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Application Note

Using RT-qPCR and Tissue Culture to Control Hop Latent Viroid in Cannabis Cultivation

Hop Latent Viroid (HLVd) was first identified as associated with "dudding" disease in Cannabis in 2019. It has since rapidly spread through Cannabis cultivation facilities worldwide, causing significant yield and potency losses. HLVd can now be found in most US cannabis cultivation operations. The annual revenue lost in the US is estimated to be up to $4 billion[1]. Strategies to control HLVd include prevention, testing, and culling infected plants.

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Here we report what we have learned about the biology of HLVd and how we approach HLVd control in one of the largest Cannabis cultivation companies in Michigan. THC (formerly Fluresh) operates a 120,000 sq/ft mixed indoor-greenhouse cultivation facility that produces 1,000 lbs. of dry flower weekly. By using a combination of tissue culture and RT-qPCR testing, we have mostly eliminated crop losses to HLVd. However, controlling HLVd is an ongoing task that has become a regular part of our operations.

The Pathogen

HLVd is a member of a small group of infectious agents that have only been detected in plants. It consists of a single-stranded RNA loop of 256 bases. In contrast to viruses, the HLVd genome is not coated by a protein shell and does not code for any proteins. When entering cells of susceptible plants, the viroid multiplies and spreads throughout the plant, similarly to most plant viruses[2].

Recent studies have shown HLVd is very stable outside the plant, and it can spread mechanically through the transmission of sap, as well as through water, seeds, cuttings, and possibly insects[3].

The Challenge

Marijuana-type Cannabis is propagated primarily through cuttings (also known as cloning) and in some cases through seeds. Although both methods allow for transmission of HLVd, the viroid is particularly challenging for cultivators who rely on clonal propagation. Infected plants often show no or mild symptoms in the vegetative growing phase, so infected mother plants can often escape detection.

Oftentimes, it isn't until cuttings from an infected mother enter the flowering stage that symptoms start to emerge. Stunting, brittle stems, and horizontal branching typically appear weeks after flowering is induced. At this stage, crop losses cannot be avoided.

Cultivators can lose valuable strains when the last mother plant becomes infected with HLVd. Tissue culture propagation can be used to recover healthy clones, but success requires either starting from a non-infected part of the mother plant or culturing the tissue under conditions that inhibit or delay HLVd multiplication. Success rates depend on the strain and the severity of the infection of the mother.

When HLVd spread in our cultivation facilities in 2021, we built a tissue culture laboratory and purchased a qPCR instrument to help create and maintain a healthy mother stock and rehabilitate existing and acquired strains. We then developed and refined our tissue culture and testing protocols as we learned more about the disease.

qPCR Testing

The most effective method for detecting HLVd in cannabis plants is RT-qPCR. This method is similar to COVID testing where the first step is copying a specific part of the HLVd RNA and converting it to DNA. That DNA copy is then amplified through several repeated cycles. During each cycle, a fluorescent signal is released that can be quantified by a qPCR instrument.

Preparing a qPCR plate

At THC we use a BIORAD CFX-96 qPCR instrument and Medicinal Genomics PathoSEEK® Cannabis Virus Multiplex Detection Assay, which contains additional probes for Lettuce Chlorosis Virus and Cannabis Cryptic Virus.

The instrument can detect multiple fluorochromes simultaneously and has proven reliable and virtually maintenance-free. Up to 96 samples can be run at the same time. We also use the instrument and PathoSEEK assays for other applications, including:

- Screening seedlings for plant sex
- Quality control testing for Botrytis, Total Yeast and Mold, and Aspergillus
- Pest and pathogen detection (Fusarium, Powdery Mildew, TMV, Russet mites)

The HLVd testing reagents cost approximately $12-13 per sample. A new instrument costs about $30,000, with used instruments selling for much less. Reducing the reagent costs is possible through grouping samples, reducing reaction volumes, and bulk purchasing.

There are several benefits of in-house testing in addition to cost savings. Results are obtained almost instantly, samples don't have to be packed and shipped, and re-tests that are needed in case of inconclusive results or group testing can be done immediately.

