TITER AND DISTRIBUTION OF HOP STUNT VIROID AND HOP LATENT VIROID INFECTING HOPS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of NINH LIEN KHUU find it satisfactory and recommend that it be accepted.

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Abstract

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Hop stunt viroid and Hop latent viroid have proven recalcitrant to attempts to eliminate them. This project was undertaken to understand the localization of the two viroids within the hop cultivars, 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' under cold and dark stress with the goal of improving viroid elimination therapies. With micro-shoot tip culture, HSVd elimination was successful in 4% to 9% of regenerated meristems and HLVd elimination was successful 19% to 54% in 'Comet' and 'Chinook,' respectively. HLVd was not eliminated in 'Canadian Red' and 'Wuerttemberger.' *In situ* hybridization showed that HSVd was present in the ground meristem, but not the tunica, while HLVd was not present in the first ~500 µM in 'Comet' and 'Chinook.' HLVd was present in the ground meristem of 'Wuerttemberger.' This localization may explain the difficulty of eradicating the viroids from infected hops. Dark therapy increased viroid titers and is not advisable. Though cold therapy decreased viroid titers, meristems failed to regenerate. Unexpectedly, sourcing shoots from plants during spring that experienced a natural dormancy period showed an increase in HLVd elimination (ranging from 50% to 100%) in cvs. 'Cascade,' 'Centennial,' 'Triumph,' and 'Wuerttemberger.' This increase in HLVd elimination, accompanied by a 13-fold increase in HSVd elimination, was observed in 'Comet' plants that experienced an artificially induced spring.

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Dedication

Parents often make sacrifices so that their children can have a better future. I would like to dedicate this thesis in memory of my father, Quang Khuu, who has sacrificed much so that his children could live the American Dream. Like millions of immigrants before them, he and my mother, Tina, came to America with little to their name and worked hard to provide for me and three elder siblings, Nhan, Vi, and Uyen. My parents have always led by example – honesty, humility, generosity, and perseverance. It is by these principles that I have lived my life.

I know that whatever challenges I may face in life pale in comparison to the struggles my father and mother experienced in their lives – it is this knowledge that has allowed me to overcome adversity while maintaining a sense of optimism. I know he would be proud of my accomplishments, and I hope I can help be a role model to my nephew, Tyler, and nieces, Nikki and Skylar.

CHAPTER ONE: TITER AND LOCALIZATION OF HOP STUNT VIROID AND HOP LATENT VIROID

Abstract:

Micro-shoot tip therapy has been employed for nearly 70 years to free plants of viral infections. Unfortunately, in some host/virus combinations, elimination may be unsuccessful, or elimination rates are exceedingly low. Eradication of Hop latent viroid and Hop stunt viroid from hops falls into the latter category. To improve elimination therapies, *in situ* hybridization and relative quantification with RT-qPCR were performed to understand the localization and distribution of the two viroids in four hop cultivars, 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger.' *In situ* hybridization results provided evidence that neither viroid can replicate in the tunica, but HSVd is able to replicate within the ground meristem in the first 200 µm of the meristem. In order to free infected hops from viroid infection, it appears necessary to excise meristems of ~0.1 mm or shorter with just the tunica and 1 to 2 leaf primordia.

Introduction

Viral infection is a complex interaction involving a compatible host and infectious agent (Hadidi et al., 2017; Hull, 2014; Taiz et al., 2014). Infections may be spread by a vector – arthropod, nematode, fungus, or parasitic plant (Hadidi et al., 2017; Hull, 2014). Humans may also act as vectors of plant viruses and viroids through horticultural practices. Activities such as grafting (new grafts or topworking over old rootstock), importation of propagules (seeds, bulbs, rhizomes, etc.), and pruning may spread viruses to new areas (Hadidi et al., 2017; Hull, 2014; Mahaffee et al., 2009).

Fortunately, not all viral infections result in disease (Hadidi et al., 2017). These "latent" infections are characterized by lack of obvious symptoms or measured declines in growth or

yield of the host. Unfortunately, this “latent” status is not static and may be affected by changes in host species, host cultivar, temperature, abiotic stress, age of the host, or synergistic interactions with other viruses/pathogens (Barbara et al., 1990; Sanger & Ramm, 1975; Semancik & Szychowski, 1994). Two important viroids infect hops in the United States: Hop stunt viroid (HSVd) and Hop latent viroid (HLVd) (Mahaffee et al., 2009). As the names suggest, infection with the former in susceptible cultivars results in stunting of the plant along with decreased branching while infection with the latter is predominately asymptomatic. However, studies that compare plants of the same cultivar with and without HLVd infection provide evidence that it is capable of decreasing alpha-acid and beta-acid acid content in a cultivar-dependent manner (Barbara et al., 1990). In addition, infection leads to changes in other secondary metabolite concentrations and overall profile, potentially altering the flavor characteristics of the end product: beer (Mahaffee et al., 2009; Neve, 1991; Rybacek, 1991).

Unlike bacterial or fungal infections, preventive sprays or curative sprays do not exist for viral infections in the field (Mahaffee et al., 2009; Neve, 1991). Plants do not possess an active immune system to combat viral infections through production of antibodies – once infected, plants have to rely upon RNA-silencing to combat viral pathogens (Honjo et al., 2020). The best tools against viruses in plants is genetic resistance, economic tolerance for pathogen injury, and starting with certified planting material (Hadidi et al., 2017; Hull, 2014; Mahaffee et al., 2009; Skoric, 2017).

In addition to pest-resistance, hop growers must also consider agronomic traits: alpha-acid content, yields, growth habit, maturation, fungal-resistance, and secondary metabolite profile. With this in mind, the greatest practical importance falls upon starting with certified

planting stock. The first question is what is certified stock? At its most basic, certified stock is propagation material that is free of targeted graft-transmissible pathogens – which pathogens are of interest are dependent upon the crop and region (*Natl. Clean Plant Netw.*, 2021). In the United States, the National Clean Plant Network (NCPN) is an organization composed of 22 centers dedicated towards production of certified propagation material (also known as Generation 1, or G1) for clonally propagated plants (*NCPN.*, 2021). Part of Washington State University, the Clean Plant Center Northwest (CPCNW, located in Prosser, Washington) is the sole center focused on production certified hops within the network (CPCNW, 2021).

The next question is how is certified stock for hops sourced? Propagation material (straps, tissue culture plants, plugs, etc.) of the cultivars of interest are sent to the CPCNW for cultivation and subjected to virus testing. RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) is performed for targeted viruses and next-generation sequencing is performed to screen for both targeted viruses and new viruses. If the plant material passes the virus panel, the plants qualify as G1 plants for distribution to stakeholders (nurseries, growers, etc.). In the situation where the testing indicates that plants are infected, virus/viroid elimination therapies are necessary to separate the pathogen from the host.

Increased access to viral diagnostic tools and utilization of certified planting stock has largely led to the successful control of HSVd in the United States after its first detection in 2004 (Kappagantu et al., 2017). Interestingly, HLVd-infected hops are the rule rather than the exception. Studies that have assessed the effects of fungal, virus or viroid infection in hops have often used plants that are already infected with HLVd – possibly confounding results (Kappagantu et al., 2017; Momma & Takahashi, 1983). The reason for the tolerance of HLVd in

the certified stock program is two-fold: (1) infection is thought to be asymptomatic in most cultivars and (2) HLVd has proven recalcitrant to current elimination therapies (See Appendix I for a review of viroid elimination therapies) (Adams et al., 1996; Grudzińska & Solarska, 2005; Morton et al., 1993).

The goal of this project is to improve viroid-elimination therapies by understanding the fundamental properties of viroid infection in hops: accumulation of viroids and localization in tissues near the growing tip. The current hypothesis is that the viroids can invade the tunica layers preventing efficient viroid-elimination by only meristem-tip culture. This is important as excision of the apical meristem and surrounding leaf primordia of ~0.5mm in length is the current method of viral elimination for many crops, including hops (Hadidi et al., 2017; Al. Panattoni & Triolo, 2010; Valero et al., 2003). To achieve this goal, the objective of this section of the project is to localize and quantify the titer of HSVd and HLVd in the shoot tips, first internode, first node petiole, and first node leaf of potted plants. The shoot tips are of primary focus as this is the tissue commonly used to source meristems for production of G1 plants. Sampling of the other tissues allows for visualization of viroid replication dynamics distal from the shoot tip. Additionally, petiole and stem tissue act as positive controls for shoot-apical meristems *in situ* hybridization as these tissues are usually sampled for viroid detection.

Methods

Plants

Plants of cv. 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger,' were cultivated in a growth chamber in one-gallon pots at 25°C with a 16-hour photoperiod at approximately 150 PPFD provided by LED lights (General Electric, Boston, MA).

Tissue culture plants of the same cultivars were established by surface sterilizing nodal segments for 20 mins with 10% commercial bleach (v/v) and multiplied on MS+0.5 BA media (See Appendix III for recipes and protocols). Plants were cultivated at 21°C with a 16h photoperiod at 70 PPFD provided by LED lights (General Electric).

Sampling of tissue and RNA extraction

Tissue from the shoot tip, first internode, first petiole, and first leaf were sampled for RNA extraction using a Spectrum Plant Total RNA (STRN250-Kit, MilliporeSigma, Burlington, MA) extraction kit for cv. 'Comet.' As the focus of the project is on improving viroid elimination, only shoot tips were extracted for cvs. 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' because this is the tissue that will sourced for micro-shoot tip therapy.

Preceding DNase treatment, RNA concentration was quantified with the RNA Broad Range Assay Kit (Q10211, Thermo Fisher Scientific, Waltham, MA) on a Qubit 4 Fluorometer (Thermo Fisher Scientific). Then, the RNA was DNase-treated using TURBO DNA-free kit (AM1907, Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitation of viroid titer with reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Each reaction consisted of 10 µL of 2x qScript XLT 1-Step RT-qPCR ToughMix (95132, Quantabio, Beverly, MA), 400 nM each of forward and reverse primers, 100 nM dual-labeled FAM-BHQ-1 probes (LGC Biosearch Technologies, Novato, CA), and 2 µL of DNase-treated RNA with a final reaction volume of 20 µL. Each sample was run in triplicate on a CFX96 real-time thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following conditions: reverse transcription for 15 mins at 50°C and initial denaturation for 2 mins at 94°C followed by 40 cycles of 94°C for 15 seconds and 63°C for 60 seconds (For primer and probe sequences, see Appendix II). Viroid titer was calculated using ΔΔCt method for relative quantification using CFX Maestro (Version 4.1.2433.1219, Bio-Rad Laboratories) normalized to the Time-Zero comet shoot tip.

In situ *hybridization for HSVd and HLVd in hop tissue*

Localization by colorimetric *in situ* hybridization using commercially available BaseScope (Advanced Cell Diagnostics, Newark, CA). For cv. 'Comet' tissue from the adventitious bud, rhizome, shoot tip, first internode, first petiole, and first leaf were sampled. For cvs. 'Chinook,' 'Canadian Red,' and 'Wuerttemberger,' only the shoot tip was sampled for *in situ* hybridization because the focus of this project is on the tissue that will be regenerated for micro-shoot tip therapy. Tissue was sampled from plants growing in one-gallon pots in a growth chamber at 25°C with a 16h photoperiod with 150 PPFD provided by LED lights.

Tissue was fixed for 24-72 hours in the following solution: 1.25% Glutaraldehyde (V/V) (16320, Electron Microscopy Sciences [EMS], Hatfield, PA), 2% Paraformaldehyde (V/V) (15710, EMS), 1x Phosphate buffered saline (PBS) (P5493-1L, MilliporeSigma), 0.025% Tween 20 (ACC 00528/0030, Agdia, Elkhart, IN). Tissue was rinsed three times with 1x PBS for before being

dehydrated in successive washes of ethanol in water (30%, 50%, 70%, 80%, 95%, 100%, 100%; V/V) for 30 minutes each and dehydrated overnight in 100% ethanol. Tissue was rinsed in 100% ethanol before clearing tissue with Histoclear II (64111-04, EMS) in successive washes of 1:3, 1:1, and 3:1 of Histoclear II: absolute ethanol for 8-16 hours per wash. Tissue was cleared with 100% Histoclear II before permeabilization with Histoplast PE (8330, Thermo Fisher Scientific) in successive infiltrations of 1:3, 1:1, and 3:1 Histoclear II: Histoplast PE (V/V) for 24 hours each at 56°C. Tissue was infiltrated with paraffin with three incubations of 100% Histoplast PE at 56°C for 24-48 hours each. Tissue was embedded in 7x7x5mm molds (22363552, Fisher Scientific, Waltham, MA) and refrigerated at 4°C until sectioning. Approximately 8-10um sections were made using a HM 355S automatic rotary microtome (Thermo Fisher Scientific) and sections were baked onto TruBond 380 adhesion slides (63700-W1, EMS) at 56° for at least one hour. Sections were de-paraffinized in 4 washes of Histoplast PE for 30 minutes each with a final wash with absolute ethanol to dry. De-paraffinized sections were circumscribed with a hydrophobic barrier using an ImmEdge Pen (H-4000, Vector Laboratories, Burlingame, CA) and wet with Reverse-Osmosis (RO) water after the barrier set. Sections were then hybridized following the instructions in the BaseScope Red Assay with probes for HSVd, HLVd, and negative control. Slides were dried at 56°C for 15 minutes before mounting with EcoMount (EM897L, BioCare Medical, Pacheco, CA). Dried, mounted slides were imaged at 100-200x magnification with a DM1000 LED (Leica Microsystems, Wetzlar, Germany).

Results

Viroid titer in vivo *quantified by RT-qPCR*

Both HSVd and HLVd decreased towards the apical meristem (Figure 1.1). Overall, internode, petiole, and leaf tissue accumulate higher levels of HSVd than the shoot tip and HLVd accumulates more than HSVd for cv. 'Comet'. Compared to 'Comet,' 'Chinook' has a lower titer of HSVd and HLVd at one and two months in the shoot tips (HSVd at two months was not statistically significant) (Figure 1.1). Additionally, 'Canadian Red' and 'Wuerttemberger' also display a significant reduction in HLVd in the shoot tips compared to 'Comet.'

Viroid titer in vitro *quantified by RT-qPCR.*

HSVd and HLVd tiers remained the same, except during month two, where an increase was seen across all cultivars (Figure 1.2). At time point zero and month three, 'Wuerttemberger' had significantly higher HLVd titer than the other three cultivars.

In situ *hybridization of HSVd and HLVd*

In both longitudinal and transverse sections, HSVd and HLVd was detected extensively in petiole and stem tissue in 'Comet' and 'Chinook' (Figures 1.3 and 1.4). Only the HLVd assay produced a signal in sections of 'Canadian Red' and 'Wuerttemberger' in a similar manner to 'Comet' and 'Chinook' (Figures 1.3 and 1.4). All living cells (epidermis, parenchyma, collenchyma, and vessels) showed presence of HLVd.

At time point zero, HSVd was detected in the ground meristem for cvs. 'Comet' and 'Chinook' (Figure 1.5). HSVd was not detected in 'Canadian Red' and 'Wuerttemberger' meristems at time point zero. HLVd was not detected in 'Comet,' 'Chinook,' and 'Canadian Red' at time point zero (Figure 1.5). In 'Wuerttemberger,' HLVd was detected in the ground

meristem at time-point zero. At one and three months, HSVd kept the same localization in ‘Comet’ but density was increased. HLVd showed an increase density and appeared to migrate closer to apical meristem in ‘Comet,’ but does not penetrate the ground meristem like HSVd does.

In the below-ground tissues, the adventitious buds of cv. ‘Comet’ showed presence of HSVd, but not HLVd (Figure 1.6). The rhizome showed extensive presence of both HSVd and HLVd in all living tissues (Figure 1.6).

In vivo viroid titer

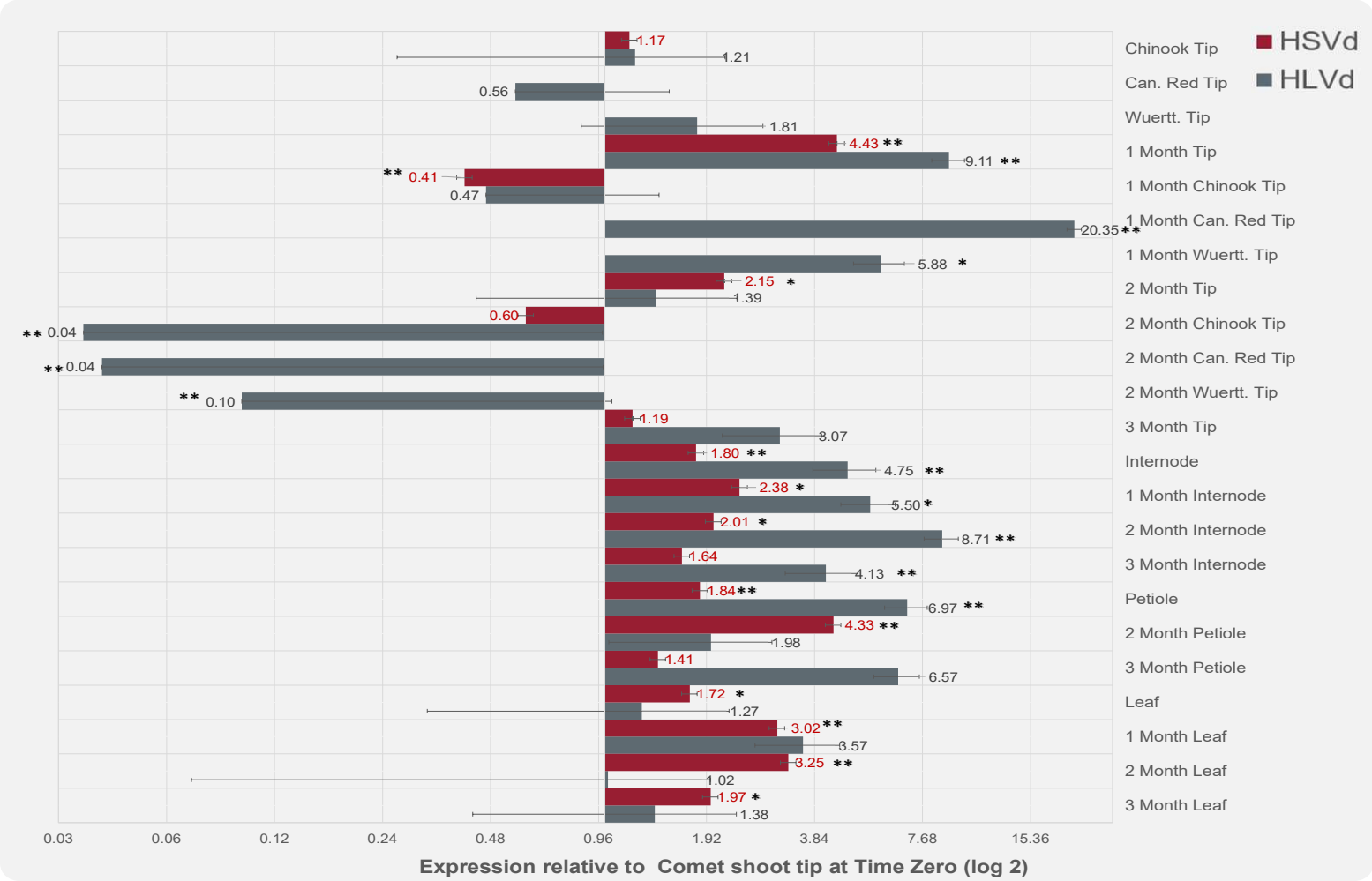

Figure 1.1. HSVd and HLVd expression normalized to 3 reference genes (POAC, TIP41, & YLS8) in potted plants grown of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' at 25°C for 1, 2, and 3 months. T-test for difference from baseline expression * p-value ≤ 0.05; ** p-value ≤ 0.01.

