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ORIGINAL ARTICLE

Risk potential of international fruit trade for viroid spreading - case study on hop viroids in Europe

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Abstract

Most hops are produced in Europe; therefore, it is alarming that the citrus bark cracking viroid (CBCVd), the causal agent of the severe hop stunt disease, was detected in different nonadjacent hop growing countries. It is still unclear how the initial infection occurred since CBCVd is typically found in citrus and not in hops. To extent data for a viroid risk assessment, potential hosts were tested for the presence of viroids in grocery stores in the hop producing areas of Slovenia and Germany. Samples positive for hop-pathogenic viroids were further used for infection studies. The surveys covered CBCVd, hop stunt viroid (HSVd), citrus exocortis viroid (CEVd), citrus dwarfing viroid (CDVd), citrus viroid V (CVdV), and citrus bent leaf viroid (CBLVd). The results show that all tested viroids can be found in fruits sold in grocery stores, thus there is a risk of introducing CBCVd, HSVd, and other viroids into the hop growing regions via imported fruits and their remains. Furthermore, the transmission study reveals that CBCVd and HSVd infected citrus fruits can lead to infected plants, irrespective of the type of inoculum whether in the form of RNA extract, injected sap, or fruit peel in the soil. Finally, the phylogenetic analysis showed that the sequence diversity within viroid samples is high and that CBCVd and HSVd sequence variants can be found, which are almost identical to variants confirmed in hop. We assumed that fruit imports contribute to international viroid spreading and inappropriate handling like fruit waste deposition to agricultural lands is a serious risk factor.

Keywords Survey · Risk assessment · Reverse transcription · Real-time quantitative PCR · Phylogenetic analysis

Introduction

Viroids are the smallest known pathogens and can cause severe symptoms in plants. Hop (*Humulus lupulus* L.) is known to be a host plant for four viroids, of which the apple fruit crinkle viroid (AFCVd) has not been found in Europe up to date. The hop latent viroid (HLVd) is mostly symptomless for hops thus it is widely spread in commercial hop production (Patzak et al. 2021). The hop pathogenic hop stunt viroid (HSVd) is latent in European grapevines but surprisingly no major outbreak has occurred in the European hop production until now (Matousek et al. 2003; Przybyś 2020). The most severe hop pathogenic viroid, the citrus bark cracking viroid (CBCVd), was detected in heavily stunted hop plants first in Slovenia in 2007 (Jakse et al. 2015). In 2019 it was also detected in the Hallertau, Germany's biggest and globally the largest contiguous hop growing region (Julius Kühn-Institut 2019). In Slovenia, since the first finding of the stunted plants in 2007, almost 500 ha

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of hop gardens have been affected of which approximately 300 ha were removed and destroyed in order to eradicate the viroid (EPPO Global Database 2021). While CBCVd is a serious threat to hop cultivation, it is tolerated or sometimes actively utilized as dwarfing agent in citrus production (Bar-Joseph 1993). Generally, members of the genus citrus host seven viroids, of which only the citrus exocortis viroid (CEVd) and certain variants of the hop stunt viroid (HSVd) have been associated with citrus diseases seriously affecting only some species or cutivars (Bagherian and Izadpanah 2010; Loconsole et al. 2013; Murcia et al. 2015; Vernière et al. 2004; Zhou et al. 2020). Other citrus viroids are the citrus dwarfing viroid (CDVd) and citrus bent leaf viroid (CBLVd), which are sometimes used to improve management of high-density citrus plantations through achieving smaller and more compact trees without negatively affecting the fruit quality (Lavagi-craddock et al. 2022; Vidalakis et al. 2010, 2011). The citrus viroid V (CVdV) is globally distributed and found mostly symptomless in different citrus species (Serra et al. 2008; Zhou et al. 2020). The appearance of CBCVd on hop plants in Slovenia present first report of this viroid outside citrus growing regions on agricultural land (Jakse et al. 2015). The analysis of primary outbreak location showed close proximity of illegal refuse dump of waste from nearby fruit distribution center what has led to the hypothesis that CBCVd transmission to hops in Slovenia resulted from citrus fruit residues (Radišek and Benko-Beloglavec 2016). Since most citrus fruits are not produced in central Europe, especially not in the temperate hop growing regions, the most obvious import route of citrus is international trade delivering infected fruits to grocery stores. There are known examples for viroid imports, such as the case of pepper chat fruit viroid infected seeds (Chambers et al. 2013), or the import of the avocado sunblotch viroid to Australia (Geering 2018), or the import of tomato chlorotic dwarf viroid to Japan (Tsuda and Sano 2014). Without screenings, viroids could be imported unknowingly and cause symptoms only years after the infection has occurred, which makes it difficult to trace them back to their origin. The challenge is that the viroid pathogenicity is typically slow and symptoms become visible only after several months or years, thus infections remain undetected for long (Geering 2018; Matsushita and Tsuda 2016; Tsuda and Sano 2014). Imported infected fruits could find their way through the usage of (household) compost or spoiled goods from retail as organic fertilizer. Thus, there is a potential risk of plant residues containing viroids for infections, but it is not known if this is possible specifically for the CBCVd-hop pathosystem.

The aim of this study is to quantify the potential of imported fruits as source of viroids generally and specifically for hop-pathogenic viroids. Further, we wanted to test if an infection of hop plants could derive from hop pathogenic viroid-infected fruit residues. Therefore, first a broad study covering six common citrus viroids was conducted in Slovenian grocery stores, then the hop pathogenic viroids have been studied in infectivity tests. A more detailed study has been conducted on three viroids in Germany in the course of a year to find possible patterns of higher risk of certain fruits, related to the country of origin or time of the year.

Materials and methods

Sample categories

The samples were categorized into either of the following groups (1) oranges (*Citrus sinensis*), (2) grapefruit (*C. paradisi*, *C. maxima*), (3) lemon (*C. limon*), (4) easy peeler (*C. reticulata*, *C. clementina*, *C. x unshiu*), (5) lime (*C. aurantifolia*, *C. latifolia*, or *C. warburgiana*, formerly *Microcitrus warburgiana*), (6) *Citrus* spp. (*Fortunella margarita*, *C. x limon*, *C. medica*, *Citrofortunella microcarpa*), (7) grape (*Vitis vinifera*), and (8) melon (*Cucumis melo*).

