THESIS

EXPLORING THE HEMP VIROME AND ASSESSING HEMP GERMPLASM FOR RESISTANCE TO EMERGING PATHOGENS

Submitted by

Laine Hackenberg

Department of Agricultural Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2024

Master's Committee:

Advisor: Punya Nachappa

Mark Stenglein
Robyn Roberts

Copyright by Laine Hackenberg 2024

All Rights Reserved

ABSTRACT

EXPLORING THE HEMP VIROME AND ASSESSING HEMP GERMPLASM FOR RESISTANCE TO EMERGING PATHOGENS

Hemp (*Cannabis sativa* L.), commonly grown for its seeds, fiber, and non-psychoactive cannabinoids, has been experiencing a resurgence in the United States with its recent legalization. While farmers across the nation have readily adopted this crop, resources for pest management are still lacking, particularly regarding the diversity and distribution of pathogens. As production increases and the crop diversifies, the emergence and spread of these pathogens are certain. To circumvent loss due to disease, research is needed understand the threats and to identify sustainable management options. The goal of this study is to describe the diversity and distribution of viruses/viroids infecting hemp in Colorado and to determine if there is genetic resistance to pathogens in hemp. The objectives of this study are to 1) characterize the virome of different hemp cultivars throughout the growing season across different locations and 2) screen a panel of genetically unique genotypes of hemp for resistance to emerging viruses/viroids of hemp.

Throughout 2021 and 2022, the hemp virome was examined in four major hemp producing regions of Colorado. In total, nine fields were sampled, and each field was visited during three phenological stages (early vegetative, late vegetative, and mature flowering) in order to characterize the virome throughout the growing season. Leaf tissue samples were collected from two cultivars of hemp from each field site. These tissue samples were submitted for High Throughput Sequencing (HTS) and upon bioinformatic analysis, candidate virus/viroid

sequences were validated. Across both years, a total of seven viruses were identified: *Alfalfa mosaic virus* (AMV), *Beet curly top virus* (BCTV), *Cannabis cryptic virus* (CanCV), *Cannabis sativa mitovirus* (CasaMV1), *Grapevine line pattern virus* (GLPV), *Opuntia umbra-like virus* (OULV), and *Tomato bushy stunt virus* (TBSV). All viruses identified had >97% nucleotide identity to the nearest GenBank accession. Between individual cultivars isolated from the same field, both similar and unique viromes were observed. Viral diversity and incidence increased as the growing season progressed for both years. The three viruses that were most commonly found across all regions were CasaMV1, GLPV, and BCTV. Dominating the virome in viral load were CasaMV1 and GLPV.

Given the prevalence of BCTV in the virome, in addition to its prevalence in hemp across the western United States, 13 genotypes of hemp were screened for resistance to this pathogen. These genotypes of hemp are genetically diverse, which will aide in the discovery of candidate genes involved with resistance. BCTV is the causal agent of curly top disease which can have drastic symptomology in hemp plants, causing malformed growth, stunted plants, and crop loss up to 100%. Varying BCTV copy number was observed across the hemp genotypes. Additionally, percent disease index (PDI) was analyzed to determine the frequency of infection of individual genotypes. Two of the genotypes were observed to have a lower PDI than the others, 4587 and 4710.

Hop latent viroid (HLVd) has been emerging as a threat to the cannabis industry. It has been described across North America but is believed to be worldwide due to its global distribution in hops. HLVd has been documented to cause drastic reduction in cannabinoid content in mature inflorescences and therefore has the potential for substantial economic losses. Although not identified within the 2021 or 2022 virome, HLVd was determined to be an

important threat facing hemp production therefore it was included in the screening. Similarly to BCTV, a panel of 14 genetically unique genotypes of hemp were analyzed for resistance to HLVd. Resistance was identified in a single genotype, 517, which had a lower frequency of infection than the others. However, no varying viroidal loads were observed between genotypes. Throughout this study, viruses associated with hemp were described as well as the identification of genetic resistance to emerging pathogens. This work will help to further integrated pest management strategies and promote sustainable agriculture.

ACKNOWLEDGEMENTS

I cannot imagine my graduate school career without my advisor Dr. Punya Nachappa. Punya is an incredible researcher and mentor. She goes above and beyond for her students to ensure they have opportunities for development of both professional and technical skills. The encouragement and support I received from Punya means the world to me. I admire how dedicated she is to her students and research. Her passion for virus-vector interaction as well as hemp pathology is contagious.