In vitro viroid titer

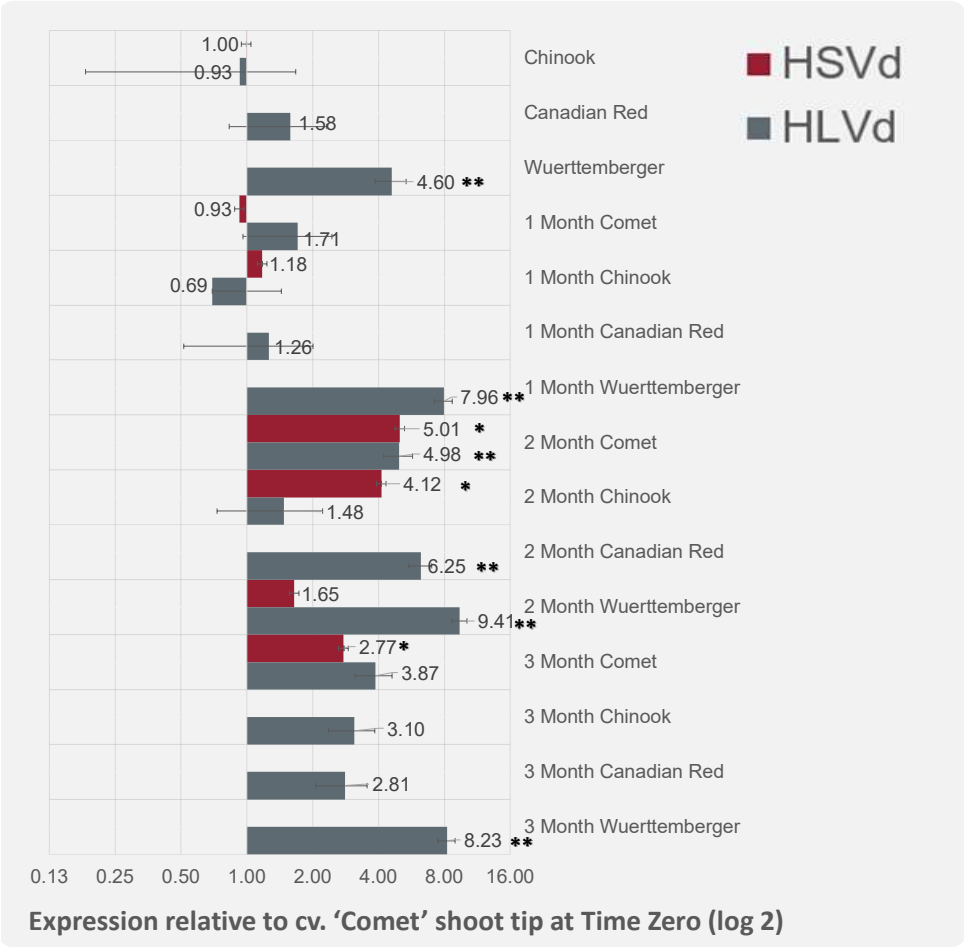

Figure 1.2. HSVd and HLVd expression normalized to 3 reference genes (POAC, TIP41, & YLS8) in tissue cultured plants of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' grown at 25°C for 1, 2, and 3 months. * p-value ≤ 0.05; ** p-value ≤ 0.01

In situ hybridization

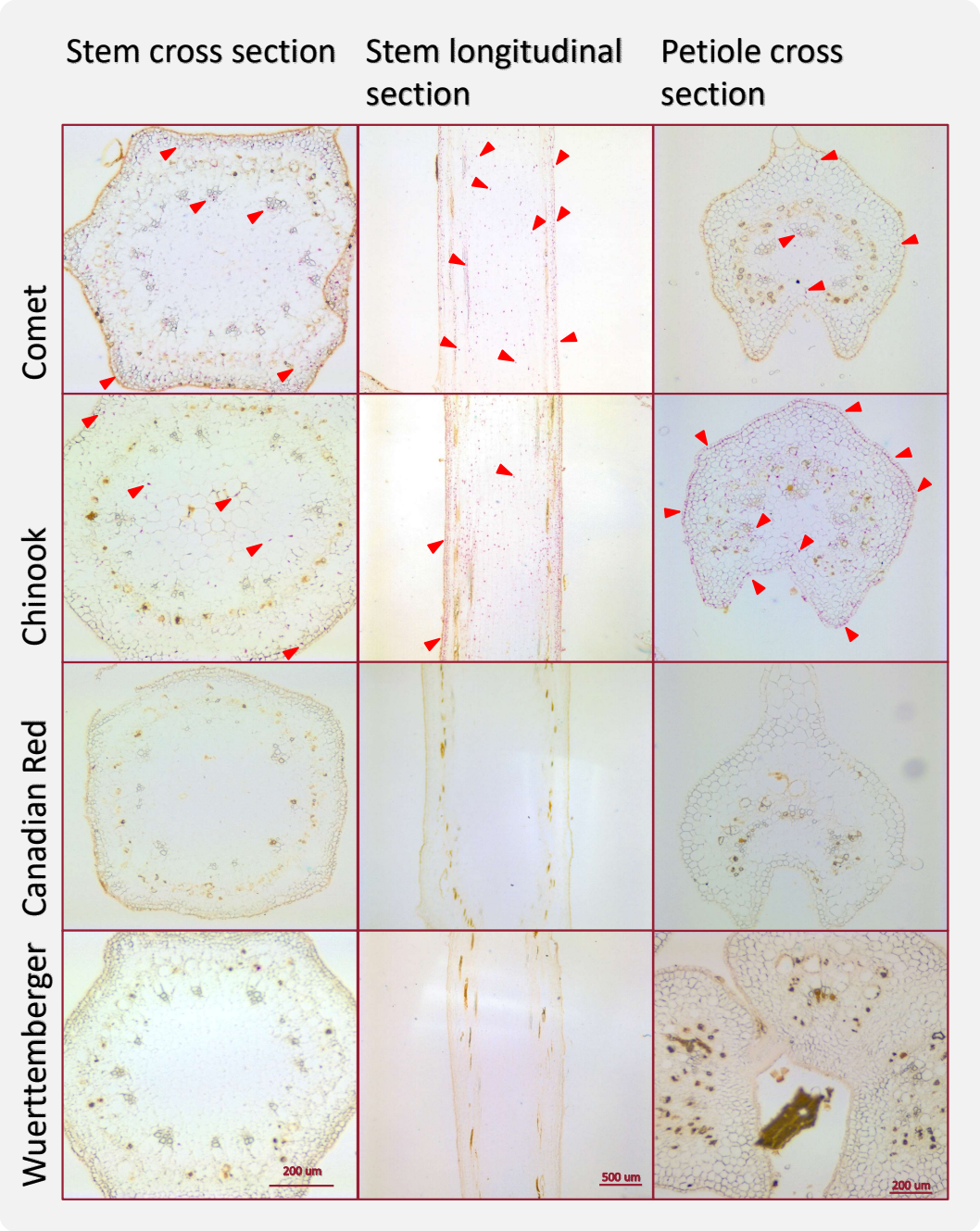

Figure 1.3. *In situ* hybridization for HSVd in stem and petiole tissue of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' grown at 25°C for 1 week. Red clusters (arrows for emphasis) indicate presence of viroid. 40x mag. in center and 100x on sides

In situ hybridization

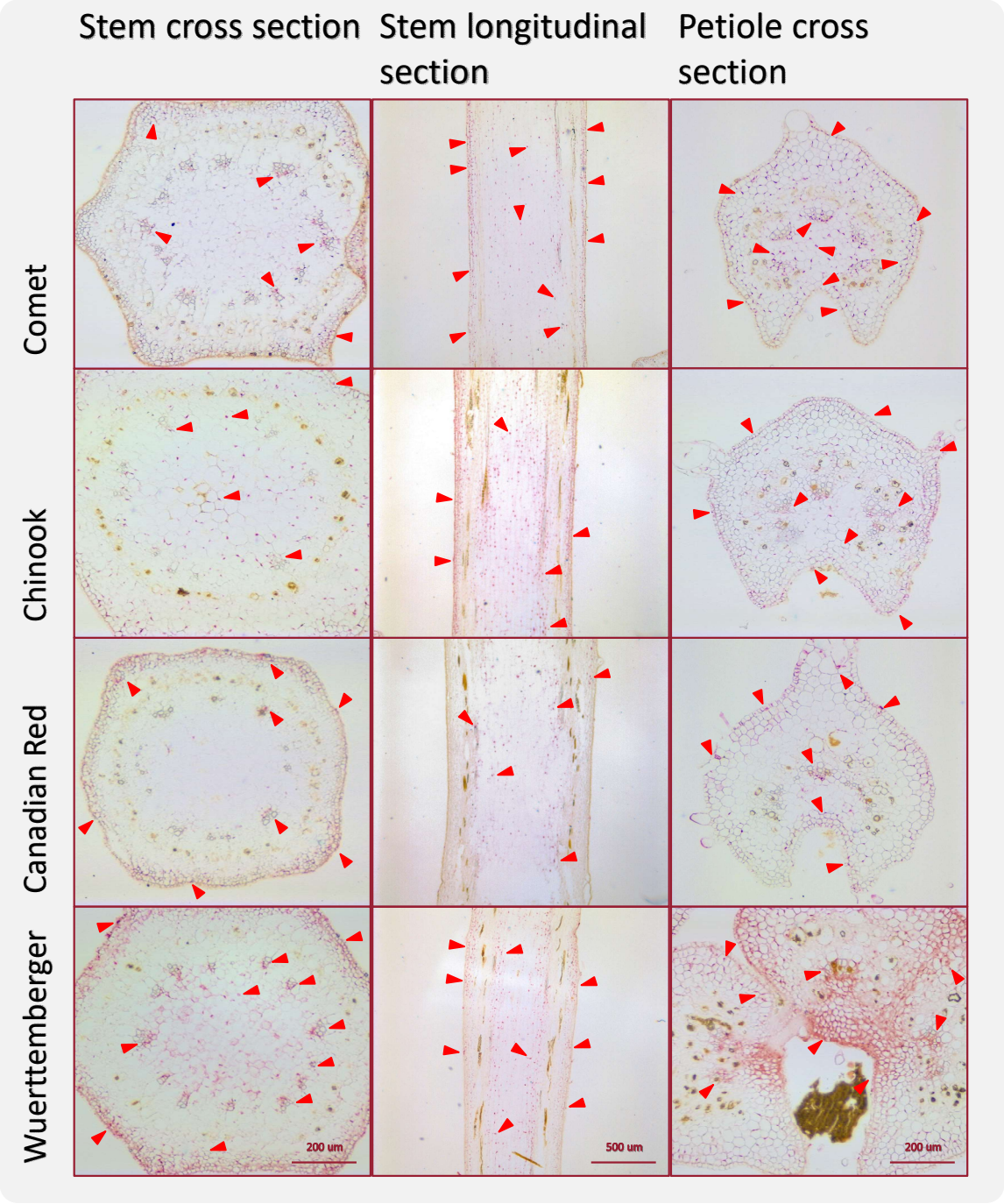

Figure 1.4. *In situ* hybridization for HLVd in stem and petiole tissue of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' grown at 25°C for 1 week. Red clusters (arrows for emphasis) indicate presence of viroid. 40x mag. in center and 100x on sides

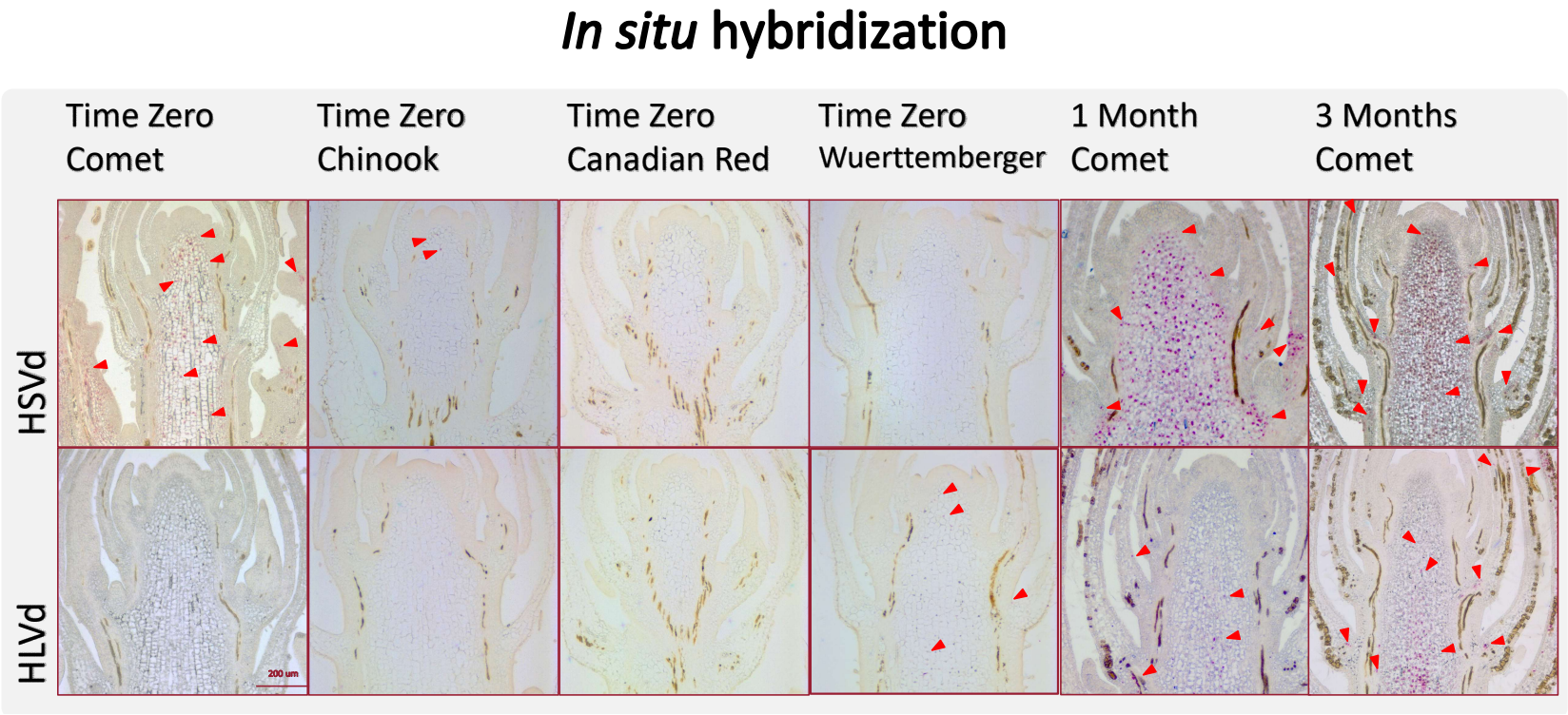

Figure 1.5. *In situ* hybridization for HSVd and HLVd in apical meristems at time zero, 1 month (cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger'), and 3 months of growth ('Comet'). Red clusters (arrows for emphasis) indicate presence of viroid. 100x magnification.

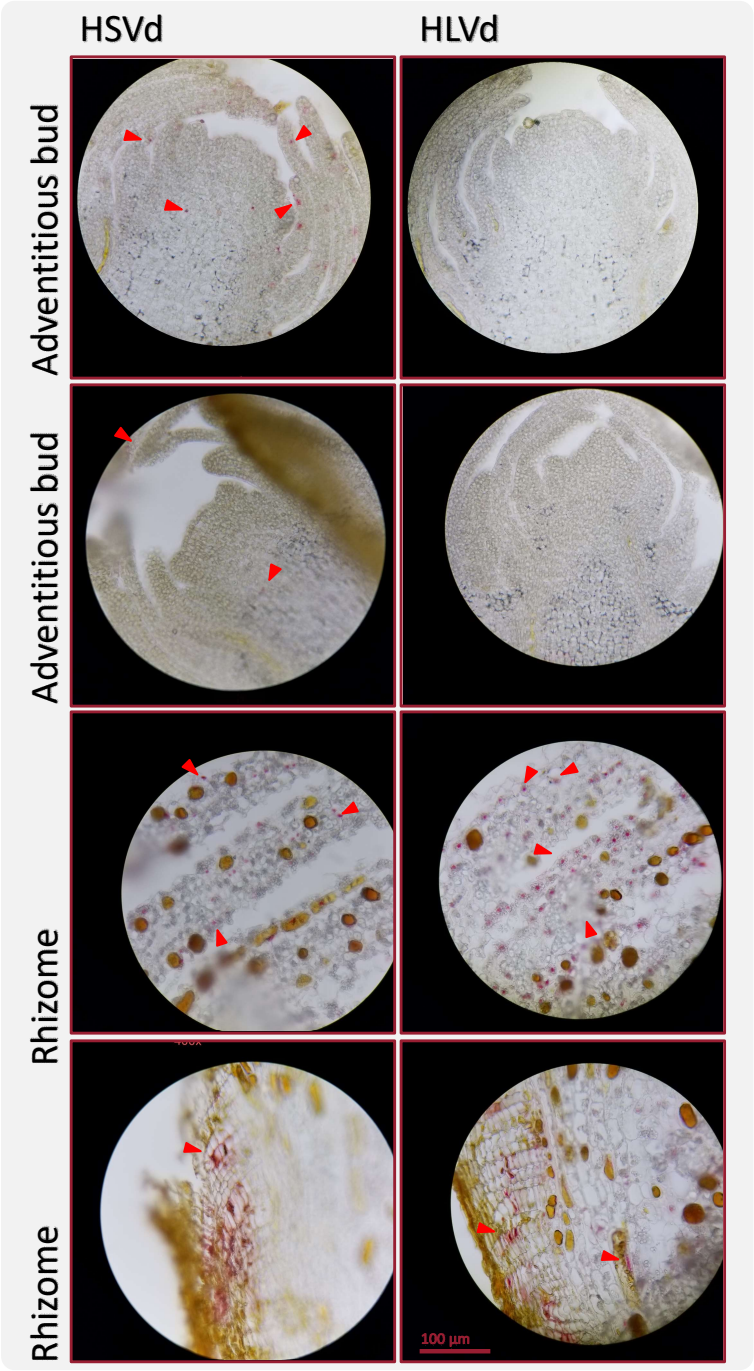

Figure 1.6. *In situ* hybridization for HSVd & HLVd in underground tissue of cv. 'Comet' grown at 25°C for 1 week. Red clusters (arrows for emphasis) indicate presence of viroid. 400x mag.

Discussion

In situ hybridization showed that both HSVd and HLVd accumulated at high levels in all living tissues in infected hops (Figures 1.3-1.6). The localization of the viroids also corresponded to their replication site within plant cells, the nucleus (Hadidi et al., 2017; Hull, 2014). In general, the RT-qPCR data supported the increase seen in the *in situ* hybridization in 'Comet' shoot tips at one and two months, but the data did not agree for month three (Figures 1.1 and 1.5). An explanation may be the accumulation of viroid RNA in bracts and tissue surrounding the apical meristem may be more consistent over time and could lead to an underestimation of viroid titer. Additionally, the increase in viroid accumulation in the stem, petiole, and leaf tissue over time may explain the efficiency at which HLVd is spread through mechanical inoculation reported by Adams et al. (1996). Assessment of viroid titers in older parts of the plant may be necessary to confirm this.

More interestingly, in the apical meristem, the two viroids exhibited differential localization. HSVd can replicate in the ground meristem (~200 µM) while HLVd was not detected until further down the stem (> 500 µM) (Figure 1.5) in cv. 'Comet.' This may account for the observations of earlier studies attempting to eliminate viroids from hops. Momma & Takahashi (1983) reported the rate of HSVd elimination was inverse to the size of the meristem excised. Only shoots regenerated from meristems of 0.2 to 0.3 mm in length tested negative for HSVd (Momma & Takahashi, 1983). Working with HLVd infected Polish hop cultivars, Grudzińska & Solarska (2005) reported HLVd elimination frequencies (~30% to 37.5%) with meristems 0.5 mm in length. Working with even longer shoots of 5.0 to 10.0 mm in length, Morton et al. (1993) reported 0% elimination of HLVd.

Though the length of the meristem appears to be important for viroid elimination, *in situ* hybridization data and RT-qPCR data for cv. 'Wuerttemberger' indicate that genotype may play an important role. Unlike 'Comet' and 'Chinook,' HLVd was detected in the ground meristem in 'Wuerttemberger' (Figure 5). Furthermore, relative quantification showed that 'Wuerttemberger' accumulated HLVd at higher levels in the shoot tips *in vitro* and *in vivo* (Figures 1.1 and 1.2). 'Canadian Red' also showed increased viroid accumulation.

In summary, the recalcitrance of HSVd and HLVd to micro-shoot tip elimination therapy is most likely due to their localization in the apical meristem. Though both viroids appear to be present co-infecting the same tissue types in the rhizome, stem and petiole, there is differential localization in the shoot apical meristem and adventitious buds. HSVd is present in the ground meristem, but not the tunica (Figure 1.5). Efforts to excise the upper most layers of the tunica (~0.1 mm in length) and regenerate them on axenic media may provide a source of HSVd-free 'Comet' and 'Chinook' hops. HLVd localization in 'Comet,' 'Chinook,' and 'Canadian Red' indicate it may be easier to eliminate than HSVd, but 'Wuerttemberger' may be more difficult due to HLVd's localization in the ground meristem for that cultivar. Furthermore, adventitious buds may also be a viable source for HSVd-free material if micro-shoot tip culture of actively growing shoots proves too difficult. Future work may include investigating how localization and titer changes in response to abiotic stress such as cold or dark treatment.

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CHAPTER TWO: EFFECT OF COLD OR DARK TREATMENT

Abstract:

The history of virus and viroid elimination therapy is rich in methodologies – many of which have led to successes, while some viroid/host combinations remain difficult to separate. Hop stunt viroid (HSVd) and hop latent viroid (HLVd) are recalcitrant to extant techniques to eradicate them from infected hops (*Humulus lupulus* L.). Cold and low-light treatment have shown to be successful in other viroid/host systems and may prove successful in hops. More importantly, understanding how the treatments affect the viroid localization and distribution sheds light upon the internal mechanisms that may be facilitating the improved viroid elimination success. Four hop cultivars, 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' were grown at 4°C or in the dark and RT-qPCR of the shoot tip, shoot, petiole, and leaves were performed to measure the viroid distribution under different treatments. This is the first report of RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) to validate viroid elimination therapy. *In situ* hybridization was performed on plants grown at 4°C to investigate the viroid localization. Dark treatment was found to increase viroid titers over time. Cold treatment reduced viroid titer but did not eliminate the viroid and the meristems that were excised from plants in cold treatment failed to develop. In conclusion, cold treatment was not successful and dark treatment may make elimination therapy more difficult.

Introduction:

Virus-elimination techniques rely upon separating virus-infected tissues from non-infected tissues (Barba et al., 2017; Hull, 2014; Paduch-Cichal & Kryczyński, 1987). This may be achieved by excising the meristematic dome and culturing it on nutrient media, or micro-grafting it onto a compatible rootstock (El-Dougdoug et al., 2010; Morel & Martin, 1952; Zhang et al., 2016). The efficiency of micro-shoot-tip culture is variable dependent not only upon the host species or cultivar, but also upon the specific research group (Barba et al., 2017). Working with the same cultivar, researchers have reported the contrasting efficiencies of tissue regeneration and virus-elimination success. This may reflect different cultural conditions (media formulation, chemical sources, growth chamber consistency, idiosyncrasies of labs, etc.), sensitivities of viral diagnostics and/or technical skills at manipulating sub-millimeter-sized tissue (Barba et al., 2017; Paduch-Cichal & Kryczyński, 1987). Furthermore, some specific virus-host combinations have proven difficult to separate solely with micro-shoot-tip culture and success rates are low (Barba et al., 2017; Hull, 2014; Matsushita & Shima, 2015).