Slovenian survey and inoculation

Samples for the Slovenian survey of imported citrus fruits were collected from seven grocery stores in the hop growing area Savinja Valley in 2013 (n = 53) and for the inoculation tests in Celje region in 2016 (n = 39). Fruits were stored at -70 °C until processing. Per fruit 100 mg of the albedo were extracted as previously described (Kump and Javornik 1996) and stored at -70 °C. For the preparation of viroid inoculum, the total RNA was extracted from 100 mg of ground frozen fruit peel using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, USA), followed by an on-column DNase digestion step. RNAs were quantified by NanoVue spectrophotometer (GE Healthcare, Chicago, USA), the integrity and quality were analyzed by formaldehyde gel electrophoresis and by the 2100 Bioanalyzer (Agilent, Santa Clara, USA). RNAs were stored at -20 °C and 10x-diluted prior to analysis.

PCR analysis and sequencing

For RT-PCR testing, RNA samples were tested for viroids by one-step reverse transcriptase PCR reaction (OneStep RT-PCR Kit, Qiagen, Hilden, Germany) using specific primers for viroids CBCVd, HSVd, CEVd, CDVd, CVdV and CBLVd (Online Resource 1). The resulting amplicons were determined with agarose gel electrophoresis based on their migration size and exemplaric amplicons were validated

by Sanger sequencing. Additionally, two sequencing verified CBCVd- and two HSVd-infected fruits were selected for inoculation. Post-inoculation PCR analysis of hop plants was also conducted to verify the infection success. A post-inoculation CBCVd full-length sequence was obtained by sequencing overlapping amplicons using the primers CVIV-1P and CVIV-1 M as well as CV4-AM3_R and CV4-AP4_F (Online Resource 1).

Viroid inoculation

Infected fruit samples with CBCVd, HSVd, or both viroids were used for three treatments to infect five plants per treatments. Rooted plantlets of the following hosts: HLVd-free hop (cv. Celeia), cucumber (*Cucumis sativus* L. cv. Cornison Paris), and tomato (*Solanum lycopersicum* cv. Heinz 1370). For hop we used one-year old rooted plants which were in phenological BBCH stage 14, and for cucumber and tomato we used seedlings in stage BBCH 12. Two inoculation treatments were done by stem injection of the inoculum (1) extracted RNA with a concentration of 200 ng/µl and an inoculation volume of 45 µL per plant, and (2) a crude fruit peel sap consisting of 22.5 µl or fruit peel sap and 22.5 µl deionized water per inoculation per plant. In the third inoculation treatment plants were removed from the pots and inoculated by using 20 g of fresh orange peel pieces (1–3 cm^2) which were directly applied to the roots up to the base of the stem. No phytotoxic effects were observed for fruit peel inoculated plants. Degradation of fruit peels was evident at the termination of the experiment. However, considering earlier research, the viroid titer should still have been high enough for infections at the first days after inoculation (Hagemann et al. 2021; Kerins et al. 2018). Plants of all treatments were replanted in 1 L pots using commercial substrate (Gramoflor S04-2004 Topf/Pikier + TonXL + Fe, Germany). Plants were maintained in growing chamber (Kambič RK-13,300, Slovenia) under the following conditions: 16 h day at 25 °C and 60% relative humidity and 8 h night at 20 °C and 60%, respectively. All plants developed normally without expression of symptoms. After four months newly formed leaves were collected from each plant and stored at -80 °C for further analysis.

German survey

Sample material

Samples for the German survey of imported fruits or leaves were collected from local grocery stores and garden centers in the hop growing area Hallertau between August 2021 and July 2022 during seven collection events (n = 368). Pretesting showed that generally the fruit exocarp, for citrus the flavedo, and for grapes the panicle, lead to high yields of high-quality RNA thus those tissues were sampled accordingly and then stored at -30 °C until extraction.

RNA extraction

For the RNA extractions the Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, USA) has been used. Per sample 100 mg was homogenized by grinding using liquid nitrogen. Thereafter, RNA purity was determined with a spectrophotometer (Nanodrop 1000, ThermoFisher, Waltham, USA). The samples were stored at -80 °C until further use.

PCR-Analysis

Samples were analyzed by two reverse transcription – duplex – real-time quantitative PCRs (RTqPCR) performed with the Bioline SensiFAST™ Probe No-ROX One-Step Kit (Bioline, London, UK) on a qPCR cycler (Rotor Gene 6000, Qiagen, Hilden, Germany) as described (Seigner et al. 2020). For every RNA sample two volumes (0.2 and 1 µl) were analyzed. The duplex-RTqPCRs included the combination of FAM- and HEX-probes for CBCVd and the internal control nad5, respectively (Seigner et al. 2020) as well as with FAM- and HEX-probes for CEVd and HSVd, respectively, in the second reaction. The CEVd detection was based on the CBCVd-probe, which specifically binds to both viroids, combined with the newly designed CEVd specific primer *CEVd_MH_F2* and the previously published CBCVd primer *CBCV_F1* (Online Resource 1). HSVd was detected with the HSVd-Probe (Luigi and Faggioli 2013) and the newly designed HSVd-consensus primers *HSVd-JK_F2* and *HSVd-JK_R2*. For the validation of the RTqPCR for all three viroids, the resulting amplicons were sent for sequencing each with the corresponding forward and reverse primer by using the Sanger SupremeTube service (Eurofins Genomics, Ebersberg, Germany).

For studying the variability of imported viroid variations, all 18 CBCVd-positive and randomly selected 18 positive samples for HSVd and CEVd were reverse transcribed and amplified in a one-step reaction with QIAGEN OneStep RT-PCR Kit (Hilden, Germany). In order to receive two complementary amplicons, the primers CVd-IV-F1 and CVd-IV-R1 as well as CBCV_1 and CBCV_1B were used for CBCVd (Online Resource 1). Different primer combinations were used for the full-length sequences (Online Resource 1). All RT-PCRs amplicons with clearly visible single band on a 1.5% agarose gel were Sanger sequenced.

