THESIS

ASSESSING THE IMPACT OF HOP LATENT VIROID (HLVD) ON HEMP (*CANNABIS SATIVA*) AND EXPLORING MITIGATION STRATEGIES

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ABSTRACT

ASSESSING THE IMPACT OF HOP LATENT VIROID (HLVD) ON HEMP (CANNABIS SATIVA) AND EXPLORING AND EXPLORING MITIGATION STRATEGIES

The emergence of hop latent viroid (HLVd) in the hemp (*Cannabis sativa L.* <0.3% THC) and marijuana (*C. sativa* >0.3% THC) industries poses a significant threat to agricultural production. Since its first identification in California in 2019, HLVd has spread across the U.S. and Canada, causing substantial yield losses due to its ability to severely disrupt crop development. Mechanically transmitted through propagation, pollen, seed, and water, HLVd is challenging to control; and its impact on emerging crops like hemp is of particular concern. The overarching goal of this thesis was to determine the impact of HLVd on hemp quality and yield and to apply new knowledge and practices to manage HLVd, reducing losses in the hemp and marijuana industries in the U.S. and worldwide. Two objectives achieved this goal: 1) assess the impact of HLVd on the growth and yield of two hemp cultivars, NWG 2463 (dual-purpose fiber grain) and Unicorn (CBD), and 2) determine the effect of chemical elicitors in reducing HLVd infection levels and symptom development. HLVd infection did not impact overall yield metrics—such as biomass, flower, or seed yield—in either the dual-purpose cultivar NWG 2463 or the CBD cultivar Unicorn under the conditions of this study. However, HLVd-symptomatic plants in the CBD Unicorn cultivar exhibited significant reductions in cannabinoid levels, specifically THCA and CBLA: CBCA. These findings highlight the critical risks that HLVd presents to producers relying on consistent cannabinoid profiles.

RT-qPCR analysis indicated that specific chemical elicitors may reduce HLVd levels in the CBD Unicorn cultivar, with 1-Triacontanol (TRIA) showing a marginally significant reduction in viroid titers compared to its ethanol (EtOH) control ($p = 0.07$), and Kinetin demonstrating a trend toward significance against its potassium hydroxide (KOH) control ($p = 0.08$). Beyond potential viroid reduction, chemical elicitors such as salicylic acid (SA), brassinolides (BR), and 6-benzylaminopurine (BAP) significantly increased biomass and flower production in the CBD Unicorn cultivar infected with HLVd. However, SA, BR, TRIA, chlormequat chloride (CCC), and methyl jasmonate (MeJA) also reduced key cannabinoid levels, notably THC and CBD, highlighting the complexity of using chemical elicitors in viroid-infected plants. The observed trade-off between enhanced plant growth and reduced cannabinoid content suggests that, although chemical elicitors may offer potential as part of an integrated management strategy, their application must be carefully optimized to avoid unintended crop losses.

This research provides critical insights into the broader agricultural implications of HLVd on hemp production and highlights the need for refined viroid management practices. The potential impact of HLVd on hemp emphasizes the urgency of developing targeted strategies that balance yield improvement with cannabinoid preservation, thereby safeguarding the financial viability of hemp cultivation in the face of viroid challenges.

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INTRODUCTION

Cannabis sativa Background

Historical Significance and Uses of Hemp

Cannabis sativa L. is a versatile plant with a long history of diverse applications and cultural significance. This species includes both industrial hemp and marijuana, which are differentiated by their chemical composition and intended uses. While industrial hemp is valued for its fibers, seeds, and oils, marijuana is cultivated for its psychoactive compounds (Small, 2016).

In the United States, hemp's legal status has fluctuated over time. Early American figures like George Washington grew hemp for its fiber on their plantations (Deitch, 2003). However, the Marihuana Tax Act of 1937 imposed strict regulations and taxes on all forms of cannabis, including hemp, causing a significant decline in production (Small & Marcus, 2002). This decline continued with further restrictions under the Boggs Act, the Narcotics Control Act of the 1950s, and later, the Controlled Substances Act (CSA) of the 1970s (Deitch, 2003; Courtwright, 2004).

Impact of Farm Bills on Hemp Production

The Agricultural Act of 2014, a landmark piece of legislation often referred to as the 2014 Farm Bill, marked a significant shift in hemp production in the United States. The 2014 Farm Bill legally distinguished hemp from marijuana by defining "industrial hemp" as *Cannabis sativa* L. plants containing no more than 0.3% THC. This represented the first significant change since the Marihuana Tax Act of 1937 (Johnson, 2018). The 2018 Farm Bill further

revolutionized hemp production by removing it from the DEA's list of controlled substances (Mark et al., 2020).

These legislative changes led to a dramatic increase in hemp production acreage. According to the USDA, hemp production acreage in the U.S. increased from virtually zero in 2011 to over 146,000 acres in 2019. The 2019 hemp boom was short-lived as the overproduction of high-CBD hemp caused a dramatic 63% decrease in planting in 2021 (2021 National Hemp Report, 2022). Currently, hemp production can be found across the United States, but in lower amounts. As of 2023, the area planted with industrial hemp totaled 27,680 acres, down 51% from 2021. The top three producers of hemp are the states of South Dakota (3,200 acres), Montana (2,900 acres), and Oregon (2,300 acres). At the same time, Colorado saw hemp production decline by 87%, from 10,100 planted acres in 2021 to 1,350 in 2023 (2023 National Hemp Report, 2024).

Hemp Secondary Metabolites

Hemp produces secondary metabolites, including cannabinoids, terpenes, and phenolic compounds (Andre et al., 2016). These metabolites play significant roles in pharmaceutical, construction, and industrial applications. Cannabinoids have been the subject of extensive research due to their considerable pharmacological potential. The significant cannabinoids include tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), and cannabichromene (CBC). These major cannabinoids are produced in substantial quantities by the plant, making them the most abundant cannabinoids available (ElSohly et al., 2017). The most recognized cannabinoid is THC, known for its psychoactive effects and its contribution to hemp’s legal complications (Johnson, 2021). The second most recognized cannabinoid is CBD, which is being studied for its therapeutic potential in treating conditions such as chronic pain,

epilepsy, and anxiety (Voicu et al., 2023). The other two cannabinoids, CBG and CBC, have also exhibited promising medicinal benefits similar to CBD, including anti-inflammatory and antibacterial properties (Devinsky et al., 2014; Pertwee, 2008).

Terpenes are aromatic compounds that contribute to the scent and flavor profiles of cannabis (Cox-Georgian et al., 2019; Russo, 2011). Beyond their sensory attributes, terpenes like myrcene, limonene, and α-pinene have shown potential therapeutic effects. Myrcene is noted for its sedative properties in the central nervous system (CNS) and the peripheral nervous system; limonene is recognized for its mood-enhancing effects in the CNS and its adaptive effects in the immune system, while pinene is known for its anti-inflammatory benefits in the CNS and the respiratory system (Russo, 2011). These compounds can enhance the pharmacological effects of cannabinoids through the entourage effect, where multiple plant compounds work synergistically (Russo, 2011).

Phenolic compounds in hemp are a diverse group of secondary metabolites known for their significant biological activities and applications in the medical and manufacturing industries (Andre et al., 2016). These compounds include flavonoids, stilbenes, lignans, and phenolic acids. Flavonoids, tannins, anthocyanins, and phenolic acids help protect plants from UV radiation and pests (Barbehenn & Peter Constabel, 2011; Gould, 2004; Imperato, 2006; Treutter, 2006). Phenolic acids and flavonoids provide significant human health benefits, including potent antioxidant, anti-inflammatory, and demonstrated antimicrobial properties that are effective against various bacteria, fungi, and viruses (Appendino et al., 2008; Flores-Sanchez & Verpoorte, 2008; Lanzoni et al., 2023). In manufacturing, phenolic compounds are used as cost-effective, renewable bio-resins derived from lignocellulosic biomass (Basafa & Hawboldt, 2023).

The versatility of hemp's potential uses extends to the industrial sector. In construction, the fibrous components of hemp, particularly the hurds, are used to produce hempcrete, a sustainable building material known for its excellent insulation properties and durability (Small & Marcus, 2002). Manufacturers can utilize hemp-derived materials, such as fibers and plant-based oils, to create biodegradable plastics and eco-friendly products, thereby reducing reliance on fossil fuels (Karus & Vogt, 2004). These more sustainable plastic materials can be developed by retting hemp fibers to extract cellulose for incorporation into reinforced polymer blends and modifying hemp seed oil through esterification to produce bio-based resins (Basafa & Hawboldt, 2023). Hemp seeds and hemp seed oil are naturally high in proteins, essential fatty acids, and micronutrients and can be used as sources of nutrition for animal feed and human consumption (Kolodziejczyk et al., 2012).

Current Knowledge of Hemp Pathogens

Like many other agricultural plants, hemp crops are susceptible to numerous pathogens that can significantly impact their health and productivity. Most of these pathogens are fungi and oomycetes, followed by viruses or viroids, with bacterial pathogens being less commonly reported but still a significant concern (Buirs & Punja, 2024; Punja et al., 2019; Punja, 2021; Punja et al., 2023; Punja et al., 2024). Three of the most common fungal pathogens in cannabis are *Botrytis cinerea*, *Fusarium sp.,* and *Golovinomyces sp.* (Punja et al., 2019). *Botrytis cinerea*, also known as gray mold, affects the flowers and buds of the plant, causing them to rot. This pathogen thrives in humid conditions and can spread rapidly, leading to significant crop losses (Garfinkel, 2020). *Fusarium* species, including *F. oxysporum*, *F. proliferatum*, and *F. solani*, are soil-borne fungi that cause root and crown rot (Punja & Rodriguez, 2018; Punja et al., 2019). These pathogens can lead to wilting, yellowing of leaves, and eventual plant death, severely

affecting the crop's yield and quality (Punja, 2021). *Golovinomyces,* also known as powdery mildew, is a widespread fungal disease characterized by white, powdery spots on leaves and stems, which can reduce photosynthesis and stunt growth (Braun et al., 2009; Punja et al., 2019; Punja, 2021; Punja et al., 2024).

Identifying viruses in hemp is particularly difficult due to insufficient research on the symptoms and transmission methods of existing and newly discovered viruses (McPartland et al., 2000). The earliest known viruses to infect hemp, including hemp streak virus (HSV) (Röder, 1941) and hemp mosaic virus (HMV) (Ceapoiu, 1958), have not yet been isolated or identified; this means they have not been confirmed using modern diagnostic methods (Nachappa et al., 2020). Additional viruses that have been found in hemp include Beet curly top virus (BCTV) Grapevine line pattern virus (GLPV), Tobacco streak virus (TSV), Opuntia umbra-like virus (OULV), and Citrus yellow vein-associated virus (CYVaV), and cannabis cryptic virus (CanCV) (Chiginsky et al., 2021; Miotti et al., 2023; Nachappa et al., 2020). Understanding of viruses affecting hemp continues to improve as more hemp virome surveys identify which viruses can utilize hemp as a host (Chiginsky et al., 2021; Miotti et al., 2023). These studies have enhanced diagnostic and management strategies for viral infections in hemp crops by developing more accurate tools for early detection, such as specific PCR primers and probes.

The least common type of pathogen among the significant pathogen groups reported in cannabis to date is bacteria (Punja, 2021). *Pseudomonas* and *Xanthomonas* are the most commonly occurring bacterial pathogens in cannabis (Jacobs et al., 2015; Punja, 2021). Both *P. syringae* and *X. campestris* cause water-soaked lesions on leaves, stems, and buds, leading to necrosis and plant death (McPartland et al., 2000). These bacteria thrive in moist conditions and spread through splashing water, contaminated tools, and plant material (McPartland et al., 2000).

These pathogens can lead to wilting, yellowing of leaves, and eventual plant death, severely affecting the crop's yield and quality (Punja, 2021; Punja et al., 2024).

There is a new pathogen threat to cannabis production that is neither bacterial, fungal, nor viral. The latest threat is a viroid, which is incredibly damaging compared to the others. Hop latent viroid (HLVd) poses a significant threat to cannabis crops, causing severe reductions in yield and quality (Punja, 2021; Punja et al., 2023; Punja et al., 2024). This viroid is particularly insidious due to its ability to remain latent and asymptomatic for extended periods, complicating detection and management efforts (Adkar-Purushothama et al., 2023; Atallah et al., 2023; Štajner et al., 2019).

HLVd Background

Origins and Initial Identification of HLVd

Hop latent viroid (HLVd), *Cocadviroid latenshumuli* was first identified in hop plants (*Humulus lupulus*) in the late 1980s (Puchta et al., 1988). HLVd belongs to the family Pospiviroidae and the genus Cocadviroid. The viroid has been characterized and consists of only a circular RNA genome (256 nucleotides) replicating autonomously using the plant host machinery (Puchta et al., 1988). HLVd's discovery occurred during investigations into latent infections in hops, which did not show apparent symptoms but adversely affected quality and yield of the crop (Barbara et al., 1990; Puchta et al., 1988). Researchers found that HLVd could persist in hop plants without causing immediate and noticeable symptoms, posing a significant challenge for detection and management (Singh et al., 2003). The impact of HLVd on hops has been well-documented, particularly its ability to remain latent and undetected while affecting plant vigor and crop yield. In addition to North America, HLVd has been found in hops globally,

including South America, Europe, Asia, and Japan (Barbara & Adams, 2003; Faggioli et al., 2017; Fonseca, 1993; Lavagi et al., 2017; Liu et al., 2008; Mahaffee et al., 2009; Singh et al., 2003). The economic implications for the hop industry are significant, prompting extensive research into detection and control methods (Pethybridge et al., 2008).

