Detection and persistence of citrus bark cracking viroid and other viroids in citrus peel oils for agricultural applications

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Online Resource 1: Pre-experiments

Pre-experiment 1: RNA extraction from citrus fruit peel

During our pre-experiment, RNA concentrations were measured in different lemon tissues. The flavedo yielded 66 ng/µL, the pulp (whole ground peel) yielded 27 ng/µL, and the pulp oil yielded 6 ng/µL. These results align with the findings of Hagemann et al. (2023) that also highlighted higher RNA yields from the flavedo compared to other citrus tissues.

Pre-experiment 2: Selection of citrus samples for viroid detection

In a second pre-experiment, 18 citrus samples were tested for viroid presence. These included lemon, lime, clementine, orange, and blood orange. The flavedo was carefully peeled using a sterile scalpel and stored at -80°C to maintain sample integrity. Two fruits were pooled together to form one treatment sample. The pooled samples were ground in liquid nitrogen and subjected to RNA extraction using the Detergent RNA extraction method (Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, USA). This method was chosen because it has been successfully used in previous studies to extract RNA from challenging matrices, such as hop leaves and compost residues (Hagemann et al., 2023; Hagemann et al., 2024).

The extracted RNA was tested for CBCVd, CEVd, HSVd, and NAD (as an internal control) using RT-qPCR, as described in Section 2.1.4. Fruits that tested negative for all viroids were processed into oil using a laboratory-scale oil press at ambient temperature. Smaller fruits did not produce enough oil for analysis, while oranges consistently yielded an adequate volume. For this reason, and due to their wider availability, oranges were selected for the main study.

References

Hagemann, M. H., Treiber, C., Born, U., Schrader, G., Stampfl, J., Jakše, J., & Radišek, S. (2023). Risk potential of international fruit trade for viroid spreading - case study on hop viroids in *European Journal of Plant Pathology*, 1(1), 1. https://doi.org/10.1007/s42161-023-01449-3

Hagemann, M. H., Treiber, C., Sprich, E., Born, U., Lutz, K., Stampfl, J., & Radišek, S. (2024). Composting and fermentation: mitigating hop latent viroid infection risk in hop residues. *European Journal of Plant Pathology*, *169*(4), 771–786. https://doi.org/10.1007/s10658-024-02869-2

Online Resource 2: Varieties of Oranges

This study included several varieties of oranges obtained from different German supermarkets, originating from multiple locations. Due to limited availability, some varieties and sources were repeated across the 32 total samples. To avoid redundancy, each variety is listed only once in Table 1, summarizing the distribution across all samples.

Table 1: Orange varieties used in the study

No.	Sample (product name)	Variety
1	Rewe best wahl orange	Navel
2	Sweet susi	Navelina
3	Navel lane late orange	Navelate
4	Loose orange	Lane late
5	Organic orange	Navelate
6	Lane late orange	Lane late
7	Sanlucar orange	Nave late
8	Bio orange	Unknown
9	Demeter orange	Navelate
10	Gut & Gunstig orange	Navel
11	loose organic orange	Lane late
12	Barberina orange	Barberina
13	Markt leibe orange	Unknown
14	Bio helden orange	Unknown
15	Sanlucar	Navelina
16	Bio orange	Navel
17	G and G orange	Unknown
18	Bw, Orange	Navelina
19	Bio orange	Valancia late
20	San lucar orange	Navelina
21	Bio orange valencia late	Valencia late
22	Sweet susi	Navelina
23	Sweet susi	Navelina e
24	Orange Demeter	Navel
25	Orange Bio	Navel
26	San Lucar	Navelina
27	SL orange	Navelina
28	BW orange	Navel
29	BW orange	Navel
30	Bio orange	Unknown
31	Organic porto orange	Valencia Late
32	Bio orange	Lane late

Online Resource 3: CBCVd dimers

Four CBCVd dimer constructs (A1a, B1a, C1a, and C1b) were designed based on full-length sequencing of variants from hops in the Hallertau, each containing polymorphisms at different genomic positions (Treiber et al., 2022). The DNA dimer construct included a T7 promoter for RNA transcription, a 7 bp spacer, two copies of the CBCVd variant, a 21-bp spacer, and a 6 bp HincII restriction site. Constructs were synthesized by Genewiz and cloned into a pUC-GW-Amp plasmid (Genewiz, South Plainfield, NJ, USA). The plasmid was dissolved in water and linearized with HincII (New England Biolabs, Ipswich, MA, USA) at 37°C for 4 hours. The reaction was inactivated at 65°C for 20 minutes. Linearized DNA was transcribed into RNA using the Megascript T7 polymerase, followed by DNA removal with Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA) through overnight incubation at 37°C. The RNA product was then purified using the MEGAclear Kit (Thermo Fisher Scientific) and used for inoculation at a final concentration of 2 µg/µl.

The RNA was used for inoculation at a concentration of 1 µg/µl, with 10 µl (20 µg RNA) per plant applied using a syringe in August and October 2022. The experiment was conducted on hop plants (cv 'Herkules'), with five plants per treatment group, while a non-infectious control was inoculated with water. Post-inoculation, plants were maintained under semi-controlled greenhouse conditions and regularly pruned to 50 cm. Sampling was conducted in July 2023, followed by winter dormancy in November 2023, during which all aboveground foliage was removed, and pots were transferred to a protected chamber with natural winter conditions. In April 2024, plants were returned to the greenhouse for further observation.

