NEW RESEARCH PAPER

TUMI Genomics

KAPRIKORN CANNABIS NURSERY

HOP LATENT VIROID LEVELS AND DISTRIBUTION IN CANNABIS PLANT TISSUE

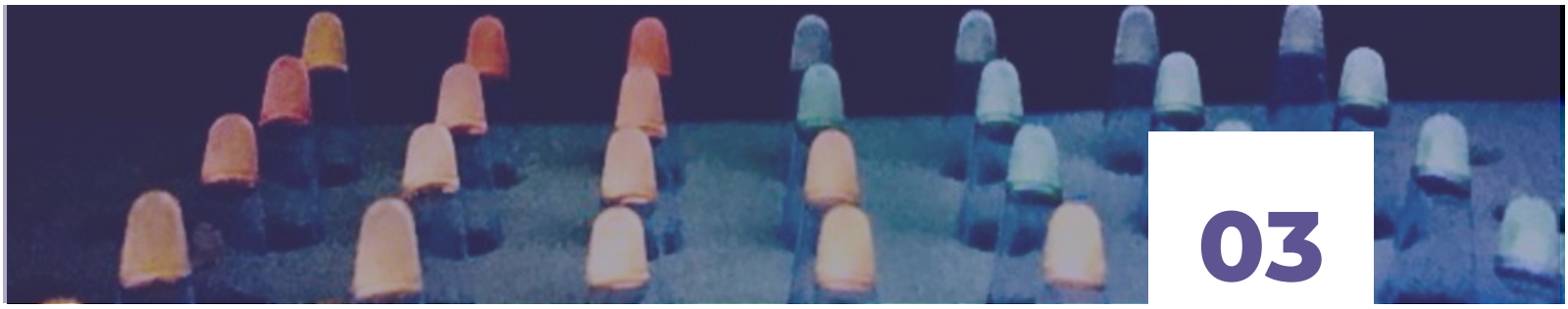
02

Summary

The purpose of this study was to determine the variability of Hop Latent Viroid (HLVd) load throughout infected cannabis plants as well as the tissue-type that most reliably predicts infection. While the number of plants assayed in this preliminary study was limited, the **data presented below strongly suggest that upper root tissue has the highest viroid load and shows consistent detection of HLVd genetic material in infected cannabis plants.**

Overview

Four cannabis plants (teenage) identified as infected with HLVd were acquired by Kaprikorn Nurseries for the purposes of this study. Infected plants were kept in strict isolation. To determine variability in viroid amounts throughout the plants, approximately twenty-five individual samples were taken from distinct locations around each plant. Tissue types sampled include: petiole samples from older growth, petiole samples from newer growth, stem sample from freshly cut upper clones, upper root mass samples and lower root mass samples. A total of 95 samples were assayed in this study. A subset of samples taken from two non-infected plants were also included in this study to confirm the absence of cross-contamination.



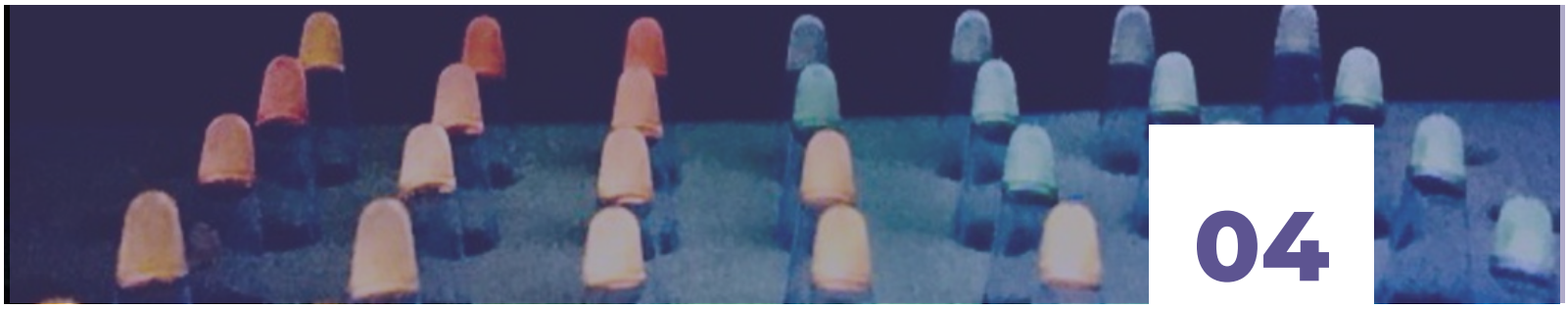
All samples were taken within the same three-day time span and were placed in identical TUMI Genomics collection tubes. Samples were mailed and processed in parallel at the TUMI Genomics Laboratory using a triplex TaqMan qRT-PCR assay with a limit of detection (i.e. the viroid level at which the PCR test is 95% accurate) of 7.5 viroid copies per reaction. Additional information about this assay and a full validation report can be found here: HLVd_validation. All samples were assayed for an internal plant RNA control to confirm sample integrity and alongside three external controls: a non-infected control, an infected control, and a non-template control; to rule out assay dysfunction or contamination.