The viroid's presence in cannabis was first reported in 2019 in California, and since its initial identification, it has been found throughout North America (Bektaş et al., 2019; Chiginsky et al., 2021; Punja, 2021; Warren et al., 2019). HLVd infection in cannabis can be challenging to diagnose due to its latent nature and the plants appearing asymptomatic. The latent nature of HLVd allows infections to spread unnoticed, often until significant damage has occurred, underscoring the importance of early detection and effective management (Buirs & Punja, 2024; Pethybridge et al., 2008; Punja et al., 2024; Štajner et al., 2019).

Viruses can infect many organisms, including animals, plants, fungi, and bacteria. They cause diseases by disrupting normal cellular processes, leading to cell damage or death (Dimmock et al., 2016). Viroids, however, are known to infect only plants (Flores et al., 2011). While viruses and viroids share pathogenic capabilities, viroids have distinct structures, replication mechanisms, and interactions with host organisms that fundamentally differentiate them (Pethybridge et al., 2008).

HLVd Structure and Characteristics

Viroids comprise a circular, single-stranded RNA molecule lacking any protein coat or lipid envelope and do not encode proteins (Navarro et al., 2012; Navarro et al., 2021). Despite their simplicity, viroids can cause significant diseases in their host plants (Flores et al., 2011). Viroid RNA replicates utilizing host cell machinery through a straightforward mechanism known as the rolling-circle model (Ding, 2010; Flores et al., 2005). Viroid RNA directly interacts with

host enzymes, such as RNA polymerase II, to replicate within the nucleus or chloroplasts of the host plant cells. The replicated RNA strands are then processed and circularized to form new viroid molecules (Flores et al., 2009; Navarro et al., 2012; Navarro et al., 2021). Viroids move throughout the plant locally through the plasmodesmata and systemically via the phloem (Takeda & Ding, 2009; Navarro et al., 2021).

HLVd, like other viroids in the Pospiviroidae family and Cocadviroid genus, consists of a single-stranded RNA molecule (Fig. 1). The secondary structure of this genus of viroids is rod-like, consists of five domains, and has a central conserved region (CCR) and a terminal conserved hairpin (TCH) (Adkar-Purushothama et al., 2023; Di Serio et al., 2021; Puchta et al., 1988). Figure 1 (adapted from Adkar-Purushothama et al.2023) shows the predicted secondary structure of HLVd, with essential regions such as the Terminal Conserved Hairpin (TCH) and Central Conserved Regions (CCR) indicated. This family of viroids replicates in the nucleus of host cells using the host's DNA-dependent RNA polymerase II through an RNA-based rolling-circle mechanism. This replication involves synthesizing longer-than-unit strands of RNA, which are then cleaved and ligated to form mature viroid RNA molecules (Flores et al., 2009; Owens & Hammond, 2009; Flores et al., 2005).

Atallah et al. (2023) analyzed 77 HLVd isolates and found minimal genetic variability, with only three parsimony-informative sites. This limited variation suggests a highly conserved structure that supports the viroid's replication and infectivity in host plants. The stability of critical regions, such as the Central Conserved Region (CCR) and Terminal Conserved Hairpin (TCH), indicates solid evolutionary selection, as mutations in these areas could impair the viroid's life cycle. As a result, these regions remain primarily unchanged across isolates.

Host Range and Symptomatology

HLVd was first identified on hops but has also been detected in two other natural hosts, Japanese hops (*Humulus japonicus*) and common nettle (*Urtica dioica*), though these occurrences are less common and typically involve latent infections that do not manifest obvious symptoms (Knabel et al., 1999; Owens & Hammond, 2009; Pethybridge et al., 2008; Puchta et al., 1988). Since the initial detection of HLVd on cannabis plants in 2019, it has been identified across North America and Canada (Bektaş et al., 2019; Punja, 2021; Warren et al., 2019). This includes the first reports of HLVd in industrial hemp crops in Colorado, Oregon, and Washington (Chiginsky et al., 2021; Jarugula et al., 2023; Rivedal et al., 2022). Recently, HLVd has also been discovered to infect other species such as tomato *(Solanum lycopersicum)*, cucumber (*Cucumis sativus)*, chrysanthemum *(Chrysanthemum morifolium)*, *Nicotiana benthamiana*, and Arabidopsis *(Arabidopsis thaliana)* (Atallah et al., 2023).

HLVd often remains asymptomatic in hop plants, complicating detection until it becomes fully systemic and yield losses appear (Barbara & Adams, 2003). In cannabis, HLVd infections are more challenging, especially when propagated vegetatively from infected mother plants. Symptom severity in cannabis intensifies during flowering. During this period, HLVd-infected plants exhibit stunting, leaf curl, brittle stems, and significant reductions in inflorescence mass and trichome density, which lowers the plant's commercial value (Fig. 2) (Bektaş et al., 2019; Buirs & Punja, 2024; Punja et al., 2023; Warren et al., 2019). Additionally, HLVd infection leads to decreased chlorophyll in leaves associated with inflorescences, resulting in visible yellowing and chlorosis (Punja et al., 2023).

Microscopic examination of HLVd-infected stem sections reveals a thicker cortical layer and reduced xylem zone compared to healthy plants, highlighting HLVd's impact on meristem

structure (Punja et al., 2023). Overall, HLVd significantly reduces flower mass, trichome density, terpene, and cannabinoid production, directly affecting the plant's economic potential (Bektaş et al., 2019; Punja, 2021; Punja et al., 2023; Punja et al., 2024; Warren et al., 2019).

Mechanisms of HLVd Transmission

Hop latent viroid is well-known for its rapid and widespread transmission through various modes. The primary method of HLVd transmission is mechanical, particularly during pruning, harvesting, and the propagation of cuttings from infected mother plants (Buirs & Punja, 2024; Punja, 2021; Punja et al., 2023; Scheck, 2020). The transmission of HLVd through vegetative propagules from mother plants can result from both HLVd being systemic within the mother plants and the pruning shears being contaminated. This common practice in cannabis cultivation results in extensive viroid distribution if the mother plant is HLVd positive since all derived clones will also be infected (Buirs & Punja, 2024; Punja, 2021; Punja et al., 2023). Another significant vector for HLVd is water. Contaminated irrigation water can facilitate the spread of the viroid to healthy plants, especially in hydroponic systems or when runoff from infected plants reaches uninfected ones (Buirs & Punja, 2024).

Viroid transmission to crops, while often mechanical, can also occur through seed, pollen, and biological vectors, as viroids lack the proteins required for movement and rely on these carriers for dissemination. In many crops, insects such as aphids, leafhoppers, and thrips are common vectors that facilitate the spread of viroids from infected to healthy plants. These insects can acquire viroids while feeding on an infected plant and transmit them to new plants, leading to widespread infection within a crop. In citrus crops, Citrus exocortis viroid (CEVd) is commonly transmitted by contaminated pruning tools and even goats (Cohen et al., 2005; Kyriakopoulou et al., 2017). Similarly, the Potato spindle tuber viroid (PSTVd) spreads through

aphids in potato and tomato crops (Flores et al., 2011). The role of biological vectors in transmitting viroids is crucial, as they significantly contribute to the spread and epidemiology of viroid-related diseases, leading to potential economic losses in agriculture is currently being investigated in our lab.

Economic Impact of HLVd

Hop latent viroid (HLVd) can have severe impacts on both hop and cannabis crops despite often being asymptomatic in hops. HLVd significantly reduces cone yield in hops and diminishes essential chemical compounds needed for beer production, severely affecting the crop's quality and commercial value (Adams et al., 1996; Barbara et al., 1990; Hagemann et al., 2024). The increased severity of HLVd symptoms in cannabis significantly impacts plant health by reducing inflorescence mass and hindering trichome development. This leads to a deficiency in secondary metabolites, such as cannabinoids, terpenes, and phenolic compounds, which have significant economic value for both medicinal and recreational cannabis markets (Punja et al., 2023; Punja et al., 2024).

As a rapidly growing industry, cannabis sales in the United States are expected to reach between $58 and $70 million by 2030. Given this substantial economic potential, the impact of HLVd on cannabis crops is particularly significant (Venkataraman et al., 2024). This widespread presence of the pathogen has been associated with an estimated $4 billion loss in cannabis crops (Benjie, 2021). Canadian researchers have noted a wide range of HLVd positivity rates, with 5.3% to 92% of 15,947 samples from nine provinces testing positive, varying by location and year (Punja et al., 2023). Infected plants experienced a 12–42% reduction in the lengths of inflorescence stems, fresh biomass, and plant height compared to uninfected counterparts, along with a decrease in cannabinoids and terpenes (Punja et al., 2023).

Management Strategies for HLVd

Managing HLVd in cannabis production requires a multifaceted approach that encompasses prevention, detection, and mitigation strategies. Prevention starts with implementing stringent sanitation protocols, including regular tool and surface disinfection, gloves, and hand sanitizing when handling plants. Additionally, all equipment and surrounding surface areas should be sterilized with 10-20% bleach between uses to significantly reduce the risk of mechanical transmission of HLVd by transferring viroid particles from infected to healthy plants (Buirs & Punja, 2024).

Due to the ease of HLVd transmission through handling and propagation, proper plant handling techniques are crucial in preventing the spread of the viroid. When propagating cannabis plants, it is vital to use certified seeds or clones from reputable suppliers who screen for HLVd (Owens, 2023). Farmers should regularly monitor plants for symptoms of HLVd and remove and destroy infected plants immediately to prevent further spread and reduce reinfection (Buirs and Punja, 2024). Quarantining new plants before introducing them into the main growing area can also help mitigate the risk of HLVd introduction (Adkar-Purushothama et al., 2023).

Early detection of disease symptoms in stock plants is essential to prevent pathogen spread. Detection involves regular monitoring and diagnostic testing. Various diagnostic methods and commercial testing services are available to identify cannabis pathogens. Regular testing and destruction of infected plants can significantly reduce disease occurrence. While visual inspections for symptoms like stunting, leaf curl, brittle stems, and poor inflorescence development are necessary, they are insufficient because HLVd can be asymptomatic. Modern PCR-based technologies, such as RT-qPCR, ddPCR, and multiplex RT-PCR, have enhanced pathogen detection sensitivity and accuracy. ddPCR offers precise quantification of pathogen

load even at low concentrations, a significant improvement over traditional RT-PCR (Punja et al., 2023). Regular testing of mother plants, particularly those used for cloning, is recommended to confirm the presence of the viroid, even in asymptomatic plants (Adkar-Purushothama et al., 2023).

Mitigation strategies include immediately removing and destroying infected plants to prevent further spread. Comprehensive mitigation involves removing symptomatic plants and those confirmed positive through diagnostic testing (Buirs & Punja, 2024; Punja, 2021; Punja et al., 2024). Thermotherapy of plant tissues has been used in other crops to eliminate viroids and is being researched for its viability in cannabis (Torres et al., 2024). Another potential strategy is meristem tip culture, which involves culturing the shoot tip of the plant in sterile conditions to regenerate a viroid-free plant. Although it is labor-intensive and requires specialized equipment, it can preserve valuable genetic stock (Adkar-Purushothama et al., 2023).

The most effective approach is integrated pest management (IPM), which combines these strategies. Educating workers about the signs of HLVd and proper sanitation practices enhances the effectiveness of management strategies (Pethybridge et al., 2008). Additionally, breeding and genetic research aimed at developing HLVd-resistant cannabis varieties offers long-term solutions to managing HLVd in cannabis production (Buirs & Punja, 2024; Punja et al., 2024).

Chemical Elicitors

Chemical elicitors are molecules that significantly influence plant development and are typically effective at very low concentrations. There are natural elicitors that the plant itself produces, as well as synthetic elicitors; those found naturally in plants are called phytohormones or plant hormones. Synthetic elicitors, known as plant growth regulators (PGRs), are externally

applied compounds that mimic or enhance the effects of natural hormones (Rademacher, 2015). The natural elicitors found within plants play crucial roles in various physiological processes. Salicylic acid (SA) is essential for plant defense, particularly in mediating systemic acquired resistance (SAR), as it activates defense genes to establish resistance against various biotrophic pathogens (Vlot et al., 2009). Jasmonic acid (JA), in conjunction with the ethylene pathway, regulates plant responses to stress, including defense against herbivores and pathogens, while influencing processes such as senescence, fruit ripening, and wound healing (Wang et al., 2021; Wasternack & Hause, 2013). Auxins, another critical group of chemical elicitors, promote cell elongation, direct plant growth through phototropism and gravitropism, and coordinate root development and fruit growth (Farman et al., 2019; Mendel, 2020).

Brassinosteroids (BRs) are vital plant chemical elicitors that promote cell expansion and elongation (Farman et al., 2019). BRs are crucial to overall plant growth and enhance the plant's ability to withstand environmental stresses like extreme temperatures and drought (Nakashita et al., 2003). Cytokinins primarily promote cell division and differentiation, playing a crucial role in shoot initiation and growth, delaying leaf senescence, and coordinating the development of roots and shoots (Farman et al., 2019). Abscisic acid (ABA) reduces the impacts of abiotic stress, such as drought, by controlling stomatal closure to minimize water loss while playing a pivotal role in seed dormancy and germination (Farman et al., 2019).