Identifying Inconclusive qPCR Results

Another major advantage of in-house testing is the ability to review the qPCR amplification plots for each sample, which can reveal opportunities for re-testing that otherwise could have been overlooked.

Fig.1 is an example of RT-qPCR curves. The results are plotted as fluorescence intensity (RFU) over the number of amplification cycles. A typical positive result shows as an exponential curve that eventually flattens out with increasing cycles (A, B, C, D). A negative sample will not generate fluorescence above background noise (F).

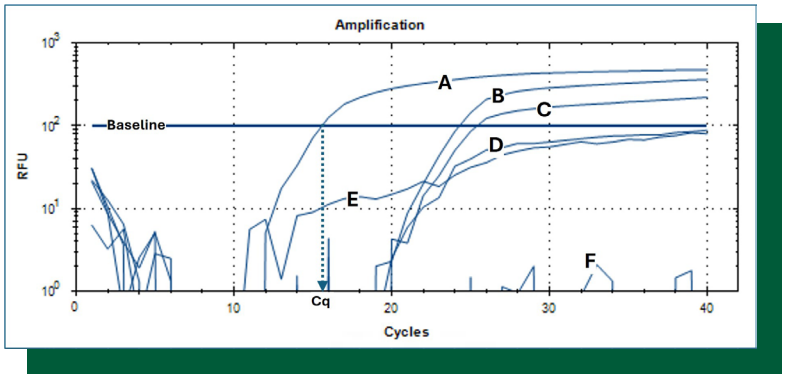

Fig. 1. Example of RT-qPCR Curves

qPCR results are usually reported at the point where the amplification curve crosses the threshold, which is set by the user or calculated by the qPCR software. This value is called the Cq (quantification cycle). Cq values allow for quantifying the amount of the target DNA or RNA in the sample under standard conditions. However, standard conditions do not apply to large-scale sampling and testing for a variety of reasons, including:

- Samples sizes vary
- The age and location of the collected tissue vary (especially when sampling roots)
- HLVd is not evenly distributed throughout the plant
- Lysates used for testing can contain components that inhibit the qPCR reactions
- Defining the baseline threshold depends on the user.

In the examples shown in Fig.1 both the low-level (D) and inconclusive (E) curves would be read as negative at the set baseline but positive if the baseline was lowered.

In our experience, Cq values are much less indicative of HLVd infections than the shapes of the fluorescence curves. When using the Medicinal Genomics multiplex assay comparing the curves for the four different targets also helps identify false positive or inconclusive results. The inconclusive samples can be re-run, or the plants re-sampled immediately to produce conclusive results.

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Tissue Culture

Tissue culture propagation is a cloning method that involves growing plants from small cuttings (explants) in a sterile environment. The resulting clones are free of pathogens and pests but not necessarily of virus and viroid diseases. Early RT-qPCR testing of tissue-culture clones allows for eliminating the infected clones before they can become a source of outbreaks.

The THC tissue culture department's mission is to supply healthy mothers to the cultivation department, maintain a selection of stock mothers representing the current portfolio of 50+ strains, and "clean up" new strains from external sources. The department also performs RT-qPCR testing.