I am indebted to my committee members Dr. Robyn Roberts and Dr. Mark Stenglein. You have both supported me throughout my time at CSU by providing clarification, technical support and advice. I am very grateful for your time and the effort you put into my education.

I would like to extend my gratitude to all members of the Nachappa lab, past and present, in no particular order: Jordan Withycombe, Max Schmidbauer, Olivia Carter, Luke Deyle, Dr. Erika Peirce, Dr. Jacob Pitt, Judith Chiginsky, Chris Hayes, Darren Cockrell, Henrique Vieira, Adam Osterholzer, Kendal Berasley, Meihua Cui, and Maria Paula. I owe a world of thanks to Dr. Jinlong Han and Dr. Jacob MacWilliams, none of this would be possible without the amazing mentorship I received from the both of you. You two are incredibly kind, patient, intelligent and absolute rockstars. Additionally, I would like to thank Marylee L. Kapuscinski for her technical support. Thank you all for helping me grow as a scientist.

I am beyond grateful for my support system I had throughout my time at CSU. Thank you to my best friend since the 6th grade Olivia Elia. I am so grateful that you moved to Colorado with me, and I appreciate that you are always willing to lend an open ear and a helping hand. Thank you, Giovanna Matos Franco, for being a great writing buddy, the final push was made

much more enjoyable with our Teams calls. A special shout-out is in order for my friend Jordan Withycombe. I am so glad we were able to go through graduate school together and have a bunch of fun along the way.

Thank you to my parents John and Lori Hackenberg for always being there for me. I would like to acknowledge my siblings Johnny and Amber Hackenberg who are truly my closest friends. Thank you both for remaining close to me, despite our physical distance. I could not have chased this dream without the support of my loving family.

Finally, I would like to thank my partner, Jacob Pitt. The love and support I received from you is immeasurable. None of this would have been possible without your advice and encouragement. Thank you for all you do for me.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS v
LIST OF TABLES ix
LIST OF FIGURES x
INTRODUCTION 1
Hemp history and its uses 1
Diseases associated with hemp 3
High throughput sequencing and metagenomic analysis for virus discovery 4
Viral disease management 5
Addressing the knowledge gap 7
MATERIALS AND METHODS 9
Virome analysis 9
Field sites 9
Tissue Collection and storage 9
Library Preparation and Sequencing 10
Bioinformatic analysis 10
Identification of Viruses 11
Beet curly top virus screening 11
Plant source and maintenance 11
Virus inoculation 12
Leaf tissue collection 13
DNA extraction and qPCR quantification 13
BCTV impacts on cannabinoid analysis 14
Plant maintenance and tissue collection 14
Ultra-Performance Liquid Chromatography Tandem Mass Spectromomy (UPLC-MS/MS) 14
Hop latent viroid screening 16
Plant source and maintenance 16
Viroid inoculation 16
Leaf tissue collection 17
RNA extraction and RT-qPCR quantification 17
Statistical analysis 19
RESULTS 20
Hemp virome summary statistics 20

Summary of hemp viromes in 2021 and 2022 20
Virus diversity by plant phenology 21
Virus diversity by cultivar and field 21
Viruses by regions 22
Viral load within virome 22
Genetic resistance for BCTV in hemp 22
Beet curly top virus impact on cannabinoids 23
Genetic resistance for HLVd in hemp 23
DISCUSSION 25
FUTURE DIRECTIONS 33
FIGURES AND TABLES 35
REFERENCES 52
APPENDICIES 62

LIST OF TABLES

Table 1: Summary of viruses and viroids known to infect hemp. 35
Table 2: County, field site and cultivar information for 2021 2022 hemp virome. 36
Table 3: BLAST results for the representative viruses found in the hemp virome. 37
Table 4: Analysis of impacts of BCTV on cannabinoid levels in high and low cannabinoid cultivars. 38

LIST OF FIGURES

Figure 1: USDA-NASS 2022 hemp report showing current hemp acreage planted and changes in acreage planted from 2021 40
Figure 2: Locations of hemp fields sampled in 2021 and 2022 41
Figure 3: Various symptoms observed in 2022 growing season. 42
Figure 4: Virome sampling and pooling methodology 43
Figure 5: Workflow for virome analysis 44
Figure 6: Viruses found in hemp virome for each year 45
Figure 7: Viral diversity and distribution by phenological timepoint 46
Figure 8: Individual viromes of hemp cultivars 47
Figure 9: Viral count by region 48
Figure 10: Viral load in hemp leaf samples collected in 2021 and 2022 49
Figure 11: Screening for BCTV resistance in diverse panel of hemp genotypes 50
Figure 12: Screening for HLVd resistance in diverse panel of hemp genotypes 51