Elicitors for Plant Defense and Growth

The chemical elicitors chosen in this study significantly influence plant growth and defense, enhancing their ability to respond to various stressors, including pathogens (He et al., 2022). Two candidate elicitors for reducing the impact of pathogens in plants are essential plant defense hormones salicylic acid (SA) and jasmonic acid (JA). Both SA and JA are the major

plant defense hormones, and their signaling pathways orchestrate the plant's responses to danger (He et al., 2022). The SA pathway protects against aphids and pathogens, while the JA pathway primarily defends against necrotrophic pathogens, herbivorous insects, and mechanical damage (Vlot et al., 2009; Wasternack & Hause, 2013). Applying either of these defense elicitors to prime the plant before HLVd infection may result in more robust plant defense responses and higher resistance levels after infection (Conrath, 2011). In addition to plant defense, exogenous applications of both SA and JA increase the synthesis of various cannabinoids (Apicella et al., 2022; Jalali et al., 2019; Mirzamohammad et al., 2021).

Other contender chemical elicitors for pathogen mitigation focus on increasing plant growth. Enhancing plant growth can reduce HLVd symptomatology, as robust plants are more resilient to pathogen pressure. By optimizing nutrient management, growth regulation, and environmental control, the plant's defense mechanisms can be strengthened, potentially mitigating the impact of HLVd. Two candidate elicitors in cell elongation and growth are auxin and gibberellins (Farman et al., 2019). Auxins are vital as they regulate gene expression for cell elongation, apical dominance, and root initiation (Mendel, 2020). Gibberellins are essential for stem elongation, seed germination, and flowering. They stimulate the production of enzymes like amylase, which mobilize nutrients stored in seeds (Farman et al., 2019).

Another contender elicitor for HLVd mitigation through plant growth is cytokinins, which promote cell division and differentiation, influence nutrient mobilization, and delay leaf senescence by promoting chlorophyll retention (Mendel, 2020). Cytokinins (CKs), such as kinetin and zeatin, are elicitors that regulate plant growth and development, potentially counteracting the stunting and poor growth caused by HLVd. Cytokinins promote cell division, elongation, and shoot formation at all growing stages, including tissue culture (Mok & Mok,

2001). They are crucial for *in vitro* propagation techniques, which help regenerate plants from explants by stimulating the formation of new shoots. Plant regeneration is particularly valuable for mass propagating disease-free plant material and conserving rare or endangered plant species (Werner et al., 2001). 6-Furfurylaminopurine, also known as kinetin, is a type of cytokinin that promotes cell division and growth. Kinetin enhances the plant's stress tolerance by delaying leaf senescence and improving nutrient mobilization. It also upregulates antioxidant enzyme activities, which help mitigate oxidative stress caused by pathogen infection (Mishchenko et al., 2022). 6-Benzylaminopurine (BAP) is another cytokinin that promotes cell division and shoot proliferation. BAP improves plant defense by enhancing the synthesis of secondary metabolites involved in pathogen resistance. It also increases the plant's photosynthetic capacity, improving overall health and resilience against stress (Werner et al., 2001).

The chemical elicitor triacontanol can also impact plant growth. This long-chain fatty alcohol acts as a plant growth stimulant by enhancing photosynthesis and nutrient uptake, thereby improving plant growth and yield (Cantaro-Segura & Huaringa-Joaquín, 2021). Triacontanol promotes plant growth by enhancing cell division, elongation, and differentiation through the auxin signaling pathway. It also interacts synergistically with gibberellins and cytokinins to stimulate overall plant growth and improve stress responses, thereby boosting plant health and resilience (Cantaro-Segura & Huaringa-Joaquín, 2021). This can help plants withstand physiological stress from HLVd infection, reducing symptom severity and improving overall plant health (Farman et al., 2019; Islam & Mohammad, 2020). Studies have shown that triacontanol treatment in crops like maize and rice increases chlorophyll content, enhances photosynthetic efficiency, and improves biomass production (Ries and Stute, 1985; Islam & Muhammad, 2020). Triacontanol also boosts the production of secondary metabolites involved

in plant defense, such as phenolic compounds and flavonoids, which enhance the plant's resistance to pathogens and pests (Kumaravelu et al., 2000).

Besides manipulating plant growth, chemical elicitors can shape plant architecture to make it more suitable for its environment. One such elicitor is chlormequat chloride, a quaternary ammonium salt commonly used as a growth retardant (Rademacher, 2000). It modifies plant architecture by inhibiting gibberellin biosynthesis, leading to shorter, sturdier stems that are less prone to bending or breaking under the weight of the grain. This results in higher yields and better-quality crops, particularly in wheat and barley crops (Rademacher, 2000). While its primary function is to control plant height, it also enhances the plant's ability to resist stress by increasing root biomass and improving water and nutrient uptake. This results in a more robust plant that can better withstand environmental stressors, such as drought or nutrient deficiencies, ultimately leading to improved growth and yield even under challenging conditions.

Brassinosteroids (BRs) are a class of polyhydroxysteroids, and another group of chemical elicitors known for playing a crucial role in promoting growth and enhancing stress tolerance (Bajguz, 2012). BRs can improve plant resistance to various stresses, including temperature extremes, salinity, and pathogen infection. Exogenous application of brassinosteroids has been shown to enhance the tolerance of rice plants to cold stress by modulating the expression of stress-responsive genes and improving cellular membrane stability (Nakashita et al., 2003). BRs enhance the plant's immune response by upregulating gene expression in pathogen defense. Brassinosteroids induce the production of pathogenesis-related proteins and improve the synthesis of phytoalexins, compounds that inhibit pathogen growth (Nakashita et al., 2003). In my study, I specifically used brassinolides, a highly active form of brassinosteroids known for

their strong growth-promoting effects, distinguishing them from other brassinosteroids that may have varying activity levels.

The biotic chemical elicitor chitosan oligosaccharides are derived from chitin and elicit plant defense responses by activating various defense-related pathways (Trono, 2024). They enhance the production of defensive enzymes and compounds, improving the plant's resistance to pathogens. They also induce the production of reactive oxygen species (ROS) and activate signaling pathways that lead to the expression of defense-related genes. Chitosan treatment enhances the synthesis of phytoalexins and PR proteins, bolstering the plant's immune system (El Hadrami et al., 2010). For example, chitosan treatment in cucumber plants has increased resistance to powdery mildew by enhancing the activity of chitinase and other defense enzymes (El Hadrami et al., 2010).

Integrating chemical elicitors into existing management strategies for HLVd has the potential to significantly enhance plant health and productivity. Timing applications that coincide with critical growth stages, such as early vegetative growth or pre-flowering, can maximize their effectiveness (Rademacher, 2015). By combining sanitation, detection, and mitigation efforts with chemical elicitors, cannabis producers can establish a comprehensive and practical framework for managing HLVd in their production systems. This multifaceted approach ensures a robust defense against the viroid, optimizing plant growth and yield.

The overarching goal of my thesis was to determine the impact of HLVd on hemp yield and quality and to apply new knowledge and practices to manage HLVd to reduce losses to the hemp and marijuana industry in the U.S. and worldwide. Two objectives achieved this: 1) assess the impact of HLVd on the growth and yield of two hemp cultivars, NWG 2463 (dual-purpose) and Unicorn (CBD), and 2) determine the effect of chemical elicitors in reducing HLVd

symptom development and infection levels. The study examined the viroid's effects on biomass, seed yield, inflorescence characteristics, and cannabinoid production by comparing HLVd-inoculated plants to mock-inoculated controls. We also evaluated whether chemical elicitors could improve overall plant health and resistance to the viroid while not negatively affecting cannabinoid profiles and yield. These findings aim to inform more targeted IPM strategies for managing HLVd in cannabis and hemp, potentially offering solutions to mitigate the impact on the industry. By improving a plant's tolerance to HLVd, chemical elicitors can reduce the viroid's impact on crop yield and cannabinoid quality, ultimately providing benefits to cultivation practices for growers.

METHODS

Plant and Viroid Source

Plant Material

Two hemp cultivars were selected for these studies: a CBD cultivar, Unicorn (Colorado Hemp Institute, Fort Collins, CO), and a dual-purpose cultivar, NWG 2463 (New West Genetics, Fort Collins, CO). Unicorn is a high-CBD cultivar (8-9%, B. Althouse, Colorado Hemp Institute personal communication) cloned from vegetative cuttings. Elite is a low-CBD cultivar (2-3%, R. Fletcher, New West Genetics personal communication) grown from seed. The plants were obtained from a certified hemp supplier with verified genetic profiles to ensure experimental uniformity. These cultivars were chosen due to their distinct production purposes, allowing for a comparison of how HLVd impacts different types of hemp crops.

Viroid Inoculum

The HLVd inoculum used in these studies was sourced from infected hemp plants confirmed to harbor the viroid via RT-PCR testing described in detail below. In the field trial experiment, viroid-infected tissue samples were collected, homogenized in a 1:5 (w/v) ratio with distilled water, and filtered to create a viroid-containing slurry. The plant tissue was cut at three meristematic points (top, middle, and bottom) to introduce the viroid sap, ensuring consistent inoculation across all plants. Control plants were mock-inoculated with distilled water in the same manner. This approach ensured consistent exposure to the viroid across all experimental groups. The chemical elicitor experiment utilized clones from HLVd-inoculated Unicorn mother plants.

Impact of HLVd on Hemp Yield and Cannabinoids

Experimental Design

The experimental design in the Cravo greenhouse aimed to evaluate the impact of HLVd infection on hemp yield and cannabinoid production. The primary factor was the presence or absence of HLVd infection, with two cultivars included as sub-factors: the dual-purpose hemp cultivar NWG 2463 and the CBD cultivar Unicorn. Each plot contained 7 to 8 plants per genotype. The greenhouse was divided into two sections to minimize the risk of accidental mechanical transmission of the pathogen: one for HLVd-inoculated plants and the other for mock-inoculated controls (Fig. 3). Rather than random assignment, plots were alternated within each section to account for potential environmental variability across the greenhouse. This arrangement enabled comparisons between treatments while reducing the influence of environmental differences on the results.

Unicorn cultivar cuttings and NWG 2463 cultivar seeds were initially started indoors, then transplanted into a 1,500 sq. ft. Cravo retractable roof greenhouse on June 14, 2023 (Cravo Retractable Roof Production System™, Cravo Equipment Ltd.) at the Agricultural Research and Development Education Center (ARDEC) South campus. Male plants of NWG 2463 were culled at 15 days and again at 36 days post-transplant to maintain an all-female population. The Cravo greenhouse at ARDEC South is open to the elements yet provides wind blocks on all sides with a transparent retractable roof, which was mechanically closed to prevent damage during potentially crop-damaging hail forecasts. A soil sample was analyzed, and organic fertilizer was added to correct nutritional deficiencies in the soil. Irrigation was administered every third day for 2 hours via drip lines running alongside the rows of plants.

At 36 days post-transplant, plants were inoculated with HLVd or mock-inoculated. A standardized sap inoculation technique was used, applying a 1:5 water: HLVd-infected plant tissue slurry to each plant's three meristematic tips (top, middle, bottom). Control plants were similarly mock-inoculated with distilled water to ensure consistency in treatment application. Each treatment (HLVd-inoculated vs. mock-inoculated) was replicated across six plots to account for variability. The dual-purpose and CBD cultivars were treated as separate experimental units to observe potential cultivar-specific responses to HLVd infection.

Sample Collection, RNA Extraction, and HLVd Testing

Before harvesting, tissue samples were collected from each plant's top, middle, and bottom sections and pooled to confirm HLVd-positive plants via RT-PCR. From here on, the HLVd-positive plants will be referred to as those that were HLVd-inoculated and tested positive with RT-PCR, while the HLVd-negative plants are the ones from mock-inoculation. Initially, samples were stored in a -80 °C freezer, and then individual plant samples were homogenized in liquid nitrogen using a mortar and pestle.

Using 50 mg of the sample, RNA was extracted using the Quick RNA ™ Fungal/Bacterial MiniPrep Zymo Kit (Research Products International, USA). The quantity and quality of RNA were evaluated using a Nanodrop One Spectrophotometer (ThermoFisher Scientific), and cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific). For the 20 µL Verso cDNA synthesis, the RNA template was mixed with molecular biology-grade water to achieve a concentration of 1,000 ng/µL and a final volume of 11 µL. The 9 µL Verso master mix consisted of 4 µL 5x cDNA Synthesis Buffer, 2 µL dNTP mix, 1 µL of primers, 1 µL Verso Enzyme Mix, and 1 µL RT Enhancer. cDNA thermocycling conditions were as follows: 42°C for 15 min, 95°C for 2 min, and hold at 4°C.

The Primers UCCR-F ‘GGGATCCCCGGGGAAACCTACTCG’ and UCCR-R ‘GGGATCCCTCTTCGAGCCCTTGCC’ were used to target the 256 bp HLVd genome (Atallah et al., 2023). 20 µL reactions were run for each sample using GoTaq Green PCR Master Mix (Promega). Reaction conditions were as follows: 4 µL 5x Green GoTaq Buffer, 1.6 µL $MgCl_2$, 0.4 µL dNTP, 0.4 µL of the forward and reverse primers, 11.1 µL molecular biology grade water, 0.1 µL Taq, and 2 µL of sample DNA. PCR thermocycling conditions were as follows: 95°C for 3 mins [95°C 1 min, 55°C 30 sec, 72°C 30 sec] x 40, 72°C for 1 min, 12°C hold. Results of PCR were visualized on a 1.5% agarose gel run at 80 volts for 50 minutes.