Results and Discussion

Root tissue gives the most consistent HLVd amplification in individual plants

Among the individual samples taken from the four test plants, upper foliage samples showed variable success for detection of HLVd (Table 1). Petiole taken from new growth showed between 10% to 100% success depending on the plant with an average success rate of 36% (9/25). Old growth and tissue from freshly cut clones showed slightly better detection success with an average of 75% (19/25) and 61% (11/18) testing positive for HLVd, respectively. Interestingly, root tissue showed an impressive level of accuracy. Every root tissue sample taken from either the upper or lower portion of the root ball showed robust amplification of HLVd (36/36 samples testing positive). We saw no HLVd amplification from samples taken from plants with no known HLVd infection (Table 2).

These results indicate that inclusion of root tissue when testing for a potential HLVd infection could improve detection accuracy.



We did note that 20 of the samples testing positive in the upper foliage were defined as low-level positive (viroid load <15 HLVd copies per reaction). This observation was prominent in both Plant 2 and Plant 3 where almost all positive samples taken from upper foliage showed very low viroid load, suggesting that detection of HLVd in foliage tissue from these plants would likely be missed by lower sensitivity assays. While it is reported that viroids reach extremely high copy numbers during infection, our results suggest that not all tissues in infected cannabis plants contain high levels of HLVd. Tissues with low viroid load may contain viroid that is trafficking through the vascular system or viroid that has just gained access to the cellular environment, but is not yet efficiently replicating. Once a system-wide infection is established, the location where the sample is taken from is likely less critical. In support of this, one of the tested plants (Plant 4) showed consistent, robust HLVd amplification in all tissues sampled suggesting development of a full systemic infection in this plant.

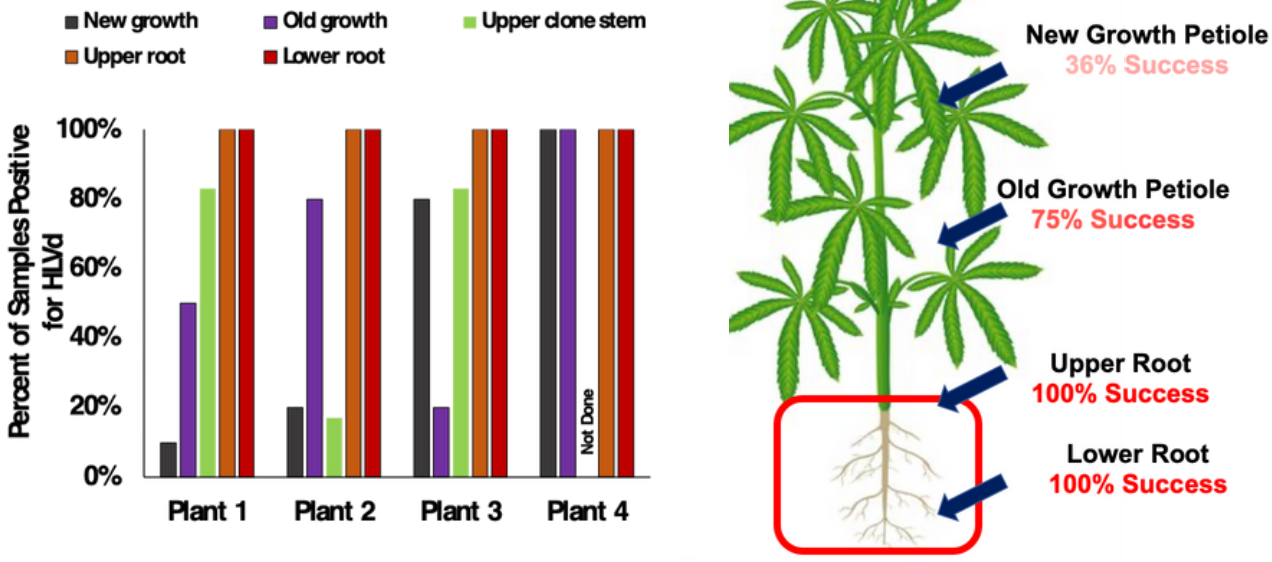
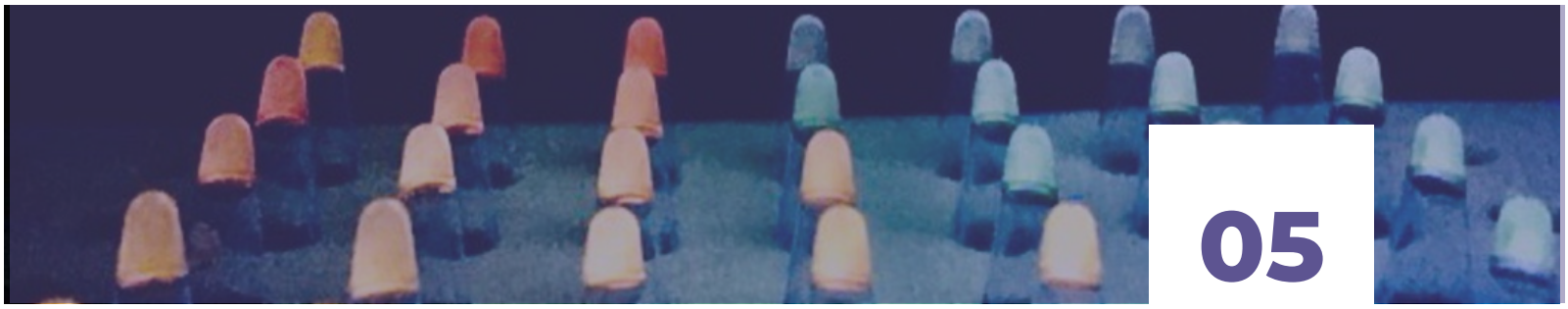


Figure 1. Root tissue contains consistent levels of HLVd in positive plants. A. the percent of samples from each of the four assayed plants where HLVd was detected in the indicated tissue. For each tissue-type, five individual samples were collected from each plant. B. the percent success of HLVd detection in indicated tissues across all plants tested. The red box highlights the root tissue, where the detection level of HLVd genetic material was 100%.