Therefore, other therapies alone, or in conjunction with micro-shoot-tip culture have been attempted to increase elimination rates (Savitri et al., 2013). A common approach has been the application of chemotherapy utilizing anti-viral compounds, such as ribavirin, amantadine, or thiouracil, to achieve viroid eradication or increase efficiency of micro-shoot-tip therapy (El-Dougdoug et al., 2010; Paduch-Cichal & Kryczyński, 1987; Savitri et al., 2013).Unfortunately, a common drawback of applying chemotherapy is the potential for phytotoxicity; there is a fine balance (milligrams per liter) between eradication of the pathogen and death of the host tissue (El-Dougdoug et al., 2010; Simpkins et al., 1981). Additionally, the

diagnostic assays used to verify the efficacy of virus-elimination have higher limits of detection than RT-qPCR leading to potential overestimation of therapies (Hollings & Stone, 1970; Kuhn et al., 2019; Papayiannis, 2014; Running et al., 1996; Stace-Smith & Mellor, 1970). This is especially a problem for chemotherapy as it may reduce titers to levels that may not be detectable by RNA-hybridization or RT-PCR, but the virus could still be present and re-accumulate with time (Sanger & Ramm, 1975). However, chemotherapy may be necessary if all other means have been exhausted or the specific germplasm is of great economic/horticultural importance. To date, no successful attempts of eradicating Avocado sunblotch viroid (ASBVd) have been recorded (Kuhn et al., 2019; Running et al., 1996; Suarez et al., 2005, 2006).

Prior to implementation of micro-shoot tip culture and chemotherapy, thermotherapy was the predominant treatment applied by scientists to obtain “virus-free” germplasm with trials dating to nearly a century ago (Kassanis, 1950). Therapies generally involved growing infected plants in containers within controlled environmental chambers under elevated (>37°C) temperatures for an extended period (30 days – 12 months) (Barba et al., 2017; Duran-Vila et al., 1988). Macro-shoots (>1cm) were excised onto rootstocks and assayed for viral infection. This rudimentary technique did not work for all virus/host combinations and improvements in viral diagnostic technologies revealed that some successes were merely infections below the detection threshold (Barba et al., 2017; Desvignes, 1986; Hosokawa, Otake, Ohishi, et al., 2004). Combining thermotherapy and micro-shoot tip therapy generally improved virus elimination therapies (Postman et al., 2005; Valero et al., 2003). Unfortunately, the previous studies that have trialed thermotherapy have not been successful (Barba et al., 2017; Schnölzer et al., 1985).

The key for viroid elimination may lie on the other end of the thermotherapy spectrum: cold treatment. Direct support for this has been provided in recent *in situ* hybridization data published by Zhang et al. (2015) and Zhang et al. (2016) working with two CSVd (Chrysanthemum stunt viroid) in *Argyranthemum* cultivars, 'Border Dark Red' and 'Yellow Empire'. For both studies, localization was performed using DIG (digoxigenin)-11-UTP probes to visualize CSVd invasion of the apical meristem and β-1,3-glucan antibodies to visualize callose deposition. These two studies provided three key insights: (1) the ability of a viroid to penetrate the meristem varied by cultivar (with cv. 'Yellow Empire' being susceptible), (2) this susceptibility may be related to viroid-dependent callose deposition in the plasmodesmata (cv. 'Yellow Empire' failed to deposit callose in response to CSVd infection), and (3) cold therapy may expand the region of non-infected tissue in the meristem (for cv. 'Border Dark Red,' but no effect was observed for cv. 'Yellow Empire'). As mentioned previously, Zhang et al. (2016) subjected the potted *Argyranthemum* plants to 1-12 months of cold treatment at 5°C before excising 0.2mm meristems and out of the 101 plants that regenerated, two explants were CSVd-negative – which is an improvement over their 0% success rate without cold treatment. In addition to cvs. 'Border Dark Red' and 'Yellow Empire,' Zhang et al. (2015) visualized CSVd localization in 'Border Pink' and 'Butterfly,' which were similar to 'Border Dark Red' at greenhouse conditions.

Work by Zhang et al. (2015 & 2016) provide a basis for understanding the positive effects of cold treatment for viroid-elimination documented in older studies. Paduch-Cicharl & Kryczynski (1986) reported an 18.5% CSVd-elimination rate for cv. 'Bonnie Jean' plants after 6 months of treatment at 5°C in pots followed by excision of apices with 1-2 leaf primordia.

Furthermore, the same authors reported other chrysanthemum viroids could also be eliminated using cold treatment. Approximately 84% and 80% elimination efficiencies were reported by Paduch-Cicharl & Kryczynski (1986) for Chrysanthemum chlorotic mottle viroid (ChCMVd) from cv. 'Deep Ridge' and Cucumber pale fruit viroid (CPFVd) from cv. 'Mistletoe,' respectively, using the same cold treatment regime described for cv. 'Bonnie Jean'. Beyond chrysanthemums, the authors reported a 45% elimination rate for PSTVd-infected potatoes by using cold therapy. Treatment of 5-7°C for six months facilitated viroid elimination in three PSTVd-infected potato cultivars compared to failure in control and 3 months of treatment (for two of the cultivars, only tubers were treated and not actively-growing plants). Other studies have reported similar results to Paduch-Cicharl & Kryczynski (1986) for PSTVd elimination in potato. Lizarraga et al. (1980) reported 29% and 54% elimination efficiencies for potted plants and *in vitro* plantlets of *Solanum tuberosum* x *S. phuerja* clone BR 63.5 when grown under cold conditions (5-8°C) for longer than 4 months (opposed to 0% elimination when grown at 25°C). For a more detailed look of the historical attempts at viroid elimination (cultivars, viroids, methodologies, diagnostic techniques, and success rates), see Appendix Table 1.1. Overall, cold therapy does appear to be more consistent in methodology and success rate than thermotherapy for viroid elimination.

In addition to antiviral chemicals, heat therapy, and cold therapy – light regimes may play a pivotal role in viroid accumulation. Authors in the 1970s observing symptoms on cv. 'Rutgers' tomato noted albinism seen only in PSTVd-infected plants (Yang & Hooker, 1977). Experiments that increased temperatures and extended the photoperiod to continuous illumination showed that these symptoms could be worsened. Furthermore, viroid titers were

also observed to increase (Yang & Hooker, 1977). Working with Chrysanthemums, Bachelier et al. (1976) reported a similar pattern of improved viroid detection and infectivity at higher light intensities and temperatures for CSVd-infected cv. 'Mistletoe.' Based on their data, the authors of both studies recommended culturing indicator plants at higher temperatures and light intensities to improve the likelihood of detection (Yang & Hooker, 1977; Bachelier et al., 1976). If increasing temperatures and light intensities worsens viroid infection, growing plants at lower temperatures and under less light may be able to reduce titers and, hopefully, facilitate viroid elimination therapy when combined with micro-shoot tip culture. Taking matters to the extreme, the effects of etiolation have not been documented viroid titer – it is worth investigating the effects of culturing viroid-infected plants in the dark.

Synthesizing the last 50 years' worth of experiments in viroid eradication, two key therapies stand out as promising for augmenting micro-shoot tip culture of hops: cold therapy and dark therapy (Appendix Table A.1.1). The hypothesis is HLVd and HSVd should behave similarly to other viroids and preferentially decrease replication under cold and dark culture conditions. With the overall goal of improving elimination efficiencies for HLVd and HSVd in hops, two goals of this experiment are to both reduce viroid titer and to change localization that facilitates viroid elimination. Towards this end, in addition to tracking regeneration and elimination success, RT-qPCR and *in situ* hybridization will be performed to illuminate how changes in cultural practices influence viroid titer and localization, respectively, in hops.

Methods:

Plants

Plants of cv. ‘Comet,’ ‘Chinook,’ ‘Canadian Red,’ and ‘Wuerttemberger,’ were grown in one-gallon pots in a growth chamber at 4°C with a 16-hour photoperiod at 150 PPFD provided by LED lights (General Electric, Boston, MA).

Tissue culture plants of the same cultivars were established by surface sterilizing nodal segments for 20 mins with 10% commercial bleach (v/v) and multiplied on MS+0.5 BA media (See Appendix III for recipes and protocols). Plants were cultivated at (1) 21°C with a 16h photoperiod at 70 PPFD provided by LED lights (General Electric) (2) 21°C in complete darkness or (3) 4°C with a 16-hour photoperiod at 70 PPFD provided by LED lights (General Electric).

Sampling of tissue and RNA extraction

Tissue from the shoot tip, first internode, first petiole, and first leaf were sampled for RNA extraction using a Spectrum Plant Total RNA (STRN250-Kit, MilliporeSigma, Burlington, MA) extraction kit for cv. ‘Comet.’ As the focus of the project is on improving viroid elimination, only shoot tips were extracted for cvs. ‘Chinook,’ ‘Canadian Red,’ and ‘Wuerttemberger’ because this is the tissue that will sourced for micro-shoot tip therapy.

Preceding DNase treatment, RNA concentration was quantified with the RNA Broad Range Assay Kit (Q10211, Thermo Fisher Scientific, Waltham, MA) on a Qubit 4 Fluorometer (Thermo Fisher Scientific). Then, the RNA was DNase-treated using TURBO DNA-free kit (AM1907, Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitation of viroid titer with reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Each reaction consisted of 10 µL of 2x qScript XLT 1-Step RT-qPCR ToughMix (95132, Quantabio, Beverly, MA), 400 nM each of forward and reverse primers, 100 nM dual-labeled FAM-BHQ-1 probes (LGC Biosearch Technologies, Novato, CA), and 2 µL of DNase-treated RNA with a final reaction volume of 20 µL. Each sample was run in triplicate on a CFX96 real-time thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following conditions: reverse transcription for 15 mins at 50°C and initial denaturation for 2 mins at 94°C followed by 40 cycles of 94°C for 15 seconds and 63°C for 60 seconds (For primer and probe sequences, see Appendix II). Viroid titer was calculated using ΔΔCt method for relative quantification using CFX Maestro (Version 4.1.2433.1219, Bio-Rad Laboratories).

In situ *hybridization for HSVd and HLVd in hop tissue*

Localization by colorimetric *in situ* hybridization using commercially available BaseScope (Advanced Cell Diagnostics, Newark, CA). For cv. 'Comet' tissue from the adventitious bud, rhizome, shoot tip, first internode, first petiole, and first leaf were sampled. For cvs. 'Chinook,' 'Canadian Red,' and 'Wuerttemberger,' only the shoot tip was sampled for *in situ* hybridization because the focus of this project is on the tissue that will be regenerated for micro-shoot tip therapy. Tissue was sampled from plants growing in one-gallon pots in a growth chamber at 25°C with a 16h photoperiod with 150 PPFD provided by LED lights.

Tissue was fixed for 24-72 hours in the following solution: 1.25% Glutaraldehyde (V/V) (16320, Electron Microscopy Sciences [EMS], Hatfield, PA), 2% Paraformaldehyde (V/V) (15710, EMS), 1x Phosphate buffered saline (PBS) (P5493-1L, MilliporeSigma), 0.025% Tween 20 (ACC

00528/0030, Agdia, Elkhart, IN). Tissue was rinsed three times with 1x PBS for before being dehydrated in successive washes of ethanol in water (30%, 50%, 70%, 80%, 95%, 100%, 100%; V/V) for 30 minutes each and dehydrated overnight in 100% ethanol. Tissue was rinsed in 100% ethanol before clearing tissue with Histoclear II (64111-04, EMS) in successive washes of 1:3, 1:1, and 3:1 of Histoclear II: absolute ethanol for 8-16 hours per wash. Tissue was cleared with 100% Histoclear II before permeabilization with Histoplast PE (8330, Thermo Fisher Scientific) in successive infiltrations of 1:3, 1:1, and 3:1 Histoclear II: Histoplast PE (V/V) for 24 hours each at 56°C. Tissue was infiltrated with paraffin with three incubations of 100% Histoplast PE at 56°C for 24-48 hours each. Tissue was embedded in 7x7x5mm molds (22363552, Fisher Scientific, Waltham, MA) and refrigerated at 4°C until sectioning. Approximately 8-10um sections were made using a HM 355S automatic rotary microtome (Thermo Fisher Scientific) and sections were baked onto TruBond 380 adhesion slides (63700-W1, EMS) at 56° for at least one hour. Sections were de-paraffinized in 4 washes of Histoplast PE for 30 minutes each with a final wash with absolute ethanol to dry. De-paraffinized sections were circumscribed with a hydrophobic barrier using an ImmEdge Pen (H-4000, Vector Laboratories, Burlingame, CA) and wet with Reverse-Osmosis (RO) water after the barrier set. Sections were then hybridized following the instructions in the BaseScope Red Assay with probes for HSVd, HLVd, and negative control. Slides were dried at 56°C for 15 minutes before mounting with EcoMount (EM897L, BioCare Medical, Pacheco, CA). Dried, mounted slides were imaged at 100-200x magnification with a DM1000 LED (Leica Microsystems, Wetzlar, Germany).

Micro-shoot tip viroid elimination therapy

Shoot tips were harvested from potted plants growing at 4°C at three time points (1 month, 2 months, and 3 months) from which five meristems ~0.1mm in length were excised and cultured aseptically on MBI media (See Appendix III for tissue culture protocols and recipes). As a control, 10-15 meristems of the same size were extracted from potted plants grown at 25°C. This was repeated twice. Survival was measured by the number of meristems that regenerated shoots. Viroid elimination was calculated by the number of plants that tested negative over the total number tested.

Results:

Viroid titer measured by RT-qPCR

In potted plants, HSVd and HLVd titers decreased with time spent in cold treatment for shoot tips of cvs. 'Comet' and 'Chinook' (Figure 2.1). 'Canadian Red' and 'Wuerttemberger' showed an increase in HLVd at one month. In the leaves, stem, and petiole tissue for 'Comet,' the data was mixed. Overall, HLVd titers significantly decreased with time, but HSVd titer was inconsistent (Figure 2.1). Petiole tissue showed an increase of HSVd titer with time, while shoot tissue displayed a decrease after one month followed by an increase followed by another decrease at three months. Leaf tissue showed a decline, but it was not statistically significant until month three (Figure 2.1).

For plants in tissue culture under cold treatment, titers for both viroids dropped immediately and stayed low, with an exception at month two with 'Comet' plants that showed an increase in HSVd titer (Figure 2.2). The other cultivars displayed a consistent drop in viroid

titer under cold treatment. Dark treatment of tissue culture plants immediately increased the titer of both viroids without exception across all cultivars.

In situ hybridization

Sections from cv. 'Comet' plants grown at 25°C showed prolific replication of both viroids in stem and petiole tissue (Figure 2.3). HSVd and HLVd signals could be detected in all living tissues. HSVd and HLVd were detected in 'Comet' plants grown at 4°C, but at a much lower density (Figure 2.3).

In the shoot tip, HLVd and HSVd were still present in after one month of cold treatment (Figure 2.4). HSVd was localized to the ground meristem below the tunica and HLVd in the parenchyma tissue below the ground meristem (Figure 2.4). With three months of cold treatment, HSVd could still be detected in the ground meristem of some shoots, while not in shoot tips (Figure 2.4). HLVd was not detected near the apical meristem at three months of cold treatment.

In vivo viroid tier

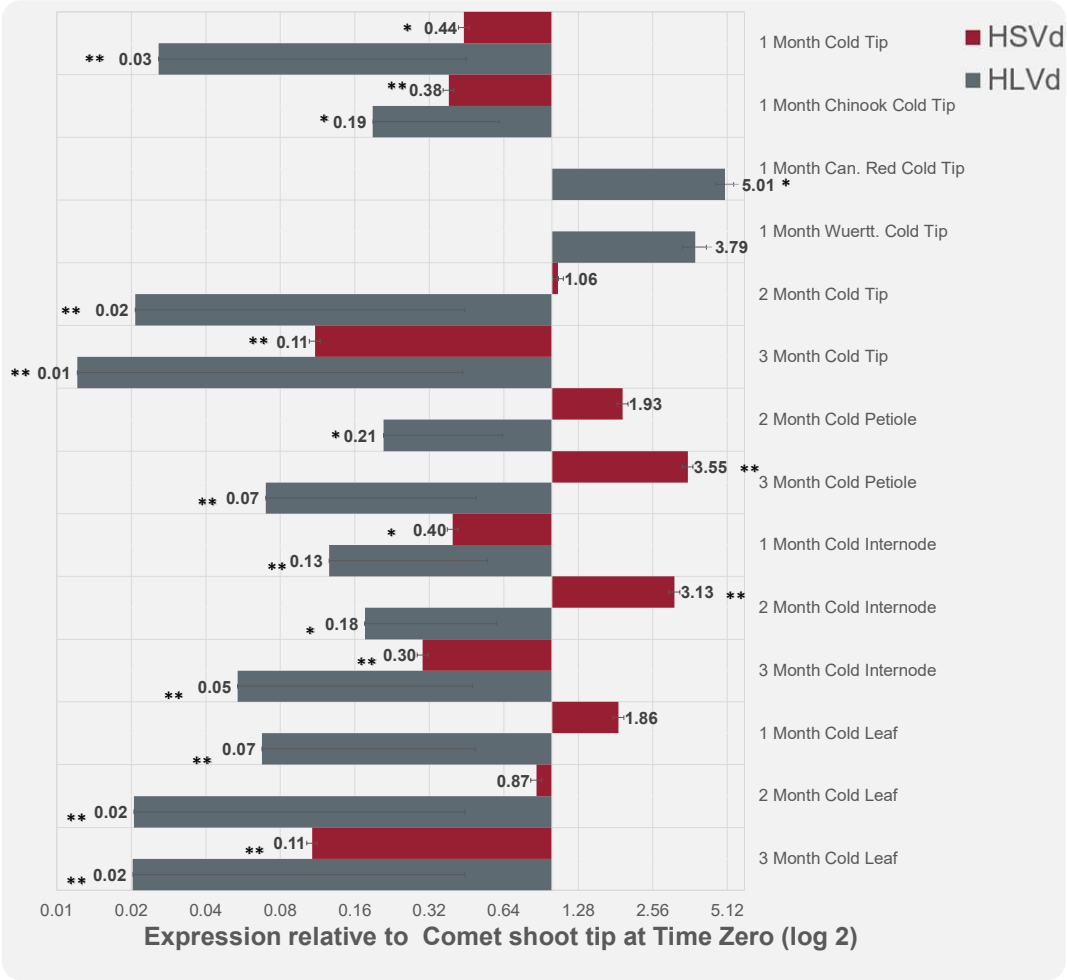

Figure 2.1. HSVd and HLVd expression normalized to 3 reference genes (POAC, TIP41, & YLS8) in potted plants of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' grown 4°C for 1, 2, and 3 months. T-test for difference from baseline expression * p-value ≤ 0.05; ** p-value ≤ 0.01.

In vitro viroid titer

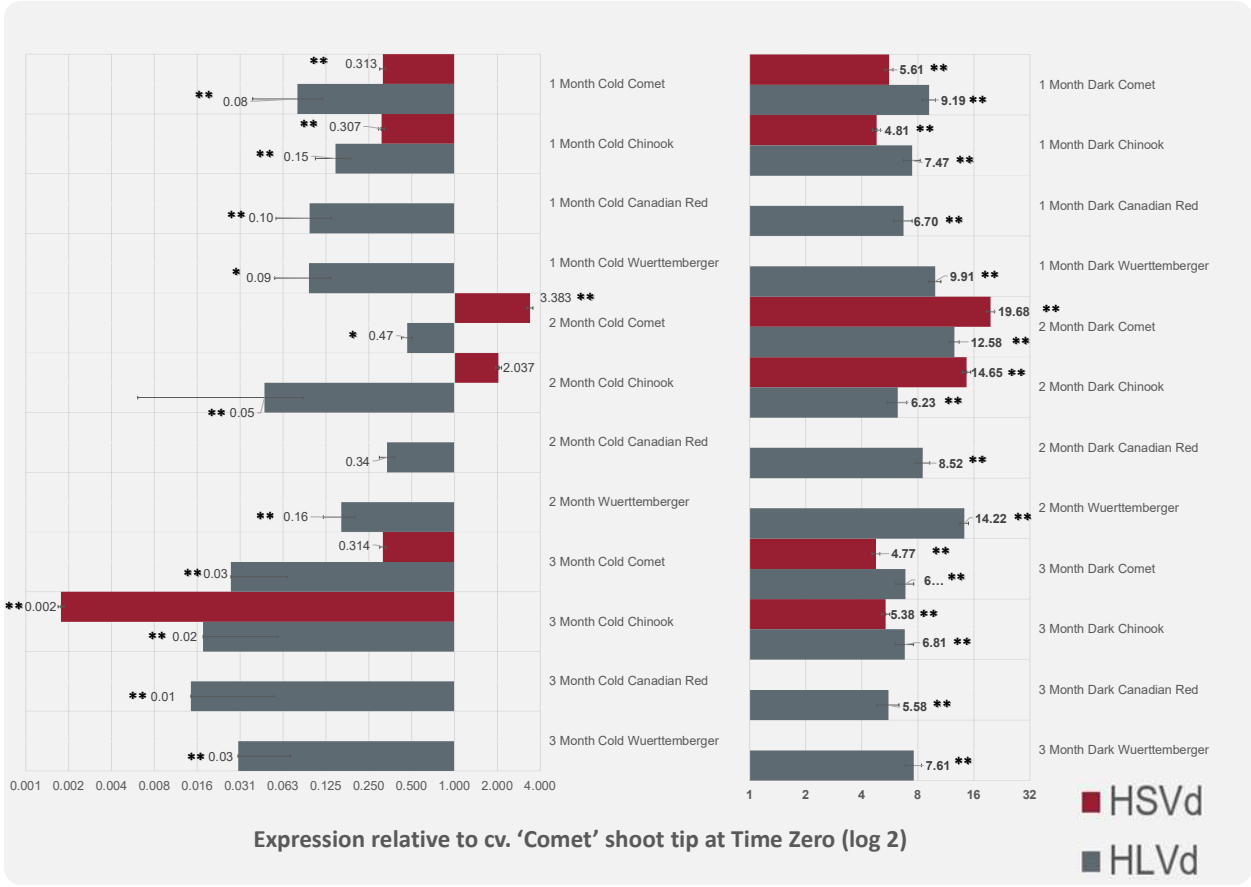

Figure 2.2. HSVd and HLVd expression normalized to 3 reference genes (POAC, TIP41, & YLS8) in tissue cultured plants of cv.s 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger' grown at 25°C, 3°C or complete darkness for 1, 2, and 3 months. * p-value ≤ 0.05; ** p-value ≤ 0.01