At harvest, the dry weight of each plant was recorded to quantify total biomass and determine the effect of HLVd on overall plant growth. Seeds were weighed to evaluate the impact of the viroid on reproductive output in the dual-purpose NWG 2463 cultivar. The weight and symptomology of inflorescences were documented to determine how HLVd affected floral development, particularly in the CBD Unicorn cultivar. Cannabinoid content, including CBD and THC levels, was measured using LC-MS/MS at CSU Bioanalysis and Omics (ARC-BIO) CORE to quantify the effect of HLVd on cannabinoid production in both cultivars.

Impact of Chemical Elicitors on HLVd Levels, Yield, and Cannabinoids

Experimental Design

This experiment investigated the effect of various chemical elicitor treatments on cannabinoid yield and viroid titers in HLVd-positive hemp plants (CBD cultivar Unicorn). By applying chemical elicitors during the vegetative growth phase, we aimed to assess whether these treatments could improve plant resilience to the HLVd pathogen and influence cannabinoid production, providing valuable insights for sustainable cultivation practices.

Chemical Elicitors Selection

Eight chemical elicitors were selected for this study based on their potential role in enhancing plant defense and promoting secondary metabolite production. In cannabis cultivation, specific chemical elicitors enhance plant defense mechanisms or promote growth and biomass accumulation. Defense-oriented elicitors strengthen the plant's resilience to stress and pathogens. In contrast, growth-oriented elicitors enhance vegetative vigor and regulate structural growth, promoting lateral branching and managing apical dominance. Collectively, these elicitors support optimized quality and yield in cannabis production.

Defense-oriented elicitors include salicylic acid (SA), methyl jasmonate (MeJA), and chitosan oligosaccharide (Table 1). Salicylic acid activates systemic acquired resistance (SAR), enhancing the plant's immune response against pathogens. This compound plays a pivotal role in strengthening endogenous defense pathways. Like other defense-oriented elicitors, methyl jasmonate modulates stress responses, particularly to necrotrophic pathogens. It upregulates defense genes and often increases terpene and resin biosynthesis, which are critical compounds for plant defense and commercially valuable secondary metabolites. Likewise, chitosan oligosaccharide elicits defense responses by mimicking pathogen attacks, stimulating the plant's innate immune system, and enhancing its resistance to biotic stressors such as pests and pathogens. Chitosan oligosaccharides, derived from chitin, have shown potential in triggering mechanisms like the hypersensitive response. Collectively, these elicitors augment the plant's defense capacity, enabling improved resilience against environmental and biotic stress conditions.

Conversely, growth-oriented elicitors such as 1-Triacontanol, 6-furfurylaminopurine, 6-benzylaminopurine, brassinolides, and chlormequat chloride primarily enhance vegetative

growth and structural development. Triacontanol acts as a photosynthesis enhancer, driving increased carbon assimilation and biomass production, which is advantageous for optimizing yields (Sarwar et al., 2021). Kinetin and BAP, both cytokinins, facilitate cell division and delay senescence, resulting in larger, more robust plants with prolonged productivity. Brassinolides promote lateral meristem growth and confer stress tolerance, supporting structural development and adaptability under suboptimal growth conditions. Chlormequat chloride, a gibberellin biosynthesis inhibitor, is particularly beneficial during the flowering stage, as it restricts apical growth, preventing excessive elongation and supporting a more compact morphology.

The application of elicitors is hypothesized to improve plant resilience to HLVd by regulating growth under stress conditions and promoting recovery through enhanced cellular responses. Although it remains unclear whether these substances can directly prevent the systemic spread of viruses or viroids, they enhance the host's defense by stimulating the production of reactive oxygen species and activating other defense pathways. This could alleviate the severity of HLVd symptoms and support overall plant health. The rationale behind these chemical elicitors is based on their known influence on plant physiology and their potential to improve resilience to HLVd. However, their efficacy in mitigating viroid transmission has yet to be fully established.

Experimental Setup

The experiment employed a non-randomized blocked design rather than a fully randomized setup to prevent cross-contamination between treatments. HLVd-positive Unicorn plants were divided into eight phytohormone treatment groups and three control groups, with two replications per group. Plants were vegetatively propagated from pathogen-confirmed Unicorn donor plants to ensure uniform HLVd infection. Each treatment group was housed in separate

cages to mitigate the risk of drift or cross-contamination during foliar spray applications. The cages were made from PVC and covered in thrips-proof netting, and the dimensions of the cages are 2 ft x 2 ft x 5 ft. A small 60 mL pump sprayer (Hydior) applied 100 mL treatments of the respective chemical elicitors for three consecutive weeks during the vegetative growth phase. Control groups received 100 mL applications of distilled water, ethanol, or potassium hydroxide, depending on the solvent required for the corresponding chemical elicitors, to account for any effects of the solvents used in the chemical elicitor solutions. There were six plants per treatment group, with eight treatment groups and three control groups, resulting in 66 plants per round. The experiment was replicated twice, totaling 132 plants across both rounds.

The foliar applications were conducted over three weeks from January 2024 to February 2024 during vegetative growth, consisting of an 18:6 day/night photoperiod. This was followed by two plants from each treatment randomly selected to mature over an eight-week flowering period from February 2024 to April 2024, consisting of a 12:12 day/night cycle to assess the treatments' impact on cannabinoid and terpene production. The experiment was conducted in a controlled greenhouse with 430-watt HID supplemental lighting. The temperature was maintained within a range of 76 to 82°F, with daytime temperatures at 82°F and nighttime temperatures at 76°F. Plants were grown in a soilless substrate to ensure precise control over nutrient inputs. The fertilizer solution's electrical conductivity (EC) was carefully monitored, with a maximum EC of 1.0 to avoid nutrient imbalances, and feeding occurred as necessary.

Data Collection

Data collection was performed at multiple stages of the experiment to monitor the effects of phytohormone treatments on plant health, viroid titers, and cannabinoid production. Plant tissues were sampled before and after treatment, and viroid titers were quantified using qPCR to

evaluate the impact of phytohormone treatments on the viroid load in the plants. Flower samples were collected after eight weeks of a 12:12 day/night photoperiod, and the inflorescence cannabinoid profiles were analyzed using LC-MS/MS after the flowering phase to assess the effects of the treatments on secondary metabolite production, including critical compounds such as CBD and THC.

RNA extraction and RT-qPCR quantification

Using 50 mg of the sample, RNA was extracted with the Quick RNA ™ Fungal/Bacterial MiniPrep Zymo Kit (Research Products International, USA). The quantity and quality of RNA were evaluated using a Nanodrop One Spectrophotometer (ThermoFisher Scientific). RT-qPCRs were performed with the Taq-Path 1-Step Multiplex Master Mix (No ROX) (Applied Biosystems) using 20 µL reactions. Reaction conditions were as follows: 5 µL Taq-Path 1-Step Multiplex Master Mix, 1 µL primer/0.1 µL probe mix, 7.5 µL molecular biology-grade water, and 4 µL RNA. Samples were run in duplicate. Primers and probes were multiplexed and targeted a ~100 bp region of the HLVd and hemp genome. These primers and probes are proprietary and were provided by Dr. Tassa Saldi at TUMI Genomics. This primer set was validated and optimized for this experiment (https://21668670.fs1.hubspotusercontent-na1.net/hubfs/21668670/Marketing/Resources/Product%20Resources/Hop%20Latent%20Viroid/HLVd%20Validation-Final.pdf). The following cycling conditions were used: Hold at 25 °°C for 2 min, hold at 53 °C for 10 min, hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min using the Quant Studio 3 Real-Time PCR Machine (Thermo Scientific). Cq values were used to determine positive samples; all samples with a Cq value of <30 were considered positive. The standard curve was produced by creating a tenfold serial dilution of an extracted bacterial plasmid containing the HLVd genome. This six-point standard curve was

performed on each plate, along with a positive control that remained consistent from plate to plate. The standard curves were averaged into one curve. The standard curve equation y = - 3.1091x + 47.488 was used to determine primer efficiency and virus quantification. The copy number was determined for individual samples using the equation $10^{(Cq - y\text{-intercept})/Slope}$. The average viroid load was then calculated for each plant in the chemical elicitor experiment.

Analysis of cannabinoids using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

Flower samples were collected after 8 weeks of a 12:12 day/night photoperiod, and the cannabinoid profiles were analyzed using LC-MS/MS at CSU Bioanalysis and Omics (ARC-BIO) CORE. Samples were transported to a -80°C freezer for 2 hours immediately before lyophilization. Samples were lyophilized for 49 h. After lyophilization, samples were stored in a -20°C freezer. Lyophilized samples were then homogenized for 5 minutes using a bead beater (Next Advance, Troy, NY, USA). After homogenization, about 40 mg of tissue for each sample was weighed into 2 mL Eppendorf tubes, along with 1 mL of cold 80% methanol in water. Samples were then vortexed vigorously for 30 minutes at 4°C, followed by 15 minutes of sonication in an ice bath and another 30 minutes of vigorous vortexing at 4°C. After mixing, samples were centrifuged at 15,000 g and 4°C for 10 minutes. Supernatants were recovered and diluted 10 times in cold 100% methanol. Then, 50 µL of the diluted sample was mixed with 50 µL of the internal standard (IS) and stored at -80°C until analysis. An aliquot (10 µL) from each study sample was pooled to generate a quality control (QC) sample prepared using internal standards. The IS, THC-d3, was purchased from Cerilliant (TX, USA) and then diluted in 100% methanol to obtain 0.1 µg/mL for spiking.

UPLC-MS/MS analysis was performed on a Waters ACQUITY UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on an ACQUITY UPLC HSS T3 column (1 x 100 mm, 1.8 μm, Waters, MA, USA). The capillary voltage of the MS detector was set to 0.7 kV in positive mode. The inter-channel delay was set to 3 msec. The source temperature was 150°C, and the desolvation temperature was 450°C. The desolvation gas flow was 1000 L/h, the cone gas flow (nitrogen) was 150 L/h, and the collision gas flow (argon) was 0.15 mL/min. The nebulizer pressure (nitrogen) was set to 7 bar. The autodwell feature was set for the collection of 12 points across the peak. The cone voltage and collision energy (CE) of each MRM were optimized. Several high-abundance compounds (CBDVA, CBGA, CBDA) in the current sample set were analyzed using a "de-optimized" cone and CE voltage. Raw data files were imported into the Skyline open-source software package (MacLean et al., 2010) for processing. Each target analyte was visually inspected for retention time and peak area integration. Peak areas were extracted for target compounds detected in biological samples and were normalized to the peak area of the appropriate internal standard or surrogate in each sample. Absolute quantitation of dry weight (μg/g) was calculated using the linear regression equation generated for each compound from the calibration curve. Cannabinoids were analyzed using R software (Version 2023.06.1+524 (2023.06.1+524). Outlier tests using a Bonferroni adjustment were run for all cannabinoid levels; no outliers existed. The difference in cannabinoid levels between chemical elicitor treatment groups and their control solvent was analyzed for statistical significance using a *t*-test.

RESULTS

Impact of HLVd on Hemp Yield and Cannabinoids

Yield

In the Cravo greenhouse experiment, the effect of HLVd infection on biomass yield was assessed in both dual-purpose and CBD hemp cultivars. In the dual-purpose cultivar, a comparison between HLVd-negative and HLVd-positive plants, confirmed to be positive or negative using RT-PCR, showed no significant difference in biomass yield (n = 10, t = 0.03, p = 0.48, df = 8), indicating that HLVd infection did not substantially affect biomass production in this cultivar (Fig. 4A). Similarly, for the CBD cultivar, HLVd-negative, and HLVd-positive plants exhibited no significant differences in biomass yield (n = 16, t = 0.33, p = 0.37, df = 14), suggesting that HLVd had minimal impact on the CBD cultivar's biomass production (Fig. 4B).

The impact of HLVd infection on flower yield was also evaluated in the Cravo greenhouse experiment. In the dual-purpose cultivar, comparisons of flower yield between HLVd-negative and HLVd-positive plants showed no statistically significant difference (n = 10, t = 0.04, p = 0.48, df = 8), suggesting that HLVd did not significantly affect flower yield in this cultivar (Fig. 5A). Similarly, in the CBD cultivar, no significant difference in flower yield was found between HLVd-negative and HLVd-positive plants (n = 16, t = 0.31, p = 0.37, df = 14) (Fig. 5B).

Despite the lack of statistical significance, symptomatic HLVd-infected CBD plants exhibited clear visual symptomology, including reduced and deformed inflorescences, stunted growth, and abnormal floral development compared to the healthy, well-developed inflorescences of mock-inoculated controls (Fig. 6).

Seed yield was assessed in the dual-purpose hemp cultivar as part of the Cravo greenhouse experiment. An initial unpaired t-test was used to compare seed yield between HLVd-positive and HLVd-negative plants, assuming normal distribution and equal variance across groups. However, significant differences in seed yield variability between the groups violated the assumptions necessary for a valid t-test. Due to this violation, a non-parametric Mann-Whitney U test was conducted, which does not require normal distribution or equal variances, making it more appropriate for this comparison. The analysis, conducted in RStudio R version 4.3.1 (2023-06-16) without a continuity correction, indicated no statistically significant difference in seed yield between HLVd-positive and HLVd-negative plants ($n = 14$, $U = 21$, $z = -0.44$, $p = 0.47$), suggesting that HLVd infection did not significantly affect seed production in the dual-purpose hemp cultivar (Fig. 7).

