



Evaluation of hop varieties (*Humulus lupulus* L.) for their response to HLVD, HSVd, and CBCVd viroids in tissue cultures

Helena Volk · Patricija Lap ·
Andreja Čerenak · Jernej Jakše

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Abstract This study evaluates the susceptibility of various hop varieties to three viroids: Hop Latent Viroid (HLVd), Hop Stunt Viroid (HSVd), and Citrus Bark Cracking Viroid (CBCVd), all of which pose significant threats to Slovenian hop production. The experiment was conducted under *in vitro* conditions, involving the cultivation of 13 different hop varieties in tissue cultures, alongside the *in vitro* synthesis of viroid transcripts and subsequent inoculation of the plants. The plants were then monitored for growth, development, and their responses to viroid infection. The findings reveal the complex interactions between hops and viroids, with successful inoculation observed in all varieties. However, infection rates varied, with HSVd showing the highest rates of infection, followed by CBCVd and HLVd. This research enhances the understanding of viroid dynamics in hop plants, which is crucial for developing effective disease management strategies. Despite the insights gained, no resistance or tolerance to the viroids was identified in any of the tested hop varieties.

Keywords Hops · Tissue culture · Viroid · CBCVd · HLVd · HSVd

The common hop (*Humulus lupulus* L.) is classified as an herbaceous perennial plant and belongs in the hemp family (Cannabaceae). Hop cultivation plays a crucial role for the brewing industry, providing the essential oils and bitter acids necessary for flavouring and preserving beer.

Viroids are small, single-stranded, circular RNA molecules with a size of 246 to 401 nucleotides. These molecules do not encode proteins, but can replicate, move, and evade the host's defence system (Darós, 2016). To date, four different hop viroids have been discovered: Hop Latent Viroid (HLVd) (Puchta et al., 1988), Hop Stunt Viroid (HSVd) (Sasaki & Shikata, 1977), Apple Fruit Crinkle Viroid (AFCVd) (Sano et al., 2004) and Citrus Bark Cracking Viroid (CBCVd) (Jakše et al., 2015). All these viroids were found in European hop fields (Radišek et al., 2012; Jakše et al., 2015), except for AFCVd, which only occurs in Japan. HLVd spreads among hop plants primarily by vegetative propagation and often shows no clear symptoms of infection (Barbosa et al., 2005). This has made HLVd the most widespread viroid in hop fields (Hadidi et al., 2003). Notably, while asymptomatic in hop, HLVd induces severe symptoms in its close relative, *Cannabis sativa* (Punja et al., 2023; Atallah et al. 2023). In contrast, HSVd, though less widespread, infects a broader

H. Volk · P. Lap · J. Jakše (✉)
Biotechnical Faculty, Department of Agronomy,
University of Ljubljana, Ljubljana, Slovenia
e-mail: jernej.jakse@bf.uni-lj.si

A. Čerenak
Slovenian Institute of Hop Research and Brewing, Žalec,
Slovenia

range of hosts and significantly impacts hop production economically. Interestingly, Atallah et al. (2024) observed no evidence of HSVd co-infection in HLVd-infected *C. sativa* samples. CBCVd was identified in 2007 in Slovenian hop fields as the cause of a previously unknown disease characterized by visible and distinct symptoms that lead to plant death within 3 to 5 years (Jakše et al., 2015). The mechanisms by which viroids cause disease are still unclear, though their symptoms resemble viral infections, suggesting transcriptional or post-transcriptional gene silencing as a mode of action. Since viroids are present in small quantities, they are not thought to deplete RNA nucleotides in host cells. Many hosts show no symptoms and are considered "latent," but viroids can multiply rapidly in susceptible plants. A single nucleotide change can alter the severity of the disease. Mechanical transmission through contaminated tools is common, while seed or pollen transmission is rare. Viroids infect plants by entering and replicating in the chloroplast or nucleus, then spreading between cells via plasmodesmata and through the phloem (Navarro et al., 2021). Infected plants activate RNA silencing mechanisms, including both transcriptional and post-transcriptional silencing, to defend against viroids (Mishra et al., 2018; Navarro et al., 2021).