References

Treiber, C., Majer, A., Born, U., Kamp, J., Schrader, G., Stampfl, J., & Hagemann, M. H. (2022). Possible import routes and sequence variations of the citrus bark cracking viroid in German hops. *Proceedings of the Scientific-Technical Comission of the International Hop Growers Convention*, *July*, 71–78. Accessed at 19.01.2025 https://www.researchgate.net/publication/361742402_Possible_import_routes_and_sequence_variations_of_the_citrus_bark_cracking_viroid_in_German_hops

Online resource 4: Ct values of results from Table 2

Table 2: Direct use of the samples for RT-qPCR for NAD and CBCVd detection using spiked RNA-oil samples, tested in separate upper and lower phases, across three time points. The analysis was performed at two replicates (I, II) at two volumes (a, b), with CBCVd and NAD detection indicated in red and non-detection in green. Ct values are color-coded, with green indicating negative samples and red representing positive detection. Values marked in bold with (*) had lower Ct values but exhibited amplification curves that did not resemble the positive control. Based on this, these values were interpreted as negative.

Incubation		**1Hour**								**1Day**								**1Week**							
Target		**NAD**				**CBCVd**				**NAD**				**CBCVd**				**NAD**				**CBCVd**			
Repetition		Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb
Treatment	**Phase**																								
RNA (-80°C)	Homogen	30,0	27,4	29,7	27,3	31,0	28,3	31,2	28,4	29,1	27,2	31,1	27,9	30,4	28,3	31,9	29,3	29,2	27,4	30,7	28,5	30,3	28,4	32,2	29,5
RNA (21°C)	Homogen	29,5	26,8	30,3	27,7	30,6	28,1	32,1	29,2	29,3	27,3	30,1	27,6	30,3	28,4	31,5	28,9	29,5	27,5	30,7	27,7	31,1	28,8	31,5	28,6
R90/O10	Upper	39,4	0,0	37,1	35,9	0,0	36,1	0,0	36,8	0,0	0,0	0,0	0,0	38,4	35,0*	0,0	0,0	30,2	30,6	31,7	31,8	32,6	32,6	34,1	33,9
R90/O10	Lower	30,6	27,2	30,3	27,7	33,6	28,5	31,3	28,9	30,2	27,4	29,6	27,6	32,3	29,0	30,8	29,2	30,7	28,0	31,6	28,4	32,4	29,6	34,3	30,2
R10/O90	Upper	0,0	0,0	39,5	0,0	0,0	32,81*	37,0	35,8	0,0	0,0	39,0	0,0	34,5*	31,21*	0,0	37,6	0,0	0,0	0,0	0,0	0,0	31,1*	36,9	32,1*
R10/O90	Lower	35,6	31,6	32,7	0,0	0,0	31,9	33,9	0,0	0,0	0,0	35,8	33,8	33,1*	17,23*	36,1	33,3	0,0	0,0	0,0	0,0	39,3	27,6*	0,0	0,0
O100	Homogen	0,0	0,0	0,0	0,0	36,0*	36,4*	0,0	0,0	0,0	0,0	0,0	0,0	36,8*	37,0*	0,0	0,0	0,0	0,0	0,0	0,0	0,0	20,0*	0,0	25,5*

Online Resource 5: Ct Values from Table 3

Table 3: Chaotropic RNA extraction followed by RT-qPCR for NAD and CBCVd detection using RNA extracted from spiked oil samples across three time points. The analysis was performed at two replicates (I, II) at two volumes (a, b), with CBCVd and NAD detection indicated in red and non-detection in green. Ct values are color-coded, with green indicating negative samples and red representing positive detection. Values marked in bold with an asterisk (*****) had lower Ct values but exhibited amplification curves that did not resemble the positive control. Based on this observation, these values were interpreted as negative.

Incubation	**1Hour**								**1Day**								**1Week**							
Target	**NAD**				**CBCVd**				**NAD**				**CBCVd**				**NAD**				**CBCVd**			
Repetition	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb
Treatment																								
RNA (-80°C)	28,8	27,1	30,4	28,5	30,2	28,3	32,1	29,9	28,9	27,0	29,0	27,8	29,8	27,9	30,4	29,2	29,4	27,2	28,0	27,7	30,5	28,3	30,0	29,4
RNA (21°C)	29,2	27,6	28,8	27,8	29,7	28,0	29,0	28,2	30,0	27,6	29,2	27,0	29,6	27,5	29,8	27,7	29,1	27,0	29,3	27,4	29,9	27,6	29,9	28,3
R90/O10	29,9	27,9	29,8	27,9	30,6	28,3	29,7	28,1	29,3	26,8	0,0	0,0	29,4	27,2	0,0	0,0	29,6	27,4	30,9	28,6	30,0	28,0	30,8	28,8
R10/O90	31,1	28,5	33,7	31,8	31,3	29,4	33,7	32,1	33,9	31,7	38,5	36,2	32,5	31,1	0,0	0,0	0,0	0,0	0,0	37,8	0,0	0,0	0,0	0,0
O100	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	38,2	0,0	0,0	0,0	0,0	36,6