Upper root tissue shows higher, less variable levels of HLVd

Because commercial assays detect HLVd with variable sensitivity, understanding the level of HLVd accumulation in different tissues is helpful to estimate the success rate of HLVd detection across different tests. Using standard curve data generated from known concentrations of HLVd sequence (done in triplicate) we estimated the median viroid load across positive samples from each tissue type (Figure 2). Due to the low number of new growth samples with detectable levels of HLVd, we did not include this tissue in the analysis. As shown in Figure 2, we found that upper root tissue had substantially higher viroid load than other plant tissue analyzed. Upper root tissue had an average of ~500-fold to ~2000-fold more HLVd than foliage tissue analyzed in this study. Even when only considering samples taken from the root, upper root tissue amplified an average of 36-fold more HLVd than samples taken from the lower root.

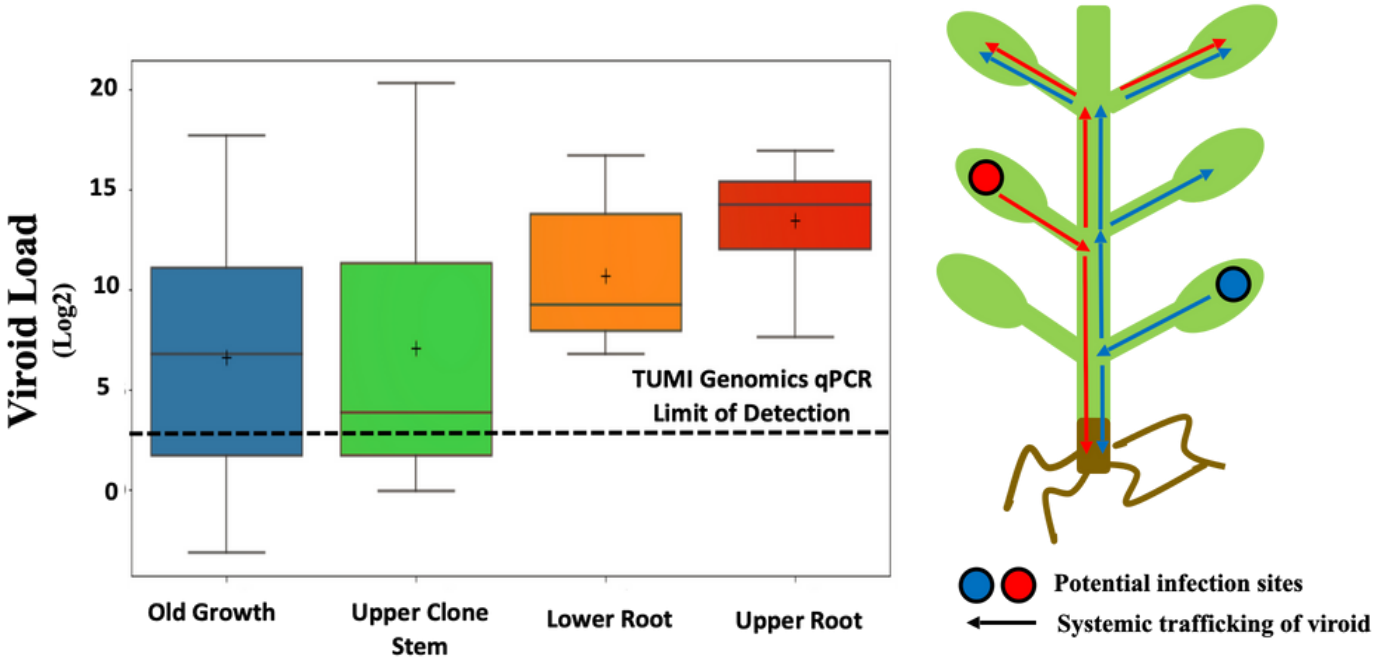
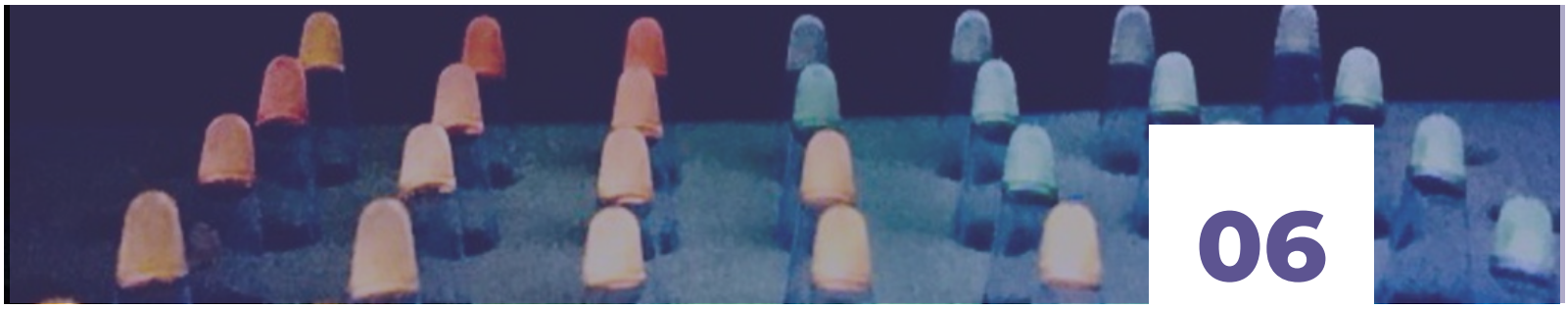