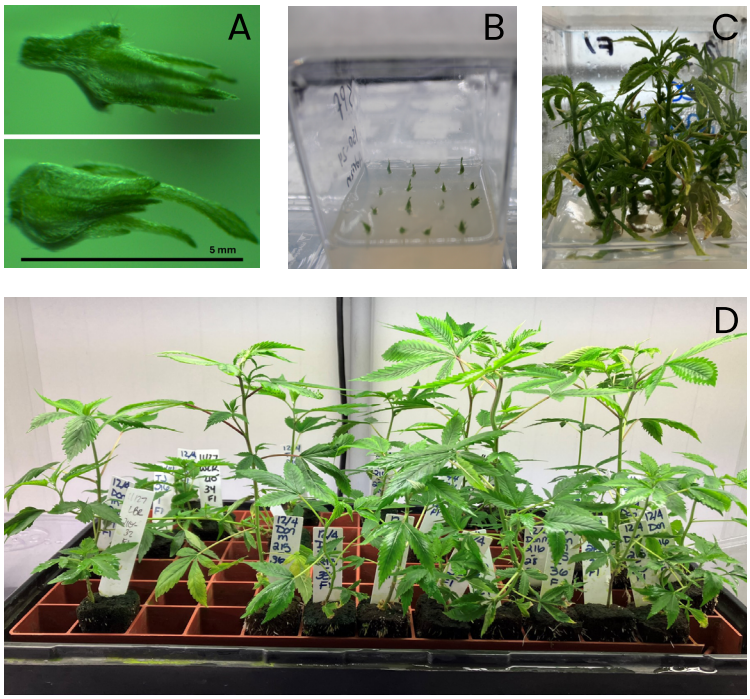


Fig. 2. Tissue Culture protocol

We start cultures from 5mm explants cut from shoot tips or axillary buds that contain as little leaf tissue as possible (Fig.2A). The explant size is a compromise between pure meristems and whole buds or nodes. Explants are sterilized in bleach solution, washed with distilled water, and then planted on solidified agar media in culture boxes (Fig 2B).

Depending on the strain, explants grow into 1-2.5 in shoots within 4-7 weeks (Fig.2C). These are removed from the culture boxes, planted on rooting cubes, and kept in domed trays until roots emerge and the shoots grow (Fig.2D). After transplanting and METRC tagging, the 4-6in tall plants are first tested for HLVd. Healthy plants are then grown to become production or stock mothers. If all plants emerging from a culture test positive, we use treatments such as heat or antiviral compounds to create conditions that impede viroid multiplication and obtain healthy tissue. This process needs to be optimized for each strain and does not always succeed.

Our tissue culture protocol does not include maintaining strains long-term in culture. We found that long-term cultures are at risk of contamination and often grow in irregular shapes. Instead, we keep a stock mom for each strain, and replace them regularly. This approach requires grow space and maintenance. We also skip the shoot-multiplication step because additional transplanting steps are not tolerated well by all strains and add to the contamination risk.

Symptoms and Distribution

Symptoms of HLVd infection vary widely between strains and during plant development. In our experience, brittle stems, horizontal branching, and stunting could sometimes be observed during the vegetative state but most often symptoms do not appear until flowering. We rarely observed smaller, discolored, or crooked leaves. Even mature mother plants that tested positive for HLVd usually looked healthy.

Poor rooting of clones and poor development of shoots in tissue culture can occur for a variety of reasons, and these plants normally do not make it into production. However, these symptoms may indicate that the mother plant is infected with HLVd.

How and when HLVd is transmitted affects how it is distributed within an infected plant. It has been shown back in 1934[2] that when virus transmission occurs through an injured leaf it first travels to the roots, then to the top, and then gradually throughout the plant. This pattern was confirmed recently for HLVd in Cannabis and applies to cases where HLVd is spread through contaminated tools or gloves[3].

Reduced root emergence in HLVd infected clone (left)
Photo credit: Zamir Punja, PhD

We have found that flowering plants showing signs of HLVd infection will test positive both in the roots and leaves. However, large mother plants that test positive in the roots often test negative in the leaves. When multiple leaves from different locations are tested from root-positive plants, it is common for some or even none of the leaf samples to test positive. Young leaves from the top of the plant are less likely to be infected than older leaves. This differs from the expected pattern of HLVd spread within infected plants and might be due to the repeated topping.

