods, including polyacrylamide gel electrophoresis (PAGE), hybridization (dot- and northern-blots and micro–/macroarrays), amplification (reverse transcription-polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP)) and sequencing (next-generation sequencing and Sanger sequencing), offer rapid cost-effective, and reliable diagnosis of viroids [60].

PAGE is considered as the first molecular technique used for the rapid (2–3 day period) identification of viroid infected plants. This technique continues to play a crucial role in the identification of new viroids since it is the only diagnostic method that is sequence-independent. PAGE analysis under denaturing conditions showed that many *Citrus* species may harbor numerous viroids including CEVd and HSVd [57]. PAGE and ethidium bromide or silver staining is considered as the first molecular technique applied for CEVd detection [22, 68]. However, it seems that the sensitivity of this technique requires an adequate viroid accumulation level [22]. In other terms, the PAGE procedure was used successfully to directly detect CEVd from field-grown sweet orange and grapefruit trees. The key was reported to be the use of large (50 g) samples of succulent, expanding-flush tissue collected during the summer season. However, samples collected from field-grown trees in January and February did not give consistent detection in trees known to be CEVd-infected, presumably because lack of new growth and low temperatures do not favor CEVd replication [69]. PAGE analysis can routinely resolve as many as four different viroids in the same sample. For instance, it has been shown that this technique can resolve two HSVd variants differing in length by only four nucleotides i.e. 303 nts vs. 299 nts [57].

Since the beginning of their use in the 1980s, dot blot hybridization and hybridization of tissue imprints began to replace PAGE for routine viroid detection. This is mainly because these methods allow the processing of a large number of samples [57]. A northern hybridization protocol, which relied on the analysis of preparations from bark tissues, was proved to be more sensitive than PAGE to detect CEVd and HSVd from field-grown plants of different citrus species and cultivars [70]. A citrus viroids-multiprobe composed of full-length clones of HSVd, CEVd, and two other citrus viroids has been constructed for the simultaneous detection of viroids associated with citrus trees. All the tested viroids were effectively detected with this multiprobe when tested by both northern hybridization and dot blot methods. It is important to highlight that this multiprobe does not allow the identification of the viroid type species resulting in a positive signal [71].

Due to the small size of viroids, numerous RT-PCR approaches can be applied for both their detection and subsequent characterization. In the case of CEVd and HSVd, numerous RT-PCR and real-time RT-PCR approaches have been developed and proved to allow the detection of CEVd and HSVd in both singleplex or multiplex assays [63, 72–76]. A list of some RT-PCR and related tests developed to detect CEVd and HSVd are presented in **Table 2**. Some of these tests allow also the discrimination between mild and severe CEVd strains and the identification of HSVd isolates associated with cachexia symptoms [77]. The multiplex one-step RT-PCR assay developed by Wang et al. [75] is considered a good tool streamlining the simultaneous detection of up to five citrus viroids, including CEVd and HSVd. This enables to reduce time and labor without affecting sensitivity and specificity. Indeed, serial dilution experiments showed that the singleplex RT-PCR sensitivity was similar to that of multiplex RT-PCR for all the tested viroids [75]. This type of assay could be used in high throughput screenings of viroids associated with citrus in field surveys, germplasm banks, nurseries, as well as in other viroid disease management programs [74]. Similarly, the multiplex RT-TaqMan PCR assay developed by Papayiannis [76] enables accurate discrimination between CEVd and HSVd with a diagnostic sensitivity and specificity of 100%. It is important to emphasize that in conventional RT-PCR tests, the overall sensitivity and specificity were lower and varied between 97 and 98% for HSVd, and 94 and 95% for CEVd. Therefore, this essay presented 1000-fold more analytical sensitivity [76]. The specificity of the tests described previously was confirmed by including healthy controls and/or plant tissue infected with other citrus graft transmissible virus and bacteria pathogens and non-targeted citrus viroids. Both singleplex and multiplex assays did not cross-react with any non-inoculated negative controls or other citrus pathogens [63, 74, 76]. To date, PCR-based approaches have been proven efficacy on viroid direct detection. However, false positives and negatives due to amplicon contamination and failure to generate cDNA of suitable size during reverse transcription, respectively, are not uncommon and therefore preclude the application of RT-PCR for large scale indexing [70].