Figure 2: Upper Root tissues accumulates high levels of HLVd. A. Median viroid load and variation across the indicated tissue types. The dashed black line shows the threshold of reliable (>95%) HLVd detection for the TUMI Genomics HLVd PCR assay. New growth samples were not included in this analysis due to a small number of positive samples obtained from this tissue. B. Model of viroid trafficking from the site of inoculation to “sink” plant tissues. Image shows two potential sites of viroid introduction (red and blue circles). Note that the location the viroid travels in the foliage is dependent on where the viroid was introduced in the plant. However, HLVd will always travel down to the root system regardless of where HLVd was introduced. Image adapted from Takeda and Ding, 2009.

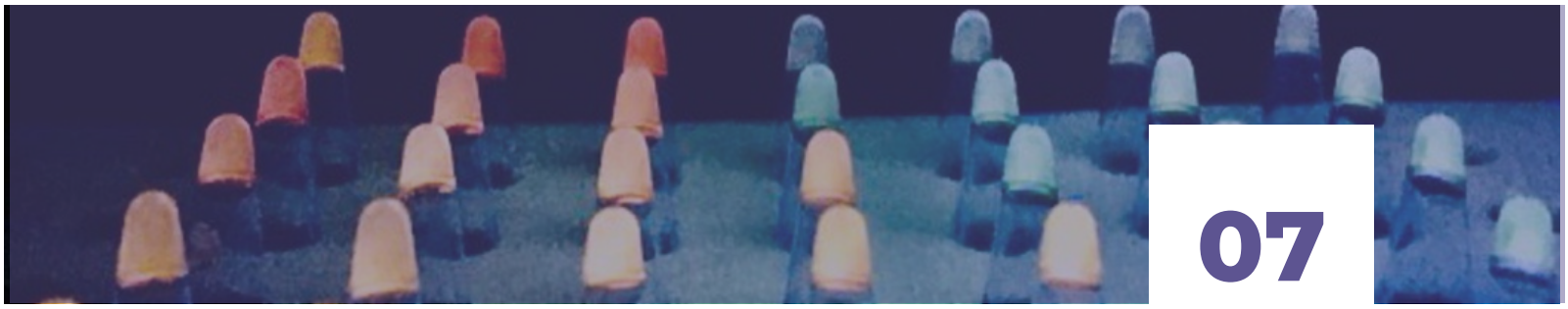


We also noted that root tissue (both upper and lower) showed less variability in viroid load both within each individual plant and when all tissue samples of each type were considered together. These results indicate that upper root tissue accumulates high-levels of viroid titer and therefore inclusion of upper root tissue when screening for HLVd infection could improve detection sensitivity.

These findings are consistent with previous studies showing that viroids accumulate to high levels in root tissue, including HLVd in Hops plants (Matousek et. al. 1995, Palukaitis, 1987, Lin et. al, 2015, Nabeshima et. al, 2017, Antignus, et. al. 2007). Because viruses and viroids traffic through the vascular system of plants, they traffic from the site of introduction to areas of active growth, i.e. growth sinks. Because the location of viroid introduction is not usually known, it is difficult to determine which leaves and petioles represent growth sinks at the time of initial infection and are therefore likely to contain spreading viroid. However, the root system is always growing and receiving energy from the upper portion of the plant, so viroids from the site of inoculation will consistently traffic to the root system (Figure 2A) (Takeda and Ding, 2009).

Follow-up Study

To confirm the findings described above, we performed a follow-up study using additional cannabis varieties collected from a distinct, off-site location. Individual samples from sixteen plants of unknown HLVd infection status we collected. For each plant, two types of samples were prepared: 1) three petiole samples from foliage along with a single pooled samples from the same petiole tissues and 2) three root samples along with a single pooled root sample from the same root tissue. Samples collected were within the same day, placed in collection tubes and mailed to TUMI Genomics where they were tested for the presence of HLVd using our RT-PCR assay.