The aim of this study was to test 13 hop varieties for their susceptibility to the viroids HLVd, HSVd and CBCVd, three viroids which pose a threat to European hop fields, in tissue culture conditions. Hop cultivation ends in late August and the plant remains dormant until late spring. As a tall plant cultivated on special trellises, evaluating symptoms throughout the year in a smaller space using tissue culture could expedite the assessment process. The expectation was that symptoms would develop earlier in tissue cultures and that we would discover new resistant or tolerant hop varieties and confirm the resistance and tolerance of two hop varieties (Styrian Wolf and Styrian Cardinal, respectively) that had shown these characteristics in initial field studies (Radišek et al., 2022).

A Murashige and Skoog medium was used for micropropagation of hop (Ferant et al., 2001) varieties: Atlas, Blisk, Buket, Cekin, Cicero, Styrian Eureka, Styrian Gold, Styrian Kolibri, Wye Target, Bobek, Styrian Wolf, Styrian Cardinal, and Celeia. The dimeric RNA transcripts used for viroid inoculation were prepared from plasmids containing the corresponding viroid sequence in dimeric form, enabling the formation of the viroid transcript's secondary structure, which is important for stability and mechanism of action following inoculation. Dimeric form also ensures proper formation of monomeric form *in vivo*. Plasmids were isolated from overnight cultures of *E. coli* DH5 α using the High Pure Plasmid Isolation Kit (Roche), linearized with a restriction enzyme, and transcribed using a transcription kit following the manufacturer's protocols. Specifically, 1 μ g of linearized plasmid was used in a 20 μ L transcription reaction, incubated at 37 °C for 4 h in a thermocycler. Table 1 lists the combinations of restriction enzymes and transcription kits used to produce the positive strand of the viroid transcript.

Inoculation of hop plants with HLVd, HSVd and CBCVd transcripts was carefully performed on selected uniform plants to allow accurate monitoring of disease symptoms. Inoculum was prepared in a volume of 50 μ L by mixing 150 μ g of each viroid transcript with 0.5 mM K₂HPO₄, with an additional viroid-free control inoculum prepared only with 0.5 mM K₂HPO₄. The largest and healthiest leaf sitting lowest on the plant was selected for inoculation. Each plant was inoculated with 1 μ L of the desired inoculum, which was smeared over the leaf with an L-shaped plastic rod to create small lesions through which the viroid could enter and spread in the plant (Fig. 1). Four plants per variety were inoculated, while two plants served as controls. The tissue cultures were maintained in a growth chamber at 21 °C under a 16-h light and 8-h dark photoperiod.

Eight weeks after inoculation, total nucleic acids were isolated (Kump & Javornik, 1996) from newly

Table 1 Enzymes and transcription kits used for viroid transcript synthesis

Viroid	Plasmid carrier	Enzyme used	Transcription kit
CBCVd	pBSII-CBCVd	HincII (Thermo Scientific)	MEGAscript™ T7 Transcription Kit (Thermo Scientific)
HLVd	pBSII-HLVd	Eco53kI (NEB)	MEGAscript™ T3 Transcription Kit (Thermo Scientific)
HSVd	pGEM-T-Easy-HSVd	BmrI (NEB)	MEGAscript™ SP6 Transcription Kit (Thermo Scientific)



Fig. 1 Inoculation of hop tissue cultures with viroid RNA transcripts

developed shoots and viroid presence was evaluated by RT-PCR (Jakše in Radišek, 2005) using the appropriate primer sequences (Bernad & Duran-Vila, 2006 for CBCVd; Hataya et al., 1992 for HLVd and Matousek et al., 2003 for HSVd).

We confirmed that all hop cultivars were successfully infected with all transcripts (Figs. 2, 3 and 4).

We also noted that the varieties 'Bobek' and 'Wye Target' were not viroid free plants since HLVd infection was confirmed in starting material. HSVd showed the highest mean infection rate with 94% of inoculated plants successfully infected. The mean infection rate of CBCVd was also high, 90% and the mean infection rate of HLVd, the most widespread viroid in hop naturally, was surprisingly low at 55%. A more detailed overview of the infection rates is presented in Table 2.