Next-generation sequencing (NGS) technologies are currently becoming routinely applied in different fields of virus and viroids studies. These advanced technologies have therefore contributed to a revolution in both the detection and discovery of plant viruses and viroids [78–81]. NGS has also provided an alternative method to identify viroids in the citrus cultivars. In other words, transcriptome sequencing has shown efficacy in citrus viroid diagnostics. Indeed, this method enabled the simultaneous identification of numerous viroids from various citrus samples, including CEVd and HSVd [82]. A deep sequencing approach, combined with bioinformatics analysis, is already being implemented for HSVd detection in *C. lemon* in China. This finding suggests that HSVd could infect this host and potentially be a pathogen that causes disease on *C. lemon* trees [56].

Name of RT-PCR test Primer/Probe Name	Sequence 5′-3′	Tm (°C)	Genomic coordinates	Size of the expected product	References
RT-PCR					
Singleplex					
CEVd-R	GGGGATCCCTGAAGGACTT	60	80-98[a]	371 bp	[72, 85]
CEVd-F	GGAAACCTGGAGGAAGTCG		99-117[a]		
HSVd-F 27-mer VP-20	CGCCCGGGGCAACTCTTCTCAGAATCC	60	78–102	251 bp	[37, 73]
HSVd-R 26-mer VP-19	GCCCCGGGGCTCCTTTCTCAGGTAAG		60–85		
HSVd VP-98	CTCCAGAGCACCGCGGCCCTC	DN	120–140	140 bp	[37]
HSVd VP-99	CTGGGGAATTCTCGAGTTGCCGC		1–23		
Multiplex					
CEVd-F194	TTTCGCTGCTGGCTCCACA	58	194–212	196 bp	[63]
CEVd-R18	ACCTCAAGAAAGATCCCGA		371–18		
HSVd-F1	GGGGCAACTCTTCTCAGAATCC		81–102	302 bp	
HSVd-R1	GGGGCTCCTTTCTCAGGTAAGTC		58–80		
CEV-R	CCGGGGATCCCTGAAGGACTT	58	78-98[a]	371 bp	[75]
CEV-F	GGAAACCTGGAGGAAGTCGAG		99-119[a]		
HSVd-R	CCGGGGCTCCTTTCTCAGGTAAGT		59-82[b]	302 bp	
HSVd-F	GGCAACTCTTCTCAGAATCCAGC		83-105[b]		

Name of RT-PCR test Primer/Probe Name	Sequence 5′-3′	Tm (°C)	Genomic coordinates	Size of the expected product	References
Real time-RT-PCR					
Singleplex					
CEVd −161 F	GTCCAGCGGAGAAACAGGAG	60	181-200[c]	105 bp	[74]*
CEVd −258 R	AGAGAAGCTCCGGGCGA		270-286[c]		
CEVd −187 P FAM	TCCTTCCTTTCGCTGCT		212-228[c]		
HSVd-208 F	GAGACGCGACCGGTGG	60	216-231[d]	88 bp	
HSVd-295 R	GCTCAAGAGAGGATCCGCG		286-304[d]		
HSVd-226 P TET	TCACCTCTCGGTTCGTC		234-250[d]		
Multiplex					
CEVd-RTR_F	GTCGCCGCGGATCACT	60	142–159	64 bp	[63]
CEVd-RTR_R	CCAGCAGCGAAAGGAAGGA		187–205		
HSVd-RTR_F	GGAATTCTCGAGTTGCCGCA		5–24	127 bp	
HSVd-RTR_R	CCGCGGCCCTCTCT		118–131		
CEVd-RTR_P	CCAGCGGAGAAACAG		163–177	—	
HSVd-RTR_P	CAACTCTTCTCAGAATCC		85–102	—	

Name of RT-PCR test Primer/Probe Name	Sequence 5′-3′	Tm (°C)	Genomic coordinates	Size of the expected product	References
CEVdF	GCGTCCAGCGGAGAAACA	60	158–175[e]	68 bp	[76]
CEVdR	CAGCGACGATCGGATGTG		226–208[e]		
CEVdTAQ	{FAM}-TCGTCTCCTTCCTTTCGCTGCTGG-{BHQ1}		181–204[e]		
HSVdF	GCCTTCGAAACACCATCGA		159–177[f]	71 bp	
HSVdR	CACCGGTCGCGTCTCATC		230–213[f]		
HSVdTAQ	{HEX}-CGTCCCTTCTTCTTTACCTTCTCCTGGCTC-{BHQ2}		179–208[f]		

[*] *The same primers have been also tested in multiplex Real time-RT-PCR test.*
[a] *GenBank Accession no. NC-001464.*
[b] *GenBank Accession no. NC-001351.*
[c] *GenBank Accession no. CEVd-HQ284019.*
[d] *GenBank Accession no. HSVd-KJ810553.*
[e] *GenBank Accession no. U21126.*
[f] *GenBank Accession no. GQ249348. R: antisense primer. F: sense primer.*

Table 2.
Primer sequences and their annealing temperature (Tm), primer/probe location, and expected size of PCR products for each primer pair when used to amplify CEVd and HSVd by RT-PCR and related tests (this is not a full or exclusive list).