INTRODUCTION

Hemp history and its uses

Hemp, *Cannabis sativa* L., is a crop with which humans share a rich history (McPartland et al., 2019). Cannabis can be divided into two different groups depending on its phytocannabinoid content, hemp and marijuana. While they are the same species, hemp and marijuana have vastly different intended uses. Hemp is commonly grown for seed, fibers, and non-psychoactive cannabinoid cannabidiol (CBD) (e.g. Johnson, 2018; Mark et al., 2020) while marijuana is grown for its psychoactive cannabinoids to be used medicinally or recreationally.

Hemp has had a long and unique history in the US. In colonial times, it was commonly grown for its strong and durable fibers [reviewed in (Johnson, 1999)]. The Marihuana Tax Act (MTA) passed in 1937 placed constraints on the cultivation of all *Cannabis*, to prevent to cultivation of cannabis with psychotropic properties that were feared to become abused. While the goal of the MTA was not meant to hurt the hemp fiber industry, it did place constraints and taxes on producers, manufacturers and distributors thus making alternative fibers more appealing [reviewed in (Johnson, 1999; Fike et al., 2020)]. During World War 2, interest in hemp was revitalized when fiber supplies from abroad were interrupted, the slogan growing “hemp for victory” was used to incentivize farmers to adopt this crop for the war effort (Johnson, 1999). After the end of the war, cannabis was again criminalized in the Controlled Substances Act in 1971 which did not differentiate between hemp and marijuana and therefore declared all cannabis a schedule 1 drug [reviewed in (Fike et al., 2020)]. Hemp remained in prohibition until the 2014 Farm Act that legitimized industrial hemp research in section 7606 by providing a definition for hemp: “the plant *Cannabis sativa* L., and any part of such plant, whether growing

or not with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis" (Agricultural Act of 2014). Thus, formally differentiating hemp from marijuana. This act allowed for institutions of higher education as well as individual state departments of agriculture to cultivate hemp for research purposes. Then with the passing of the 2018 Farm Act, hemp was able to be cultivated in states where laws permitted (Agricultural Improvement Act of 2018). After nearly 70 years of prohibition, hemp is currently experiencing a resurgence in the US.

There are 48 states in the US that allow for the cultivation of hemp as of 2022. In 2022 there were 28,314 acres of industrial hemp planted in the US contributing to a production that was valued at $283 million (National Hemp Report, NASS). The acreage of planted industrial hemp as well as the abundance compared to that of the previous year is disclosed for most states (Figure 1). Of the available information, the top states in production are Montana, South Dakota, Oregon, Missouri, and Colorado.

With the legalization of hemp for cultivation came the ability for universities to research this crop. Understandably, research on hemp has been lacking for the past 70 years and now scientists were presented with the opportunity to apply modern scientific techniques to the study of this crop. One of the most important areas of research involving hemp production is the identification of associated diseases, due to their unknown economic impacts. As the production of hemp increases and spreads throughout the US, the emergence of diseases and their outbreaks are certain (Fike, 2016).

Diseases associated with hemp

Given the resurgence of hemp research, there has been a flux of studies relating to the pathogens of hemp. Numerous first reports of fungi, bacteria, and viruses/viroids have been published in this cropping system since its legalization (e.g. Schappe et al., 2020; Beckerman et al., 2018; Szarka et al., 2019; Giladi et al., 2020; Jarugula et al., 2023; Bektaş et al., 2019; Warren et al., 2019). Historically, there has been over 100 diseases associated with this crop, however their economic importance is unknown (McPartland et al., 2000). Recent reviews on cannabis pathology identify the major pathogens of concern (Punja, 2021; Punja et al., 2023a; Miotti et al., 2023a).

However, viruses associated with hemp are still relatively under studied. Chiginsky et al. 2021 broke new grounds in cannabis virology by publishing the first comprehensive virome report of hemp, observing 7 viruses and 1 viroid infecting hemp in Colorado. Recently, a complete literature review of viruses and viroids infecting cannabis was published (Miotti et al., 2023a). Currently, there are 18 viruses and 1 viroid known to infect cannabis in published literature (Table 1). For more information on most of these viruses and their presumed threat level, readers are directed to the most recent review of cannabis virology (Miotti et al., 2023a).