The symptoms of plants infected with HLVd, HSVd, and CBCVd viroids can be challenging to recognize, especially under certain conditions like tissue culture, where plants do not fully develop visible symptoms such as cones or tall growth. In the field, HLVd infections can affect metabolic pathways, leading to chlorosis, slower growth, and smaller cones, but these symptoms were not observed in our tissue-cultured plants, which did not significantly differ from the controls. Similarly, HSVd symptoms,

Fig. 2 Results of agarose gel electrophoresis of RT-PCR of HLVd inoculation of hop plants

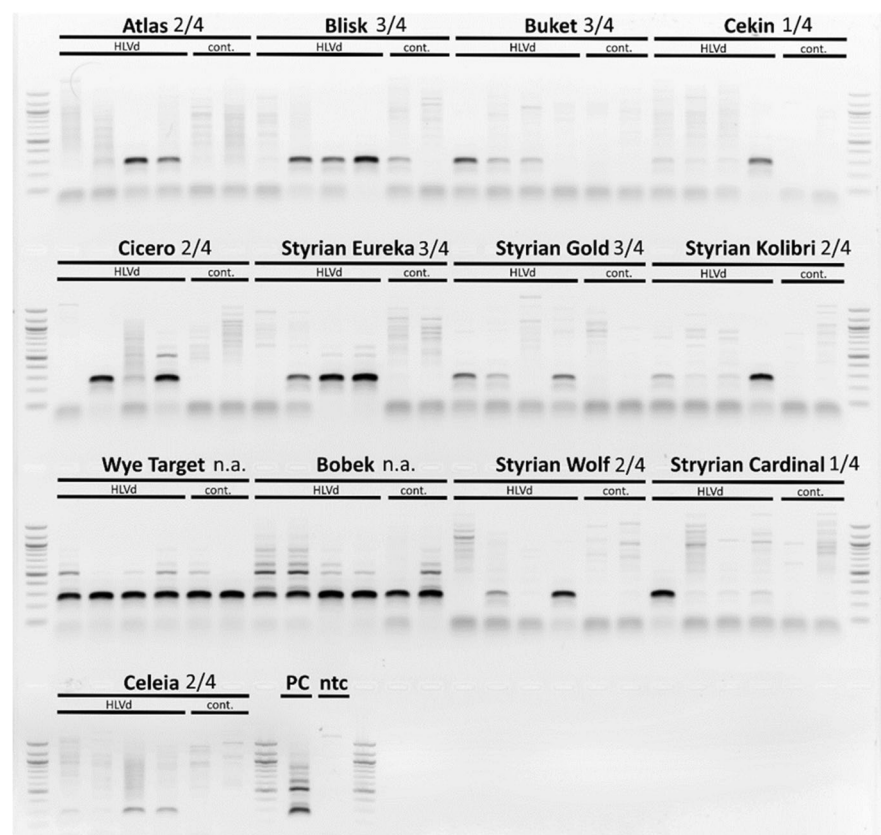
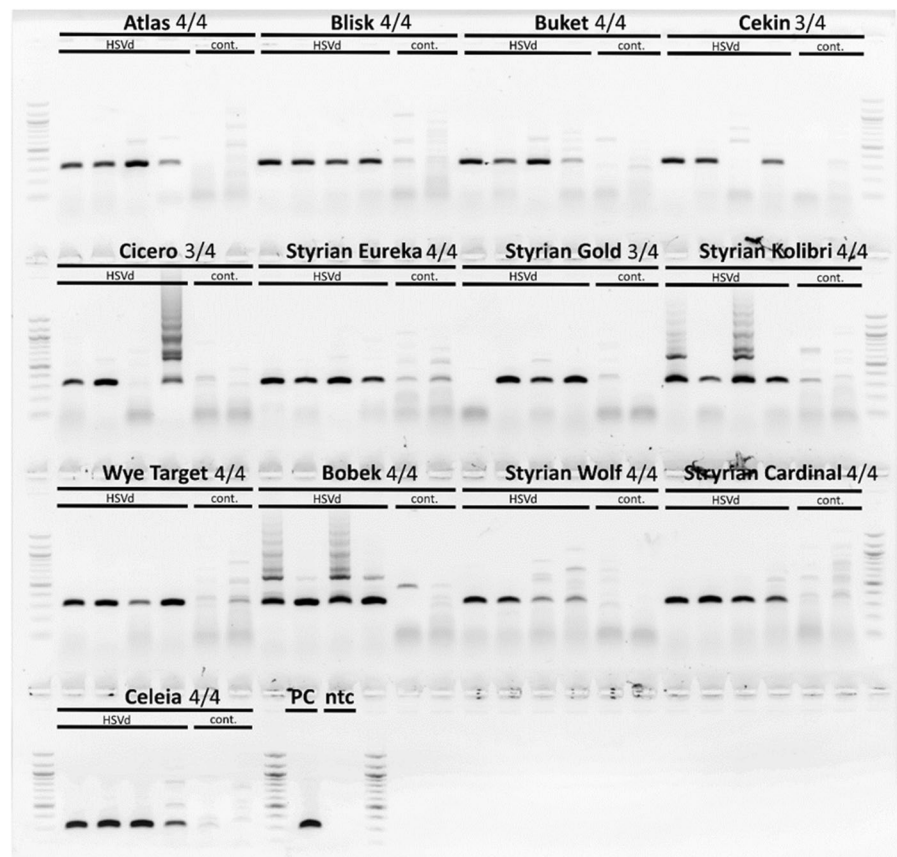