6. Control strategies

In vitro somatic embryogenesis, from both style and stigma cultures, has been proved to be a highly effective sanitation method leading to the complete elimination of the main virus and virus-like diseases associated with citrus. Furthermore, it has been shown efficacy to eliminate diseases induced by viroids, and the production of healthy citrus plants [83–85]. This method was applied to eliminate CEVd and HSVd from some *Citrus* species [83]. For example, somatic embryogenesis has been tested on 13 genotypes, belonging to the Algerian germplasm collection of two different *Citrus* species, lemon and sweet orange, infected by at least one graft-transmissible agent, including CEVd and HSVd. This method has shown efficacy to eliminate CEVd from 12/13 tested genotypes. However, HSVd was proved to be the most infectious viroid since it has been eradicated only from 5/13 tested genotypes. It is important to emphasize that no somaclonal variability has been highlighted in lemon regenerated plants. However, a genetic instability has been observed in some of the regenerated orange plants Washington navel 251 [83]. Sanitation by *in vitro* shoot-tip grafting has also been proved to be a very effective method for citrus graft-transmissible diseases eradication including citrus viroids (success rate of about 100%) [86–88]. For instance, it has been reported that CEVd and HSVd can be routinely eliminated from citrus by shoot-tip grafting. Since citrus viroids are extremely tolerant of heat, the use of thermotherapy as a sanitary method is not effective in eliminating viroids from citrus budwoods [9].

No naturally occurring durable resistance has been observed in most species, despite non-hosts for viroids exist. Therefore, the effective control methods for viroid diseases consist mainly of detection and eradication, and cultural controls [50].

7. Viroids situation in the mediterranean region: focus on Morocco

Exocortis and cachexia are widespread diseases in the Mediterranean region. CEVd and HSVd have been reported in most Mediterranean countries and are among the most prevalent citrus viroids in the region [9]. The development of reliable diagnostic methods facilitated extensive surveys for CEVd and HSVd in different parts of the region. Both viroids were successively identified in many countries, including Morocco [89–92], Cyprus [33], Spain [26], Egypt [43, 93], Italy [61, 94], Tunisia [46, 95], France [21], Syria [96], and Turkey [42].

In Morocco, exocortis and cachexia are among the major citrus viroid diseases [90, 91]. These diseases are prevalent in citrus orchards and can be found in all *Citrus* species and varieties [89–92, 97]. Mechanical transmission of citrus viroids, including CEVd and HSVd, via working tools seems to be behind the widespread of these phytopathogens and their detection in both old and young plantings in all surveyed citrus orchards [92]. Research, recently completed from 2008 to 2018, to monitor CEVd and HSVd prevalence, in the main citrus-growing areas of Morocco (Gharb, Haouz, Loukkos, Moulouya, Souss, and Tadla), showed that CEVd and HSVd are omnipresent in almost all citrus-growing areas of the country with relatively high prevalence. That is the case for example for the Gharb area where CEVd and HSVd were detected at a prevalence of 85% [89] and 21% [92], respectively. Concerning genetic analysis, a first sequence comparison among six Moroccan HSVd isolates collected in the six main citrus-growing areas of Morocco has been recently reported by Afechtal et al. [92]. Phylogenetic analysis showed that the six HSVd isolates are clustered into one group within the "citrus-type". Furthermore, it seems that sequence variability is neither a function of host plant nor a function of the symptoms [92].

8. Conclusions

Citrus viroids, including CEVd and HSVd, are distributed mainly by the introduction and propagation of infected budwoods, by top working, and by mechanical transmission [9, 11]. Both viroids are known for their ability to infect a large number of host plants [36]. CEVd and HSVd are destructive to certain citrus varieties and, can cause yield losses that may be as high as 34 to 76 percent depending on the combination viroid-rootstock-scion [21, 46]. The mechanisms through which CEVd and HSVd interact with their hosts and induce pathogenesis are beginning to be deciphered. In other words, the involvement of RNA-silencing and basic defense mechanisms following CEVd and HSVd infection has been highlighted [54, 56].