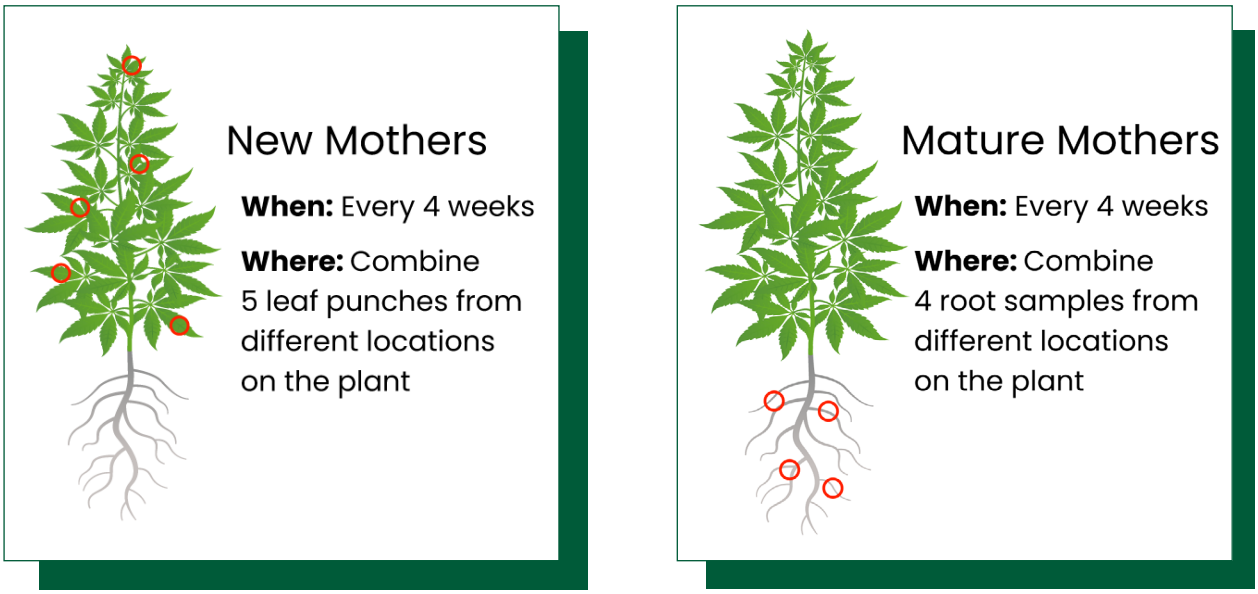
We studied the distribution of HLVd in young plants that had been propagated through tissue culture. HLVd positive clones that had recently rooted always tested positive in the leaves but not always in the roots. The same was also observed in some plants in the early vegetative state before topping. However, most early vegetative plants tested positive both in the roots and leaves. Once plants have grown to a size where roots could be sampled from near the top of the grow media and extensive foliage had grown, root samples produced more reliable results than leaf samples.

When and Where to Sample

HLVd is not evenly distributed throughout the infected plant, which makes sampling tricky. Small numbers of HLVd copies can be present in parts of the plants during the vegetative stage, providing a reservoir from which the infection can spread later. These low-level infections can be easily missed in early-stage testing.

Testing all plants in a commercial grow is cost- and time prohibitive. An effective testing strategy must consider the sources and patterns of HLVd transmission. The two most common origins of HLVd infection in our facilities have been infected mother plants and seeds or clones from outside sources.

Testing Mother Plants

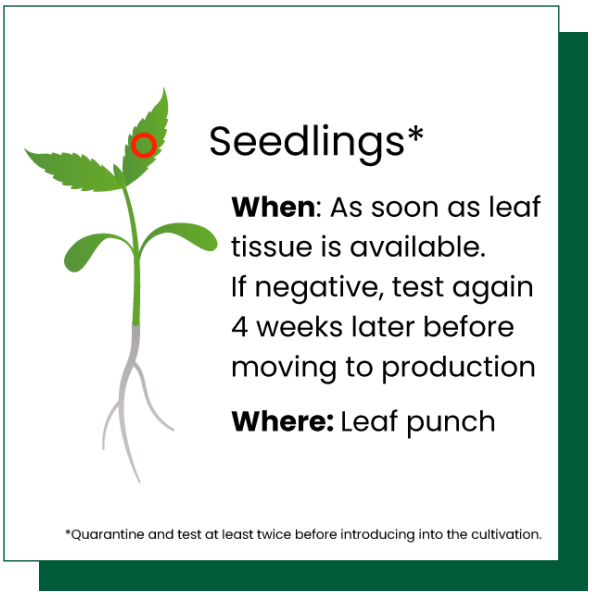


Testing recommendations for mother plants

The key to controlling HLVd is keeping it out of the mother room. Most of the plants that showed symptoms in the vegetative or flowering stages could be traced back to infected mother plants. We test all our mother plants every 4 weeks. New mothers are tested by combining 5 leaf punches from different locations per assay. Four root samples are collected from each of the larger and mature mother plants. Collecting root samples requires disturbing the surface of the grow media and cutting the roots. This presents a risk of not only transmitting HLVd but also other pathogens. We use 10% bleach to soak the tweezers and shears between plants. Bleach in the lysis tube can inhibit the PCR reaction. We spray our tools with isopropanol after soaking and shake off excess liquid between samples. Gloves are changed between plants. The root sections or leaf punches are transferred to 100ul lysis tubes and processed according to the Medicinal Genomics protocol.