Sequencing

Amplicons of all 18 samples per viroid resulting from successful RT-PCRs as previously described, were purified by Exo-CIP Rapid PCR Cleanup Kit (New England Biolabs, Ipswich, USA) before Sanger sequencing. Four or more trimmed sequences resulting from sequencing of two overlapping amplicons were mapped to the corresponding viroid NCBI nucleotide reference sequences: CBCVd to NC_003539, HSVd to NC_001351, and CEVd to NC_001464 with the Geneious mapper (Geneious Prime® 2022.1.1, Biomatters Ltd, New Zealand). In some samples ambiguous bases were verified by multiple sequences and then called following the IUPAC code of nucleotides (nt).

Phylogenetical analysis

The obtained sequences from the survey (Online Resource 2) were aligned to the curated viroid sequences published in the NCBI Genbank, whereas only full-length sequences were considered and aligned using MAFFT (v7.490) at the following settings: determination of algorithm set to auto, score set to 200PAM, gap opening penalty set to 3, offset value set to 1, and automatic determine direction (Katoh et al. 2002). Typically, the RNA polymerase II starting point in the left terminal loop is set as the start of the linear sequence in viroid databases (Kolonko et al. 2006). Since several sequences had a different start, they were rotated or edited accordingly, and all sequences were realigned with MAFFT and used as input for the *randomized axelerated maximum likelihood* (RAxML) phylogenetic analysis with the nucleotide model GTR Gamma and the rapid hill-climbing algorithm at a bootstrap value of 100 (Stamatakis 2006).

Results

Slovenian case study

Slovenian survey

The results show that all six tested viroids could be found (Fig. 1). In particular, CBCVd was detected in 10% of sampled fruits, mostly in grapefruit (Fig. 1). HSVd was found in two thirds of all samples and almost all were oranges. CEVd was found in a quarter of all samples, mostly in lemons and oranges. The CDVd was the overall most dominant viroid with 43 findings out of 53 samples, while CBLVd and CVdV were detected only in a few samples. The number of viroid-free fruits was only six, while 13 were single infected. Most samples were either double, triple, quadruple infected, and one grapefruit contained five of the six viroids tested. The double infection, especially of the combination of HSVd and CEVd, was most frequent in oranges, while the triple infection of HSVd, CEVd and CDVd was dominant in lemons.

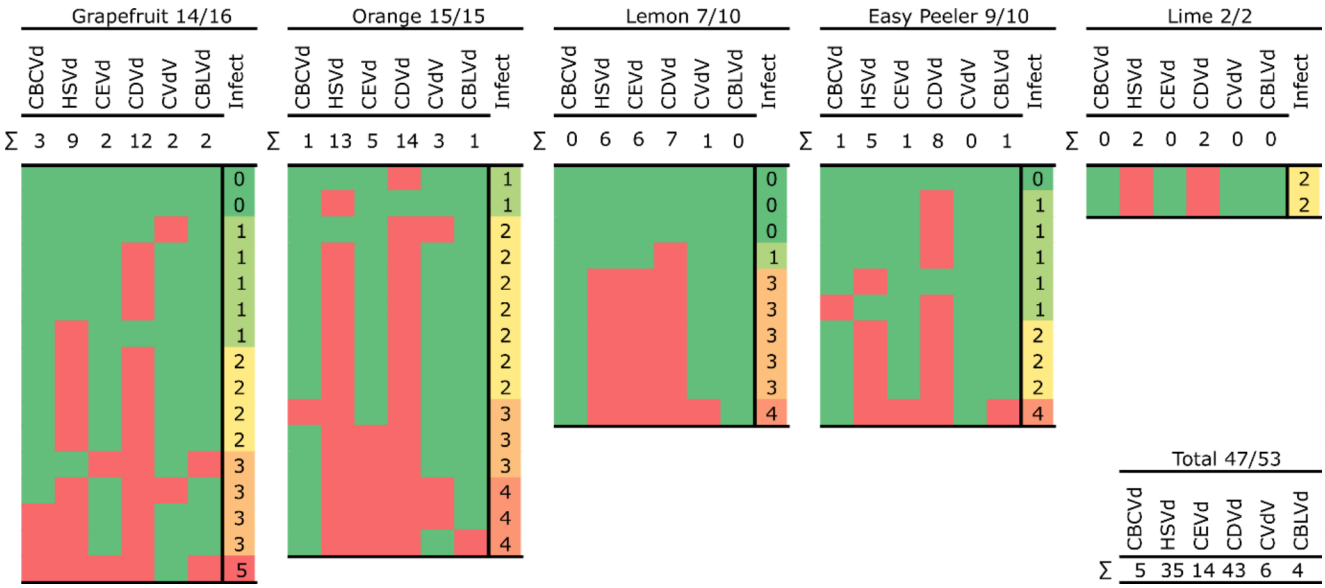

Fig. 1 Slovenian survey on viroid infected citrus fruit in grocery stores around the hop production area Savinja Valley. The sum of viroid-infected compared to the total number of sampled fruits is indicated in the headline of each block. Each block represents one fruit category tested for six viroids, whereas each row within one block represents a single sample with green indicating the absence and red the presents of a viroid. The number of infections per sample is summed in the column "infect"

Table 1 Infection of hop, tomato, or cucumber with single HSVd or CBCVd single, or HSVd CBCVd double infected orange sample RNA extract, sap, or fruit peel mixed in the soil of treated plants. Treatment effect was assessed by RT-PCR four months after inoculation

Infected isolate	Inoculum type	Target	Number of infected plants		
			Hop	Tomato	Cucumber
HSVd from orange *Red Taracco*	RNA	HSVd	0 / 5	0 / 5	0 / 5
	Plant sap	HSVd	0 / 5	2 / 5	0 / 5
	Peels	HSVd	0 / 5	0 / 5	0 / 5
CBCVd from *Navel* orange	RNA	CBCVd	1 / 5	2 / 5	2 / 5
	Plant sap	CBCVd	2 / 5	1 / 5	2 / 5
	Peels	CBCVd	1 / 5	1 / 5	2 / 5
HSVd + CBCVd from *Navel* orange	RNA	HSVd	0 / 5	0 / 5	0 / 5
		CBCVd	1 / 5 *	2 / 5	1 / 5
	Plant sap	HSVd	1 / 5	1 / 5	1 / 5
		CBCVd	0 / 5	0 / 5	0 / 5
	Peels	HSVd	1 / 5	0 / 5	1 / 5
		CBCVd	0 / 5	2 / 5	0 / 5