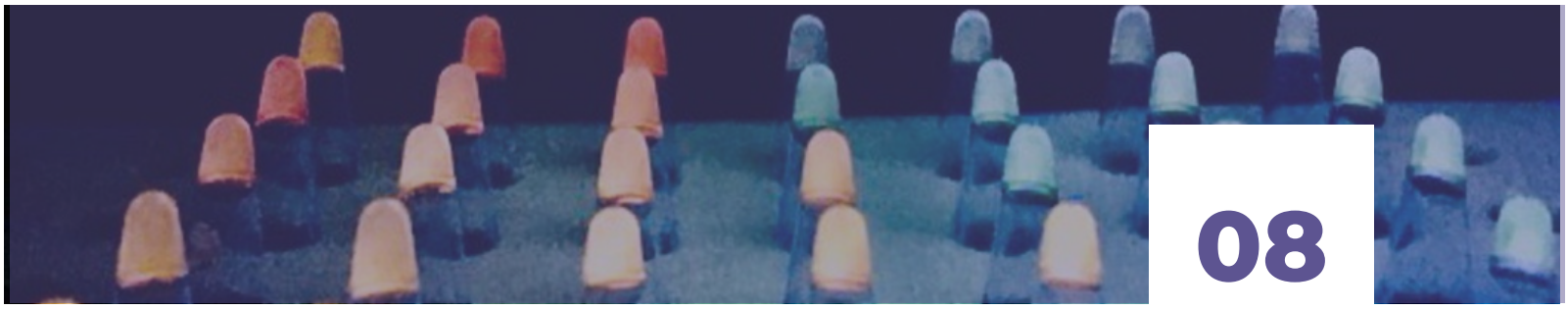


Among the tested plants, three out of the sixteen tested positive for HLVd. The success rate of HLVd detection in each root sample and the pooled root samples remained 100%, replicating the data presented above. The foliage samples again showed poor individual success at detecting HLVd infection (Table 1). On one plant, HLVd was not detected in any of the petiole sample but was still present in all three of the individual root samples and the pooled samples.

Table 1: Success rate of HLVd detection among samples taken from positive plants

Sample	Individual petiole success rate	Pooled petiole	Individual root success rate	Pooled root
Plant 1	33%	Positive	100%	Positive
Plant 2	0%	Negative	100%	Positive
Plant 3	66%	Positive	100%	Positive

We also noted that the TUMI Genomics test was able to detect HLVd in pooled samples without a loss in sensitivity (see supplemental tables), allowing inclusion of multiple samples from a single root mass which can increase sensitivity and accuracy of the test results.

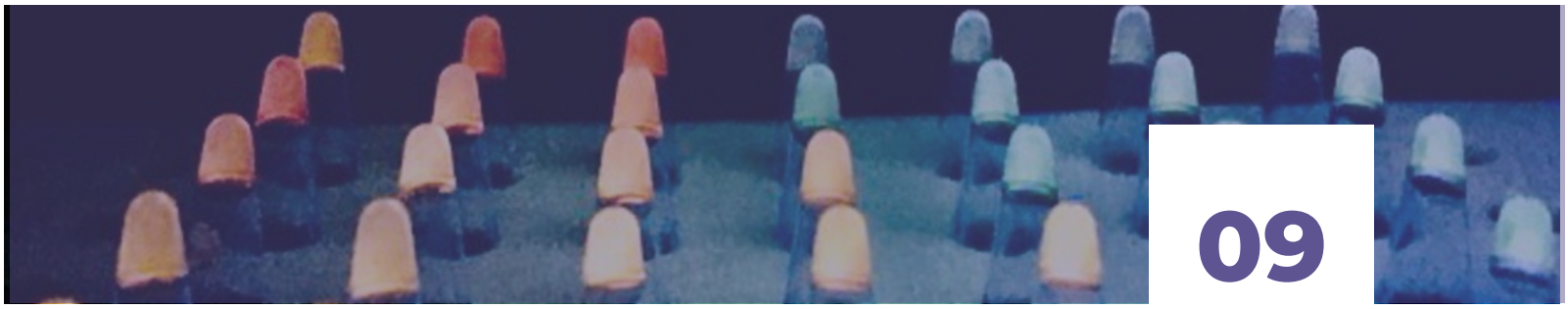


Recommendations

HLVd can exist at dramatically different levels across different tissues within an infected plant and data shown here confirms this assertion. For reliable diagnostic testing, it is best to collect multiple tissue samples from around the plant. This ensures that if the infection is early and/or local (not spread system-wide), diagnostic assays can still detect HLVd in the submitted tissue. Based on the data presented here, along with findings in published literature from different viroids in other crops, we recommend that upper root tissue be included when submitting tissue samples for HLVd testing. Because of the limited sample size of this study, we recommend growers continue to *also* include petiole samples from older growth among the tissue submitted for testing. If root tissue is not available (such as in a young clone), care should be taken to submit multiple tissue samples from older growth areas of the plant.

Limitations

While this study strongly suggests that root tissue is the most reliable tissue to identify HLVd infection, the sample size tested was limited. Seven plants were assayed originating from five different varieties. We cannot completely rule out the possibility that the viroid distribution in the tested plants is cultivar specific. However, given the 100% detection rate of hop latent viroid in every root sample assayed (45 individual samples total), the data clearly indicates that inclusion of root tissue when testing for HLVd can dramatically improve identification of true positive plants.



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Supplementary data

Tables Show the cycle threshold values and estimated viroid load obtained for all samples tested in this study. Samples that tested at average to high positive levels are indicated in red. Samples that tested low-level positive (<15 viroid copies per microliter) are shown in blue. NaN indicates non-detectable. Run controls are shown at the end of Table 2.