Cannabinoids

No significant differences in cannabinoid levels were observed between HLVd-infected plants and mock-inoculated controls regardless of symptom expression (Table 2). Significant differences in cannabinoid levels were observed when comparing HLVd-positive symptomatic plants to their HLVd-positive asymptomatic counterparts. THCA concentrations in symptomatic plants were notably lower (2,152 ± 100.2 µg/g) than in asymptomatic plants (2,550 ± 141.0 µg/g, $t = 2.29$, $df = 6$, $p = 0.03$). Similarly, CBLA levels showed a significant reduction from 1,338 ± 66.13 µg/g in asymptomatic plants to 1,130 ± 17.90 µg/g in symptomatic plants ($t = 3.02$, $df = 6$, $p = 0.01$) (Supplementary Table 1).

Impact of Chemical Elicitors on Yield, HLVd Levels, and Cannabinoids

Yield

In greenhouse experiments assessing the effects of various chemical elicitors on HLVd-infected CBD hemp plants (Unicorn cultivar), distinct differences in yield were observed between treatments. For biomass yield, salicylic acid (14.92 ± 2.68 g, $p = 0.02$), brassinolides (12.82 ± 1.95 g, $p = 0.03$), and 6-benzylaminopurine (17.58 ± 4.73 g, $p = 0.04$) showed significant increases compared to their respective controls, indicating a positive impact of these chemical elicitors on biomass production in HLVd-infected plants (Table 3). Other chemical elicitor treatments, including triacontanol, kinetin, chitosan oligosaccharide, chlormequat chloride, and methyl jasmonate, did not significantly differ in biomass yield compared to controls.

In terms of flower yield, similar patterns were observed. Salicylic acid (8.13 ± 1.47 g, $p = 0.02$), brassinolides (6.87 ± 0.60 g, $p = 0.01$), and 6-benzylaminopurine (8.24 ± 1.54 g, $p = 0.02$) exhibited significant increases in flower production compared to their controls (Table 4). These results suggest that these specific chemical elicitors may enhance flower yield in HLVd-infected hemp plants. In contrast, triacontanol, kinetin, chitosan oligosaccharide, chlormequat chloride, and methyl jasmonate did not show statistically significant effects on flower yield compared to their corresponding controls.

Chemical Elicitor Impact on HLVd Levels

Initial RT-qPCR results from half of the samples (Fig. 8) indicate that 1-Triacontanol (TRIA) treatment demonstrated marginal significance in reducing HLVd titers compared to its ethanol (EtOH) control ($p = 0.07$). Similarly, Kinetin treatment showed a trend toward significance when compared with its potassium hydroxide (KOH) control ($p = 0.08$). The y-axis

in the data plot represents the elicitors and solvents, while the x-axis reflects the log copy number of HLVd detected in tissue samples.

Chemical Elicitor Impact on Cannabinoids

Chemical elicitors exhibited varied effects on cannabinoid profiles in HLVd-infected plants (Table 5). Treatments with salicylic acid (SA), methyl jasmonate (MeJA), brassinolides, and triacontanol significantly reduced specific cannabinoid levels compared to ethanol controls. Notably, SA treatment caused a marked decrease in Δ9-THC levels (237.5 ± 10.19, $p = 0.01$) compared to the EtOH control (354.3 ± 42.49). Similar reductions were observed with MeJA (225.2 ± 28.86, $p = 0.02$), brassinolides (263.5 ± 8.51, $p = 0.04$), and triacontanol (228.3 ± 26.81, $p = 0.02$). Additionally, both triacontanol and MeJA led to significant reductions in CBDA levels (37485 ± 5814, $p = 0.04$ for triacontanol; 37984 ± 5864, $p = 0.04$ for MeJA), suggesting a broad impact of these elicitors on cannabinoid biosynthetic pathways. Furthermore, chlormequat chloride (CCC) notably reduced CBDVA (1388 ± 146.2, $p = 0.03$) and THCA (1007 ± 105.1, $p = 0.04$) when compared to water controls. Other treatments, such as kinetin, chitosan oligosaccharide, and BAP, did not significantly affect individual cannabinoid concentrations.

In contrast, when examining the total cannabinoid profile, statistical analysis was performed using t-tests in GraphPad Prism, revealing no significant differences across treatments for % total THC, % total CBDV, or % total cannabinoids (Supplementary Table 2). However, analysis of % total CBD indicated significant differences in specific treatments: 1-Triacontanol (TRIA; 3.45 ± 0.528, $p = 0.03$) and methyl jasmonate (MeJA; 3.49 ± 0.537, $p = 0.04$) showed statistically significant reductions in CBD levels compared to their respective control EtOH (5.55 ± 0.824). Supplementary Table 2 presents the mean percentages and standard errors for % total

THC, % total CBDV, % total CBD, and % total cannabinoids in HLVd-infected CBD hemp plants treated with various chemical elicitors.

DISCUSSION

The current study is the first comprehensive assessment of the impact of HLVd on hemp biomass, seed yield, inflorescence characteristics, and cannabinoid production under field conditions. Additionally, this study is the first to investigate the effect of chemical elicitors in reducing HLVd symptom development and infection levels in a greenhouse. The results of this research will guide more targeted IPM strategies for managing HLVd in cannabis and hemp, potentially providing solutions to reduce the viroid's impact on the industry.

The results demonstrated that HLVd infection did not affect overall yield metrics—such as biomass, flower, or seed yield—in either the dual-purpose cultivar NWG 2463 or the CBD cultivar Unicorn under the conditions of this study. While trends toward reduced yield were observed in symptomatic plants, particularly in the CBD cultivar, these reductions were not statistically significant. Biomass comparisons between HLVd-negative and HLVd-positive plants showed no significant differences in the dual-purpose or CBD cultivar. Flower yield did not differ significantly between HLVd-negative and HLVd-positive plants. The impact of HLVd on cannabinoid production was more pronounced in symptomatic CBD Unicorn plants, as these plants exhibited significant reductions in cannabinoid levels, specifically THCA and CBLA: CBCA. Although HLVd infection may not significantly impact biomass or flower production, it potentially disrupts cannabinoid biosynthesis in symptomatic plants.

In contrast, Punja et al. (2023) reported 12–42% reductions in inflorescence stem lengths, fresh weights, and plant heights in infected cannabis (>0.3% THC) plants, along with significant declines in THC and terpene levels. They also found that HLVd infection negatively impacted glandular trichome development, resulting in shorter stalks and smaller glandular head sizes,

corresponding with lower cannabinoid accumulation. The discrepancy between our findings and those of Punja et al. (2023) may be due to differences in genotype susceptibility; cannabis clones grown under hydroponic conditions could be more vulnerable to HLVd than genetically diverse, field-grown hemp. Additionally, environmental conditions and the specific hemp cultivars used can influence symptom severity and the viroid's impact. While this study focused on CBD and dual-purpose varieties, it is crucial to recognize that other CBD or dual-purpose cultivars may respond differently to HLVd infection.

In contrast to hemp and cannabis, HLVd in hops can cause a significant reduction of alpha bitter acids and other secondary metabolites in cones (Puchta et al. 1988; Barbara et al. 1990; Patzak et al. 2001). The insights from Patzak (2001) and Patzak (2021) add further context to these observations. In their research on hops, Patzak (2001) documented how HLVd led to reductions in essential oils, which directly impacted secondary metabolites like humulones and lupulones, crucial compounds for aroma and flavor in hops. The viroid’s ability to impair the synthesis of these key metabolites was linked to disrupted biosynthetic pathways, and a reduction in glandular trichome functionality was similarly observed. This mirrors findings in cannabis, where HLVd affects the development of glandular trichomes, leading to reduced cannabinoid production in symptomatic plants.

Patzak (2021) further explored the impact of HLVd on secondary metabolites and demonstrated that viroid infection in hops induced stress responses in the plants, leading to shifts in metabolic pathways prioritizing defense rather than secondary metabolite production. The reduction of terpene and flavonoid levels in hops due to disrupted trichome development and biosynthesis aligns with the observed cannabinoid reductions in cannabis. The impact on glandular trichomes, which play a central role in secondary metabolite storage and production,

underscores the broader implications of HLVd infection on crops that rely on these structures for their value.

The parallels between hops and cannabis suggest that the influence of HLVd on trichome development and secondary metabolite biosynthesis may be a common mechanism across different plant species. In cannabis, this could explain the reduced cannabinoid concentrations, such as THCA and CBLA, observed in symptomatic plants in our study. Despite the absence of significant biomass or flower yield reductions, the viroid's specific disruption of secondary metabolite pathways has a more pronounced impact on the biochemical composition of the plant, which is critical for high-value cultivars like CBD-dominant strains.

Applying chemical elicitors, including salicylic acid (SA), brassinolides, and 6-benzylaminopurine (BAP), significantly increased biomass and flower yield in HLVd-infected plants compared to the ethanol control. Garrido et al. (2022) also found that 0.1 mM and 1.0 mM of chemical elicitors like SA and MeJA enhanced growth and biomass in medical cannabis. The observed yield increases are likely a result of the chemical elicitors' ability to promote cell division, delay senescence, and enhance stress tolerance, thereby mitigating the adverse effects of HLVd infection. Brassinosteroids, known for their diverse roles in regulating plant architecture and adaptability (Tong & Chu, 2018), likely contributed to this increased growth by improving stress tolerance and plant vigor under viroid infection. Moreover, brassinosteroids are recognized for regulating disease resistance and promoting growth under stress conditions (Nakashita et al., 2003). Mishchenko et al. (2022) demonstrated that BAP significantly enhances biomass production in cannabis, supporting the role of chemical elicitors in promoting growth. These results suggest chemical elicitors can promote biomass accumulation and improve flower production in HLVd-infected plants.

While chemical elicitors such as SA, BAP, and brassinolides positively impacted flower and biomass yield in HLVd-infected plants, they significantly reduced cannabinoid levels. SA and triacontanol led to a marked decrease in Δ9-THC compared to the ethanol control, while chlormequat chloride and brassinolides similarly reduced cannabinoid concentrations. Additionally, both MeJA and triacontanol significantly reduced CBDA levels. These findings suggest that chemical elicitors can negatively influence secondary metabolite production, including cannabinoids, primarily when used in concentrations of 10 mM or higher (Garrido et al., 2022).

A recent review by Trono (2024) examined the effects of critical elicitors, SA and MeJA, on secondary metabolite biosynthesis in cannabis, focusing on their impact across different chemotypes, specifically Chemotype I (high-THC varieties with THC > 0.3% and low CBD) and Chemotype II (plants with roughly equal amounts of THC and CBD, typically near or below 0.3% THC). The review did not include Chemotype III (low-THC < 0.3% and high CBD > 0.5%). In Chemotype I, low concentrations of SA (0.3–1.5 mM) and MeJA (1–15 mM) increased THC levels, while CBD levels remained unchanged or decreased depending on the elicitor and concentration. Conversely, low concentrations of these elicitors in Chemotype II plants enhanced cannabinoid and phenolic compound accumulation. However, at higher concentrations, both SA and MeJA reduced THCA and CBDA levels, a trend reflected in this study. These findings illustrate the dose-dependent effects of elicitors and highlight the need to optimize their concentrations to balance yield and cannabinoid content.

This dynamic can be explained through ecological theory, particularly the growth-defense trade-off, as discussed in the review by He et al. (2022). When plants face stress, such as pathogen invasion or herbivory, their metabolic resources are often redirected from growth and

secondary metabolite production to defense mechanisms. High concentrations of chemical elicitors like salicylic acid (SA) and methyl jasmonate (MeJA) likely activate stress-related pathways that prioritize survival, reducing cannabinoid biosynthesis in favor of plant defense. Although these elicitors may improve plant resilience and growth under stress, they suppress valuable cannabinoids, particularly in CBD-dominant cultivars, when overapplied. This reduction in cannabinoids observed highlights the inherent trade-off between enhancing plant vigor and maintaining high levels of critical secondary metabolites (Züst & Agrawal, 2017).

Trono (2024) further supports this trade-off, showing that elicitors can upregulate enzymes involved in cannabinoid biosynthesis, such as THCA synthase, but only when concentrations are finely controlled. Overapplying these elicitors induces stress responses that divert metabolic resources away from cannabinoid production toward defense mechanisms. Garrido et al. (2022) and Trono (2024) caution that altering a plant's hormonal balance with elicitors can carry fitness costs, potentially impairing development or the production of secondary metabolites when overused. Therefore, while chemical elicitors offer the potential for managing HLVd-induced stress and boosting growth, careful optimization of dosage and timing is critical to avoid unintended reductions in cannabinoid yields.

Our study revealed a significant trade-off in using chemical elicitors on HLVd-infected plants. While SA and BAP significantly reduced cannabinoid levels, they also led to substantial increases in biomass and flower yield compared to the ethanol control. These highlights are a vital consideration for cannabis cultivation. Though chemical elicitors may reduce cannabinoids like THCA and CBDA, the simultaneous increase in biomass and flower yield presents a benefit for growers prioritizing plant productivity. In specific contexts, such as dual-purpose cultivars or

environments focused on overall plant mass, this increased vegetative and reproductive output could compensate for lower cannabinoid levels.

The ability of chemical elicitors to boost growth under stress, as shown in prior research (e.g., Garrido et al., 2022; Mishchenko et al., 2022) and further validated by Trono (2024), indicates their value in managing HLVd infections by improving plant vigor. However, the potential trade-offs in cannabinoid biosynthesis highlight the need for careful optimization in commercial cultivation. Future research should aim to balance the yield benefits with the importance of maintaining high cannabinoid content, especially in CBD-dominant cultivars where cannabinoid production is paramount.