In situ hybridization

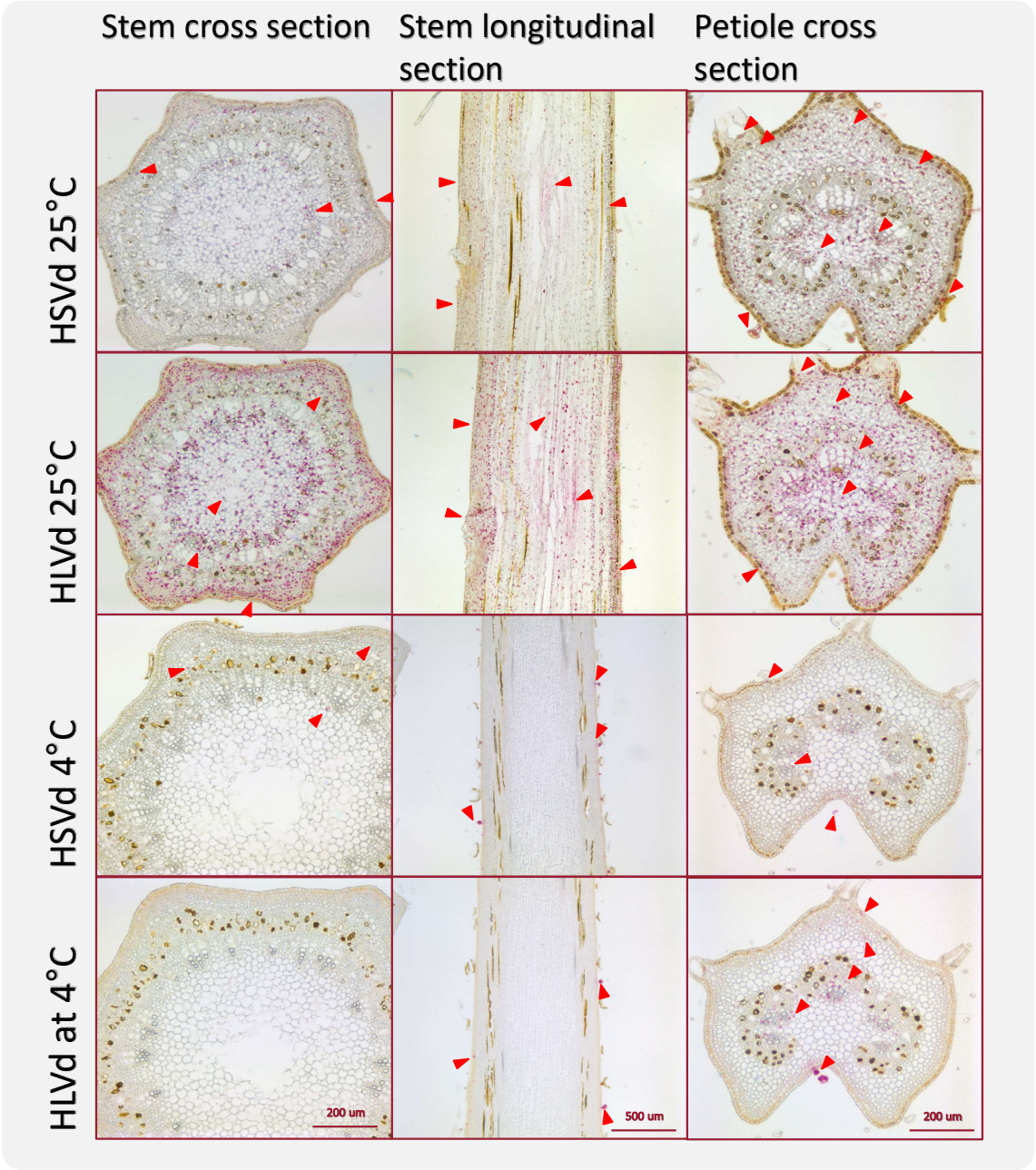

Figure 2.3. *In situ* hybridization for HSVd and HLVd in stem and petiole tissue of cv. 'Comet' grown at 4°C or 25°C for 3 months. Red clusters (arrows for emphasis) indicate presence of viroid. 40x mag. in center and 100x on sides

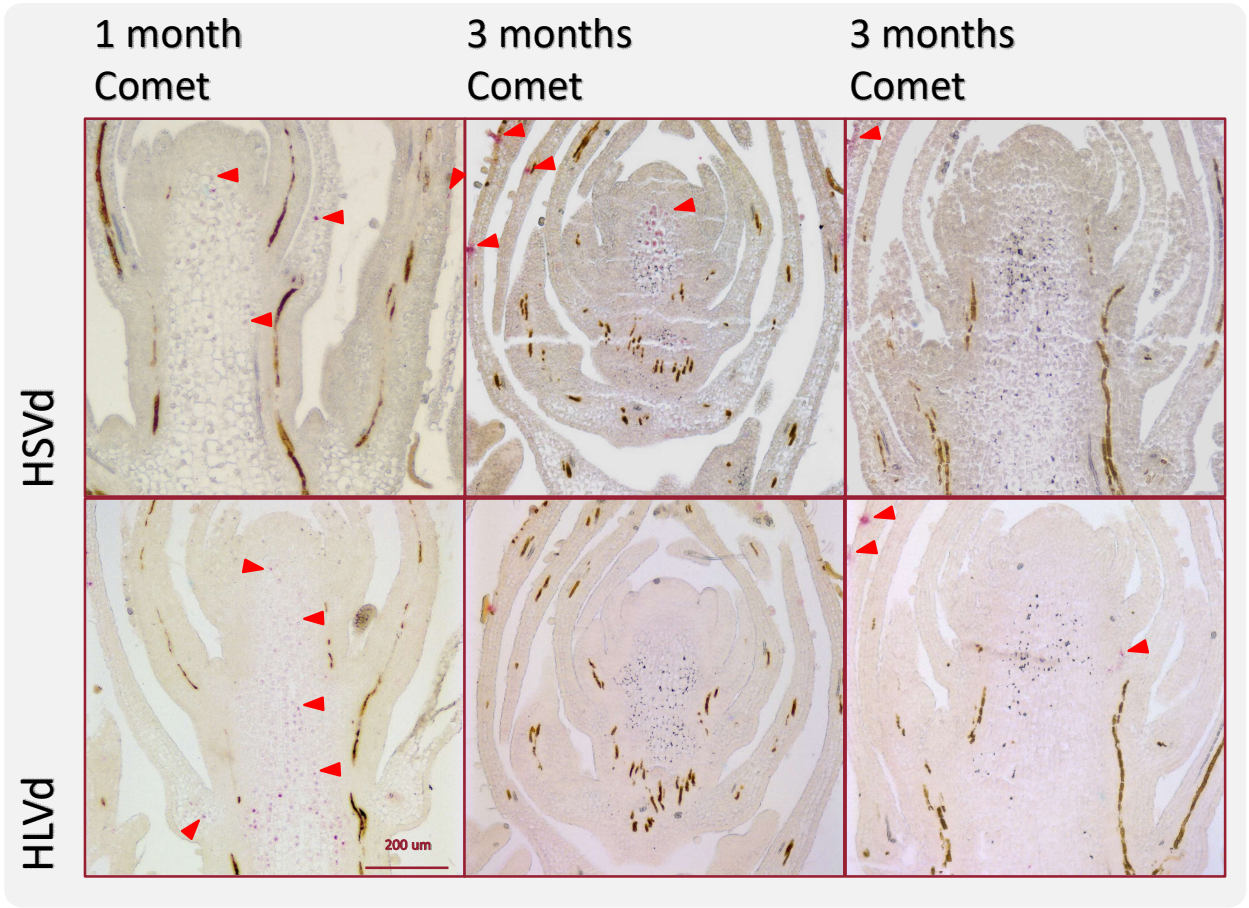

Figure 2.4. *In situ* hybridization for HSVd and HLVd in apical meristems at 1 month and 3 months of growth in cold conditions (4°C) for cv. 'Comet'. Red clusters (arrows for emphasis) indicate presence of viroid. 100x magnification.

Viroid elimination success

All meristems that were initiated from cold treatment plants failed to regenerate (meristems died or only produced callus tissue) (Table 2.1). Meristems initiated from plants grown at 25°C were able to regenerate with viroid elimination rates varying from 5.6% for HSVd to 57.89% for HLVd for 'Comet' (Table 2.1). For 'Chinook,' elimination rates ranged from 14.29% for HSVd to 21.42% for HLVd (Table 2.1). All regenerated plants from 'Comet' and 'Chinook' that were positive for HLVd were positive for HSVd – no single infections of HLVd were recovered (Data not shown). There was a failure to eliminate HLVd from both 'Canadian Red' and 'Wuerttemberger' (Table 2.1).

Table 2.1 Meristem survival rate and viroid elimination rate for hop plants, cvs. 'Comet,' 'Chinook,' 'Canadian Red,' and 'Wuerttemberger,' subjected to 1 to 3 months of cold treatment at 4°C. Control plants of the same cultivars were grown at 25°C.

Variety	Meristems excised	Meristems regenerated	Survival rate	HSVd positive	HSVd elimination rate	HLVd positive	HLVd elimination rate
4°C for 1 month							
Comet	15	0	0.00%	-	-	-	-
Chinook	15	0	0.00%	-	-	-	-
Canadian Red	15	0	0.00%	-	-	-	-
Wuerttemberger	15	0	0.00%	-	-	-	-
4°C for 2 months							
Comet	15	0	0.00%	-	-	-	-
Chinook	15	0	0.00%	-	-	-	-
Canadian Red	15	0	0.00%	-	-	-	-
Wuerttemberger	15	0	0.00%	-	-	-	-
4°C for 3 months							
Comet	15	0	0.00%	-	-	-	-
Chinook	15	0	0.00%	-	-	-	-
Canadian Red	15	0	0.00%	-	-	-	-
Wuerttemberger	15	0	0.00%	-	-	-	-
25°C for 1 month							
Comet	45	38	84.44%	36	5.56%	16	57.89%
Chinook	30	14	46.67%	12	14.29%	11	21.42%
Canadian Red	30	16	53.33%	-	-	16	0
Wuerttemberger	30	15	50.00%	-	-	15	0

Discussions:

Success of micro-shoot tip therapy by itself was cultivar dependent. This is the first report of a RT-qPCR assay being used for validation of viroid elimination therapy (See Appendix I). 'Comet' and 'Chinook' plants free of HSVd and HLVd infection were produced, but efficiencies were low (Table 2.1). However, all 'Canadian Red' and 'Wuerttemberger' plants that

regenerated with positive for HLVd. This difference may be due to callose deposition. Zhang et al. (2015) worked with two cultivars of chrysanthemums infected with Chrysanthemum stunt viroid (CSVd). In one cultivar, 'Yellow Empire,' CSVd was able to replicate in the uppermost layers of the shoot meristem, invading the tunica, while 'Border Dark Red' plants showed limited invasion of the uppermost layers (Zhang et al., 2015). Immunolocalization of callose in the same cultivars showed that significantly fewer callose plugs in the plasmodesmata of CSVd infected 'Yellow Empire' compared to infected 'Border Dark Red.' Individuals of both cultivars without CSVd infection showed no callose deposition in the plasmodesmata of the shoot apical meristem (Zhang et al., 2015). Additionally, in the follow-up study by the same group that investigated the effect of cold treatment on elimination of CSVd from same two cultivars of chrysanthemum, success was low, but possible only in 'Border Dark Red' (Zhang et al., 2016). This could also indicate that the limit of detection of *in situ* hybridization techniques is not adequately sensitive to detect viroid invasion in tunica.

Unexpectedly, dark treatment resulted in increased viroid titer across all cultivars. This indicates that sourcing meristems from *in vitro* dark-treatment plants may not be advisable for viroid elimination. The caveat is that this experiment was performed with plants *in vitro* and plants in pots may respond differently. Future work to confirm the phenomenon occurs *in vivo* may be of interest.

The failure to of meristems to regenerate from plants that underwent cold therapy is unfortunate (Table 2.1). The *in situ* hybridization data and RT-qPCR data of both potted plants and tissue culture plants indicated cold therapy may be the best option for eliminating viroids.

Work with chrysanthemums by Zhang et al., (2016) reported very high regeneration rates after one to twelve months of cold therapy at 5°C. In that study, *in vitro* plants were treated, rather than plants in pots. This may be worth experimenting with the in future as it may increase meristem survival. Unfortunately, the same study reported a maximum of 8.0% CSVd elimination (Zhang et al., 2016). Other possibilities include formulating a media specifically for regenerating cold-treated meristems or "hardening off" the meristems with a gradual increase in temperature.

In conclusion, elimination of HSVd and HLVd from 'Comet' and 'Chinook' is inefficient, but possible (Table 2.1). There appears to be a cultivar effect on the success rate of viroid elimination. To eliminate HLVd from 'Canadian Red' and 'Wuerttemberger,' additional therapies may be necessary, or a larger number of meristems may need to be excised. Potential directions include chemotherapy (El-Dougdoug et al., 2010; Savitri et al., 2013) or cryotherapy (S. M. Jeon et al., 2016).

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CHAPTER THREE: IMPROVEMENT OF VIROID ELIMINATION THERAPY DURING SPRING

Abstract:

In the United States, hops (*Humulus lupulus* L.) are infected by two viroids: Hop stunt viroid (HSVd) and Hop latent viroid (HLVd). Both viroids have proven recalcitrant to traditional therapies to eradicate them from infected hops including micro-shoot tip therapy and cold therapy. As a temperate crop, hops undergo seasonal changes in physiology and there is a flush of young shoots at the beginning of spring. In other temperate plants, spring growth corresponds with an increase in RNA-silencing genes and this increase may produce a positive effect on viroid elimination rates. Four hop cultivars, 'Cascade,' 'Centennial,' 'Triumph,' and 'Zeus' experienced 50% to 100% HLVd elimination rates from shoots harvested in spring. Shoots of cv. 'Comet' plants that were subjected to an artificially-induced spring reported 55% HSVd elimination and 90% HLVd elimination, compared to 9% and 54% without undergoing artificially induced spring. This is the first report of seasonal effects on viroid elimination.

Introduction:

In temperate regions, plants have evolved various mechanisms to endure sub-zero winter temperatures. Many crops may even require a specific number of "chilling hours" for consistent germination, flowering, or bud break (Kozlowski & Pallardy, 1997; Taiz et al., 2014; Wareing & Saunders, 1971). Research has demonstrated the changes in carbohydrate mobilization from the below-ground organs to the shoots during spring, and the reverse as autumn sets in motion (Neve, 1991; Rybacek, 1991; Wareing & Saunders, 1971). For the hop plant, *Humulus lupulus* L., regular and predictable changes in plant growth occur in both below and above-ground structures. For the rootstock, total starch content peaks around the autumn

equinox with total dry weight peaking a bit earlier in August in the northern hemisphere (Neve, 1991; Rybacek, 1991). Though rootstock dry weight stays stable from August until February, the starch content starts to gradually decline soon after its peak and is converted into soluble sugar throughout the winter (Neve, 1991). The total soluble sugar content reaches its maximum in late winter and starts to quickly decline as it is remobilized for above-ground tissue in early spring. Total rootstock dry weight, starch, and soluble sugars reach their nadir in May, corresponding to the start of the exponential phase of above-ground biomass accumulation (Neve, 1991). In most commercial settings, hops are harvested by cutting bines at the base so above-ground biomass is reduced to near zero at the end of a growing season (Neve, 1991). The mobilization of carbohydrates in hops mirrors that of other perennial crops: during the late spring into the fall, rootstocks function as the sink and in turn function as the source for late winter to early spring (Kozlowski & Pallardy, 1997; Wareing & Saunders, 1971; Wilson et al., 2008).

However, what interests plant pathologists is the effect of seasonality on distribution of viral pathogens (Davino et al., 1986). Viruses and viroids are obligate parasites that must be able to adapt to these seasonal changes to overwinter during dormancy and re-emerge in the spring (Desvignes, 1986; Honjo et al., 2020). Previous work on virus and viroid distribution have been focused on diagnostics to answer the question, which tissues provide the most reliable results at different times of the year (Davino et al., 1986; Sanger & Ramm, 1975)? Though important, a question of great practical importance arises: do annual changes in plant physiology have a positive effect success of viroid elimination by micro-shoot tip culture?

To investigate this question, I examined selected hop varieties grown in a near-natural setting and excised meristems during spring. Additionally, to verify whether results can be replicated under artificial conditions, I artificially induced spring growth in cv. 'Comet.' The Clean Plant Center Northwest houses a collection of economically important Generation 1 (G1) plants for domestic hop industry (*Clean Plant Cent. Northwest*, 2021; *Natl. Clean Plant Netw.*, 2021). To prevent infection with viruses and viroids, individual hop plants that have passed certification are grown in isolation in raised planting boxes housed within aphid-proof screenhouses. These plants can experience the natural changes photoperiod and diurnal temperature fluctuations throughout the year and can act as the good experimental source of shoot tips.

As a companion to these naturally treated plants, dormancy can be induced by growing plants at 4°C for 7-9 months with a 16-hour photoperiod to induce emergence of adventitious buds and spring-like growth. To the author's knowledge, this is the first report of investigations of the effects of seasonal growth on viroid elimination.

Methods:

Plants

Two-year-old plants of cv. 'Cascade,' 'Centennial,' 'Triumph,' and 'Zeus' were grown in isolation in an aphid-proof screenhouse as per standard CPCNW procedures for G1 Hops. All plants had single infections with Hop latent viroid (HLVd). To artificially replicate spring growth, cv. 'Comet' plants were grown in 2-gallon pots for 3 months at 25°C prior to moving to cold storage at 4°C for 7-9 months; both temperatures had 16-hour photoperiods. The plants were naturally co-infected with HLVd and HSVd (Hop stunt viroid). As no-treatment controls, plants

of cvs. ‘Comet,’ ‘Chinook,’ ‘Canadian Red,’ and ‘Wuerttemberger’ were grown in one-to-two-gallon pots in growth chambers at 25°C with 16-hour photoperiods. ‘Comet’ & ‘Chinook’ were naturally co-infected with HLVd and HSVd. ‘Canadian Red’ and ‘Wuerttemberger’ were infected with HLVd.

Micro-shoot tip viroid elimination therapy

During spring, shoots were harvested from ‘Cascade,’ ‘Centennial,’ ‘Triumph,’ and ‘Zeus’ and 14 apical meristems with leaf primordia ~0.1mm in length were excised onto MBI media (See Appendix III for recipes). Temperatures averaged 2.6°C to 5.50°C from December 2019 to February 2020. This was repeated once.

From the ‘Comet’ plants that underwent artificial spring growth, approximately 40-60 apical meristems of ~0.1mm in length were excised from shoots sprouted from the taproot and cultured on MBI media. This was repeated twice.

As a control, approximately 16-30 apical meristems with first leaf primordia ~0.1mm in length were excised onto MBI media from ‘Comet,’ ‘Chinook,’ ‘Canadian Red,’ and ‘Wuerttemberger’ plants grown at 25°C with a 16h photoperiod. This was repeated twice.

RT-qPCR viroid detection

In addition to testing for the two viroids, an RT-qPCR assay was designed for a hop gene, *POAC*, as an RNA extraction control (Appendix II). Tissue culture plants were sampled for viroid testing at six months of age. Approximately ~100mg of plant tissue was used for RNA extraction using a Spectrum Plant Total RNA (STRN250-Kit, MilliPore Sigma, USA) extraction kit. Each RT-qPCR consisted of 10 µL of 2x qScript XLT One-Step RT-qPCR ToughMix (95132, QuantaBio, Beverly, MA), 400 nM each of forward and reverse primers, 100 nM dual-labeled FAM-BHQ-1

probe (LGC Biosearch Technologies, Novato, CA), and 2 µL of total RNA with a final volume of 20 µL. See Appendix II for primer and probe sequences for HSVd, HLVd, and *POAC*. Reactions were loaded onto a qPCR plate and run on a CFX96 (Bio-Rad Laboratories, Hercules, CA) with the following parameters: Reverse transcriptase for 15 mins at 50°C and initial denaturation for 2 mins at 94°C followed by 40 cycles of 94°C for 15 seconds and 63°C for 60 seconds. In addition to viroid elimination samples, a negative control, no template control, and positive control were run. CFX Maestro (Version 4.1.2433.1219, Bio-Rad Laboratories) software was used to interpret the RT-qPCR results. A subsample of plants was transferred *ex vitro* into soil and tested for HSVd and HLVd at six and twelve months.

Results:

The plants that experienced natural dormancy (avg. temperatures 2°C-5.50°C) under screenhouse conditions developed vigorous, purple-pigmented, flexuous shoots in spring (Figure 1). Plants that underwent artificially induced spring growth with straight shoots that were yellow with irregular greening (Figure 2).

Figure 3.1. Newly emerged hop shoots during spring in March 2020. Temperatures averaged 2.6°C to 5.50°C from December 2019 to February 2020. Cv. 'Zeus' (A) shoots emerging during spring and (B) shoot tips harvested for extraction of apical meristems.