While the list of viruses associated with cannabis has been growing, it was only recently that researchers have adopted high throughput sequencing (HTS) to aide in this discovery. Only a few of these first reports of viruses and viroids in hemp have been detected utilizing HTS (Nibert et al., 2018; Hadad et al., 2019; Bektaş et al., 2019; Warren et al., 2019; Giladi et al., 2020; Chiginsky et al., 2021; Miotti et al., 2023b; Gezovitch et al., 2023). For the rapid identification of pathogens associated with this emerging crop, the use of HTS technologies is essential.

High throughput sequencing and metagenomic analysis for virus discovery

Metagenomics is the study of microbial communities within individual samples through the analysis of genetic materials. For metagenomic analysis to occur, genetic material in the form of nucleotide sequences must first be obtained. In the early days of metagenomics, these sequences of nucleotides were obtained from Sanger sequencing [reviewed in (Thomas et al., 2012)]. The arrival of new technologies that built off Sanger sequencing known as high throughput sequencing (HTS), or next generation sequencing (NGS), revolutionized metagenomics by increasing the amount of nucleotide sequences obtained from a single instrument run making metagenomic studies more productive (Villamor et al., 2019). There are many sequencing technologies that qualify as HTS/NGS, but all these technologies follow the same essential steps: DNA or cDNA fragmentation for library creation, ligation of adapters to fragments, followed by the sequencing of each fragment (Villamor et al., 2019).

Plant pathologists quickly adopted this technology for the use of microbe discovery in plants. Unlike previous diagnostic tests like enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) that require specific antibodies or primers for microbe detection, HTS can be used to identify all microbes within a given sample, known or unknown, pathogenic or benign in a single assay. Since its first implementation in 2009 (Adams et al., 2009; Al Rwahnih et al., 2009; Donaire et al., 2009; Kreuze et al., 2009), there have been countless metagenomic analyses performed via HTS for virus discovery in cultivated plants. Metagenomic analyses that are specifically investigating virus/viroid communities are referred to as virome studies. It is suspected that given the trend of HTS for plant virus discovery, the virome of all agricultural crops is likely to be documented (Villamor et al., 2019). This assumption seems to be holding true, as there has been many agricultural crops with their virome

described. For example wheat, tomato, grapevine, potato, peppers watermelon, peaches and sweet potato just to name a few (e.g. Albrecht et al., 2022; Lee et al., 2023; Redila et al., 2021; Singh et al., 2020; Ma et al., 2020; Rivarez et al., 2023; Turco et al., 2018; Xu et al., 2017; Burger & Maree, 2015; Czotter et al., 2018; Hily et al., 2018; Miljanić et al., 2022; Nuzzo et al., 2022; Elwan et al., 2023; Lai et al., 2022; Turco et al., 2018; Jo et al., 2017, 2022; Luria et al., 2019; Dias et al., 2022; Jo et al., 2018; Gu et al., 2014; Jo et al., 2020; Orfanidou et al., 2022).

Recently, HTS has been utilized for virus and viroid discovery in cannabis (Bektaş et al., 2019; Chiginsky et al., 2021; Gezovitch et al., 2024; Giladi et al., 2020; Grunwald et al., 2023; Hadad et al., 2019; Jarugula et al., 2023; Lopez-Jimenez et al., 2023; Miotti et al., 2023b; Nibert et al., 2018; Punja et al., 2023a; Warren et al., 2019). These studies successfully characterized known viruses/viroids of cannabis (Chiginsky et al., 2021; Grunwald et al., 2023; Jarugula et al., 2023; Lopez-Jimenez et al., 2023; Miotti et al., 2023b; Punja et al., 2023a), viruses/viroids never before reported to infect cannabis (Bektaş et al., 2019; Chiginsky et al., 2021; Gezovitch et al., 2024; Giladi et al., 2020; Hadad et al., 2019; Nibert et al., 2018; Warren et al., 2019), and were used for novel virus discovery (Miotti et al., 2023b).

Viral disease management

The predominant goal of virome analyses is virus/viroid identification for the management of disease. In order to prevent a disease from decimating a crop, its causal agent must be identified, and the proper control methods must be put in place. Unfortunately, there are no ways to cure a plant (on a field scale) once it has become infected with a virus (Singh & Srivastava, 2020). Therefore, viral disease management relies heavily on preventing infection.

There are numerous avenues to choose from when preventing the introduction of a virus or the prevention of a viral disease in a cropping system. However, it not recommended to rely on just one of these prevention measures for fear of developing resistance to the approach. Rather, it is best to diversify the approach by employing several of the following tactics.