Fig. 3 Results of agarose gel electrophoresis of RT-PCR of HSVd inoculation of hop plants



typically appearing years after infection and including curling leaves, smaller cones, and reduced alpha acid content, were absent in our study. Both viroids infected all cultivars with no resistance or tolerance, with infection rates for HLVD and HSVd ranging from 25 to 100%, as shown in Table 2. Generally, the first symptoms of CBCVd infection in hop plants become visible several months after infection or even after dormancy, manifesting as early mild yellowing and curling of leaves in the field (Jakše et al., 2015). However, our CBCVd-infected plants in tissue culture did not exhibit significant symptoms. While field observations suggest that Styrian Wolf is the only hop that cannot be infected with CBCVd (Radišek et al., 2022), our study successfully infected it with 100% efficiency using *in vitro* viroid transcripts. We hypothesize that the differences between our results and field conditions stem from varying viroid transmission methods and plant growth environments, with field infections primarily occurring mechanically through low levels of infected sap, whereas our

study used high concentrations of synthetic viroid transcripts. Future research should explore lower concentrations and RNA extracts from infected plants to better replicate field conditions.

The tissue cultures were maintained for an additional 3 months until the plants depleted the cultivation media and required subculturing. A visual evaluation conducted at that time again yielded no significant information on viroid symptoms due to uneven growth. This aligns with previous studies, such as Marton et al. (1982), who found no significant effect of Citrus Exocortis Viroid (CEVd) on the growth of tomato callus and suspension cultures. Recent research on viroids and *in vitro* plant cultivation has primarily focused on viroid elimination (Bettoni et al., 2022; Yang et al., 2024) or detection (Wah & Symons, 1997; Lee et al., 2022), rather than symptom development.

Our findings further highlight that visual symptoms may not reliably indicate viroid presence in tissue culture, emphasizing the need for molecular

Fig. 4 Results of agarose gel electrophoresis of RT-PCR of CBCVd inoculation of hop plants