Although the chemical elicitor studies' preliminary RT-qPCR findings are promising, they are limited by the current sample size, as only half of the total samples have been analyzed to date. This limited dataset constrains the statistical power of the observations, necessitating additional data for more definitive results. The preliminary trends observed for 1-Triacontanol (TRIA) and Kinetin treatments, showing marginal significance in reducing HLVd titers, will be re-evaluated once the remaining samples are processed. The potential for specific chemical elicitors to reduce viroid levels aligns with the broader goals of Objective 2 in this study: assessing the impact of elicitors on HLVd levels, yield, and cannabinoids. If confirmed, these findings could inform viroid management strategies in cannabis cultivation by integrating elicitors as a dual-function approach—both as growth promoters and stress mitigators.

Implications for IPM

The findings from this study offer valuable insights into potential IPM strategies for managing HLVd in hemp cultivation. Traditional IPM methods, such as sanitation, pathogen

monitoring, and pathogen-free planting material, remain the cornerstone of HLVd control. Strict sanitation measures, such as sterilizing tools and regular testing for viroid contamination, are essential for preventing the spread of HLVd within and between cultivation sites. Buirs and Punja (2024) emphasize that destroying symptomatic plants and disinfecting tools with sanitizing agents such as bleach can significantly reduce viroid incidence in large-scale production environments. These measures are critical given the ability of HLVd to spread mechanically through contaminated equipment or plant material. Additionally, quarantine protocols and frequent testing of incoming plant materials are essential for preventing the introduction and spread of HLVd (Adkar-Purushothama et al., 2023). These proactive measures are fundamental to any comprehensive IPM strategy for managing HLVd in cannabis.

While traditional methods remain critical, the Trono (2024) study highlights the potential role of chemical elicitors, such as salicylic acid (SA) and methyl jasmonate (MeJA), in managing plant health under viroid stress. The study demonstrated that these elicitors can enhance plant resilience at low concentrations by modulating secondary metabolite pathways and upregulating defense-related genes. However, the findings also emphasize that elicitor concentration must be carefully controlled to avoid negative impacts on valuable cannabinoids, such as THC and CBD.

Although sanitation measures and pathogen-free planting materials are established components of IPM, chemical elicitors could offer additional protection by strengthening plant defenses even without viroid-free stock. However, as Trono (2024) noted, using SA and MeJA in IPM frameworks must be finely tuned to avoid unintended losses in cannabinoid yields, particularly in high-CBD cultivars.

The findings from Trono (2024) stress the need for a comprehensive approach when integrating chemical elicitors into IPM strategies. While these elicitors can enhance plant

resilience to stress, including HLVd infection, their potential to reduce cannabinoid levels at higher concentrations poses a challenge for growers focused on maximizing cannabinoid production. Optimizing dosages, treatment timings, and combinations of chemical elicitors will be essential for developing sustainable cultivation practices that balance plant health, yield, and cannabinoid quality. Future research should investigate the interactions between chemical elicitors, viroid infections, and cannabinoid biosynthesis to develop IPM strategies that are both effective and feasible for the cannabis industry.

FIGURES AND TABLES

Table 1: Chemical Treatments to Increase Plant Tolerance to HLVd in High-CBD (Unicorn) Hemp Plants.
The table outlines the chemical elicitor treatments applied to HLVd-infected plants. All infected plants were from infected mother plants. Each 100 mL treatment was administered via foliar spray once a week for three consecutive weeks during the vegetative growth phase. Treatments included 1-Triacontanol (photosynthesis enhancer/growth stimulant), 6-Furfurylaminopurine (Kinetin), 6-Benzylaminopurine (cell division promoters/senescence delay agents), Salicylic Acid (defense response activator/systemic acquired resistance inducer), Methyl Jasmonate (stress response regulator/defense gene activator), brassinolides (growth promoter/stress tolerance enhancer), Chlormequat Chloride (gibberellin biosynthesis inhibitor), and Chitosan Oligosaccharide (defense response elicitor). Control groups were treated with ethanol, water, or potassium hydroxide (solvent controls). [*]EtOH was the negative for comparisons with TRIA, SA, BR, BAP, and MeJA; [#]KOH was the negative for comparison with KIN; [@]Water was the negative for comparisons with Chi and CCC.

Chemical Elicitor	**Dosage (mM)**	**Solvent**	**Mode of Action**
1-Triacontanol	0.5	EtOH	Photosynthesis enhancer/ growth stimulant
6-Furfurylaminopurine (Kinetin)	50	KOH	Cell division promoter/ senescence delay agent
6-Benzylaminopurine	50	EtOH	Cell division promoter/ senescence delay agent
Salicylic Acid	10	EtOH	Defense response activator/ systemic acquired resistance inducer
Methyl Jasmonate	100	EtOH	Stress response regulator/ defense gene activator
Brassinolides	0.2	EtOH	Growth promoter/ stress tolerance enhancer.
Chlormequat Chloride	10	H2O	Gibberellin Biosynthesis inhibitor
Chitosan Oligosaccharide	1	H20	Defense response elicitor
Ethanol[*]	Control	-	
Water[@]	Control	-	
Potassium Hydroxide[#]	Control	-	

Table 2: Comparison of Cannabinoid Levels in HLVd-Infected and Mock-Inoculated High-CBD (Unicorn) Hemp Plants.

No significant differences in cannabinoid levels were observed between HLVd-infected plants, regardless of symptom expression, and mock-inoculated controls.

Cannabinoid	HLVd Status	Mean ± SE (ug/g)	t-test$_{(df)}$	P-value[a]
CBDVA	-	25,844 ± 3,738	0.43$_{(14)}$	0.33
	+	23,417 ± 4118		
CBD	-	7,435 ± 1,353	0.30$_{(14)}$	0.38
	+	8,116 ± 1,795		
THCV	-	30.25 ± 4.40	0.23$_{(14)}$	0.40
	+	32.0 ± 5.90		
CBG	-	639.1 ± 51.64	0.71 $_{(14)}$	0.24
	+	693.5 ± 55.53		
CBDA	-	93,325 ± 6,633	0.58$_{(14)}$	0.28
	+	87,860 ± 6,628		
CBGA	-	3,120 ± 1,034	0.16$_{(14)}$	0.43
	+	3,325 ± 748.1		
THCVA	-	176.3 ± 11.20	0.67 $_{(14)}$	0.25
	+	164.1 ± 14.10		
Δ^9-THC/Δ^8-THC	-	910.0 ± 134.5	0.34$_{(14)}$	0.36
	+	985.5 ± 170.4		
CBDV	-	209.4± 33.5	0.16$_{(14)}$	0.43
	+	218.8 ± 47.16		
CBLA:CBCA	-	1,319 ± 72.76	1.56$_{(14)}$	0.07
	+	1,149 ± 80.92		
CBCV	-	60.25 ± 9.49	0.13$_{(14)}$	0.44
	+	62.38 ± 12.15		
CBN	-	4.00 ± 0.46	0.37 $_{(14)}$	0.35
	+	4.25 ± 0.49		
CBC	-	1,476 ± 242.5	0.19$_{(14)}$	0.42
	+	1,548± 267.1		

CBL	-	3.75 ± 0.52	0.15(14)	0.43
	+	3.62 ± 0.59		
THCA	-	2,501± 127.7	1.20 (14)	0.12
	+	2,257 ± 157.1		
CBT	-	24.25 ± 5.8	0.66(14)	0.25
	+	32.13 ± 10.27		

Table 3: Impact of Chemical Elicitors on Biomass Yield in HLVd-Infected CBD (Unicorn) Hemp Plants Grown in a Greenhouse Environment.

The table displays the mean biomass yield (g) ± standard error of HLVd-infected plants treated with different chemical elicitors compared to their respective controls (EtOH, KOH, or H2O). Statistical significance was determined using a t-test, with significant differences ($p < 0.05$) observed for Salicylic Acid ($p = 0.02$), brassinolides ($p = 0.03$), and 6-Benzylaminopurine (BAP) ($p = 0.04$). Other chemical elicitor treatments did not show significant differences compared to controls. Data represent mean biomass yield ± standard error for six replicates per treatment. [*]EtOH was the negative for comparisons with TRIA, SA, BR, BAP, and MeJA; [#]KOH was the negative for comparison with KIN; [@]Water was the negative for comparisons with Chi and CCC.

Chemical Elicitor	**Biomass Mean ± SE (g)**	**t-test$_{(df)}$**	**P-value[a]**
1-Triacontanol	17.47 ± 6.36	$1.43_{(6)}$	(0.10)
6-Furfurylaminopurine (Kinetin)	17.19 ± 3.45	$0.729_{(6)}$	(0.24)
Salicylic Acid	14.92 ± 2.68	$2.44_{(6)}$	**(0.02)**
Chitosan Oligosaccharide	15.61 ± 2.21	$0.52_{(6)}$	(0.30)
Brassinolides	12.82 ± 1.95	$2.27_{(6)}$	**(0.03)**
Chlormequat Chloride	10.68 ± 1.69	$0.97_{(6)}$	(0.18)
6-Benzylaminopurine	17.58 ± 4.73	$1.94_{(6)}$	**(0.04)**
Methyl Jasmonate	9.6 ± 1.57	$0.79_{(6)}$	(0.22)
Ethanol[*]	8.330 ± 0.31	-	-
Water[@]	13.77 ± 2.6	-	-
Potassium Hydroxide[#]	14.18 ± 2.25	-	-

Table 4: Impact of Chemical Elicitors on Flower Yield in HLVd-Infected CBD Cultivar (Unicorn) Hemp Plants Grown in a Greenhouse Environment.

The table presents the mean flower yield (g) ± standard error of HLVd-infected plants treated with different chemical elicitors compared to their respective controls (EtOH, KOH, or H2O). Statistical significance was determined using a t-test, with significant differences ($p < 0.05$) observed for Salicylic Acid ($p = 0.02$), brassinolides ($p = 0.01$), and 6-Benzylaminopurine (BAP) ($p = 0.02$). Other chemical elicitor treatments did not show significant differences in flower yield compared to controls. Data represent mean flower yield ± standard error for six replicates per treatment. [*]EtOH was the negative for comparisons with TRIA, SA, BR, BAP, and MeJA; [#]KOH was the negative for comparison with KIN; [@]Water was the negative for comparisons with Chi and CCC.

Chemical Elicitor	**Flower Mean ± SE (g)**	**t-test$_{(df)}$**	**P-value[a]**
1-Triacontanol	10.46 ± 5.26	$1.11_{(6)}$	(0.15)
6-Furfurylaminopurine (Kinetin)	9.30 ± 1.99	$0.64_{(6)}$	(0.27)
Salicylic Acid	8.13 ± 1.47	$2.38_{(6)}$	**(0.02)**
Chitosan Oligosaccharide	8.45 ± 0.89	$0.04_{(6)}$	(0.48)
Brassinolides	6.87 ± 0.60	$3.53_{(6)}$	**(0.01)**
Chlormequat Chloride	6.77 ± 0.78	$0.80_{6)}$	(0.22)
6-Benzylaminopurine	8.24 ± 1.5	$2.35_{(6)}$	**(0.02)**
Methyl Jasmonate	5.82 ± 0.69	$1.70_{(6)}$	(0.06)
Ethanol[*]	4.57 ± 0.24	-	-
Water[@]	8.35 ± 1.81	-	-
Potassium Hydroxide[#]	7.76 ± 1.27	-	-

Table 5: Impact of Chemical Elicitor Treatments on Cannabinoid Profiles in HLVd-Infected CBD Cultivar (Unicorn) Hemp Plants. Treatments showed varied effects on cannabinoid levels. Salicylic Acid (SA), Methyl Jasmonate (MeJA), brassinolides, and 1-Triacontanol significantly reduced cannabinoid concentrations compared to ethanol controls. SA led to a marked decrease in Δ9-THC (237.5 ± 10.19, $p = 0.01$), with similar reductions seen for MeJA (225.2 ± 28.86, $p = 0.02$), brassinolides (263.5 ± 8.518, $p = 0.04$), and 1-Triacontanol (228.3 ± 26.81, $p = 0.02$). Both 1-Triacontanol and MeJA also significantly reduced CBDA levels. Chlormequat Chloride (CCC) decreased CBDVA (1388 ± 146.2, $p = 0.03$) and THCA (1007 ± 105.1, $p = 0.04$). Other treatments, such as Kinetin, brassinolides, Chitosan Oligosaccharide, and BAP, did not significantly affect cannabinoid concentrations. [*]EtOH was the negative for comparisons with TRIA, SA, BRASS, 6BAP, MeJA: [#]KOH was the negative for comparison with KIN: [@]Water was the negative for comparisons with Chi and CCC.