Insects are phenomenal vectors of disease, most plant viruses are transmitted by insects (Whitfield et al., 2015). To avoid the introduction of a particular virus, its insect vector(s) can be targeted. The planting date of the crop can be altered to miss the heaviest population densities of the vector [reviewed in (Hilje et al., 2001)]. Deterrents can be used to repel insects away from the area, such as reflective mulches (e.g. McLean et al., 1982). Beneficial insects or microbes can be employed to control the target insect [reviewed in (Baker et al., 2020)]. Additionally, if appropriate, insecticides can be used to kill the insect before viral transmission occurs (Perring et al., 1999).

Another area of concern is the sources of inoculum. While insect vectors are the primary mode of horizontal transmission, they first must acquire the virus by feeding on infected plants. Additionally, some viruses are known to be seed transmissible, meaning if plants are infected, they have the possibility of producing seeds that contain the virus and cause infection in the next generation (Johansen et al., 1994). It is essential to obtain clean seeds and other propagative material to avoid the introduction of viral pathogens in a cropping system. Other ways to avoid the introduction or spread of inoculum is to control weeds in the area that may be source of the virus or habitat for the insect vector, test plants regularly for the presence of pathogens and remove those that are positive, as well as integrating other appropriate phytosanitary practices on farm (e.g. Varma, 1993; Jones, 2004; Singh & Srivastava, 2020).

The use of resistant or tolerant cultivars is a great way to mitigate disease if they are available for the crop and pathogen or vector of concern. Resistant cultivars detect the pathogen of interest through a multitude of ways and once it is recognized, mount a defense to impede replication and/or movement of the pathogen (e.g. Wu et al., 2019; Varma, 1993). Additionally, plants may also have resistance to the insect vector, reducing transmission events (e.g. Yao et al., 2019). Resistant cultivars can therefore evade infection from the pathogen and/or reduce the amount of pathogen within them. While tolerant cultivars maintain a normal viral load, but do not have any fitness costs associated with this infection thus inhibiting disease (Cooper & Jones, 1983).

Addressing the knowledge gap

This study aims to provide a comprehensive overview of hemp virome and to screen for disease resistance in cultivars of hemp. The objectives of this study are to systematically characterize the diversity and distribution of viruses associated with hemp in Colorado using HTS as well as identify disease resistant cultivars of hemp through screening a diverse set of hemp germplasm for pathogen resistance. The virome was systematically characterized by visiting four hemp producing regions of Colorado, sampling from a variety of cultivars at three distinct phenological timepoints throughout the growing season. This provided insight on the diversity and distribution of viruses in hemp as well as when they occur throughout the growing season, promoting understanding of their epidemiology. Resistance to prominent pathogens of hemp were evaluated in a panel of genetically distinct hemp germplasm. Resistance was identified; therefore these genetics will be implemented in breeding programs to help mitigate these diseases in production. The long-term goals of this project are to aide in the development

of robust and effective diagnostic tools for early pathogen detection in hemp, to provide a basic understanding of epidemiology of hemp diseases as well as identify disease mitigation strategies.

MATERIALS AND METHODS

Virome analysis

Field sites

In 2021, hemp leaf samples were collected from field sites in Montrose, Mesa, Larimer and Morgan counties in Colorado (Figure 2). In these counties, two fields were sampled in Montrose, one in Mesa, three in Larimer, and one in Morgan. These field sites were visited three times throughout the growing season and samples were collected from plants in early vegetative (2-4 weeks), advanced vegetative (7-10 weeks), and mature flowering (12-16 weeks) phenology stages (Supplemental Figure 1) except for field ME3 which was only able to be sampled at the first and last timepoint. From each field, two cultivars were sampled when available. In 2022, hemp leaf samples were collected from field sites in Montezuma and Rio Grande counties in Colorado (Figure 2). One field was visited in each of these counties. Again, these field sites were visited throughout the growing season to assess the same stages of plant phenology as described for 2021 (Table 2).

Tissue Collection and storage

For both years, the location in the field where sampling occurred remained consistent for all timepoints. Plants were selected at random within these specific locations, following a ‘Z’ pattern throughout the area. Plants were selected regardless of symptomology. Anecdotally, in 2021 very few symptoms were observed in the field. In 2022, when symptoms were observed on plants being sampled, they were documented (Figure 3). Tissue samples were collected in the form of three leaflets from around the plant, focusing on the newest growth from each plant. There were 10 plants sampled from each cultivar at each field. For every timepoint, samples

were pooled by field and cultivar. Resulting in pools consisting of 10 plants for each cultivar per field at every timepoint. To make these pools, the three leaflets from each plant were stacked, and a section was taken from the middle of leaflets through a transverse cut. Then all the samples for a given pool were added to the same 1.5mL Eppendorf tube stored for RNA extraction. This resulted in 36 pools for 2021 and 12 pools for 2022 (Figure 4). In 2021, samples were collected then pooled and stored in -80°C for RNA extraction. In 2022, samples were collected and pooled then stored in RNALater (Thermo Fisher Scientific) and kept at -80°C for RNA extraction.