Table 1: Amplification results among samples taken from HLVd infected plants

Plant	Tissue	Result	Control CT Value	HLV Target 1 CT Value	HLV Target 2 CT Value	Viroid Load (copies per microliter)
Plant 1	New Petiole	Positive	30.16	18.92	21.69	88090.82
Plant 1	New Petiole	Positive	34	18.28	20.86	138731.51
Plant 1	New Petiole	Negative	33.77	NaN	NaN	Not Detected
Plant 1	New Petiole	Negative	33.97	NaN	NaN	Not Detected
Plant 1	New Petiole	Negative	34.16	NaN	NaN	Not Detected
Plant 2	New Petiole	Low Positive	31.81	40.6	NaN	0.02
Plant 2	New Petiole	Negative	32.76	NaN	NaN	Not Detected
Plant 2	New Petiole	Negative	32.34	NaN	NaN	Not Detected
Plant 2	New Petiole	Negative	31.84	NaN	NaN	Not Detected
Plant 2	New Petiole	Negative	31.23	NaN	NaN	Not Detected
Plant 3	New Petiole	Low Positive	31.14	39.03	NaN	0.06
Plant 3	New Petiole	Negative	32.57	NaN	NaN	Not Detected
Plant 3	New Petiole	Negative	30.63	NaN	NaN	Not Detected
Plant 3	New Petiole	Negative	30.22	NaN	NaN	Not Detected
Plant 3	New Petiole	Negative	30.4	NaN	NaN	Not Detected
Plant 4	New Petiole	Positive	30.36	18.05	21.05	163327.70
Plant 4	New Petiole	Positive	30.38	19.63	22.59	53224.62
Plant 4	New Petiole	Positive	30.45	21.45	24.59	14628.46
Plant 4	New Petiole	Positive	30.18	18.73	21.7	100806.28
Plant 4	New Petiole	Low Positive	30.25	35.7	NaN	0.59
Plant 1	Old Petiole	Positive	30.1	24.19	26.83	2092.89
Plant 1	Old Petiole	Positive	33.07	27.42	30.44	211.48
Plant 1	Old Petiole	Positive	30.21	20.52	24.15	28302.15
Plant 1	Old Petiole	Positive	29.35	24.68	27.16	1478.19
Plant 1	Old Petiole	Negative	29.44	NaN	NaN	Not Detected
Plant 2	Old Petiole	Negative	28.17	NaN	NaN	Not Detected
Plant 2	Old Petiole	Positive	28.36	29.27	35.41	56.90
Plant 2	Old Petiole	Low Positive	28.17	38.03	NaN	0.11
Plant 2	Old Petiole	Low Positive	28.21	36.71	NaN	0.29

Table 1: Amplification results among samples taken from HLVd infected plants

Plant 2	Old Petiole	Low Positive	28.63	33.32	NaN	3.21
Plant 3	Old Petiole	Low Positive	28.11	31.36	36	12.91
Plant 3	Old Petiole	Positive	28.66	28.34	33.9	110.09
Plant 3	Old Petiole	Low Positive	29.13	37.7	NaN	0.14
Plant 3	Old Petiole	Low Positive	29.52	35.97	NaN	0.49
Plant 3	Old Petiole	Negative	29.34	NaN	NaN	Not Detected
Plant 4	Old Petiole	Positive	27.94	20.63	22.55	26176.89
Plant 4	Old Petiole	Positive	29.6	25.52	28.52	814.42
Plant 4	Old Petiole	Positive	28.35	17.71	21.06	207895.93
Plant 4	Old Petiole	Positive	29.47	21.45	24.52	14628.46
Plant 4	Old Petiole	Low Positive	27.64	31.84	NaN	9.18
Plant 1	Root Lower	Positive	28.49	26.15	29.76	520.82
Plant 1	Root Lower	Positive	31.65	27.9	31.86	150.43
Plant 1	Root Lower	Positive	27.35	21.88	25.42	10781.39
Plant 1	Root Lower	Positive	25.45	19.63	22.37	53224.62
Plant 2	Root Lower	Positive	25.75	19.77	22.45	48190.93
Plant 2	Root Lower	Positive	24.09	18.62	21.41	108990.59
Plant 2	Root Lower	Positive	27	23.51	26.07	3390.92
Plant 2	Root Lower	Positive	27.12	25.72	29.1	706.66
Plant 3	Root Lower	Positive	26.04	26.23	28.11	492.07
Plant 3	Root Lower	Positive	30.27	26.98	30.14	288.99
Plant 3	Root Lower	Positive	28.25	24.09	26.98	2246.80
Plant 3	Root Lower	Positive	24.7	20.4	23.54	30817.88
Plant 4	Root Lower	Positive	28.05	27.09	29.72	267.29
Plant 4	Root Lower	Positive	28.26	28.06	33.5	134.29
Plant 4	Root Lower	Positive	29.39	28.34	32.87	110.09
Plant 4	Root Lower	Positive	31.49	27.55	30.35	192.85
Plant 1	Root- Upper	Positive	32.48	25.81	28.33	662.93
Plant 1	Root- Upper	Positive	27.77	18.64	21.24	107454.62
Plant 1	Root- Upper	Positive	27.31	21.15	24.45	18099.10
Plant 1	Root- Upper	Positive	NaN	27.58	33.22	188.78