* sample selected for full-length sequencing resulting in the CBCVd hop variant KM211546 (99.6% pairwise identity)

Viroid inoculation

CBCVd or HSVd single infected as well as CBCVd and HSVd double infected orange peel samples were selected for infection experiments and all treatments; extracted RNA, sap, or fruit peel in the soil lead to infections (Table 1). Neither of the infections led to symptoms within the 4-month period until the analysis. The single infected HSVd sample led only to two positively infected tomato plants from the sap treatment. The CBCVd single inoculation showed to be the most infective viroid, which lead to CBCVd infections in one third of all cases with no clear tendency regarding the treatment or host plant (Table 1). The result shows that CBCVd-infected citrus material can lead to infections in hops and in the two experimental hosts tomato and cucumber (Table 1). Three-quarters of all inoculated plants did not show to contain either of the inoculated viroids and there was no case where the double infected sample also led to a double infected plant. Partial sequencing of CBCVd from the pre-inoculation Navel orange sample infected with HSVd and CBCVd resulted in a CBCVd sequence with a 93% pairwise identity to AB054634 from citron, whereas sequencing of leaves of inoculated hop plant resulted in a CBCVd sequence with a 99.6% pairwise identity to KM211546, which is one of the two CBCVd variants originally found in Slovenia (Jakse et al. 2015). Comparing the partial sequence of CBCVd from orange prior to inoculation with the sequences received from a hop plant 4-month after the inoculation shows 10 nucleotide differences (Fig. 2). The five regions typic for Pospiviroids were annotated for the hop-derived CBCVd sequence based on the work of Xu et al. (2012). Nucleotide differences occur in all regions except in the variable region, which however could not be fully sequenced in this study.

German case study

German survey

Less viroids but more samples were analyzed in the German compared to the Slovenian survey, in order to cover different citrus supplying regions throughout the year. CBCVd was found in 5% of the 368 samples, or in 6% of the 304 samples excluding the non-CBCVd hosts melon and grape (Fig. 3). In detail, CBCVd was found in nine out of 74 lemons, as well as in samples of grapefruits, easy peelers and in one orange fruit. More CBCVd infected fruits were found during the European winter from November to April (Fig. 4, a). Further, most CBCVd infected fruits came from the Mediterranean countries Turkey and Israel (Fig. 4, b). HSVd was the dominant viroid from the German survey with a total of 110 findings from 368 samples (Fig. 3). It was found mostly in lemon, grapefruit, and oranges. Two thirds of all grapes panicles contained HSVd, while all melon samples were free from viroids, even though is showed to be a reliable experimental HSVd host (Yaguchi and Takahashi 1984). Most HSVd positive samples came either from Italy, Spain, or Turkey. However, since overall, most citrus fruit came from Spain, the relative incidence of HSVd-infections is lower for Spain than for Italy and Turkey. CEVd was found in 49 out of 368 samples, mostly in lemon and lime throughout the year (Figs. 3 and 4 and b). The incidence for double infections of CEVd and CBCVd was low, while the HSVd and CEVd double infection was frequent especially

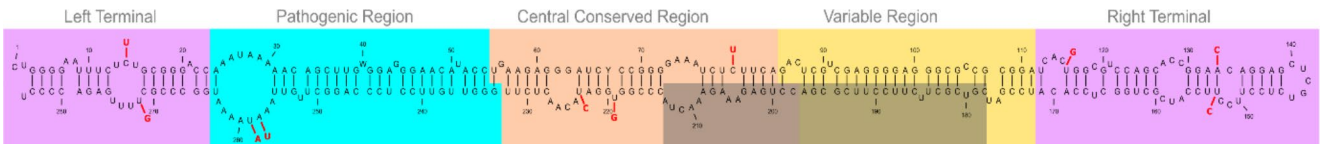

Fig. 2 Predicted secondary structure of CBCVd received from a hop plant 4-month after the inoculation with RNA from a HSVd and CBCVd-infected orange fruit. The five regions typic for Pospiviroids have been annotated (Xu et al. 2012) as well as sequence differences (red) from the partially sequenced orange CBCVd sequence as determined prior to inoculation. Missing part of the partial sequence is indicated as grey area spanning from the lower central conserved to the variable region