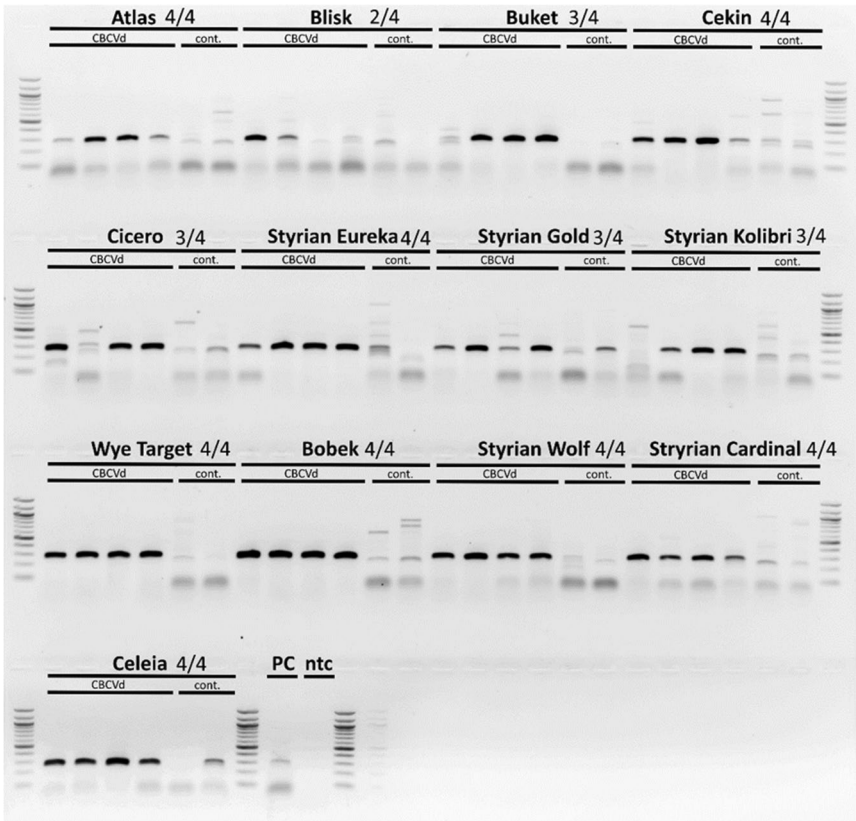


Table 2 Number and % of infected plants inoculated with HLVd, HSVd or CBCVd viroid

Legend: “no.” – number of viroid positive plants out of 4 inoculated per viroid; “n.a.” – not applicable, the plants have been HLVd-infected prior the experiment

Hop cultivar	HLVd		HSVd		CBCVd		All 3 viroids	
	no	%	no	%	no	%	no	%
Atlas	2	50	4	100	4	100	10	83
Blisk	3	75	4	100	2	50	9	75
Buket	3	75	4	100	3	75	10	83
Cekin	1	25	3	75	4	100	8	67
Cicero	2	50	3	75	3	75	8	67
Styrian Eureka	3	75	4	100	4	100	11	92
Styrian gold	3	75	3	75	4	100	10	83
Styrian Kolibri	2	50	4	100	3	75	9	75
Wye Target	n.a		4	100	4	100	8	100
Bobek	n.a		4	100	4	100	8	100
Styrian Wolf	2	50	4	100	4	100	10	83
Styrian Cardinal	1	25	4	100	4	100	9	75
Celeia	2	50	4	100	4	100	10	83
All cultivars	24	55	49	94	47	90	120	81

detection techniques like RT-PCR for accurate identification. Hop growers face significant challenges from various pathogens, including viroids, which

can be mitigated by using viroid-free planting material. To further ensure effective viroid management, implementing regular surveillance programs for

viroid detection across different parts of the plant and various tissue developmental stages within both regulatory frameworks and commercial operations is essential. Breeding efforts also focus on developing virus- and viroid-free genotypes (Oset Luskar et al., 2021). This research aimed to assess the susceptibility of various hop cultivars to HLVd, HSVd, and CBCVd infections, which threaten European hop fields. While we expected to find resistance or tolerance in some cultivars, all tested varieties were infected by the three viroids. Surprisingly, HLVd, the most widespread viroid, had the lowest infection potential (55% average), while CBCVd and HSVd exhibited higher infection rates, with averages of 90% and 94%, respectively. RT-PCR confirmed successful infection in nearly all samples inoculated with HSVd.

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Declarations

Competing interests The authors declare that they have no financial or non-financial competing interests to disclose.

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