Table 1: Amplification results among samples taken from HLVd infected plants

Plant 1	Root- Upper	Positive	25.09	18.41	21.67	126505.62
Plant 2	Root- Upper	Positive	24.48	19.36	21.95	64465.16
Plant 2	Root- Upper	Positive	23.91	19.05	22.11	80327.70
Plant 2	Root- Upper	Positive	25.66	20.13	23.28	37326.33
Plant 2	Root- Upper	Positive	24.48	23.64	25.58	3092.09
Plant 2	Root- Upper	Positive	26.23	20.41	23.54	30599.96
Plant 3	Root- Upper	Positive	28.23	23.03	26.13	4767.07
Plant 3	Root- Upper	Positive	26.09	23.02	26.07	4801.02
Plant 3	Root- Upper	Positive	24.51	20.62	22.93	26363.31
Plant 3	Root- Upper	Positive	27.28	22.84	25.14	5455.17
Plant 3	Root- Upper	Positive	27.54	21.02	24.3	19848.25
Plant 4	Root- Upper	Positive	27.23	23.61	26.56	3158.63
Plant 4	Root- Upper	Positive	25.43	19.21	22.88	71705.79
Plant 4	Root- Upper	Positive	25.28	20.24	22.81	34523.43
Plant 4	Root- Upper	Positive	28.65	25.12	27.69	1081.74
Plant 4	Root- Upper	Positive	26.78	23.17	26.05	4316.22
Plant 1	Stem- Upper Clone	Positive	25.54	15.66	17.04	890522.17
Plant 1	Stem- Upper Clone	Positive	25.39	15.12	17.13	1306380.74
Plant 1	Stem- Upper Clone	Low Positive	26.26	31.26	34.48	13.86
Plant 1	Stem- Upper Clone	Positive	25.12	16.85	18.09	382730.67
Plant 1	Stem- Upper Clone	Negative	26.69	NaN	NaN	Not Detected
Plant 1	Stem- Upper Clone	Low Positive	26.32	35.07	NaN	0.93
Plant 2	Stem- Upper Clone	Negative	25.51	NaN	NaN	Not Detected
Plant 2	Stem- Upper Clone	Negative	24.09	NaN	NaN	Not Detected
Plant 2	Stem- Upper Clone	Negative	26.48	NaN	NaN	Not Detected

Table 1: Amplification results among samples taken from HLVd infected plants

Plant 2	Stem- Upper Clone	Negative	26.14	NaN	NaN	Not Detected
Plant 2	Stem- Upper Clone	Negative	25.23	NaN	NaN	Not Detected
Plant 2	Stem- Upper Clone	Low Positive	25.96	34.17	NaN	1.76
Plant 3	Stem- Upper Clone	Negative	25.49	NaN	NaN	Not Detected
Plant 3	Stem- Upper Clone	Low Positive	26.31	33.16	NaN	3.60
Plant 3	Stem- Upper Clone	Low Positive	25.04	31.66	NaN	10.44
Plant 3	Stem- Upper Clone	Low Positive	25.55	30.98	43.89	16.91
Plant 3	Stem- Upper Clone	Low Positive	24.46	33.5	NaN	2.83
Plant 3	Stem- Upper Clone	Low Positive	25.94	31.16	NaN	14.88

Table 2: Amplification results among samples taken from plants with no known infection by HLVd

Plant	Tissue	Result	Control CT Value	HLV Target 1 CT Value	HLV Target 2 CT Value	Viroid Load (copies per microliter)
Plant 5	Root	Negative	26.03	NaN	NaN	Not Detected
Plant 5	Old Petiole	Negative	27.16	NaN	NaN	Not Detected
Plant 5	Old Petiole	Negative	26.50	NaN	NaN	Not Detected
Plant 5	Old Petiole	Negative	27.34	NaN	NaN	Not Detected
Plant 5	Old Petiole	Negative	27.23	NaN	NaN	Not Detected
Plant 5	New Petiole	Negative	28.22	NaN	NaN	Not Detected
Plant 5	New Petiole	Negative	28.41	NaN	NaN	Not Detected
Plant 5	New Petiole	Negative	27.86	NaN	NaN	Not Detected
Plant 5	New Petiole	Negative	30.06	NaN	NaN	Not Detected
Plant 5	New Petiole	Negative	28.31	NaN	NaN	Not Detected

Table 2: Amplification results among samples taken from plants with no known infection by HLVd