We have found that it is essential to sample from several locations around the plant and repeat the testing on a regular schedule. HLVd can be present in low concentrations in small “reservoirs” within the plant and is unevenly distributed in the vegetative state. We do not know what triggers HLVd multiplication and spread in non-flowering plants. A mother that had tested negative repeatedly can test positive later without an obvious cause. Replacing the mothers 2-3 times yearly with new tissue-culture clones has helped minimize HLVd spread within the mother room and from mothers to clones.

Testing Seedlings and Incoming Clones



Testing recommendations for seedlings and clones

Seedlings from outside sources that were grown for evaluation as new production strains sometimes tested positive at a very early stage. Clone batches from outside sources frequently contained HLVd-positive plants, which were immediately culled. However, if a strain is considered especially valuable, we will attempt to recover healthy clones through tissue culture. This worked for most, but not all strains.

All incoming clones and all seedlings should be quarantined and tested as soon as enough leaf tissue is available. Clones and seedlings that test positive for HLVd should be culled, and plants that test negative should be tested again 4 weeks later before being introduced into the cultivation facility.

Main Findings and Recommendations

- HLVd is here to stay. Controlling its spread and the losses it causes is an ongoing task.
- The mother room is the key battleground. Keeping the mothers healthy is essential. Replace mothers with young, healthy stock 2-3 times yearly. Test mothers as frequently as possible.
- Any clones or seeds from outside sources should be considered a risk and must be quarantined and tested at least twice before being introduced into the cultivation.
- Bleach is your friend. Use it with good hygiene practices to prevent plant-to-plant spread.
- There are different methods of tissue culture cloning. We use it to maintain and restore our genetic assets and minimize HLVd and other pathogens.
- HLVd symptoms in vegetative stage plants can be mild or absent. Once flowering, the damage is irreversible. Understanding the origins and spread of infection is important for effective diagnosis.
- RT-qPCR is currently the only reliable method of detecting HLVd. Testing is expensive, but the yield and potency losses caused by HLVd infection are much costlier.
- Acquiring in-house PCR testing equipment helps lower the cost per assay and can also be used for other applications in cultivation (pests, pathogens, gender) and post-harvest quality control (microbial and mold contamination).

References

1: Adkar-Purushothama, Charith Raj; Sano, T.; Perreault, J-P. Hop Latent Viroid: A Hidden Threat to the Cannabis Industry. Viruses 2023, 15, 681.
2: Samuel, G. (1934). The movement of Tobacco Mosaic Virus within the plant. Ann. Appl. Biol. 21, 90-111.
3: Punja, Zamir. "Understanding and Managing Hop Latent Viroid in Cannabis". YouTube, uploaded by Medicinal Genomics, June 8, 2023. https://youtu.be/xQTRWQuUCzY.

Acknowledgments
Many thanks to the THC leadership for their support of the TC and PCR labs, Mae Beach and the TC lab crew, and James Haley and the cultivation crew.

About the Author

Ulrich has a PhD in Plant Science from the University of Kiel, Germany, where he worked on plant proteins that function in plant defense. He has conducted research on the biochemistry and molecular biology of plant-pathogen and plant-insect interactions at Washington University, the Scripps Research Institute, and the University of Oklahoma. Ulrich entered the Cannabis industry in 2014. He has been involved in starting and operating cultivation facilities in Nevada, Louisiana, and California before joining Fluresh in Michigan in 2021. Ulrich has designed and operated tissue culture laboratories and developed methods for micro-propagating Cannabis at an industrial scale. His responsibilities at Fluresh include running the tissue culture laboratory, maintaining a healthy genetic stock, diagnosing and remediating viroid and virus infections, and supporting the cultivation and production teams with in-house laboratory services.