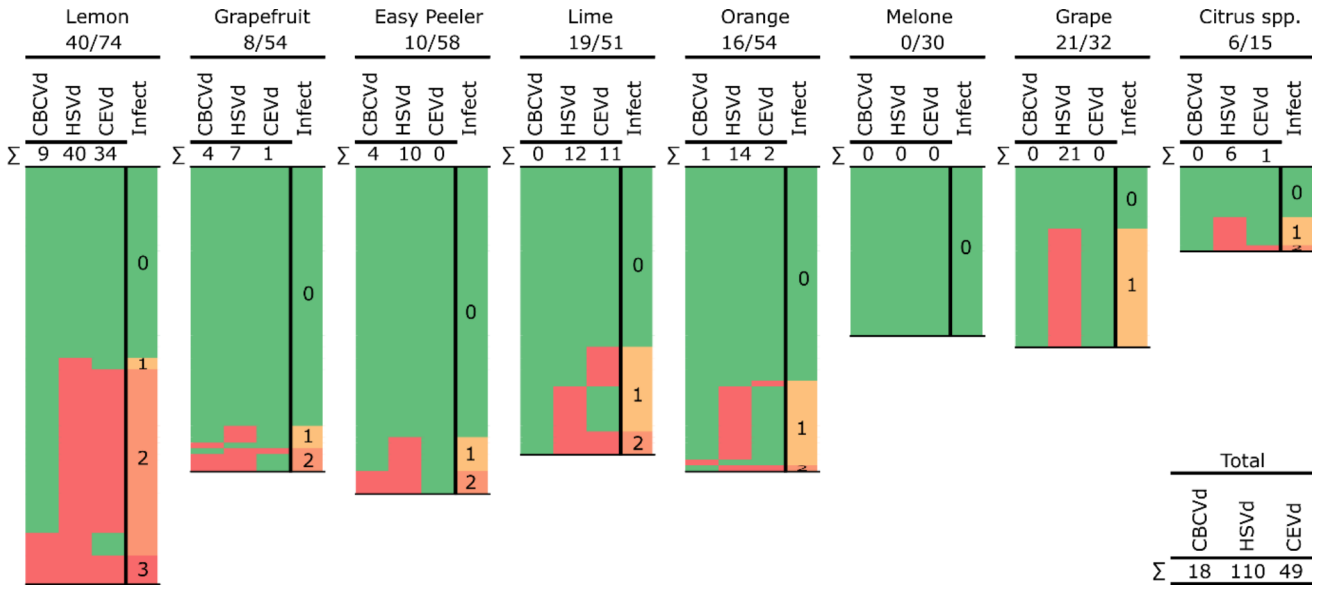

Fig. 3 German survey on viroid in grocery stores around the hop production area Hallertau. The sum of viroid-infected compared to the total number of sampled fruits is indicated in the headline of each block. Each block represents one fruit category tested for three viroids, whereas each row within one block represents a single sample with green indicating the absence and red the presents of a viroid. The number of infections per sample is indicated by the column "infect"

in lemon. The CBCVd, HSVd, and CEVd triple infection also occurred in the German survey, however only in a few lemon samples.

Phylogenetic analysis

The phylogenetic analysis was conducted on all 18 CBCVd infected samples as well as on 18 randomly selected samples infected with either HSVd or CEVd to equally estimate the sequence variability of all three viroids. We analyzed the sequences from our survey with the curated full-length sequences obtained from the NCBI database including metadata for characterizing the phylogenetically similar associations. The CBCVd-pistachio accession was used as an outgroup, since it is on average only 87% identical to the other CBCVd variants (Rwahnih et al. 2018) and 65 nt different from the CBCVd accessions identified in this study (Fig. 5). The alignments of CBCVd variants from this study did not show to be identical to either CBCVd-hop variants found in Slovenia (Jakse et al. 2015) or in Germany (data unpublished). One CBCVd-citrus accession had only 3 nt differences from the Slovenian accession, while two quite different CBCVd-citrus variants showed to be similar to a CBCVd-hop variant found in Brazil (Eiras et al. 2023). The phylogenetic analysis shows that there is a small group of highly similar CBCVd accessions from a study from Pakistan, however, no further biological, host- or origin related pattern could be identified from the clustering branches (Online Resource 3). However, the whole diversity of CBCVd sequences could be captured in our survey (Fig. 5, a), which, excluding the CBCVd-pistachio variant, ranges between 83 and 100% pairwise difference. The analysis of HSVd sequences also show that samples from German grocery stores have a high diversity and can be found in very distant branches of the HSVd phylogeny. HSVd shows to have a branch of mostly pome fruits accessions, which however were not considered in this study. Citrus and grapes samples were distributed in all branches of the HSVd phylogeny including branches typical for the genera *Vitis* or *Citrus* (Fig. 5, b). Also, the data show that there is a branch consisting of grape and hop accessions. Specifically, the lemon accession RNA1138 from a German grocery store is only 5 or 6 nt different from two accessions isolated from hops in Slovenia (Radisek et al. 2012). Within the citrus accessions of HSVd we annotated the cachexia expression motif, which is an indicator for the cachexia disease affecting sensitive citrus hosts such as mandarin (Bar-Joseph 2015; Loconsole et al. 2013). This led to the identification of cachexia clusters; however, the cachexia motif was rarely found in citrus accessions but in more than 20 accessions isolated from the genera *Prunus*, *Morbus*, *Ficus*, *Pistacia*, or *Vitis* (Online Resource 4). The phylogenetic analysis of CEVd shows several phylogenetic branches of accessions isolated from citrus or grapes, but also a branch characterized by hosts belonging to the genus *Solanum* and one belonging to the genera *Vicia* and *Impatiens* (Fig. 5, c and Online Resource 5). The samples from this study are similar or identical to samples from all branches of the CEVd tree except from the solanaceaen branch, since we did not sample plants from that genus.

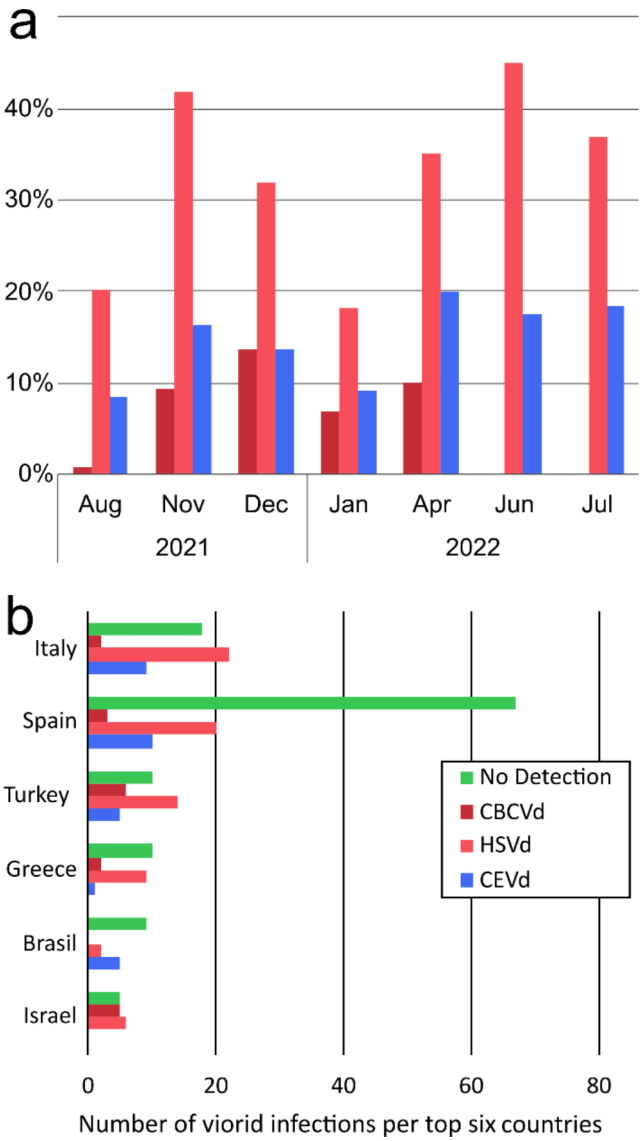

Fig. 4 Results of the German viroid survey, with (**a**) showing the seasonal variability of viroid incidence from August 2021 until July 2022. Percentage was calculated to account for sample number imbalance per sample date, which were n = 119 in August and n = 41 ± 3 from November to July, depending on the availability of fruits from different origins. (**b**) is showing the top six countries of origin of viroid infected samples compared to samples of which no viroid was detected (green)