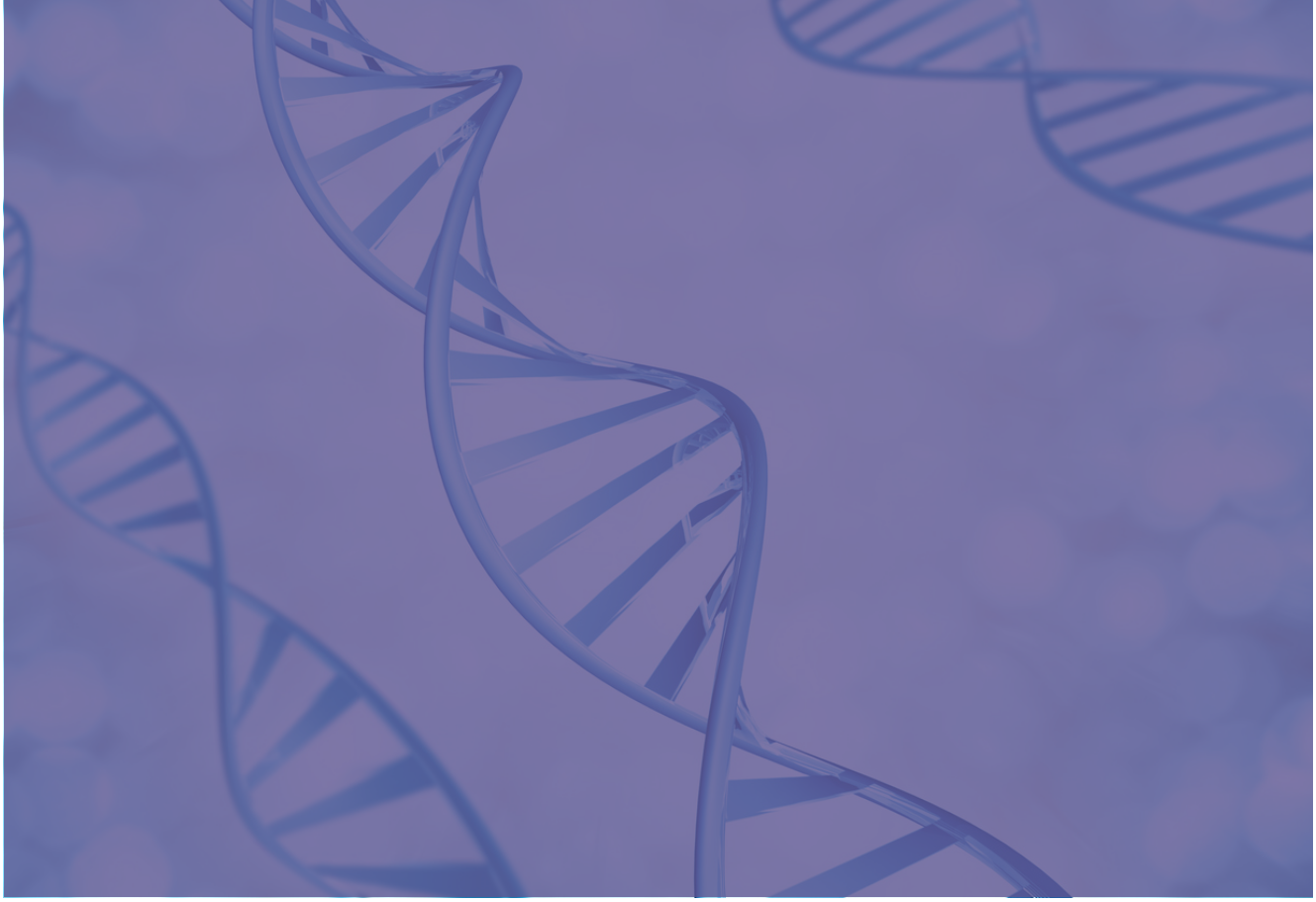
Plant 6	Root	Negative	25.23	NaN	NaN	Not Detected
Plant 6	Old Petiole	Negative	31.0	NaN	NaN	Not Detected
Plant 6	Old Petiole	Negative	28.86	NaN	NaN	Not Detected
Plant 6	Old Petiole	Negative	28.98	NaN	NaN	Not Detected
Plant 6	Old Petiole	Negative	29.43	NaN	NaN	Not Detected
Plant 6	New Petiole	Negative	32.27	NaN	NaN	Not Detected
Plant 6	New Petiole	Negative	30.44	NaN	NaN	Not Detected
Plant 6	New Petiole	Negative	32.88	NaN	NaN	Not Detected
Plant 6	New Petiole	Negative	33.28	NaN	NaN	Not Detected
Plant 6	New Petiole	Negative	33.30	NaN	NaN	Not Detected
Control- non-infected	-	Not-Infected	27.11	NaN	NaN	Not Detected
Control-Infected	-	Infected	28.1	15.47	18.29	1019064.54
Non-Template Control	-	NTC	NAN	NAN	NAN	Not Detected

Table 3: Amplification results from plants assayed in the follow-up study. Only plants with at least one sample testing positive for HLVd are shown.

Plant	Tissue	Result	Control CT Value	HLV Target 1 CT Value	HLV Target 2 CT Value	Viroid Load (copies per microliter)
Plant 1	Petiole	Positive	25.05	17.22	17.69	294348.14
Plant 1	Petiole	Negative	26.93	NaN	NaN	NaN
Plant 1	Petiole	Positive	22.31	18.48	19.22	120374.98
Plant 1	Petiole - pool	Positive	26.73	21.04	22.33	19568.53
Plant 1	Root	Positive	26.69	22.44	24.05	7245.79
Plant 1	Root	Positive	25.09	24.38	25.58	1828.89
Plant 1	Root	Positive	27.13	23.95	25.08	2481.49
Plant 1	Root- pool	Positive	25.05	17.22	17.69	294348.14
Plant 2	Petiole	Negative	27.25	NaN	NaN	NaN
Plant 2	Petiole	Negative	25.92	NaN	NaN	NaN

Table 3: Amplification results from plants assayed in the follow-up study. Only plants with at least one sample testing positive for HLVd are shown.

Plant 2	Petiole	Negative	26.43	NaN	NaN	NaN
Plant 2	Petiole - pool	Negative	24.39	NaN	NaN	NaN
Plant 2	Root	Positive	25.08	23.6	25.1	3181.12
Plant 2	Root	Positive	28.19	23.32	24.18	3880.38
Plant 2	Root	Positive	27.57	26.23	30.03	492.07
Plant 2	Root- pool	Positive	22.89	20.96	23.68	20711.61
Plant 3	Petiole	Positive	25.07	17.74	18.18	203516.75
Plant 3	Petiole	Positive	23.16	16.48	17.18	497651.41
Plant 3	Petiole	Negative	26.01	NaN	NaN	NaN
Plant 3	Petiole - pool	Positive	23.08	15.83	16.53	789317.39
Plant 3	Root	Positive	28.73	21.58	22.85	13339.31
Plant 3	Root	Positive	30.02	24.2	26.44	2078.09
Plant 3	Root	Positive	29.48	23.36	24.98	3771.79
Plant 3	Root- pool	Positive	26.49	24.03	25.72	2344.53



At TUMI Genomics we recommend consistent and periodic testing to effectively control for HLVd.

In upcoming publications, we will provide Standard Operating Procedures (SOPs) that will help professional growers unlock their grow potential.

info@tumigenomics.com

320 E. Vine Dr, Suite 213 - Fort Collins, CO 80524

(720) 807-8864

www.tumigenomics.com