Chemical Elicitor	**CBDA (ug/g)**	**CBDVA (ug/g)**	**CBD (ug/g)**	**THCA (ug/g)**	**THCVA (ug/g)**	**Δ9-THC** (ug/g)
1-Triacontanol	37485 ± 5814 (***p* = 0.04**)	1695 ± 319.4 ($p = 0.12$)	1658 ± 246.4 ($p = 0.06$)	1113 ± 190.5 ($p = 0.06$)	192.1 ± 33.75 ($p = 0.11$)	228.3 ± 26.81 (***p* = 0.02**)
6-Furfurylaminopurine (Kinetin)	35869 ± 4525 ($p = 0.19$)	1558 ± 302.9 ($p = 0.20$)	1605 ± 217.7 ($p = 0.29$)	1059 ± 131.3 ($p = 0.17$)	181.6 ± 31.78 ($p = 0.23$)	217 ± 26.88 ($p = 0.22$)
Salicylic Acid	45821 ± 3699 ($p = 0.10$)	2141 ± 277.4 ($p = 0.24$)	1633 ± 85.31 (***p* = 0.05**)	1392 ± 135.7 ($p = 0.13$)	229.5 ± 20.93. ($p = 0.19$)	237.5 ± 10.19 (***p* = 0.01**)
Chitosan Oligosaccharide	41403 ± 5543 ($p = 0.16$)	1885 ± 353.6 ($p = 0.17$)	1646 ± 450.7 ($p = 0.24$)	1228 ± 151.6 ($p = 0.14$)	204.4 ± 31.30. ($p = 0.12$)	236.2 ± 42.66 ($p = 0.19$)
Brassinolides	52779 ± 2391 ($p = 0.24$)	2498 ± 182.2 ($p = 0.38$)	1962 ± 154.5 ($p = 0.10$)	1620 ± 81.07 ($p = 0.25$)	262.8 ± 17.56 ($p = 0.31$)	263.5 ± 8.51. (***p* = 0.04**)
Chlormequat Chloride	36183 ± 3131 (***p* = 0.05**)	1388 ± 146.2 (***p* = 0.03**)	2433 ± 227.2 ($p = 0.15$)	1007 ± 105.1 (***p* = 0.04**)	158.3 ± 17.34. (***p* = 0.01**)	294.5 ± 20.35 ($p = 0.47$)
6-Benzylaminopurine	47438 ± 5507 ($p = 0.14$)	2146 ± 316.9 ($p = 0.24$)	1741 ± 266.7 ($p = 0.07$)	1442 ± 171.4 ($p = 0.16$)	227.5 ± 27.76 ($p = 0.19$)	267.8 ± 33.04 ($p = 0.07$)
Methyl Jasmonate	37984 ± 5864 (***p* = 0.04**)	1750 ± 300.4 ($p = 0.13$)	1621 ± 334.5 (***p* = 0.06**)	1122 ± 165.1 (***p* = 0.06**)	186.1 ± 25.27. ($p = 0.09$)	225.2 ± 28.86 (***p* = 0.02**)
Ethanol[*]	60046 ± 9631	2736 ± 742.6	2849 ± 626.9	1920 ± 413.9	304.4 ± 77.58	354.3 ± 42.49

Water[@]	47921 ± 12197	2299 ± 764.1	1942 ± 548.9	1481 ± 399.8	242.8 ± 74.65	267.1 ± 54.36
Potassium Hydroxide[#]	51686 ± 7830	2495 ± 489.2	2033 ± 282.3	1592 ± 273.8	265.0 ± 35.41	291.0 ± 41.43

Legend: Treatments had varied effects on cannabinoid levels. Significant reductions in Δ9-THC, CBDA, CBDVA, and THCA were observed with specific elicitors compared to controls (*EtOH, #KOH, @Water).

- *EtOH was the control for TRIA, SA, BR, BAP, and MeJA.
- #KOH was the control for Kinetin.
- @Water was the control for Chitosan and CCC.

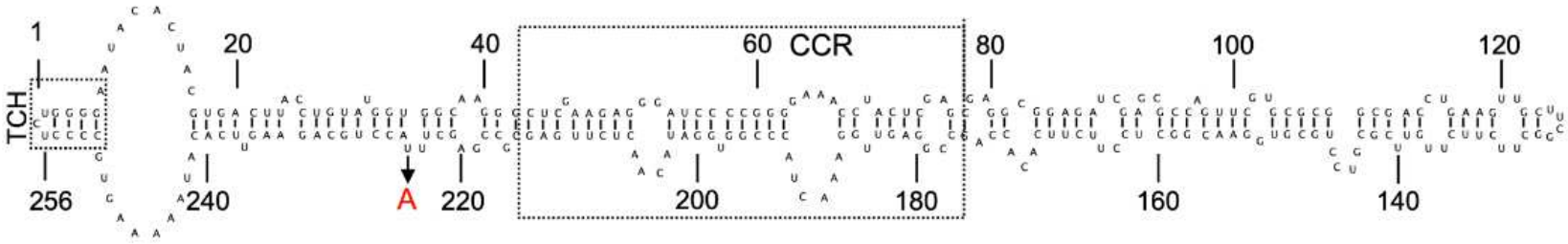

Figure 1: Predicted secondary structure of Hop Latent Viroid (HLVd).
The figure highlights key structural elements, including the Terminal Conserved Hairpin (TCH) and Central Conserved Region (CCR) (boxed). The arrow indicates the U225A point mutation identified in HLVd isolates from commercial hop gardens in China. Adapted from [Adkar-Purushothama et al., 2023].

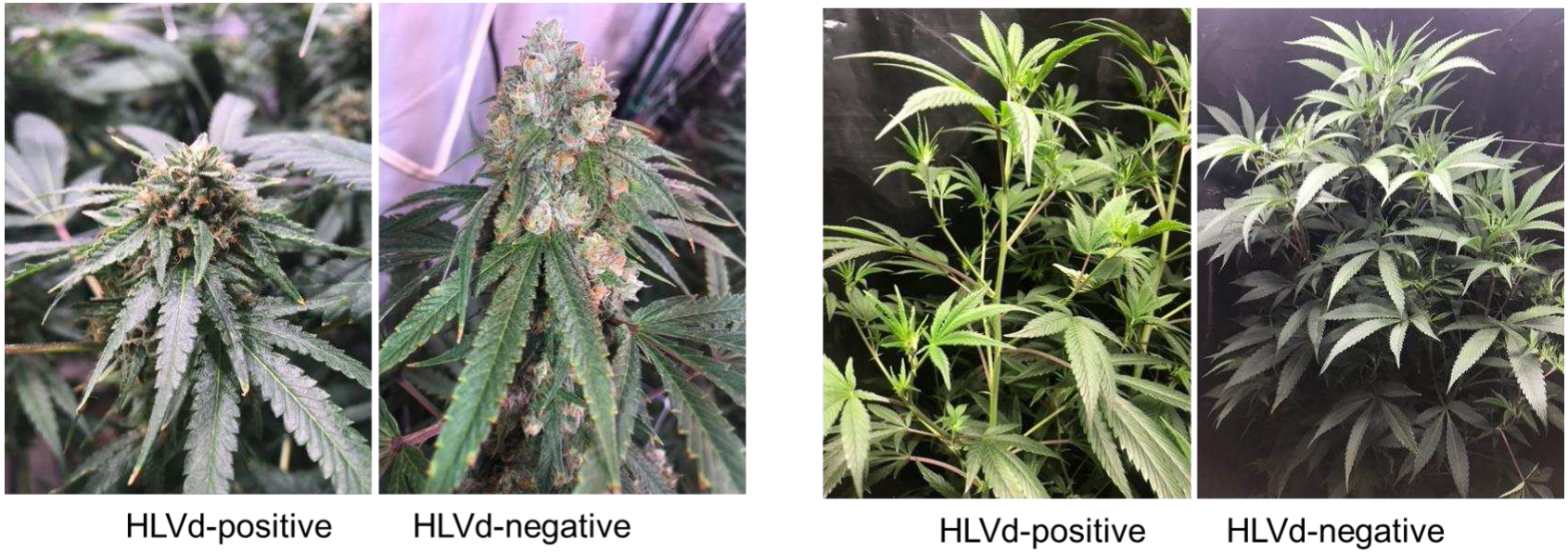

Figure 2: Visual comparison of HLVd-positive and HLVd-negative hemp plants during flowering (left) and vegetative stages (right). The HLVd-positive plants exhibit typical symptoms, including stunted growth, abnormal leaf morphology, and reduced vigor, compared to the healthy HLVd-negative plants, which show normal development and robust growth in both flowering and vegetative phases. Photo credit: L. Deyle.

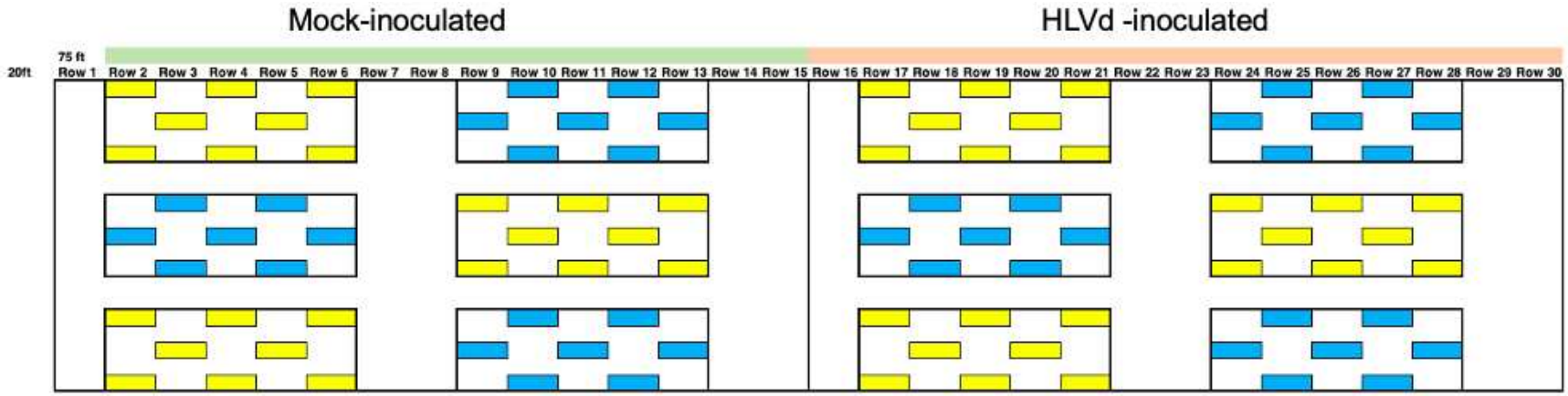

Figure 3: Experimental layout of the Cravo greenhouse study assessing the impact of HLVd on hemp yield and cannabinoids. The primary factor was the presence or absence of HLVd infection, with two cultivars included as sub-factors: the dual-purpose hemp cultivar NWG 2463 (blue) and the CBD cultivar Unicorn (yellow). Each plot contained 7-8 plants per genotype.

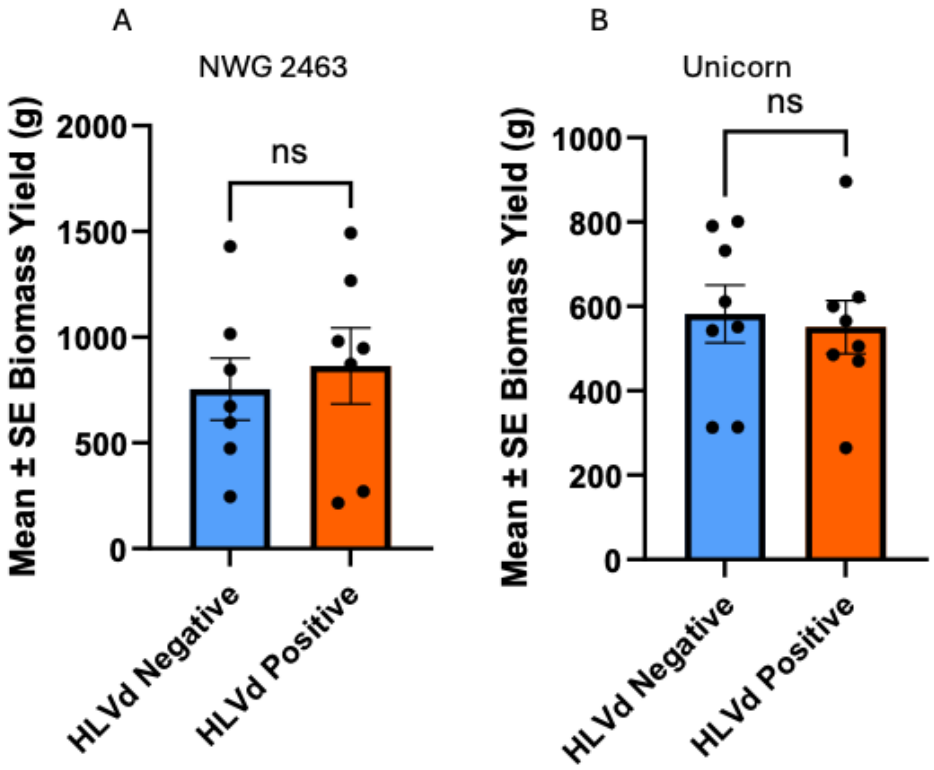

Figure 4: Impact of HLVd on hemp biomass yield in dual-purpose NWG 2463 and CBD Unicorn cultivars in a Cravo structure. Plants were grown in the Cravo greenhouse and inoculated with HLVd; samples were collected at maturity. Plants were confirmed to be positive or negative using RT-PCR. **(A)** Biomass comparison between HLVd-negative and HLVd-positive plants in the dual-purpose cultivar NWG 2463 (n = 10, t = 0.03, p = 0.48, df = 8). **(B)** Biomass comparison between HLVd-negative and HLVd-positive plants in the high-CBD cultivar Unicorn (n = 16, t = 0.33, p = 0.37, df = 14).