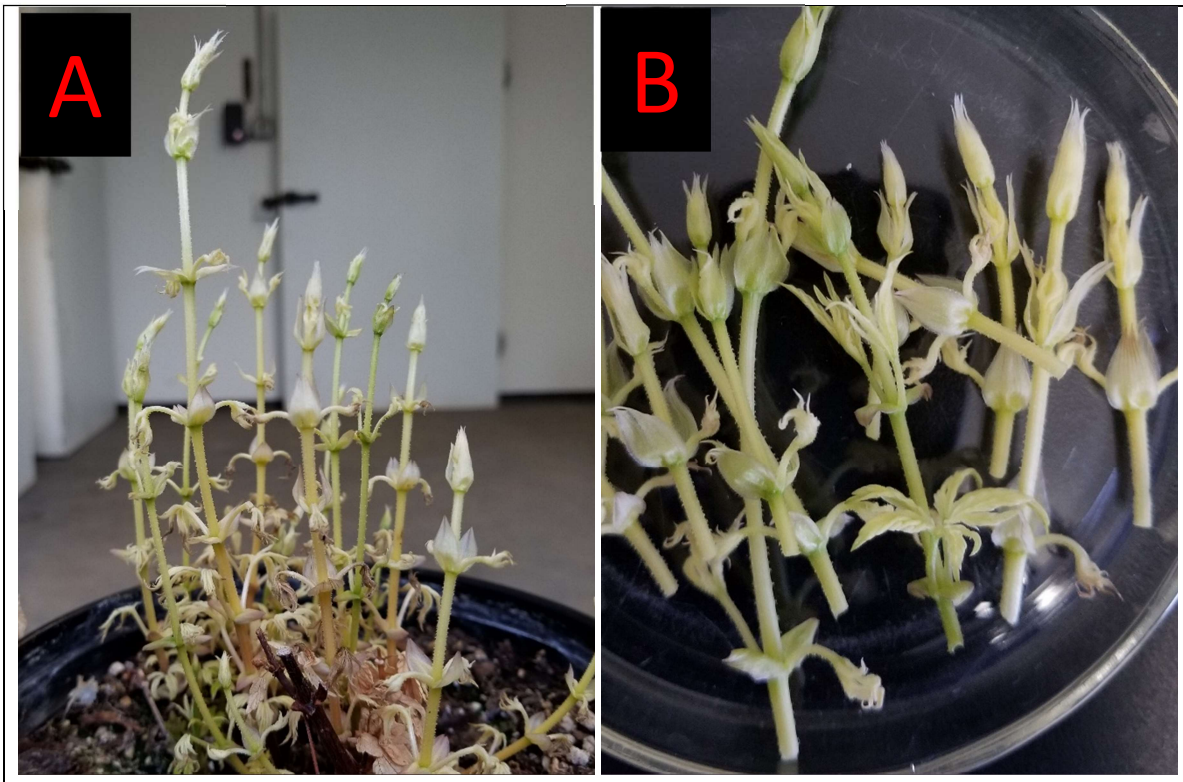

Figure 3.2. Artificially induced spring growth in cv. 'Comet' cultivated at 4°C for 7-9 months to force shoots to emerge from the rhizome. (A) New shoots and (B) shoot tips harvested for extraction of apical meristems

For the plants that experienced a natural spring, meristems that regenerated from cvs. 'Cascade,' 'Centennial,' and 'Zeus' had 100% HLVd elimination while 'Triumph' had 57% elimination. The 'Comet' plants that underwent artificially spring had 90% HLVd elimination and 55% HSVd elimination. Additionally, the tissue culture plants developed faster (differentiated shoots and leaves) than no-treatment controls. Leaves developed in 2-4 weeks after establishment compared to 8-12 weeks in the controls. Plants in soil continued to test negative at six and twelve months.

Plants that did not undergo spring growth had 0-55% HLVd elimination and 4-9% HSVd elimination. HLVd could not be eliminated from either 'Canadian Red' or 'Wuerttemberger.'

Table 3.1. Meristem regeneration rate and viroid elimination rate by cultivar and treatment. Survival and viroid elimination efficiency of HLVd single-infected plants ('Cascade,' 'Centennial,' 'Triumph,' 'Zeus,' 'Canadian Red,' & 'Wuerttemberger') or HSVd and HLVd co-infected plants ('Comet' & 'Chinook'). Spring meristems were excised in March 2020 from newly emerged shoots in raised beds. Meristems were excised from artificially induced spring growth in August and September from growth that emerged from the rootstock. Meristems from no treatment controls were excised in May from actively growing shoots in a growth chamber.

Variety	Meristems excised	Meristems regenerated	Survival rate	HSVd positive	HSVd elimination rate	HLVd positive	HLVd elimination rate
Spring							
Cascade	28	4	14.29%	-	-	0	100.00%
Centennial	28	14	50.00%	-	-	0	100.00%
Triumph	28	7	25.00%	-	-	3	57.14%
Zeus	28	27	96.43%	-	-	0	100.00%
Artificially induced spring growth							
Comet	68	42	61.76%	19	54.76%	4	90.48%
No treatment control							
Comet	142	99	69.72%	95	4.04%	45	54.55%
Chinook	91	44	48.35%	40	9.09%	36	18.18%
Canadian Red	47	13	27.66%	-	-	13	0
Wuerttemberger	48	17	35.42%	-	-	17	0

Discussion

Though the literature for both viroid and virus elimination is rich in terms of methodologies, hosts, and pathogens, only Valero et al., (2003) have investigated the effect of seasonality on virus elimination efficiency (Barba et al., 2017; Mahaffee et al., 2009; Al. Panattoni & Triolo, 2010). To this author's knowledge, this is the first report of seasonal effects on viroid elimination.

It is vital to try to understand the potential physiological responses that may underlie the improved viroid elimination efficiency attributed to spring growth. Temperate perennial crops undergo complex annual cycles of growth, senescence and regrowth (Wareing &

Saunders, 1971). Combinations of hormones such as gibberellins, cytokinins, auxins, and abscisic acid work in complex interactions to orchestrate the growth of plants in response to environmental cues (Taiz et al., 2014; Wareing & Saunders, 1971; Wilson et al., 2002). Carbohydrates accumulate below ground during the growing season and peak during autumn to be utilized to fuel the growth in spring (Neve, 1991; Rybacek, 1991). Unknown physiological changes may be occurring during spring or autumn to facilitate the preferential exclusion or degradation of HLVd and HSVd RNA. Honjo et al. (2020) recently reported on seasonality of viral dynamics of Turnip mosaic virus (TuMV) naturally infecting *Arabidopsis hallerii* populations in Japan in which accumulation of TuMV in the leaf tissue climbed quickly during spring peaked in summer and autumn followed by a drastic drop in winter. Coinciding with the peak in viral accumulation in autumn, genes related to RNA silencing, *RNA-Dependent RNA Polymerase 6 (RDR6)*, *Argonaute1 (AGO1)*, and *ARGONAUTE2* (*AGO2)* were upregulated. To help maintain the suppression, spring was associated with upregulation of genes associated with local and systemic acquired resistance (SAR), *NIM1-INTERACTING 1 (NIM1N1), WRKY 70,* and *PATHOGENESIS-RELATED PROTEIN 2 (PR2)* (Honjo et al., 2020). A similar phenomenon may be occurring in hops during autumn to degrade HSVd and HLVd RNA and continue to suppress replication into the spring, facilitating viroid elimination.

Viruses may behave different from viroids in response to seasonal effects. The only study that has investigated the effects of seasonality on virus elimination success was Valero et al. (2003). They reported 96% to 100% elimination of Grapevine leafroll associated virus-3 (GLRaV-3) and Grapevine fanleaf virus (GFLV) from co-infected cv. 'Napoleon' grape clones after plants were subjected to summer heat in Murcia, Spain. The improved virus elimination was

attributed to “natural heat therapy” (Valero et al., 2003). What is more interesting is decrease in GLRaV-3 elimination when shoot tips were sourced in spring. Valero et al. (2003) reported a 20-60% drop in virus elimination depending on the distance of the source material from the shoot apex. This decrease in efficiency is opposite what was observed for HSVd and HLVd in hops during spring treatment. This is to be expected as viroids, such as Potato spindle tuber viroid (PSTVd), have shown preferential accumulation in elevated temperatures (Sanger & Ramm, 1975; Schnölzer et al., 1985). Lizarraga et al. (1980) provided further support against heat therapy for viroid elimination by showing that cold therapy was able to facilitate PSTVd elimination from an infected potato hybrid by subjecting the *in vitro* plantlets to cold treatment of 5°C-8°C; elimination did not occur at 25°C. Altogether, this adds to the mounting evidence that there is no one method guaranteed to eliminate all viroids and viruses and a combination of techniques may be necessary depending on host and pathogen(s).

In additional to seasonal effects, it may be important to assess if this effect may be cultivar dependent. Previous studies that have investigated viroid elimination have reported a cultivar effect for success in hops and other crop plants (Adams et al., 1996; Barbara et al., 1990; Momma & Takahashi, 1983; Paludan, 1980). The HLVd elimination data from the no treatment controls (Table 3.1) agree with Adams et al., (1996) which reported that male hop selections and two female hops had higher viroid elimination than cv. ‘Fuggle’ trialed in the study and (Morton et al., 1993) (See Appendix I for a summary of viroid elimination studies). While elimination of HLVd in ‘Chinook’ and ‘Comet’ were low (18% and 54%, respectively) without treatment, ‘Canadian Red’ and ‘Wuerttemberger’ could not be freed of HLVd infection under the same cultural conditions. Future work to examine the elimination efficiency of HLVd

from ‘Cascade,’ ‘Centennial,’ ‘Triumph,’ and ‘Zeus,’ under growth chamber conditions and HLVd & HSVd from ‘Chinook,’ ‘Canadian Red,’ and ‘Wuerttemberger’ under natural or artificial spring conditions warrants further investigation.

The key difference between this study and previous works is its simplicity. Experiments by other authors attempting to eradicate viroids recalcitrant to micro-shoot tip culture have employed complicated temperature regimes alone, or in combination with anti-viral chemicals, have resulted in mixed results (El-Dougdoug et al., 2010; S.-M. Jeon et al., 2012; Momma & Takahashi, 1983; Zhang et al., 2016). In contrast, this study has found that timing the micro-shoot tip culture with right physiological stage of hop growth can drastically increase viroid elimination efficiency. HSVd elimination was increased by 13-fold while HLVd elimination was increased to >90% for ‘Comet.’ In summary, this work provides a simplified framework for eliminating recalcitrant viroids from hops. Timing micro-shoot tip culture of 0.1mm apices at shoot emergence during spring efficiently frees plants from HSVd and HLVd infection without additional therapies. Elimination of HLVd from germplasm provides a foundation on which to perform experiments without the confounding factor of a viroid replication. More importantly, advances the sustainability of U.S. hop production by reducing inefficiencies associated with viroid infection (Flores et al., 2017; Hataya et al., 2017). Future work to elucidate the host genes involved in degradation or suppression of viroid accumulation will provide a clearer image of the underlying mechanisms. Furthermore, practical experiments in other temperate host-viroid combinations will need to be performed to validate this technique for eliminating viroids recalcitrant to elimination therapies.

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APPENDIX

Appendix I: Review of the past 50 years of viroid elimination experiments

Introduction:

A complete history of viroid elimination therapies has been lacking. This is an attempt to fill in the holes present in reviews of both virus and viroid elimination publications (Barba et al., 2017; Faggioli & Barba, 2008; Hull, 2014; Panattoni et al., 2013). To understand what therapies may be appropriate for eliminating viroids, two questions must be answered (1) how successful were the therapies (regeneration rate and viroid elimination rate) and (2) what technologies were implemented to verify elimination therapies were successful? The following review hopes to provide a more comprehensive view of the studies that have been performed, in chronological order, that answer those two questions. One of the oldest diagnostic tools in the plant pathology tool box to detect obligate parasites is the bio-assay, also referred to as biological indexing (Hadidi et al., 2017; Hull, 2014). At its most basic, bio-assays rely upon the compatible reaction between a susceptible host and a virulent pathogen. For economically important pathogens, specific cultivars of hosts that are known to be sensitive to viroid infection are cultivated for diagnostics. For example, 'Suyo' cucumber is sensitive to Hop stunt viroid (HSVd) and is used as an indicator plant to assay for presence of HSVd (Hataya et al., 2017; Momma & Takahashi, 1983). Rub inoculation of HSVd-positive sap results in symptoms that include leaf epinasty and curling along with overall stunting at 25°C-30°C. As diagnostic technologies evolve, it is necessary to look back at historical studies that relied upon older technologies such as bio-assay, gel-electrophoresis, and dot-blot hybridization with a more critical eye (Hosokawa et al., 2004; Kuhn et al., 2019; Papayiannis, 2014; Running et al., 1996).

Modern technologies, such as RT-PCR, directly detect viroids based on nucleotide sequence and do not rely upon outward symptoms to provide precise and sensitive results. The last 50 years of viroid elimination therapy, successes and failures, have been compiled in Table A.1.1.

Methods:

In addition to the relevant sources referenced in Barba et al. (2017), google scholar (scholar.google.com) was queried for articles related to viroid-elimination. Experiments related to viroid elimination in hops or experiments related to HSVd were emphasized in the search as they are most relevant to the project. A table was compiled to summarize the work done in each study to record the host, cultivar, viroid, viroid-elimination therapy employed, diagnostic technology employed, regeneration rate, elimination rate, and citation (including all author names) in chronological order (Table A.1.1). Viroid names were updated to reflect the current taxonomic classifications, e.g. Potato spindle tuber virus (Stace-Smith & Mellor, 1970) is now recognized as a viroid while cachexia virus in citrus and pale fruit disease in cucumber is caused by Hop stunt viroid (HSVd) (Roistacher et al., 1976). With regards to the elimination therapy, focus was put upon meristem size, plant source (e.g., *in vitro* plants, cultivation photoperiod, temperature, and light intensity prior to excision), temperature regimes, photoperiod/light intensity, and any nuances in experimental design. Diagnostic technology was broken down into four broad groups: bio-assay (biological indexing utilizing rub inoculation, grafting, etc.), gel electrophoresis (usually PAGE), nucleic acid hybridization (dot-blot, spot-blot, southern blot, etc.), and RT-PCR (reverse transcriptase polymerase chain reaction). Studies were reviewed specifically for the age, status (*in vitro,* in potting soil, planted in the field, etc.), type of tissue (leaf, bark, etc.), quantity, and cultivation temperature of the plant source used to validate

viroid elimination therapy efficacy. Regeneration rates were listed as number of plants that regrew over attempted ('unknown' is recorded if the number of attempts was omitted). Success rates were calculated based on the number of plants tested. In the case where only a subsample of the total number of regenerated plants were tested, the number of tested plants is listed in parentheses.

Results:

A total of 26 articles were reviewed to compile Table A.1.1. Studies were published on the subject of viroid elimination prior to their recognition as a distinct group of pathogens in 1971 by Ted Diener (1971). Hollings & Smith (1970) and Stace-Smith & Mellor (1970) attempted to use thermotherapy to eliminate Chrysanthemum stunt viroid (CSVd) and Potato spindle tuber viroid (PSTVd), respectively, from each viroid's namesake host with little success. Over the past 50 years, various therapies have been attempted: heat therapy (Hollings & Stone, 1970; Howell & Mink, 1992; Paludan, 1980; Stace-Smith & Mellor, 1970), micro-shoot tip culture (Bachelier et al., 1976; Duran-Vila & Semancik, 1986; Momma & Takahashi, 1983; Paludan, 1980), micrografting (M. Barba et al., 1995; Howell et al., 1998; Roistacher et al., 1976; Roistacher & Kitto, 1977), cold therapy (Lizarraga et al., 1980; Morton et al., 1993; Paduch-Cichal & Kryczyński, 1987), alternating temperature regimes (Postman & Hadidi, 1995), nucellus culture (Suarez et al., 2006), chemotherapy (El-Dougdoug et al., 2010), combination chemotherapy & cold therapy (El-Dougdoug et al., 2010), and cryotherapy (S. M. Jeon et al., 2016) (Table A.1.1).

Diagnostic technologies have evolved alongside elimination methods. Biological indexing was augmented with PAGE (Lizarraga et al., 1980), dot-blot hybridization (Morton et

al., 1993), RT-PCR (Postman & Hadidi, 1995), and nested-PCR (M. Hosokawa, Otake, Sugawara, et al., 2004).

Regarding hop viroid elimination experiments, results have been mixed. Morton et al., (1993) reported 0% success in HLVd elimination from cv. 'Fuggle' out of 132 regenerated plants (detection was performed by dot-blot hybridization) (Table A.1.1). Adams et al., (1996) reported better elimination with frequencies of 7.1% to 100% using the same diagnostic technique. An important caveat for Adams et al. (1996) is the small sample sizes that may inflate viroid elimination rates (Table A.1.1). It is also not clear if the improvement was due to a longer duration of cold storage, excision of a smaller apical meristem, genotypic response, or a combination of any or all factors compared to Morton et al. (1993). Solarska & Grudzińska (2001) reported that dot-blot hybridization may not be as sensitive as RT-PCR for detection of HLVd as RT-PCR. Out of 108 samples (covering nine cultivars), dot-blot hybridization reported 104 false negatives compared to RT-PCR. Grudzińska & Solarska, (2005) attempted to eliminate HLVd from hop cultivars in Poland by micro-shoot culture (~0.3-0.5mm length meristems) without heat therapy and used RT-PCR to validate the elimination success. They reported 30% to 37.5% elimination rates for four Polish hop cultivars (sample sizes 8 to 10, Table A.1.1) (Grudzińska & Solarska, 2005). The only study that has attempted to eliminate HSVd from hops is Momma & Takahashi (1983). The study reported 0.0% to 33.3% elimination rates with micro-shoot therapy in combination with cold therapy, with a two important caveats: 6-10 plants were tested per treatment and HSVd elimination was validated by bio-assay (rub inoculation of sap onto cv. 'Suuyuu' cucumber leaves) (Table A.1.1) (Momma & Takahashi, 1983).

To date, no studies on viroid elimination have implemented RPA (recombinase polymerase amplification), LAMP (loop-mediated isothermal amplification), RT-qPCR (intercalating dye, hydrolysis probe, multiplexing, etc.), sequencing, or digital-droplet PCR for diagnostics. Even as recently as 2016, Zhang et al., (2016) performed RT-PCR for validation of CSVd elimination and Jeon et al., (2016) performed a nested PCR for validation of CSVd and CChMVd elimination. The future implementation of more sensitive assays (coupled with more efficient extraction methods) may further push the boundaries of viroid elimination therapies towards the goal of producing the cleanest germplasm possible.

Table A.1.1. Studies of viroid elimination therapies over the past 50 years listed in chronological order. Information regarding treatment (e.g., culturing conditions, therapies, therapy duration, therapy dosage, meristem size, presence of leaf primordia, or any remarkable treatments), diagnostic technology (Biological assay, gel-electrophoresis, dot-blot hybridization, or reverse transcriptase polymerase chain reaction), regeneration rate, elimination rate (with the number of meristems tested if fewer the number that regenerated), and citation listing all authors. [a] Experiments by the same authors have the same citation number listed in the 'Elimination rate' column. CSVd = Chrysanthemum stunt pospiviroid; PSTVd = Potato spindle tuber pospiviroid; CEVd = Citrus exocortis pospiviroid; HSVd = Hop stunt hostuviroid; CChMVd = Chrysanthemum chlorotic mottle pelamoviroid; PLMVd = Peach latent pelamoviroid; ASSVd = Apple scar skin apscaviroid; HLVd = Hop latent cocadviroid; ASBVd = Avocado Sunblotch avsunviroid; PPFD = photosynthetic photo flux density measured in µmol per meter2 per second.