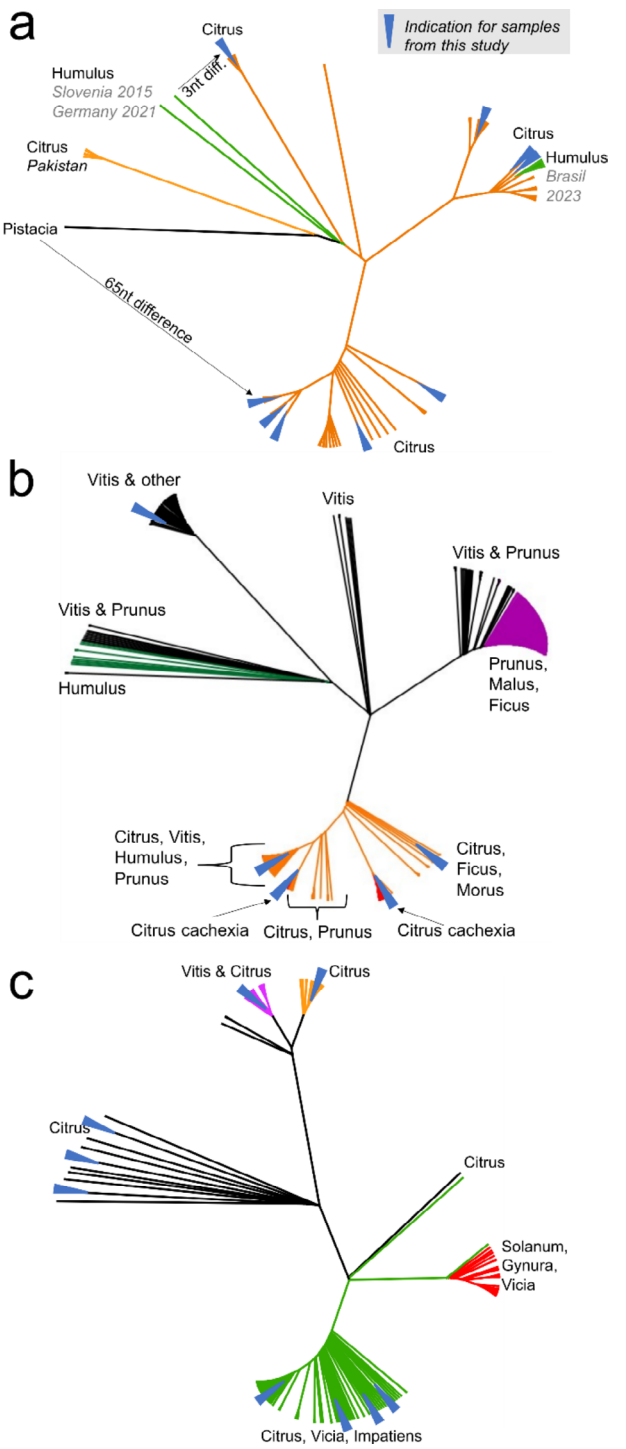

Fig. 5 Phylogenetic analysis of 18 full-length viroid variants (**a**) from CBCVd positive samples analyzed together with 73 curated CBCVd accessions, (**b**) from HSVd positive samples analyzed together with 953 curated HSVd accessions with indication (red) of the cachexia expression motif (Loconsole et al. 2013), and (**c**) from CEVd positive samples analyzed together with 291 curated CEVd accessions (all accessions have been downloaded in September 2022 from NCBI). Groups of accessions were labeled with the genus of the main host plants and colored accordingly. RAxML trees with 100x bootstraps are transformed for visualization, but the fully expanded untransformed tree is provided online (Online Resource 3, 4, and 5)

Discussion

Survey

The survey on viroids in 2013 in Slovenia was a reaction to the finding that CBCVd is likely the causal agent of the stunting disease of hops in the Savinja valley, which was proven shortly after (Jakse et al. 2015). The survey in Germany was a reaction to the finding of several hectares of CBCVd-infected hops in the Hallertau in 2019 (Julius Kühn-Institut 2019). Besides CBCVd also HSVd and the

CEVd were included in both studies, while the Slovenian study was further extended to the citrus viroids CDVd, CBLVd, and CVdV (Vernière et al. 2007). The citrus viroid VI, which has been found only in Japan, China, and Australia was not included since there was only one sample from those countries (Cao et al. 2017; Chambers et al. 2020; Ito et al. 2001). The viroid-like RNA referred to as citrus viroid VII was not included, because it is rare and not verified as viroid (Chambers et al. 2018).

Both surveys showed similar results; all viroids tested, including the hop pathogenic CBCVd and HSVd could be found in at least 5% of the samples (Figs. 1 and 3). In detail, CBCVd was found mainly in grapefruit and to a lesser extent in oranges and easy peelers in both studies. CBCVd was found in some lemons in Germany, while no CBCVd infected lemon was found in Slovenian (Figs. 1 and 3). This may be due to different origins of fruits or cultivar specific viroid tolerance; it was shown that CBCVd-infected trees of Persian lime *Citrus latifolia* develop bark cracking, while Australian lime *Citrus warburgiana* stay symptomless after CBCVd-inoculation (Barbosa et al. 2002). The German survey shows that the risk for CBCVd-infected fruits is highest during the European winter (Fig. 4, a), which corresponds with the citrus harvest season in the Mediterranean. This coincides with the finding that most CBCVd-infected fruits came from Mediterranean countries (Fig. 4, b), of which at least the citrus industry in Israel was promoting CBCVd-based citrus dwarfing for in the past (Bar-Joseph 1993).