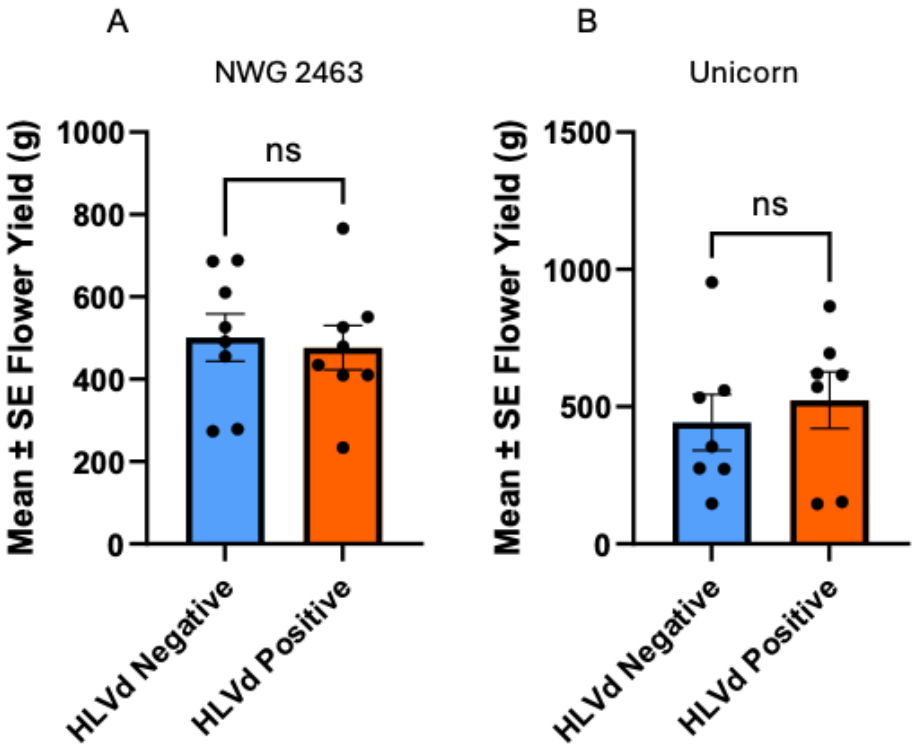

Figure 5: Impact of HLVd on hemp flower yield in dual-purpose NWG 2463 and CBD Unicorn cultivars in a Cravo structure. Plants were grown in the Cravo greenhouse and inoculated with HLVd; samples were collected at maturity. Plants were confirmed to be positive or negative using RT-PCR. **(A)** Flower yield comparison between HLVd-negative and HLVd-positive plants in the dual-purpose cultivar NWG 2463 (n = 10, t = 0.04, p = 0.48, df = 8). **(B)** Flower yield comparison between HLVd-negative and HLVd-positive plants in the high-CBD cultivar Unicorn (n = 16, t = 0.31, p = 0.37, df = 14).

Figure 6: Impact of Hop Latent Viroid (HLVd) on hemp inflorescences from the Cravo greenhouse experiment.
HLVd-infected plants (right) display reduced and deformed inflorescences, with signs of stunted growth and abnormal floral development, compared to the healthy, well-developed inflorescences of HLVd-free plants (left).

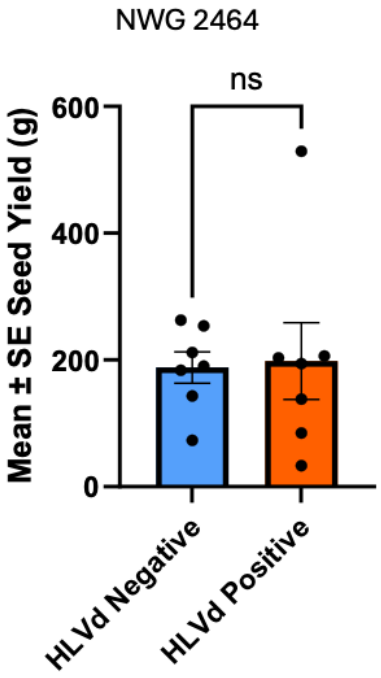

Figure 7: Impact of HLVd on hemp seed yield in dual-purpose NWG 2463 in a Cravo structure. Plants were cultivated in the Cravo greenhouse and inoculated with HLVd. At maturity, samples were collected to compare seed yield between HLVd-positive and HLVd-negative plants in the dual-purpose cultivar. An initial unpaired t-test, which assumes normal distribution and equal variance, revealed significant differences in variance between the two groups, violating the test's assumptions. Consequently, a non-parametric Mann-Whitney test was conducted to compare the ranks of the observations between groups. The Mann-Whitney test indicated no significant difference in seed yield between HLVd-positive and HLVd-negative plants (n = 14, U = 21, z = - 0.44, p = 0.47)

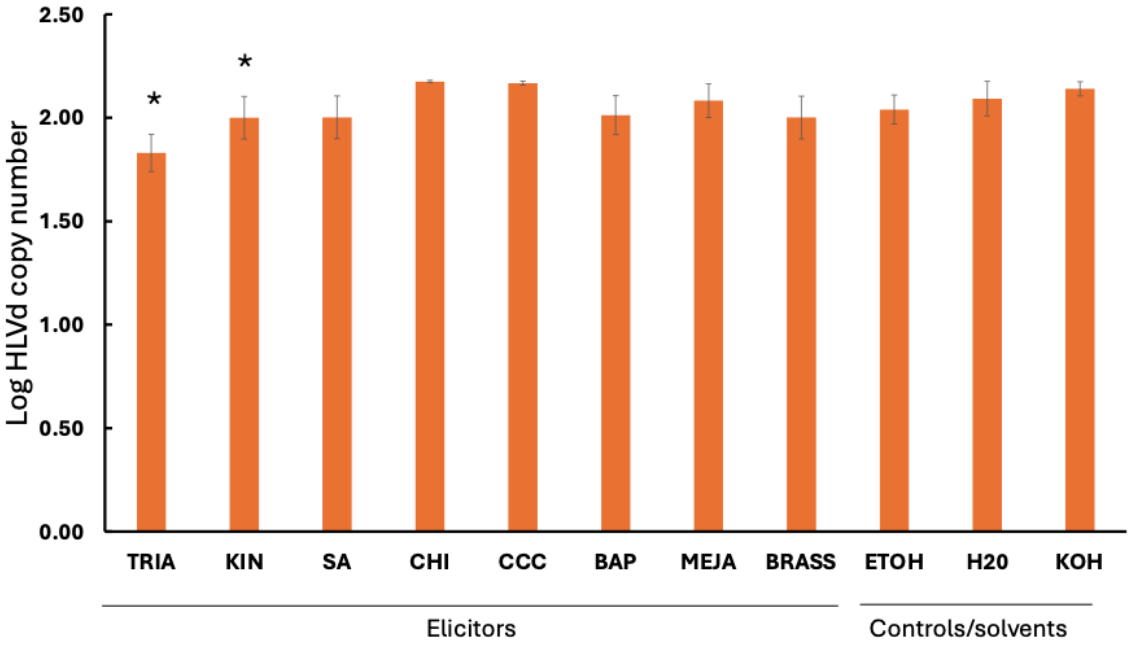

Figure 8: Impact of Chemical Elicitors on HLVd Levels.
This figure presents preliminary RT-qPCR results on the effect of chemical elicitors in reducing HLVd titers. Data from half of the samples indicate that 1-Triacontanol (TRIA) treatment showed a marginally significant reduction in HLVd levels compared to its ethanol (EtOH) control ($p = 0.07$). In contrast, Kinetin treatment exhibited a trend toward significance against its potassium hydroxide (KOH) control ($p = 0.08$). The y-axis represents the elicitors and corresponding solvents, and the x-axis shows the log copy number of HLVd detected in tissue samples. Although these trends suggest a potential impact of TRIA and Kinetin treatments on lowering HLVd titers, the incomplete dataset limits statistical power. Complete data analysis is needed to confirm the efficacy of these treatments.

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APPENDICES

Supplementary Table 1: Effect of HLVd Symptoms on Cannabinoid Production in CBD Hemp Cultivar 'Unicorn' During the Cravo Greenhouse Experiment.
THCA levels in symptomatic plants were significantly lower (2,152 ± 100.2 µg/g) compared to asymptomatic plants (2,550 ± 141.0 µg/g, $t = 2.29$, $df = 6$, $p = 0.03$). Similarly, CBCA levels dropped from 1,338 ± 66.13 µg/g in asymptomatic plants to 1,130 ± 17.90 µg/g in symptomatic plants ($t = 3.02$, $df = 6$, $p = 0.01$), indicating a significant disruption in cannabinoid biosynthesis in symptomatic plants.

Cannabinoid	**HLVd Status**	**Mean ± SE (ug/g)**	**t-test$_{(df)}$**	**P-value[a]**
CBDVA	-	25,496 ± 5,546	0.68$_{(6)}$	0.25
	+	20,764 ± 4,105		
CBD	-	8,549 ± 2,341	0.55$_{(6)}$	0.29
	+	10,697 ± 3,064		
THCV	-	33.0 ± 5.98	0.56$_{(6)}$	0.29
	+	39.50 ± 9.71		
CBG	-	672.3 ± 64.48	0.39 $_{(6)}$	0.35
	+	701.1 ± 34.06		
CBDA	-	93,021 ± 7,492	0.97$_{(6)}$	0.18
	+	85,484 ± 1,913		
CBGA	-	4,278 ± 1,942	0.46$_{(6)}$	0.32
	+	3,200 ± 1,259		
THCVA	-	176.3 ± 15.25	0.96 $_{(6)}$	0.18

	+	156.3 ± 14.16		
Δ^9-THC/Δ^8-THC	-	1,010 ± 192.5	0.57(6)	0.29
	+	1,205 ± 278.3		
CBDV	-	233.5± 54.06	0.46(6)	0.32
	+	279.3 ± 81.56		
CBLA:CBCA	-	1,338 ± 66.13	3.02(6)	**0.01**
	+	1,130 ± 17.90		
CBCV	-	67.00 ± 15.49	0.46(6)	0.32
	+	78.75 ± 20.09		
CBN	-	3.75 ± 0.75	1.12 (6)	0.15
	+	4.75 ± 0.47		
CBC	-	1,674 ± 412.8	0.35(6)	0.36
	+	1,887± 443.4		
CBL	-	3.75 ± 0.75	0.19(6)	0.42
	+	4.0 ± 1.08		
THCA	-	2,550± 141.0	2.29 (6)	**0.03**
	+	2,152 ± 100.2		
CBT	-	29.50 ± 10.04	0.86(6)	0.21
	+	47.25 ± 17.89		

Supplementary Table 2: Impact of Chemical Elicitor Treatments on % Total THC, CBDV, CBD, and Cannabinoid Profiles in HLVd-Infected CBD Cultivar (Unicorn) Hemp Plants.

This table presents the mean percentage and standard error for total THC, total CBDV, total CBD, and total cannabinoids in HLVd-infected CBD hemp plants treated with various chemical elicitors. Although slight variations are observed between treatments, no significant differences ($p > 0.05$) were found when comparing the effects of the elicitors against control treatments. For % Total CBD, however, significant differences were observed in specific treatments, with p-values indicating statistical significance levels. Notably, CBD levels differed significantly in specific treatments: % Total CBD for 1-Triacontanol (3.45 ± 0.528, $p = 0.03$) and Methyl Jasmonate (3.49 ± 0.537, $p = 0.04$) showed statistically significant reductions compared to their respective controls. Values are reported as means ± standard error, with *p*-values indicating the significance level for each treatment comparison. [*]EtOH was the negative for comparisons with TRIA, SA, BR, BAP, and MeJA; [#]KOH was the negative for comparison with KIN; [@]Water was the negative for comparisons with Chi and CCC.

Chemical Elicitor	% Total THC	% Total CBDV	% Total CBD	% Total Cannabinoids
1-Triacontanol	0.115 ± 0.0170 ($p = 0.40$)	0.152 ± 0.0311 ($p = 0.33$)	3.45 ± 0.528 (**$p = 0.03$**)	0.152 ± 0.0311 ($p = 0.33$)
6-Furfurylaminopurine (Kinetin)	0.140 ± 0.0178 ($p = 0.28$)	0.197 ± 0.034 ($p = 0.41$)	3.30 ± 0.417 ($p = 0.19$)	0.197 ± 0.034 ($p = 0.41$)
Salicylic Acid	0.140 ± 0.0248 ($p = 0.15$)	0.170 ± 0.040 ($p = 0.23$)	4.18 ± 0.324 ($p = 0.08$)	0.170 ± 0.040 ($p = 0.23$)
Chitosan Oligosaccharide	0.157 ± 0.011 ($p = 0.16$)	0.210 ± 0.021 ($p = 0.17$)	3.79 ± 0.529 ($p = 0.16$)	0.210 ± 0.021 ($p = 0.17$)
Chlormequat Chloride	0.205 ± 0.039 ($p = 0.29$)	0.252 ± 0.067 ($p = 0.44$)	3.41 ± 0.287 ($p = 0.06$)	0.252 ± 0.067 ($p = 0.44$)
6-Benzylaminopurine	0.135 ± 0.033 ($p = 0.24$)	0.175 ± 0.055 ($p = 0.26$)	4.33 ± 0.507 ($p = 0.12$)	0.175 ± 0.055 ($p = 0.26$)
Brassinolides	0.125 ± 0.006 ($p = 0.11$)	0.147 ± 0.006 ($p = 0.21$)	4.82 ± 0.213 ($p = 0.21$)	0.147 ± 0.006 ($p = 0.21$)
Methyl Jasmonate	0.130 ± 0.021 ($p = 0.20$)	0.1575 ± 0.033 ($p = 0.29$)	3.49 ± 0.537 (**$p = 0.04$**)	0.1575 ± 0.033 ($p = 0.29$)
Ethanol[*]	0.110 ± 0.009	0.137 ± 0.010	5.55 ± 0.824	0.137 ± 0.010
Water[@]	0.180 ± 0.017	0.242 ± 0.024	4.73 ± 0.714	0.242 ± 0.024

Potassium Hydroxide[#]	0.160 ± 0.027	0.210 ± 0.043	4.39 ± 1.11	0.210 ± 0.043