Crop	**Viroid**	**Therapy**	**Diagnostic technology**	**Regeneration rate (survive/ excised)**	**Elimination rate [Citation Number][a]**	**Citation [Number]**
Chrysanthemum cv. 'Mistletoe'	CSVd	35°C for 14-37 weeks followed by excision of 0.1-0.25mm length meristem with 1-2 leaf primordia	Bio-assay: Self-indexed *ex vitro* at 18-25°C with 16h photoperiods in the greenhouse.	10/95	0.0% [1]	Hollings & Stone (1970) [1]
		35°C for 14-37 weeks followed by excision of 025-0.5mm length meristem with 1-2 leaf primordia		56/221	3.6% [1]	
		35°C for 14-37 weeks followed by excision of 0.5-0.75mm length meristem with 1-2 leaf primordia		4/19	0.0% [1]	
		35°C for 14-37 weeks followed by excision of 0.75-1.0mm length meristem with 1-2 leaf primordia		2/2	0.0% [1]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		35°C for 37 weeks followed by propagation of >1cm cuttings		30/41	0.0% [1]	
Potato cv. 'White Rose'	severe-PSTVd	33°C-36°C for 2-12 weeks with 16h photoperiod followed by excision of 0.5-1.0mm meristems (0.3-0.8mm for m-PSTVd)	Bio-assay: Leaves from established *ex vitro* plants were rub inoculated onto cv. 'Rutgers' tomato seedlings	62/66	0.0% [2]	Stace-Smith & Mellor (1970) [2]
Potato cv. 'White Rose'	mild-PSTVd			242/248	2.5% [2]	
Chrysanthemum cv. 'Mistletoe'	CSVd	0.25 mm length meristem with one leaf primordium maintained at 23°C with a 16h photoperiod.	Bio-assay: *Ex vitro* plants chip-budded onto cv. 'Mistletoe' (cv. 'Mistletoe' mericlones were self-indexed)	74/160	13.5% [3]	Bachelier, Monsion, & Dunez (1976) [3]
Chrysanthemum cv. 'Flamand rose'				20/30	30.0% [3]	
Chrysanthemum cv. 'Dede Aulen'				3/30	66.7% [3]	
Chrysanthemum cv. 'Soeur Marthe'				2/20	50.0% [3]	
Chrysanthemum cv. 'Miss Katy'				unknown	0.0% [3]	
Chrysanthemum cv. 'Blanche Poitevine'				unknown	0.0% [3]	
Chrysanthemum cv. 'Crepuscule'				unknown	0.0% [3]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Chrysanthemum cv. 'Fanete'				unknown	0.0% [3]	
Citrus x aurantium cv. 'Cadenera' sweet orange	CEVd (co-infected with Citrus concave gum-associated bunyavirus)	0.14-0.18mm meristems micrografted onto *in vitro* germinated Troyer citrange seedlings cultured for 3-5 weeks illuminated with 13.5 PPFD followed by culture for 2-3 weeks with 135 PPFD (16h photoperiod for both light regimes)	Bio-assay: 2 buds or stem pieces of *ex vitro* plants grafted onto 2 or 3 'Arizona 861' citron scion on rough lemon rootstock and grown at 25-40°C - indexing was carried out over 2 years.	25/unknown	0.0% [4]	Roistacher, Navarro, & Murashige (1976) [4]
Citrus x aurantium cv. 'Monreal' clementine				6/unknown	100.0% [4]	
Citrus x aurantium cv. 'Cadenera Fina' sweet orange	CEVd (co-infected Citrus tristeza closterovirus)			33/known	100.0% [4]	
Citrus x aurantium cv. 'Robertson' navel orange	CEVd (Citrus psorosis ophiovirus)			26/unknown	92.3% [4]	
Citrus x aurantium cv. 'Temple' tangor	CEVd			32/unknown	100.0% [4]	
Citrus x aurantium cv. 'Genetic dwarf' grapefruit				3/unknown	100.0% [4]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Citrus x aurantium cv. 'Frost' navel orange				3/unknown	100.0% [4]	
Citrus retticulata cv. 'Willowleaf' mandarin	CEVd (co-infected with HSVd)			55/unknown	98.2% [4]	
	HSVd (co-infected with CEVd)		Bio-assay: 2 buds or stem pieces of ex vitro plants grafted onto 3 'Parsons Special Mandarin' scion on rough lemon rootstock and grown at 25-40°C - indexing was carried out over 2 years.	55/unknown	0.0% [4]	
Citrus limon cv. 'Ricote' lemon	HSVd (co-infected with CEVd)	0.14-0.18mm meristems micrografted onto *in vitro* germinated rough lemon seedlings cultured for 3-5 weeks illuminated with 13.5 PPFD followed by culture for 2-3 weeks with 135 PPFD (16h photoperiod for both light regimes)		11/unknown	100.0% [4]	
	CEVd (co-infected with HSVd)		Bio-assay: 2 buds or stem pieces of ex vitro plants grafted onto 2 or 3 'Arizona 861' citron scion on rough lemon rootstock and grown at 25-40°C - indexing was carried out over 2 years.	11/unknown	0.0% [4]	
Citrus limon cv. 'Santa Teresa' lemon	CEVd			1/unknown	100.0% [4]	
Citrus x aurantiifolia cv. 'Bearss' lime				2/unknown	100.0% [4]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Citrus medica selection S-1 citron				1/unknown	100.0% [4]	
Citrus medica cv. 'Fingered' citron				8/unknown	87.5% [4]	
Citrus reshni cv. 'Cleopatra mandarin'	CEVd (co-infected with Dweet mottle citrivirus)	0.14mm meristems micrografted onto *in vitro* germinated Troyer citrange and Arizona 861 Etrog citron seedlings cultured at 27°C for 3-5 weeks illuminated with 135 PPFD 'Gro Lux' lights	Bio-assay: 2 buds grafted onto at least 2 'Arizona 861' citron indicators	14/unknown	78.6% [5]	Roistacher & Kitto (1977) [5]
Citrus x meyeri cv. 'Meyer Lemon'	CEVd (co-infected Citrus tristeza closterovirus and Citrus tatter-leaf capillovirus)			21/unknown	95.2% [5]	
Solanum tuberosum x S. phureja clone BR 63.5	severe-PSTVd	5°C-6°C for 6 months *in vitro* under 7 PPFD fluorescent lights *in vitro* followed by excision of meristems with 1 leaf primordium cultured at 25°C	Gel-electrophoresis: RNA extracted from 1g *ex vitro* tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: Leaves from e*x vitro* plants were rub inoculated onto	13/48	53.8% [6]	Lizarraga, Salazar, Roca, & Schilde-Rentschler (1980) [6]

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		illuminated at 13.5 PPFD with a 16h photoperiod	cv. 'Rutgers' tomatoes at >25°C under continuous illumination at 27 PPFD			
		25°C for 2 months *in vitro* under 20 PPFD fluorescent lights followed by excision of meristems with 1 leaf primordium cultured at 25°C illuminated at 13.5 PPFD with a 16h photoperiod		16/48	0.0% [6]	
		8°C for 4 months under greenhouse conditions with 93 PPFD followed by excision of meristems with 1 leaf primordium cultured at 25°C illuminated at 13.5 PPFD with a 16h photoperiod		17/48	29.4% [6]	
		22°C for 4 months under greenhouse conditions with 93 PPFD followed by excision of length meristem with 1 leaf primordium cultured at 25°C illuminated at 13.5 PPFD with a 16h photoperiod		16/48	0.0% [6]	
Chrysanthemum cv. 'Mistletoe'	CSVd	Constant illumination of potted plants at 34°C for 16 hours and 20°C for 8 hours or 37°C for 7 months followed by 0.25mm length meristems with 1 or 2	Bio-assay: tissue from *ex vitro* plants grafted onto cvs. 'Mistiletoe' or 'Fanfare' - repeated twice 2 months apart	210/ unknown	2.4% [7]	Paludan (1980) [7]

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Chrysanthemum cv. 'Deep Ridge'	CChMVd	leaf primordia and grown at 22°C, 27°C, and 30°C for 60-150 days.	Bio-assay: tissue from *ex vitro* plants grafted onto cv. 'Deep Ridge' - repeated twice 2 months apart	682/ unknown	0.1% [7]	
Chrysanthemum cv. 'Mistletoe'	CSVd	0.25mm length meristems excised and grown for 3 weeks prior to heat treatment for 4 months (34°C for 16h with illumination and 20°C for 8h in the dark)	Bio-assay: tissue from *ex vitro* plants grafted onto cvs. 'Mistletoe' or 'Fanfare' - repeated twice 2 months apart	54/unknown	1.9% [7]	
Chrysanthemum cv. 'Deep Ridge'	CChMVd		Bio-assay: tissue from *ex vitro* plants grafted onto cv. 'Deep Ridge' - repeated twice 2 months apart	125/ unknown	0.0% [7]	
Chrysanthemum cv. 'Mistletoe'	CSVd	0.2mm length meristems	Bio-assay: tissue from ex vitro plants grafted onto cvs. 'Mistletoe' or 'Fanfare' - repeated twice 2 months apart	2/unknown	100.0% [7]	
		0.25mm length meristems		29/unknown	41.4% [7]	
		0.05mm length meristems		26/unknown	23.1% [7]	
Chrysanthemum cv. 'Mistletoe'		0.2mm length axillary meristems		19/unknown	63.2% [7]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		0.25mm length axillary meristems		75/unknown	14.7% [7]	
		0.05mm length axillary meristems		16/unknown	0.0% [7]	
Chrysanthemum cv. 'Deep Ridge'	CChMVd	0.20mm length meristems	Bio-assay: tissue from ex vitro plants grafted onto cv. 'Deep Ridge' - repeated twice 2 months apart	21/ unknown	0.0% [7]	
		0.25mm length meristems		55/unknown	0.0% [7]	
		0.50mm length meristems		20/unknown	0.0% [7]	
Chrysanthemum cv. 'Mistletoe'	CSVd	0.25mm length meristems excised on media supplemented with 0.1 or 0.2 mg/l 2,4-D for	Bio-assay: tissue from ex vitro plants grafted onto cvs. 'Mistletoe' or 'Fanfare' - repeated twice 2 months apart	12/unknown	8.3% [7]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Chrysanthemum cv. 'Deep Ridge'	CChMVd	one month at 20°C with a 16h photoperiod.	Bio-assay: tissue from ex vitro plants grafted onto cv. 'Deep Ridge' - repeated twice 2 months apart	19/unknown	0.0% [7]	
Hop cv 'Shinsuwase'	HSVd	0.2mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.	Crude extract rubbed onto the first true leaves of cv. 'Suuyou' cucumber	6/8	33.3% [8]	Momma & Takahashi (1983) [8]
		0.3mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		8/33	12.5% [8]	
		0.4mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		1/10	0.0% [8]	
		0.5-1.0mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		4/27	0.0% [8]	
		1.1-2.0mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		7/11	0.0% [8]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		2.1-3.0mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		20/28	0.0% [8]	
		3.1-4.0mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		3/5	0.0% [8]	
		4.1-5.0mm length meristems taken from field grown plants. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		6/7	0.0% [8]	
		10±2°C for 1-4 months with 16h photoperiod at 59.4-86.4 PPFD followed by excision of 0.2-3.0mm meristems. Explants cultured at 25°C with continuous illumination at 13.5-40.5 PPFD.		10/103	10.0% [8]	
Tomato cv. 'Rutgers'	CEVd	0.2-0.4mm length meristems excised from greenhouse-grown plants. *In vitro* plants kept at 28°C with 16h photoperiod at 40 PPFD provided by cool light fluorescent lights	Gel-electrophoresis: Nucleic acid extracted from 3-4g *ex vitro* tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: Nucleic acid extracted from 3-4g *ex vitro* tissue and stem-puncture inoculated onto cv. 'Rutgers' tomato	6/67	100.0% [9]	Duran-Vila & Semancik (1986) [9]

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Potato cv. 'Prosna'	severe-PSTVd	3 months or 6 months at 5°C with 16h photoperiod at 60.5 PPFD followed by excision of meristematic dome with 1-2 leaf primordia. *In vitro* plants cultured at 16°C-20°C with 14h photoperiod at 27 PPFD.	2,4-, and 6-months *ex vitro:* Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Rutgers' tomatoes.	16/24	37.5% [10]	Paduch-Cichal & Kryczynski (1986) [10]
Potato cv. 'Azalia' - tubers		Tubers stored for 3 or 6 months at 6°C-7°C in a refrigerator followed by excision of meristematic dome with 1-2 leaf primordia. *In vitro* plants cultured at 16°C-20°C with 14h photoperiod at 27 PPFD.		9/18	11.1% [10]	
Potato cv. 'Irys' - tubers				17/42	11.8% [10]	
Potato cv. 'Azalia' - tubers	mild-PSTVd			22/44	22.7% [10]	
Potato cv. 'Irys' - tubers				18/40	22.2% [10]	
Potato cv. 'Azalia' - tubers	Severe-PSTVd	Tubers stored at room temperature followed by excision of meristematic dome with 1-2 leaf primordia. *In vitro* plants cultured at 16°C-20°C		15/22	0.0% [10]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Potato cv. 'Irys' - tubers		with 14h photoperiod at 27 PPFD.		22/48	0.0% [10]	
Potato cv. 'Azalia' - tubers	mild-PSTVd			36/48	0.0% [10]	
Potato cv. 'Irys' - tubers				22/48	0.0% [10]	
Chrysanthemum cv. 'Deep Ridge.'	CChMVd	3 months or 6 months at 5°C with 16h photoperiod at 60.5 PPFD followed by excision of meristematic dome with 1-2 leaf primordia. *In vitro* plants cultured at 16°C-20°C with 14h photoperiod at 27 PPFD.	2,4-, and 6-months *ex vitro*: Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Deep Ridge'	65/114	40.0% [10]	
Chrysanthemum cv. 'Bonnie Jean'	CSVd		2,4-, and 6-months *ex vitro*: Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Bonnie Jean'	59/150	8.5% [10]	
Chrysanthemum cv. 'Mistletoe'	HpSVd-cuc			20/60	40.0% [10]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Potato cv. 'Prosna'	severe-PSTVd	Plants grown at greenhouse conditions followed by excision of meristematic dome with 1-2 leaf primordia. *In vitro* plants cultured at 16°C-20°C with 14h photoperiod at 27 PPFD.	2,4-, and 6-months *ex vitro:* Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Rutgers' tomatoes	25/60	0.0% [10]	
Chrysanthemum cv. 'Deep Ridge.'	CChMVd		2,4-, and 6-months *ex vitro*: Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Deep Ridge'	54/95	0.0% [10]	
Chrysanthemum cv. 'Bonnie Jean'	CSVd		2,4-, and 6-months *ex vitro*: Gel-electrophoresis: RNA extracted from 1g tissue and electrophoresed on 5% Polyacrylamide gel. Bio-Assay: indexed on cv. 'Bonnie Jean'	50/125	0.0% [10]	
Chrysanthemum cv. 'Mistletoe'	HpSVd-cuc			40/55	0.0% [10]	
Hop cv. 'Fuggle'	HLVd	Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 10°C for 28 days followed by excision of 0.5cm-1cm buds (20°C-25°C with 16h photoperiod)	Nucleic acid hybridization: Tissue from ex vitro plants was ground with buffer for dot-blot hybridization	132/ unknown	0.0% [11]	Morton, Barbara, & Adams (1993) [11]

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 15°C for 11 days followed by excision of 0.5cm-1cm buds (20°C-25°C with 16h photoperiod)				
		Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 25°C for 8 days followed by excision of 0.5cm-1cm buds (20°C-25°C with 16h photoperiod)				
		Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 10°C for 28 days followed by excision of 0.5mm-1.0mm meristems (20°C-25°C with 16h photoperiod)				
		Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 15°C for 11 days followed by excision of 0.5mm-1.0mm meristems (20°C-25°C with 16h photoperiod)				
		Dormant rhizomes stored at 2°C for 3 months and potted up and grown at 25°C for 8 days followed by excision of 0.5mm-1.0mm meristems (20°C-25°C with 16h photoperiod)				

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Peach cv. 'Maygrand'	PLMVd	0.3-0.4mm length meristems with 3 leaf primordia excised from 2-year-old potted plants and micrografted onto cv. 'Nemaguard' seedlings at 24°C with 16h photoperiods at 27 PPFD	2-month *ex vitro* plants used for Gel-electrophoresis: Nucleic acid was extracted from 20g tissue and used for 2 cycles of 5% polyacrylamide gel electrophoresis. Nucleic acid hybridization: Total nucleic acid was extracted from 0.4g leaf tissue and blotted onto nylon membranes for slot-blot hybridization. Bio-assay: 50–60-day old cv. 'GF305' seedlings were double chip budded and inoculated with severe-PLMVd.	12/unknown	66.7% [12]	Barba, Cupidi, Loreti, Faggioli, & Martino (1995) [12]
Peach cv. 'Armking'		0.4-0.5mm length meristems with 3 leaf primordia excised from 2-year-old potted plants and micrografted onto cv. 'Nemaguard' seedlings at 24°C with 16h photoperiods at 27 PPFD		8/unknown	12.5% [12]	
Peach cv. 'Firebrite'				5/unknown	20.0% [12]	
Peach cv. 'Stark Redgold'		0.6-0.8mm length meristems with 3 leaf primordia excised from 2-year-old potted plants and micrografted onto cv. 'Nemaguard' seedlings at 24°C with 16h photoperiods at 27 PPFD		10/unknown	0.0% [12]	
Peach cv. 'GF677'				8/unknown	0.0% [12]	
Pear cv. 'Liu Yue Shian'	ASSVd	22°C *in vitro* for 55 days followed by excision of 0.5mm length apical meristems	RT-PCR: Plants were tested twice. *In vitro* plants were tested 12 weeks after initiation followed by testing of e*x vitro* plants that were micrografted onto *Pyrus betufolia* seedlings 12 weeks later. RNA was extracted from 400-800mg of tissue with Gene Release (BioVentures, Inc.). The extract	7/unknown	14.3% [13]	Postman & Hadidi (1995) [13]
		Alternates 30°C and 38°C every 4 hours *in vitro* for 55 days followed by excision of 0.5mm length apical meristems		7/unknown	85.7% [13]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		Alternates 22°C (8 hours) and -1°C (16 hours) *in vitro* for 7 days followed by 4°C for 49 days in the dark followed by excision of 0.5mm length apical meristems	was used for a modified two-step RT-PCR was performed and visualized on a 5% native polyacrylamide gel.	13/unknown	84.6% [13]	
Hop cv. 'Fuggle'	HLVd	Rhizomes stored at 2°C for 6 months in sealed plastic bags followed by excision of <0.5mm meristems with 1st leaf primordia. *In vitro* plants grown at 20°C-25°C with a 16h photoperiod provided with cool Phillips white fluorescent tubes	Nucleic acid hybridization: Petiole tissue from ex vitro plants was ground with buffer for dot-blot hybridization	4/unknown	0.0% [14]	Adams, Barbara, Morton, & Darby (1996) [14]
Hop cv. 'Fuggle'		Rhizomes stored at 2°C for 17 months in sealed plastic bags followed by excision of <0.5mm meristems with 1st leaf primordia. *In vitro* plants grown at 20°C-25°C with a 16h photoperiod provided with cool Phillips white fluorescent tubes		23/unknown	17.4% [14]	
Hop cv. 'Fuggle'		Rhizomes stored at 2°C for 21 months in sealed plastic bags followed by excision of <0.5mm meristems with 1st leaf primordia. *In vitro* plants grown at 20°C-25°C with a 16h photoperiod provided with cool Phillips white fluorescent tubes		14/unknown	7.1% [14]	
Male hop 24/68/103		Rhizomes stored at 2°C for 7 months in sealed plastic bags		5/unknown	100.0% [14]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Male hop 24/68/8		followed by being potted and grown at 4°C in the dark for 1 month. This was followed by excision of <0.5mm meristems with 1st leaf primordia. *In vitro* plants grown at 20°C-25°C with a 16h photoperiod provided with cool Phillips white fluorescent tubes		1/unknown	100.0% [14]	
Male hop 1/63/45				14/unknown	80.0% [14]	
Hop cv. 'Cobbs Golding'		Rhizomes stored at 2°C for 9 months in sealed plastic bags followed by excision of <0.5mm meristems with 1st leaf primordia. *In vitro* plants grown at 20°C-25°C with a 16h photoperiod provided with cool Phillips white fluorescent tubes		12/unknown	25.0% [14]	
Hop cv. 'Wye Challenger'				4/unknown	75.0% [14]	
Apple cv. 'Red delicious'	ASSVd	Dormant buds grafted onto Malus domestica seedling rootstock for 70 or more days at 38°C (16h photoperiod 202.5 PPFD) followed by excision of 5mm length shoots budded onto new rootstock growing in clay pots at 25°C	Testing was performed after plants were established for 1 year in the field. RT-PCR: 10g of bark or fruit tissue was homogenized for RNA extraction with phenol-chloroform. 10ng of viroid-enriched RNA was used for 2-step RT-PCR. Nucleic acid hybridization: 0.1-0.2g of tissue was ground with buffer for dot-blot hybridization.	10/10	40.0% [15]	Howell, Burgess, Mink, Skrzeczkowski, and Zhang (1998) [15]
Apple cv. 'Heiya'				5/5	40.0% [15]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Apple cv. 'Red delicious'		Dormant buds grafted onto Malus domestica seedling rootstock for 70 or more days at 25°C followed by excision of 5mm length shoots budded onto new rootstock growing in clay pots at 25°C	Bio-assay: Performed for 4 consecutive years. Cv. 'Stark Earliest' were budded onto 1-year old apple seedlings and inoculum buds were grafted under. Foliar epinasty indicated ASSVd infection.	4/4	0.0% [15]	
Apple cv. 'Heiya'				1/1	0.0% [15]	
Apple positive control isolates PK13, P112, and X4843	ASSVd	36°C-37°C for 41 days followed by excision of 2-4mm apical tips grafted onto cv. 'Virginia crab'	Nucleic acid hybridization: Nucleic acid was extracted from fruits, bark, or leaves (2 months or older) and used for dot-blot hybridization. Bio-assay: Buds were inoculated on cv. 'Starkrimson'	30/unknown	3.3% [16]	Desvignes, Grasseau, Boye, Cornaggia, Aparicio, Serio, & Flores (1999) [16]
Apple positive control isolate X4573		36°C-37°C for 99 days followed by excision of 2-4mm apical tips grafted onto cv. 'Virginia crab'		8/unknown	12.5% [16]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Apple positive control isolates PK13, X4530, X4533, X4843		4°C-5°C for 3 months followed by 48 days at 36°C-37°C followed by excision of 2-4mm apical tips grafted onto cv. 'Virginia crab'		43/unknown	88.4% [16]	
Chrysanthemum cv. 'Piato'	CSVd	Apical meristems were excised with leaf primordia grown at 20°C (+/- 3°C) with 16h photoperiod at 40.5 PPFD provided by cool white fluorescent tubes	Nested-PCR: Total RNA was extracted from 0.2g fresh tissue for reverse transcription. cDNA was used for nested-PCR.	Unknown	0% (14 tested) [17]	Hosokawa, Otake, Ohishi, Ueda, Hayashi, & Yazawa (2004) [17]
		Apical meristems were excised without leaf primordia and attached to root tips of Chrysanthemum cvs. 'Mistletoe' and 'Sei Alps' grown at 20°C (+/- 3°C) with 16h photoperiod at 40.5 PPFD provided by cool white fluorescent tubes		Unknown	14.3% (21 tested) [17]	
		Apical meristems were excised without leaf primordia and attached to *in vitro* root tips of cabbage cv. 'Shikidori' grown at 20°C (+/- 3°C) with 16h photoperiod at 40.5 PPFD provided by cool white fluorescent tubes		79/180	3.1% (64 tested) [17]	
Chrysanthemum cv. 'Piato' - low viroid titer				6/30	16.7% [17]	
Chrysanthemum cv. 'Piato'	CChMVd	Apical meristems were excised without leaf primordia and attached to *in vitro* root tips of cabbage cv. 'Harunami' grown at	Nested-PCR: Total RNA was extracted from 0.2g fresh tissue from 3-month-old plants with TRIzol (Invitrogen) for reverse	29/unknown	3.4% [18]	Hosokawa, Matsu-shita, Ohishi, &