HSVd was found in one third of the German and in two thirds of the Slovenian samples, mostly in lemon, grapefruit, and oranges (Figs. 1 and 3). In addition, two thirds of all grapes tested in Germany contained HSVd, which underpins the result of a Czech HSVd survey on grapevines (Matousek et al. 2003), where the authors state that HSVd is latent in grapes but possesses a high risk for neighboring hop gardens since the transmission is possible (Matousek et al. 2003; Sano et al. 2001). Thus, we recommend to keep grape residues, tools, and machinery out of hop gardens. Most HSVd-positive samples came either from Italy, Spain, or Turkey, whereas Spain had relatively the lowest viroid-incidence (Fig. 4, b). This might result from the Spanish citrus tree nursery program, which started in 1979 and since then provided more than 142 million certified viroid-free plants to citrus growers (Pina et al. 2015). HSVd can induce bark cracking, also called cachexia, which is a concern especially for sensitive citrus species such as mandarins (Bar-Joseph 2015; Loconsole et al. 2013). The symptom was associated with a specific cachexia sequence motive, which we annotated at HSVd accessions (Fig. 5, b). Thereby we could reconstruct specific cachexia clusters as identified before (Loconsole et al. 2013). Further, we could show that cachexia expression motif containing HSVd-variants were present in grocery stores in Germany, which could point to further cachexia distribution pathways. As the name suggests HSVd is also a concern for hop production even though to a lesser extent than CBCVd; US studies on HSVd-infected hops reported yellowing, reduced vigor (Eastwell and Nelson 2007) as well as yield losses between 14 and 62% for the cultivar 'Cascade' and 'Glacier', respectively (Kappagantu et al. 2017). The authors also analyzed the disease progression over time, showing a lack of obvious symptoms for the cultivar 'Nugget' but a slow upbuilding yield depressions for 'Willamette' leading to a 50% reduction after 5-years (Kappagantu et al. 2017). This slow disease progression and latency in some cultivars and other crops make the globally distributed HSVd to a maybe even more economically concerning viroid for hop production compared to CBCVd since it causes "hidden losses".

CEVd is not known to infect hops, however, it is a globally widely distributed viroid in citrus plants. It was present in a quarter of all Slovenian and in 13% of all German samples in all fruit types except melons (Figs. 1 and 3). This again could be a concern for citrus producers, since also the exocortis symptoms caused by some CEVd-citrus host combinations can negatively affect citrus production (Zhou et al. 2020). However, CEVd and the other non-hop pathogenic viroids can be a threat in a different way – there is evidence that CBCVd is a chimeric viroid consisting partly of CEVd fragments (Puchta et al. 1991). Similarly, the Australian grapevine viroid has also been described as CEVd-containing viroid chimera (Rezaian 1990), thus it seems that CEVd has the tendency for recombination. Hops, which are typically infected with the HLVd, might be suitable incubator for the next CEVd-HLVd chimeric potentially (hop) pathogenic viroid.

Three other citrus viroids have been analyzed only in the Slovenian survey, the CDVd present in 80% of the tested fruits, along with the rarer CBLVd and CVdV (Fig. 1). CDVd and CBLVd are promoted as dwarfing agent for citrus producers (Lavagi-craddock et al. 2022; Vidalakis et al. 2010, 2011), thus it was unexpected to find CBLVd less frequent compared to CDVd. We also expected to find more samples containing CVdV, since it is a globally distributed viroid (Serra et al. 2008). Maybe this is origin related, since a viroid survey in Thailand showed high incidence of CDVd and CBLVd in citrus fruits imported from China and Cambodia (Tangkanchanapas et al. 2018). Further, a recent survey in Greece showed that e.g. CBLVd was restricted to Crete, while all CEVd, HSVd, and CDVd, was commonly found in all growing regions (Mathioudakis et al. 2023). This shows that the origin of import of high importance when creating a viroid risk assessment in general.

Further, our results show that multiple infections do not just occur occasionally but are more frequent than single

infections in most fruits (Figs. 1 and 3), which was shown to be typical for different types of citrus hosts (Mathioudakis et al. 2023). Two third of the Slovenian samples were either double, triple, or quadruple infected, while one grapefruit contained even five viroids (Fig. 1). The double infection was most frequent in both surveys, however not all viroids were tested in both surveys. As in the recent Greek citrus viroid survey, HSVd and CDVd were often found together in grapefruit and orange (Mathioudakis et al. 2023). In lemons HSVd and CEVd was present in half of the German samples and as triple infection with CDVd in half of the Slovenian samples (Figs. 1 and 3). The study of Lin et al. (2015) and the literature therein shows that the HSVd and CEVd viroids can coexist in some citrus hosts and even enhance their titer synergistically, but at least in one study on clementine, severe symptoms were detected in the HSVd and CEVd combination (Vernière et al. 2007). In line with those results, only 1 out of 10 easy peelers did show to have a HSVd and CEVd double infection in the Slovenian survey. The combination of HSVd and CBCVd was rare, probably due to the observation that this combination leads to the severe bark cracking in clementine (Vernière et al. 2004). The Greek citrus survey, however, showed that at least in one growing region the CEVd, HSVd, and CBCVd triple infection was predominant (Mathioudakis et al. 2023), which again shows how much the specific citrus host (cultivar) as well as probably also scion and rootstock combination seems to matter for the viroid biology (Černi et al. 2020).