Once introduced and established in a country, both viroids can spread relatively rapidly because of their ability to be transmitted via mechanical means [9, 11]. Since CEVd and HSVd have a high resistance to heat, the chemical treatment appears to be the best method to disinfect CEVd- and HSVd-contaminated tools. For instance, a 0,25 to 0,5 and a 1 percent solution of sodium hypochlorite appears to be the best option to eliminate CEVd and HSVd, respectively, from contaminated hedging and budwood cutting tools [11, 98]. Like all citrus viroids, CEVd and HSVd seem to be successively eliminated from propagative material by shoot-tip grafting or by the deployment of nucellar budlines. Being extremely tolerant of heat, CEVd and HSVd have not been successfully eliminated from budwood by applying thermotherapy [9]. Certification programs must include measures to control viroid spread in nurseries [32]. The majority of rootstocks that are tolerant to the citrus tristeza virus [*Closteroviridae*; *Closterovirus*; CTV] are susceptible to citrus viroids. Therefore, in the absence of a certification program, exocortis disease usually follows upon the replantation of these rootstocks [9]. Since no useful sources of natural resistance to viroid disease are known, diagnostic tests continue to play a key role in efforts to control viroid diseases [67]. Nowadays, several nucleic acid-based methods for detecting CEVd and HSVd exist, including PAGE, hybridization, amplification, and sequencing [60]. Although biological assay has several disadvantages, it will always play a pivotal role in viroid research. Indeed, Cuban Shaddock has been proved to be the best rootstock for symptom expression on Arizona 861 S-1 citron and Clemeline 11–20 indicator plants for indexing exocortis and cachexia, respectively [64]. Besides, gynura seems to be an excellent indicator for CEVd [9]. A combination of both molecular and biological assays should lead to the most effective means for viroid identification and characterization [60].

Complicated interactions, including antagonism and synergy, occur between viroids coinfecting the same citrus host. These interactions may lead to different symptoms, canopy volumes, fruit yields, and commercial performance. Although no obvious physiological changes in citrus hosts have been described in mixed infections of CEVd and HSVd and both viroids do not induce severe symptoms in citrus [24, 99], their interaction was intriguing because they are commonly found simultaneously infecting different citrus cultivars and they have identical biological properties within the same host. The relationship between the two viroids has been investigated over 3 years (from 2011 to 2013). Results showed a positive correlation between CEVd and HSVd in specific tissues of two citrus cultivars (blood orange and Murcott mandarin). This result has been supported by three findings: titer enhancement, localization similarity, and lack of symptom aggravation under mixed-infection conditions. Compared to their concentrations under single-infection conditions, a significant increase in the CEVd and HSVd population has been observed under mixed-infection during 6 and only 1 season of the 12 monitored seasons, respectively. This result is somewhat surprising because no competition phenomenon for host resources occurs between the two viroids although they have

the same biological functions and share identical cellular and subcellular spaces [27]. This issue merits consideration in future research.

Regarding the current situation of CEVd and HSVd in Morocco, this chapter provides a general overview of their spread in the Moroccan citrus-growing areas. Preventing the introduction and the establishment of exocortis and cachexia diseases in the Moroccan citrus orchards can be set up through the use of viroid-free (certified) planting material, disinfection of pruning tools, regular monitoring of citrus orchards to ensure early detection of both diseases, and by avoiding top working practice. This review pointers to new research avenues in exocortis and cachexia diseases in Morocco or elsewhere. These research fields could include for instance the characterization of CEVd and HSVd isolates, searching for secondary hosts, and developing sustainable control strategies. Investigating the prevalence of CEVd and HSVd infection in numerous natural host plants, and the characterization of the viroid sequence variants is valuable especially that a cross-transmission phenomenon between different hosts seems to be possible for HSVd [100].

Studying functional genomics through transcriptomic analysis and/or proteomic approaches in citrus-CEVd/HSVd interaction would be an interesting approach to shed more light on the full mechanisms underlying the complex and varied events associated with such interactome, and thus contribute to the development of novel diagnostic methods and plant protection strategies. This further advanced research will expand our understanding of CEVd and HSVd epidemiology and the mechanisms behind their spread across the world in general and Morocco in particular, and could potentially help in devising innovative management strategies of both viroids.

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Conflict of interest

The authors declare no conflict of interest.

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