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Chrysanthemum cv. 'Sttetsuman'		20°C (+/- 3°C) with 16h photoperiod at 40.5 PPFD provided by cool white fluorescent tubes	transcription. cDNA was used for PCR and nested-PCR.	6/unknown	33.3% [18]	Yazawa (2005) [18]
Hop cv. 'Marynka'	HLVd	0.3-0.5mm length meristems with 1-2 leaf primordia *in vitro* at 24°C with 16-hour photoperiod	RT-PCR: Total nucleic acid extract from leaves & petioles of 3 months *ex vitro* plants	8/30	37.5% [19]	Grudzinska & Solarska (2005) [19]
Hop cv. 'Lomik'				8/30	37.5% [19]	
Hop cv. 'Lubelski'				3/30	33.3% [19]	
Hop cv. 'Zbyszko'				10/30	30.0% [19]	
Avocado cv. 'Donaldson'	ASBVd	<0.5mm Meristems with 2-3 leaf primordia micrografted onto cv. 'Wilson Popenoe' rootstock *in vitro* at 25°C with 16-hour photoperiod produced by cool white fluorescent tubes at 40-50 PPFD	RT-PCR: 6-7 months after micrografting, RNA was extracted and used for modified 2-step RT-PCR	5/48	0.0% (3 tested) [20]	Suarez, Schnell, Kuhn, & Litz (2005) [20]
		0.5mm-1.0mm meristems with 2-3 leaf primordia micrografted onto cv. 'Wilson Popenoe' rootstock *in vitro* at 25°C with 16-hour photoperiod produced by cool white fluorescent tubes at 40-50 PPFD		32/48	0.0% (12 tested) [20]	
Avocado cv. 'Vero Beach' GRD		<0.5mm Meristems with 2-3 leaf primordia micrografted onto cv. 'Simmonds' rootstock *in vitro* at 25°C with 16-hour photoperiod produced by cool white fluorescent tubes at 40-50 PPFD		2/20	0.0% [20]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		0.5mm-1.0mm meristems with 2-3 leaf primordia micrografted onto cv. 'Simmonds' rootstock *in vitro* at 25°C with 16-hour photoperiod produced by cool white fluorescent tubes at 40-50 PPFD		8/20	0.0% (2 tested) [20]	
Avocado cv. 'Vero Beach' SE2	ASBVd	Nucellar tissue from avocado seed was established *in vitro* at 25°C with 16-hour photoperiod produced by cool white fluorescent tubes at 40-50 PPFD	RT-PCR: 12 months after micrografting, RNA was extracted RNA was extracted and used for modified 2-step RT-PCR	4/164	0.0% [21]	Suarez, Schnell, Kuhn, & Litz (2006) [21]
Peach cv. 'Florida Prince'	HSVd	37°C for 3 weeks *in vitro* followed by excision of 1-2mm shoots onto fresh medium	Nucleic acid hybridization: Petioles were printed directly 3 times on 3 separate nitrocellulose membranes (0.45µm).	40/40	0.0% [22]	El-Dougdoug, Osman, Hayam, Rehab, & Reham (2010) [22]
Pear cv. 'Balady'				40/40	0.0% [22]	
Peach cv. 'Florida Prince'		4°C for 1 month *in vitro* with a 16h photoperiod at 67.5 PPFD followed by excision of 1-2mm shoots onto fresh medium at 25°C with the same light regime.		28/40	5.0% [22]	
Pear cv. 'Balady'				31/40	5.0% [22]	
Peach cv. 'Florida Prince'		4°C for 2 months *in vitro* with a 16h photoperiod at 67.5 PPFD followed by excision of 1-2mm shoots onto fresh medium at 25°C with the same light regime.		25/40	12.0% [22]	
Pear cv. 'Balady'				24/40	12.0% [22]	
Peach cv. 'Florida Prince'		4°C for 3 months *in vitro* with a 16h photoperiod at 67.5 PPFD followed by excision of 1-2mm shoots onto fresh medium at 25°C with the same light regime.		10/40	18.0% [22]	
Pear cv. 'Balady'				12/40	18.0% [22]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 10mg of Virazole per liter for 1 month followed by excision of 1-2mm shoots onto fresh medium		29/40	18.0% [22]	
Pear cv. 'Balady'				32/40	15.0% [22]	
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 20mg of Virazole per liter for 1 month followed by excision of 1-2mm shoots onto fresh medium		28/40	33.0% [22]	
Pear cv. 'Balady'				31/40	35.0% [22]	
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 30mg of Virazole per liter for 1 month followed by excision of 1-2mm shoots onto fresh medium		14/40	41.0% [22]	
Pear cv. 'Balady'				8/40	40.0% [22]	
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 10mg of thiouracil per liter for 1 month followed by excision of 1-2mm shoots onto fresh medium		22/40	10.0% [22]	
Pear cv. 'Balady'				26/40	10.0% [22]	
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 20mg of thiouracil per liter for 1		16/40	12.0% [22]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Pear cv. 'Balady'		month followed by excision of 1-2mm shoots onto fresh medium		24/40	25.0% [22]	
Peach cv. 'Florida Prince'		*In vitro* plants were grown on media supplemented with 30mg of thiouracil per liter for 1 month followed by excision of 1-2mm shoots onto fresh medium		7/40	18.0% [22]	
Pear cv. 'Balady'				8/40	30.0% [22]	
Peach cv. 'Florida Prince'		4°C for 1 month *In vitro* supplemented with 20mg of Virazole per liter followed by excision of 1-2mm shoots onto fresh medium		25/40	40.0% [22]	
Pear cv. 'Balady'				30/40	40.0% [22]	
Chrysanthemum cv. 'Ency'	CSVd	0.2-0.4mm meristems with 1 leaf primordium were grown at 22°C± 2°C with a 16h at 40 PPFD	RT-PCR: Total RNA was extracted from 0.1g *in vitro* plants with TRIzol (Invitrogen) or RNeasy Mini Kit (Qiagen) for reverse transcription. cDNA was used for PCR.	7/107	28.6% [23]	Jeon, Savitri, Park, Chung, & Kim (2012) [23]
		0.2-0.4mm meristems with 2 leaf primordia were grown at 22°C± 2°C with a 16h at 40 PPFD		32/91	22.2% [23]	
		≥0.5mm meristem with 3 leaf primordia were grown at 22°C± 2°C with a 16h at 40 PPFD		23/39	0.0% [23]	
		≥0.5mm meristem with 4 leaf primordia were grown at 22°C± 2°C with a 16h at 40 PPFD		41/50	0.0% [23]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		In vitro plantlets grown at 24°C followed by excision of 0.2-0.4mm with 1 or 2 leaf primordia grown at 22°C± 2°C with 20 PPFD for 1 week before reverting to 40 PPFD		11/32	27.3% [23]	
		37°C for 2 weeks with 16h photoperiod at 400 PPFD *In vitro* followed by excision of 0.2-0.4mm with 1 or 2 leaf primordia grown at 22°C± 2°C with a 16h at 20 PPFD for 1 week before reverting to 40 PPFD		17/45	29.4% [23]	
		37°C for 4 weeks with 16h photoperiod at 400 PPFD *In vitro* followed by excision of 0.2-0.4mm with 1 or 2 leaf primordia grown at 22°C± 2°C with a 16h at 20 PPFD for 1 week before reverting to 40 PPFD		16/45	25.0% [23]	
		37°C for 6 weeks with 16h photoperiod at 400 PPFD *In vitro* followed by excision of 0.2-0.4mm with 1 or 2 leaf primordia grown at 20PPFD for 1 week before reverting to 40 PPFD		6/25	4.0% [23]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		37°C for 8 weeks with 16h photoperiod at 400 PPFD *In vitro* followed by excision of 0.2-0.4mm with 1 or 2 leaf primordia grown at 22°C± 2°C with a 16h at 20PPFD for 1 week before reverting to 40 PPFD		5/27	3.7% [23]	
Chrysanthemum cv. 'Ency'	CSVd	0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C with 16h photoperiod at 70 PPFD (provided by cool white fluorescent lights) and maintained at the same culture conditions	Nested PCR: Total RNA was extracted from 0.1g *in vitro* plants with TRI Reagent (Ambion) for reverse transcription. cDNA was used for PCR of a 350bp and 237bp product. PCR product was then used for nested PCR for 205bp and 214 bp product.	70/70	50% (40 tested) [24]	Savitri, Park, Jeon, Chung, Han, & Kim (2013) [24]
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 1 month *in vitro* (same light regime) followed by excision 3mm shoot tips cultured at 24°C± 2°C with the previous light regime.		19/19	60% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights)		14/19	90% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		followed by culture at 4°C for 2 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured at 24°C± 2°C with the previous light regime.				
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 3 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured at 24°C± 2°C with the previous light regime.		16/20	80% (10 tested) [24]	
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems were grown on media supplemented with 25mg/L of Amantadine at the same temperature and light regime		29/30	50% (10 tested) [24]	
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems		21/24	50% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		were grown on media supplemented with 50mg/L of Amantadine at the same temperature and light regime				
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems were grown on media supplemented with 100mg/L of Amantadine at the same temperature and light regime		29/30	20% (10 tested) [24]	
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems were grown on media supplemented with 25mg/L of Ribavirin at the same temperature and light regime		28/29	40% (10 tested) [24]	
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems were grown on media supplemented with 50mg/L of		27/30	20% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		Ribavirin at the same temperature and light regime				
		0.5-0.7mm meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C± 2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights). Meristems were grown on media supplemented with 100mg/L of Ribavirin at the same temperature and light regime		26/30	60% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 1 month *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Amantadine at 24°C±2°C and the same light regime		10/20	80% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 1 month *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Amantadine at 24°C±2°C and the same light regime		12/20	50% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 1 month *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Ribavirin at 24°C±2°C and the same light regime		17/17	70% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 1		19/19	70% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		month *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Ribavirin at 24°C±2°C and the same light regime				
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 2 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Amantadine at 24°C±2°C and the same light regime		9/20	77.8% (9 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 2 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Amantadine at		7/20	100% (7 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		24°C±2°C and the same light regime				
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 2 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Ribavirin at 24°C±2°C and the same light regime		17/20	80% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 2 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Ribavirin at 24°C±2°C and the same light regime		13/20	70% (10 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 3 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Amantadine at 24°C±2°C and the same light regime		6/20	100% (6 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 3 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Amantadine at 24°C±2°C and the same light regime		4/18	75% (4 tested) [24]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 3 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 50mg/L of Ribavirin at 24°C±2°C and the same light regime		17/20	100% (10 tested) [24]	
		0.5-0.7mm length meristems with 2-3 leaf primordia were excised from *in vitro* plants cultured at 24°C±2°C (16h photoperiod at 70 PPFD with cool white fluorescent lights) followed by culture at 4°C for 3 months *in vitro* (same light regime) followed by excision 3mm shoot tips cultured on media supplemented with 100mg/L of Ribavirin at 24°C±2°C and the same light regime		18/20	100% (10 tested) [24]	
Argyranthemum cv. 'Border Dark Red'	CSVd	4-week-old *in vitro* plants (grown at 23°C, 18h photoperiod with 50 PPFD provided by cool white	Tissue was taken from 6-month-old *in vitro* plants RT-PCR: Total RNA was extracted from 0.1g tissue with a Plant RNA	30/30	0.0% [25]	Zhang, Lee, Sivertson, Skjeseth,

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Argyranthemum cv. 'Yellow Empire'		fluorescent tubes) were put into cold treatment at 5°C for 1 month (same light regime) followed by excision of 0.2mm length meristems cultured at 23°C (same light regime)	mini kit (Omega Bio-Tek) and used for 1-step RT-PCR. Nucleic acid hybridization: 0.1g of tissue was crushed with buffer and used according to the manufacturer's instructions (Agdia, USA).	25/30	0.0% [25]	Haugslien, Clarke, Wang, & Blystad (2016) [25]
Argyranthemum cv. 'Border Dark Red'		4-week-old *in vitro* plants (grown at 23°C, 18h photoperiod with 50 PPFD provided by cool white fluorescent tubes) were put into cold treatment at 5°C for 2 months (same light regime) followed by excision of 0.2mm length meristems cultured at 23°C (same light regime)		13/30	0.0% [25]	
Argyranthemum cv. 'Yellow Empire'				11/30	0.0% [25]	
Argyranthemum cv. 'Border Dark Red'		4-week-old *in vitro* plants (grown at 23°C, 18h photoperiod with 50 PPFD provided by cool white fluorescent tubes) were put into cold treatment at 5°C for 3 months (same light regime) followed by excision of 0.2mm length meristems cultured at 23°C (same light regime)		25/30	18.2% (11 tested) [25]	
Argyranthemum cv. 'Yellow Empire'				15/30	0.0% [25]	
Argyranthemum cv. 'Border Dark Red'		4-week-old *in vitro* plants (grown at 23°C, 18h photoperiod with 50 PPFD provided by cool white		19/30	0.0% [25]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
Argyranthemum cv. 'Yellow Empire'		fluorescent tubes) were put into cold treatment at 5°C for 6 months (same light regime) followed by excision of 0.2mm length meristems cultured at 23°C (same light regime)		23/30	0.0% [25]	
Argyranthemum cv. 'Border Dark Red'		4-week-old *in vitro* plants (grown at 23°C, 18h photoperiod with 50 PPFD provided by cool white fluorescent tubes) were put into cold treatment at 5°C for 12 months (same light regime) followed by excision of 0.2mm length meristems cultured at 23°C (same light regime)		14/30	0.0% [25]	
Argyranthemum cv. 'Yellow Empire'				26/30	0.0% [25]	
Argyranthemum cv. 'Border Dark Red'		0.2mm length meristems from 4-week-old *in vitro* plants cultured at 23°C with an 18h photoperiod at 50 PPFD provided by cool white fluorescent tubes		21/30	0.0% [25]	
Argyranthemum cv. 'Yellow Empire'				22/30	0.0% [25]	
Chrysanthemum cv. 'Borami'	CSVd	Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 1h in liquid nitrogen (excised from *in vitro* plants and precultured and pretreated at -20°C for 1h before cryotherapy).	Nested-RT-PCR: 6 weeks after culturing, total RNA was extracted with an RNeasy Plant Mini Kit for a 2-step RT-PCR. An aliquot of the RT-PCR product was used for the nested PCR (expected size of 204-bp for CSVd & 242 for CChMVd)	Unknown	13.3% (15 tested) [26]	Jeon, Naing, Kim, Chung, Lim, & Kim (2016) [26]

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 3h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	13.3% (15 tested) [26]	
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 5h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 7h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 10h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	
		Shoot tips with no primordia were subjected to cryotherapy for 1h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 1h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	13.3% (15 tested) [26]	
		Shoot tips with 3-4 leaf primordia were subjected to cryotherapy for 1h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	
		Shoot tips with 1-2 leaf primordia were subjected to cryotherapy for 1h in liquid nitrogen (excised from in vitro plants and precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	13.3% (15 tested) [26]	
		In vitro plants stored at -20°C for 1h prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	0% (15 tested) [26]	
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and		Unknown	20% (15 tested) [26]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		pretreated at -20°C for 1h before cryotherapy).				
		In vitro plants stored at 4°C for 8 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy).		Unknown	6.7% (15 tested) [26]	
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Chilgok region of the Republic of Korea.		Unknown	0% (15 tested) [26]	
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gumi region of the Republic of Korea.		Unknown	20% (15 tested) [26]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gyeongsan region of the Republic of Korea.		Unknown	0% (15 tested) [26]	
Chrysanthemum cv. 'Secret Pink'		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Chilgok region of the Republic of Korea.		Unknown	0% (15 tested) [26]	
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gumi region of the Republic of Korea.		Unknown	6.7% (15 tested) [26]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gyeongsan region of the Republic of Korea.		Unknown	0% (15 tested) [26]	
Chrysanthemum cv. 'Yellow Cap'	CChMVd	In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Chilgok region of the Republic of Korea.		Unknown	0% (15 tested) [26]	
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gumi region of the Republic of Korea.		Unknown	13.3% (15 tested) [26]	

Crop	Viroid	Therapy	Diagnostic technology	Regeneration rate (survive/ excised)	Elimination rate [Citation Number][a]	Citation [Number]
		In vitro plants stored at 4°C for 4 weeks prior to excision of shoot tips with 1-2 leaf primordia for cryotherapy for 1h in liquid nitrogen (precultured and pretreated at -20°C for 1h before cryotherapy). Shoot tips harvested from the Gyeongsan region of the Republic of Korea.		Unknown	0% (15 tested) [26]	

Appendix II: RT-qPCR primer design and optimization

Introduction:

Quantification of target RNA using reverse-transcriptase quantitative PCR (RT-qPCR) can be performed as an absolute count of the target, or measured relative to reference genes (Ruijter et al., 2013; Štajner et al., 2013). While absolute quantification can tell you how many copies of the viroid are present, it relies on several assumptions: that (1) total RNA extraction efficiency is the same between samples, tissue types, and experimental conditions; and that (2) RNA samples with the same concentrations have the same distribution of host-to-viroid RNA (Ruijter et al., 2013). For the purposes of understanding titer and distribution of viroids in plant tissue, relative quantification is more informative as viroid titers are normalized against expression of internal controls, usually a constitutively expressed housekeeping gene or genes (Ruijter et al., 2013; Štajner et al., 2013). Ideal reference gene candidates are genes that are expressed in all tissues and have minimal variability in expression between samples and experimental treatments.

For the purposes of this study, primers and probes for three viroids, Hop stunt viroid (HSVd), Hop latent viroid (HLVd), and Citrus bark cracking viroid (CBCVd), and three host reference genes, *POAC, TIP41,* and *YLS8* were designed and optimized.

Methods:

Sample collection and extraction

Petiole and leaf tissue were collected from cv. 'Comet' plants naturally coinfected with HSVd and HLVd in a growth chamber at 25°C (16h photoperiod; 150 PPFD provided by blue/red LEDs). Green bark tissue from CBCVd-infected citrus was kindly provided by Dr. Georgios Vidalakis (UC Riverside). Negative controls were petiole and leaf tissue collected from 'Comet' plants freed of detectable viroid and virus infection. Positive controls included 'Comet' and 'Chinook' plants singly infected with HSVd or naturally co-infected with HSVd, HLVd, ApMV (Apple mosaic virus), AHLV (American hop latent virus), HMV (Hop mosaic virus), and HLV (Hop latent virus). Samples of unknown status were kindly provided by Josh Havill (University of Minnesota), Dr. Kayla Altendorf (USDA-ARS, Corvallis), and Dr. Kim Hummer (USDA-ARS, Corvallis). Total RNA was extracted with a Spectrum Plant Total RNA extraction kit (STRN250, MilliporeSigma, Burlington MA) following the manufacturer's instructions.

RT-qPCR primer design

The assays for HSVd, HLVd, and CBCVd were designed by adding dual-labeled probes to published RT-PCR assays. The exception is the forward primer for the CBCVd assay by Bernad & Duran-Vila (2006), which was modified by the addition of three additional nucleotides to the 3' end to make the melting temperature closer to the reverse primer. The probes were designed using RealTimeDesign software (LGC Biosearch Technologies, Novato, CA).

In hops (*Humulus lupulus* L.), Štajner et al., (2013)evaluated 23 reference gene candidates. Using this study as a foundation, primer sets were designed for six genes (*YLS8, DRH1, TIP41,*

CAC, POAC, and *SAND*) reported by the study to be the most stably expressed under biotic stress caused by *Verticillium albo-atrum* infection (Štajner et al., 2013). RT-qPCR and dual-labeled probes were designed using the RealTimeDesign. Primers and probes used in this study are listed in Table A.2.1.

RT-qPCR optimization

Optimization of the reaction conditions for each primer and probe set was performed using qScript XLT 1-Step RT-qPCR ToughMix (95132, Quantabio, Beverly, MA) reagents on a CFX96 real-time thermocycler (Bio-Rad Laboratories, Hercules, CA). Parameters optimized included $MgSO_4$ concentration (3 mM to 6 mM) and annealing/extension temperature (57°C-68°C). Samples were run in triplicate. Each assay was optimized against cross-reactivity with host and non-target virus using known positive and negative controls.

Assay validation

The limit of detection for each assay was determined with a dilution series using *in vitro* synthesized viroid RNA. Forward primers for each assay with a T7 promoter (5'TAATACGACTCACTATAGGGAGA3' added to the 5' end of each forward primer) were designed and used to generate full-length viroid cDNAs. The PCR products were run on a 1.5% agarose gel (1613102, Bio-Rad Laboratories) and the band corresponding to the size of the expected product (Table A.2.1) was excised. The PCR product was purified using a GenElute PCR Clean-Up Kit (NA1020, MilliporeSigma). Full-length viroid RNAs were synthesized using the MAXIscript SP6/T7 kit with the T7 polymerase according to the manufacturer's instructions (AM1322, Thermo Fisher Scientific, Waltham MA). DNase treatment was performed using TURBO DNA-*free* kit (AM1907, Thermo Fisher Scientific). The RNA was quantified using the RNA

BR assay (Q10211, Thermo Fisher Scientific) on a Qubit 4 fluorometer (Thermo Fisher Scientific). A dilution series of 10^0-10^7 copies was created by diluting the synthetic RNA into viroid-free hop RNA extract. For each dilution, a RT-qPCR was performed in triplicate using the qScript RT-qPCR XLT One-Step Tough Mix with 400 µM each of the forward and reverse primers, 100 µM probe, and 2 µL of each dilution step on a CFX96 real-time thermocycler with the following conditions: reverse-transcription for 15 mins at 50°C (10 mins for CBCVd) and initial denaturation at 94°C for 2 mins, followed by 40 cycles of 94°C for 15 seconds and 63°C for 60 seconds (of 94°C for 10 seconds and 58°C for 45 seconds for CBCVd). The Bio-Rad CFX Maestro (Version 4.1.2433.1219, Bio-Rad Laboratories) software was used to create a standard curve and calculate the corresponding R^2 and efficiency. LinRegPCR (Version 11.0, Ruijter et al., 2013) was used to calculate mean PCR efficiency using non-baseline corrected data exported from CFX Maestro for HLVd, HSVd, CBCVd*, POAC, TIP41*, and *YLS8*.