Viroid inoculation

The inoculation with CBCVd and HSVd single and double infected orange peel, sap or RNA extracts did lead to symptomless infections in some plants of the hosts hop, tomato and cucumber (Table 1). Based on the results of this study the risk from HSVd and CBCVd double infected citrus fruits is lower compared to the CBCVd single infected fruits (Table 1). Three-quarters of all inoculated plants did not show to contain either of the infected viroids and there was no case where the double infected sample also induced a double infected plant. This is in line with the hypothesis that HSVd and CBCVd have an antagonistic relationship (Matoušek et al. 2017). The single infected HSVd sample did not infect hop or cucumber, which could be a temperature effect, since other viroid inoculation experiments have been successfully performed at higher temperatures, for example for HSVd cucumber inoculation at 28/25°C day/night temperatures (Xia et al. 2017). This could also have increased the infection rate in this study, but this would have been further away from the field conditions. It may as well be possible that just the variants of HSVd did not match the particular host genotype, since there are about a thousand HSVd variants with partly different biological properties known to date. Even though, it has been proposed and shown for some HSVd variants that they are able to adapt from one host to another (Matousek et al. 2003; Sano et al. 2001; Xia et al. 2017). Sequencing of CBCVd from the pre-inoculation Navel orange sample and the subsequentially inoculated hop plant showed 10 nucleotide differences, which is a similar number as observed after the inoculation of hops with a HSVd-grape variant, which resulted in one to nine nucleotide differences (Sano et al. 2001). Later work showed that different spontaneous mutations are generated when HSVd is infecting grapes compared to HSVd infecting hops (Zhang et al. 2020). However, only a small fraction of the mutations occurs at high frequency and even less are exclusive for a specific host, and thus would qualify as *host adaptation* (Zhang et al. 2020). The finding of a CBCVd variant in Brazil (Eiras et al. 2023), which is more similar to citrus variants than to the Slovenian or German hop variants of CBCVd, support the notion that most of the 10 nucleotide difference found between citrus and hops are random and not related to host adaptation. However, further in-depth analysis of CBCVd variants would be necessary to clarify this. Anyway, the result that CBCVd infected citrus peel can lead to infections in hops, tomato, and cucumber is alarming. Here we showed that not only purified viroid containing RNA or sap are potential sources of infection but also unprepared fruit residues in the soil. It has been shown for HLVd-infected hop and PSTVd-infected tomato residues that the viroids are stable for several weeks when stored at temperatures of 40 °C or less (Hagemann et al. 2021; Kerins et al. 2018). Taking those findings together with our results suggest that any unprocessed citrus residues can induce infection and are the likely cause of the primary outbreak at least in Slovenia, where a hop garden was established on one part of a former waste dump (Radišek and Benko-Beloglavec 2016). Whether or not the German outbreak was also associated with citrus fruit residues remains unclear, since in contrast to the Slovenian outbreak no reports on citrus residues were recorded in the CBCVd infected area in Germany. Since vegetative propagation is the most prevalent mode of global viroid spread (Hadidi et al. 2022) exchange of planting material between hop growers from Slovenia to Germany may be an alternative explanation.

Phylogenetic analysis

Generally, the phylogenetic analysis for the three viroids, CBCVd, HSVd, and CEVd showed that samples from the survey are highly diverse and came from distant branches of their corresponding phylogenetic tree (Fig. 5). Since pistachio and citrus are included in the same order of *Sapindales*,

it may be less surprising to find CBCVd in citrus as well as in pistachio, than it was to find CBCVd infecting hops, which belong to the order *Rosales* (Jakse et al. 2015). Further, it could be shown that CBCVd can infect plants from the order *Cucurbitales* and *Solanales*, most susceptible bittersweet nightshade (*Solanum dulcamara*) and tomato (*Solanum lycopersicum*), however, this was found out only through inoculation experiments and not under natural conditions or in the context of plant production as far as we know (Semancik and Vidalakis 2005). Regarding CBCVd in hops, no exact representation of the CBCVd-hop variant was found in this study, however, this may not be necessary for infections, since the Brazilian hop-variant found in 2022 is not very similar to the CBCVd-hop variants found in Slovenia (Eiras et al. 2023). Further, host adaption of several nucleotide differences is possible as also shown in this study (Fig. 2). The HSVd phylogenetic analysis is overall in line with earlier work identifying a divers citrus group, a prunus-malus group and several groups for grape (Sano et al. 2001) and samples from this study belong to distant branches of the HSVd tree (Fig. 5, b). The HSVd-citrus accession closest to a HSVd-hop accession found in this study belongs to a branch consisting of accessions from the genera *Citrus*, *Vitis*, *Humulus* and *Prunus*. Specifically, a lemon accession from a German grocery store showed to be only a few nt different from HSVd-hop accessions found in Slovenia (Radisek et al. 2012), which represent a genetical distance that may be easily overcome through host adaptation (Sano et al. 2001). Consequently, not only does any variant or accession get imported to hop production areas, but specifically also those, which have a great potential for host adaptation and therewith for pathogenicity. The phylogenetic analysis of CEVd shows similarly to HSVd that there is no clear host specific pattern reflected in the phylogeny, but rather tendencies of certain sequence variations being more typic for one or the other host.

Conclusion

We showed that indeed several viroid species are imported into Slovenian and German grocery stores at high rates. We assume that this is very likely true for other regions or countries as well. The inoculation studies showed that viroid infections of hops or experimental hosts are possible not only for extracted and thereby purified viroid containing total RNAs but also from sap injections or fruit peel in the soil. Consequently, the appropriate handling of citrus waste like thermal composting and total residue decomposition is important to reduce the risk of viroid transmission to hop or any other sensitive host plant. This is not only important regarding the CBCVd-citrus-hop pathosystem, but also for other viroids or viruses, which are latent and widely distributed in one host if there are sensitive hosts existing. This includes HSVd from citrus or grape potentially infecting hops, or HSVd-cachexia variants, or CBCVd from citrus infecting other sensitive citrus species. These findings should be included in phytosanitary risk assessment studies for hops, vulnerable citrus species or other viroid susceptible horticultural crops within or far away from citrus growing regions.

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Authors contribution **Michael Helmut Hagemann**: Idea and conceptualization, RTqPCR probe and primer development, data processing and sequence assembly, literature research and draft manuscript preparation. **Charlotte Treiber**: sample collection, extraction, full-length PCR and sequencing. **Ute Born**: sample preparation, extraction and RTqPCR analysis. **Gritta Schrader**: risk assessment literature research. **Johannes Stampfl**: project design and sampling. **Jernej Jakše**: data processing and sequence assembly, manuscript development and RNA folding. **Sebastjan Radišek**: PCR method adaptation, sample collection, extraction and RT-PCR analysis, infection experiments, risk assessment and manuscript review & editing.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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