Results:

Reaction optimization

For HSVd, HLVd, *POAC, TIP41,* & *YLS8*, an annealing temperature of 63°C worked well. *CAC, SAND,* and *DHR1,* showed a significant shift of 2 cycles higher at 63°C compared to the three other reference genes from the same samples. The CBCVd assay performed best at an annealing temperature of 58°C. Annealing temperatures above 64°C increased Cq values for all assays. Additional Mg^{2+} did not result in higher Cq values.

Non-target amplification was not detected in no-template controls, viroid-free samples, and virus-free samples for all three viroid assays. Cross-reactivity of HLVd was not detected in

hop RNA samples with either single infections or natural co-infections of HSVd and ApMV. Cross-reaction of HSVd and CBCVd was not detected in any hop samples naturally co-infected with any combination of ApMV, AHLV, HMV, and HLV from 'Comet' and 'Chinook' samples (HLVd was always present in these combinations). CBCVd was not detected in any hop samples and only total RNA from the citrus samples received from UC Riverside produced amplification.

Assay validation

The assays for HSVd and CBCVd were able to detect down 10^0 copies of each viroid per reaction with reaction efficiencies of 99.8% and 98%, respectively (Figure A.2.1). HLVd could reliably detect 10^1 copies per reaction with a reaction efficiency of 102.1%. Efficiencies greater than 100% indicate inhibition of the PCR (Suslov & Steindler, 2005). Mean PCR efficiencies calculated for each assay was ≥1.94 (Table A.2.3).

Optimized conditions

With the focus of the project on localization and distribution of HSVd and HLVd, the reference genes *POAC, TIP41* and *YLS8* were selected due to their compatibility with the viroid assay thermocycler conditions. The final optimized conditions for the all six assays used the following conditions per 20 µL reaction: 10 µL 2x qScript XLT One-Step RT-qPCR reagent, 400 nM forward primer, 400 nM reverse primer, 100 nM probe, and 2µL of total RNA. For HSVd, HLVd, and the three reference genes, the following thermocycling conditions were used for all reactions: reverse transcription for 15 mins at 50°C and initial denaturation at 94°C for 2 mins followed by 40 cycles of 94°C for 15 seconds and 63°C for 60 seconds. For CBCVd, the following cycling conditions were used: reverse transcription for 10 mins at 50°C and initial denaturation

at 94°C for 2 mins followed by 40 cycles of 94°C for 15 seconds and 58°C for 60 seconds.

Table A.2.1. Humulus lupulus *host gene primer and probe sequences*

Reference gene	Primer Name	Function	Sequence (5'-3')	Expected product size (bp)
POAC	POAC-F	Forward	GATCGCATAGCGAGAATTTCCTC	87
POAC	POAC-R	Reverse	GCAACGTAGTGATGTCTTGGA	
POAC	POAC-P	Probe	FAM-TGCCATTCGAGTGATCCCGAAC-BHQ-1	
TIP41	TIP41-F	Forward	GGGACTTTAAGCACTTCAGACTTG	69
TIP41	TIP41-R	Reverse	ACAGCGACCCTAGCATCA	
TIP41	TIP-P	Probe	FAM-CATGACAATGGGAAGTCTCTGGCTGA-BHQ-1	
YLS8	YLS8-F	Forward	GCCGTAGATCAGGCCATCCT	74
YLS8	YLS8-R	Reverse	GGTCTCGTCCCAGTCGTG	
YLS8	YLS8-P	Probe	FAM-CGCCGAAGAAGAGCGTCTCGTC-BHQ-1	

Table A.2.2. Viroid primer and probe sequences. a – Matousek & Patzak (2000); b – A. Wright, unpublished. c – Ito et al. (2002); d – Papayiannis (2014); Modification of Bernad & Duran-vila (2006); f – this study

Viroid	Primer Name	Function	Sequence	Expected product size (bp)
HLVd[a]	HLVd-1	Forward	ATACAACTCTTGAGCGCCGA	256
HLVd[a]	HLVd-2	Reverse	CCACCGGGTAGTTTCCAACT	
HLVd[b]	AW94	Probe	FAM-CAAGGGCTCGAAGAGGGATCCCC-BHQ-1	
HSVd[c]	HSVd-CV2-AP	Forward	GGCAACTCTTCTCAAATCCAGC	302
HSVd[c]	HSVd-CV2-AM	Reverse	CCGGGGCTCCTTTCTCAGGTAAGT	
HSVd[d]	HSVd-RT-P	Probe	FAM-CGTCCCTTCTTCTTTACCTTCTCCT GGCTC-BHQ-1	
CBCVd[e]	CBCVd-F1	Forward	GGGGAAATCTCTTCAGACTCG	284
CBCVd[e]	CBCVd-F2	Reverse	GGGGATCCCTCTTCAGGT	
CBCVd[f]	CBCVd-P	Probe	FAM-CGATCGTCGCTTCTTCCTTCGCG-BHQ-1	

Table A.2.3. Mean PCR efficiency calculated by LinRegPCR using non-baseline corrected amplification data.

Target	Samples included	Mean Efficiency	Standard Deviation
Total	156	1.992	0.035
HLVd	11	2.050	0.026
HSVd	14	1.991	0.030
CBCVd	14	1.999	0.031
POAC	22	2.000	0.027
TIP41	52	1.945	0.027
YSL8	43	1.965	0.032

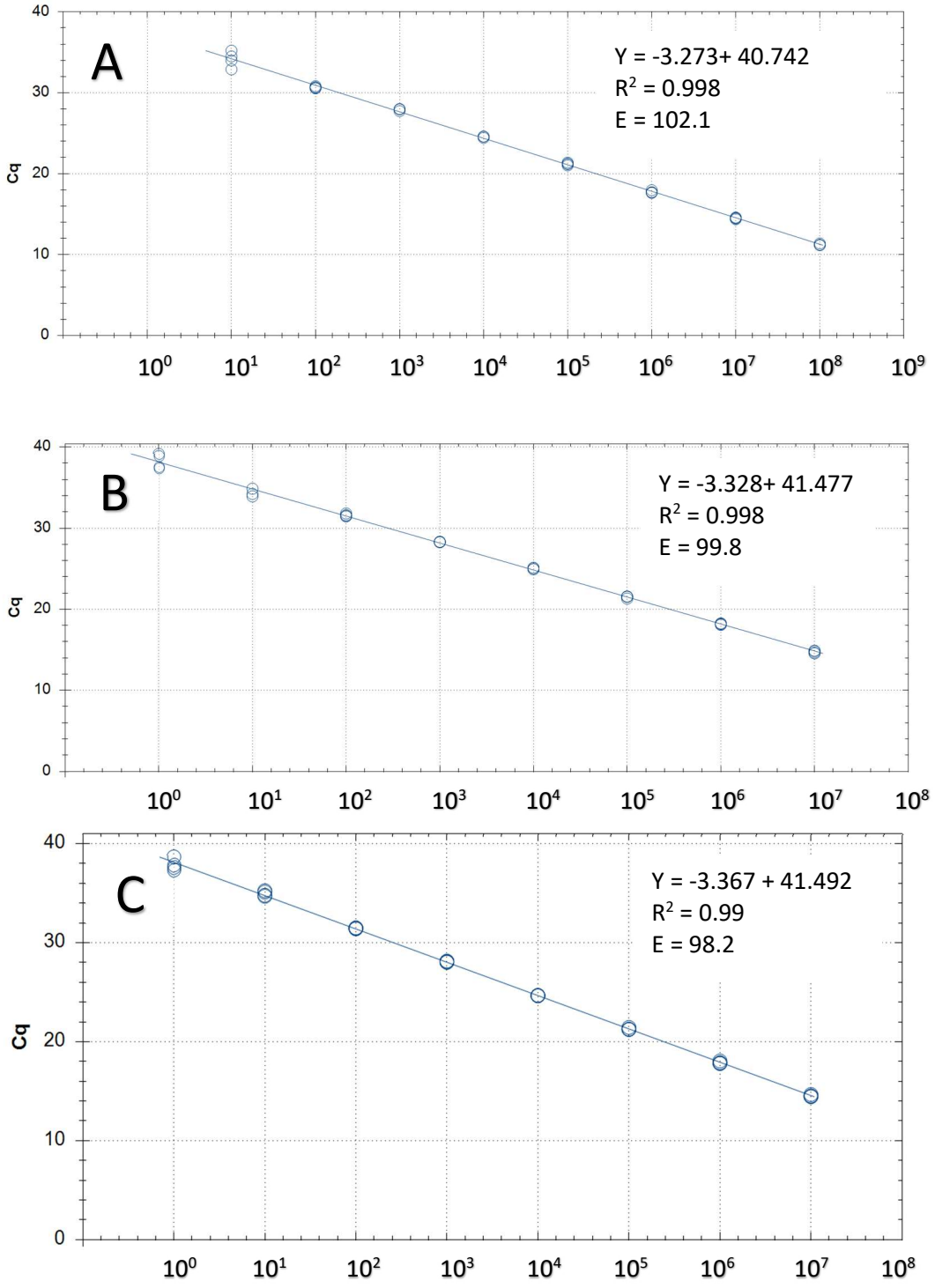

Figure A.2.3. Standard curves generated from the serial dilution of synthetic RNA standards for (A) HLVd, (B) HSVd, and (C) CBCVd diluted in hop total RNA background. Reactions were performed in triplicate. (A) was diluted 10^9 to 10^1 (B) and (C) were diluted from 10^8 to 10^0

Appendix III: Optimization of hop tissue culture

Introduction:

Viroid and virus elimination therapies have relied heavily upon regeneration of submillimeter-sized pieces of plant tissue (Barba et al., 2017; A. Panattoni et al., 2013; Wang et al., 2018). Recovery of apical meristems requires either a suitable axenic tissue culture medium or *in vitro* micrografting onto a compatible rootstock (Roistacher et al., 1976; Torres, 1989; Trigiano & Gray, 1996). At their core, tissue culture media are plant fertilizer solutions with macro (nitrogen, potassium, phosphorous, calcium, sulfur & magnesium) and micronutrients (iron, manganese, zinc, boron, iodine, copper, molybdenum, & cobalt) supplemented with a carbohydrate source that may be solidified with a gelling agent (Trigiano & Gray, 1996). Optional amendments include vitamins, amino acids, plant growth regulators, and pH regulators. Due to the complexity of media formulations, commercially available formulations have become a popular option to reduce labor and reduce variability. The leading formulation for commercial and research labs is based on Murashige & Skoog (1962) due to its broad applicability for higher plants including tobacco, tomatoes, potatoes, Arabidopsis, apples, and many others (Taiz et al., 2014; Torres, 1989; Trigiano & Gray, 1996).

Reed et al. (2003) formulated a suitable tissue culture media, named NCGR-HUM, for initiation of hop (*Humulus lupulus* L.) meristems and maintenance/multiplication of the shoots. Differences due to the idosyncracies of any particular lab and sources/lots of reagents, it is necessary to validate tissue culture recipes for your lab. Therefore, this work was done to optimize this recipe for the tissue culture lab at Clean Plant Center Northwest for regernating

hop meristems. The goal is to establish media for initiation of the meristems, maintenance, and rooting.

Methods:

Media optimization

Media were formulated based on the NCGR-HUM medium (Reed et al., 2003) (Table A.3.1). The original recipe did not list sources for reagents. To compensate for this difference in sources of gelling agent, the amount of GelZan and the pH were increased to have a firmer gel. Addition of indole-acetic acid (IAA) was trialed for the initiation media to see if a ratio of auxin-to-cytokinin may improve meristem survival (opposed to only a cytokinin) – this media was named MBI (Table A.3.1). For the maintenance media, the levels of 6-benzylaminopurine (BA) were decreased to examine the effects on callus production – this media was named MS+0.5 BA (Table A.3.1). The rooting media was based on recipes from other crops that decreased the concentrations of basal salts by half and switched the cytokinin for auxin at the same absolute quantities (mg/L) – this media was called Hop RM (Table A..3.1).

Hormone stocks were made in house. BA stock was made by dissolving 50 mg of BA powder in 1 mL of 1 M KOH followed by addition of 49 mL of de-ionized water – 10 mL aliquots were frozen at -20°C and thawed as needed. IAA stock was made by dissolving 50 mg of IAA powder in 1 mL of absolute ethanol followed by addition of 49 mL of de-ionized water – 1 mL aliquots were frozen at -20°C and thawed as needed.

The protocol for mixing one liter of media was as follows (Table A.3.1): dissolve basal salt in 800 ml of de-ionized water followed by addition of D-glucose and hormone stock. Adjust

the total volume to 1,000 mL with de-ionized water in a graduated cylinder. Adjust the pH with 0.1-1.0 M KOH and add gelling agents. Cover the solutions with foil and autoclave at 105°C for 5 minutes to dissolve the gelling agents. Dispense 7.0 mL of melted media into each 25 mM wide by 100 mM tall flat-bottom test tube. Cap all tubes and autoclave at 121°C for 15 minutes and cool over night before using.

Plant source

Shoot tips of the hop cultivar 'Comet' were harvested from potted plants growing at 25°C with a 16h photoperiod at 150 PPFD provided by LED lights (blue/red) (Figure A.3.1). Apical meristems of ~0.1 mm in length (with 1-2 leaf primordia) were excised onto initiation media (Figure A.3.1). Eighteen meristems were initiated on NCGR-HUM and 40 on MBI. Tissue culture plants were cultured at 21°C with 16h photoperiod at 70 PPFD provided by Arize horticultural LED lights (blue/red) (General Electric, Boston, MA). Explants were transferred to fresh media every two weeks. Notes on the size of the plants and number of dead plants were recorded at 2 and 3 months. Survival was measured by the number of meristems that developed shoots compared to the total number of plants that were established.

Results:

Adding a low concentration of Indole-acetic (IAA) acid helped meristems (~0.1mm length) develop faster and increased survival compared to the original NCGR-HUM media (Table A.3.1) (Table A.3.2 and A.3.3). As leaves and shoots differentiated, they developed abnormally with vitrified growth on MBI. To combat this, they were transferred to NCGR-HUM, but the plants developed large callus growth. Transferring plants from MBI or NCGR-HUM to MS+0.5BA

resulted in reduced callus growth while avoiding vitrification. Hop RM was trialed as a potential rooting media; however, plants on MS+0.5 BA produced the roots at the same rate and shoot growth was better. In conclusion, hop meristems should be initiated on MBI media and transferred every two weeks to fresh MBI until shoots have developed. Explants with shoots should be transferred to MS+0.5BA for maintenance and rooting.

Table A.3.1. Tissue culture media formulations trialed for hops. Deionized water was generated with a MilliQ Synergy system (MilliporeSigma). The reagent suppliers for the original Reed et al. (2003) publication did not list the source – the sources/product number listed here are for this experiment.

Amount	**Ingredient**	**Supplier**	**Product Number**
NCGR-HUM (Reed et al. 2003), pH 5.0			
4.43 grams	Murashige and Skoog Basal Medium with Vitamins	PhytoTech Labs	M519
20.00 grams	D-Glucose	MilliporeSigma	G7021
3.00 grams	Agarose	MilliporeSigma	A4550
1.25 grams	Gelzan	PhytoTech	G3251
4.44 µMol	6-Benzylaminopurine	PhytoTech	B800
up to 1 liter	Deionized water	N/A	N/A
MBI, pH 5.50			
4.43 grams	Murashige and Skoog Basal Medium with Vitamins	PhytoTech Labs	M519
20.00 grams	D-Glucose	MilliporeSigma	G7021
3.00 grams	Agarose	MilliporeSigma	A4550
1.50 grams	Gelzan	PhytoTech	G3251
4.44 µMol	6-Benzylaminopurine	PhytoTech	B800
0.571 µMol	Indole-Acetic acid	PhytoTech	I885
up to 1 liter	Deionized water	N/A	N/A
MS + 0.5BA, pH 5.50			
2.22 grams	Murashige and Skoog Basal Medium with Vitamins	PhytoTech Labs	M519
20.00 grams	D-Glucose	MilliporeSigma	G7021
3.00 grams	Agarose	MilliporeSigma	A4550
1.50 grams	Gelzan	PhytoTech	G3251

Amount	Ingredient	Supplier	Product Number
2.22 µMol	6-Benzylaminopurine	PhytoTech	B800
up to 1 liter	Deionized water	N/A	N/A
Hop RM, pH 5.50			
4.43 grams	Murashige and Skoog Basal Medium with Vitamins	PhytoTech Labs	M519
20.00 grams	D-Glucose	MilliporeSigma	G7021
3.00 grams	Agarose	MilliporeSigma	A4550
1.50 grams	Gelzan	PhytoTech	G3251
5.71 µMol	Indole-Acetic acid	PhytoTech	I885
up to 1 liter	Deionized water	N/A	N/A

Table A.3.2. Micro-shoot regeneration after two months of growth on MBI or NCGR-HUM media. Approximately 40 and 18 apical meristems (~0.1mm in length) were excised on MBI and NCGR-HUM media, respectively.

Meristem size	MBI		NCGR-HUM	
	Explants	Percentage	Explants	Percentage
Dead	0	0%	1	5.6%
<1mm	4	10%	1	5.6%
1-3mm	20	50%	11	61.1%
>3mm	4	10%	3	16.7%
callus+shoot formed	5	12.5%	0	0%
shoot formed	7	17.5%	2	10.1%

Table A.3.3. Micro-shoot regeneration after three months of growth on MBI or NCGR-HUM media. Approximately 40 and 18 apical meristems (~0.1mm in length) were excised on MBI and NCGR-HUM media, respectively.

Meristem size	MBI		NCGR-HUM	
	Explants	Percentage	Explants	Percentage
Dead	4	10%	2	11.1%
<1mm	0	0%	0	0%
1-3mm	9	22.5%	7	38.9%
callus+shoot formed	5	12.5%	3	16.7%
shoot formed	22	55%	6	33.33%

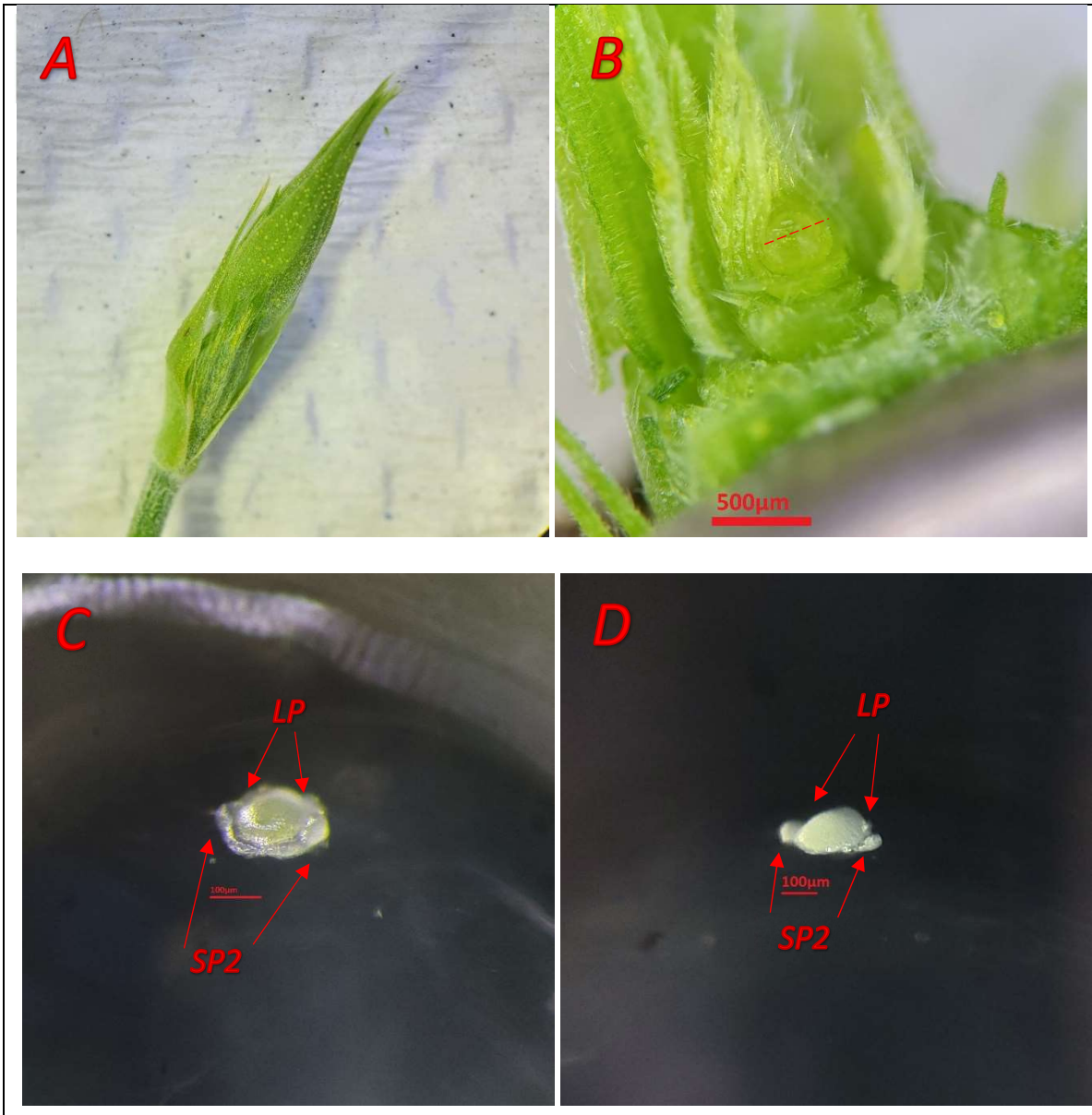

Figure A.3.1. Extraction of hop meristems from shoot tip to excised meristem. (A) Hop shoot. (B) 50x magnification of hop shoot with apical meristem exposed. Dashed red line indicates excision location (C & D) 160x magnification of hop meristem with first leaf primordia (LP) and second stipule primordia (SP2).

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