Library Preparation and Sequencing

Pooled leaf tissue samples were ground in liquid nitrogen. From this ground tissue, approximately 50mg was taken from each pool and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufactures instructions with the following modification. All centrifuge steps marked at 15 seconds were increased to 30 seconds. DNA contamination was removed from the RNA using the TURBO DNA-Free kit (Invitrogen) in 15µl reactions following the manufacturer's instructions. RNA quantity and quality were assessed using a Nanodrop One spectrophotometer (ThermoFisher Scientific) and a Qubit 3.0 flurometer (ThermoFisher Scientific). Library preparation was preformed using the KAPA RNA HyperPrep kit (Roche) using half scale reactions. No enrichments or depletions were completed. Library quality control was performed using Agilent TapeStation 4140 and KAPA library quant qPCR. Libraries were sequenced with Illumina NextSeq500 (single end, 150 cycles).

Bioinformatic analysis

Metagenomic analysis was performed using CLC Genomics Work Bench (Qiagen). Reads were demultiplexed, adapters trimmed, and quality trimmed (quality limit = 0.01, maximum number of ambiguities = 2, automatic read-through adapter trimming, discard short

reads: minimum length = 30). Reads were mapped to hemp reference genome cs10 (NCBI RefSeq: GCF_900626175.2) and unmapped reads were collected. Contigs were assembled through de novo assembly algorithm from the unmapped reads (minimum contig length = 200, map reads back to contigs: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fication = 0.5, similarity fraction = 0.8).

Identification of Viruses

Contigs were BLAST searched against NCBI data base for the characterization of the virome. Contigs were first BLAST searched at the nucleotide level then at the protein level. From these BLAST alignments, percent identity, percent coverage, and E-value were recorded (Table 3). Once contigs were identified as viral, they were manually assessed to determine if the complete genome could be recovered (Figure 5).

Beet curly top virus screening

Plant source and maintenance

BCTV was identified in both the 2021 and 2022 viromes and is commonly found throughout the western United States. Given its prevalence and potential to cause disease, this pathogen was included in the resistance screening. In order to facilitate the discovery of candidate genes involved in BCTV resistance, 13 unique genotypes of hemp were obtained from the hemp breeding facility, New West Genetics (Fort Collins, Colorado) to screen for BCTV resistance. Hemp seeds were germinated in plug trays located in a growth chamber (16:8 28°C day, 24°C night) using Promix HP+ soil (Premier Tech Horticulture). Seven seeds were planted per genotype. Once germinated and root systems established, seedlings were transplanted into one-gallon pots and moved to a greenhouse (16 day:8 night, 23°C day and 18°C night) placed in

large PVC cages surrounded in organza netting (20in x 22in x 5ft) and separated by genotype. Plants were fertilized with Jack's Professional 15-16-17 at a rate of 1tsp/gallon and were fertilized at that same rate bimonthly for the remainder of the experiment.

Virus inoculation

A colony of beet curly top virus infected-beet leafhoppers (BLH, *Circulifer tenellus*) was maintained to provide a consistent source of inoculum to be used in this and other experiments. This colony was maintained on sugar beet (*Beta vulgaris*) variety BPA9000. Individual colony plants and a group of insects (5 adults and 5 nymphs) were tested bimonthly through PCR to confirm 100% viral status of the colony. Primers used: BCTV2-F: 'GTGGATCAATTTCCAGACAATTATC' BCTV2-R: 'CCCATAAGAGCCATATCAAACTTC'. These primers target a 496bp fragment of the coat protein (Strausbaugh et al. 2017). 20µl DreamTaq Green PCR Master Mix (Thermo Fisher) reactions were utalized. Reaction conditions were as follows: 10 µl DreamTaq, 1 µl of the forward and reverse primers, 7 µl molecular biology grade water, and 2 µl of diluted sample DNA. PCR thermocycling conditions were as follows: 94°C 2mins [94°C 45sec 58°C 45sec 72°C 45sec] x35 72°C 5min 4°C hold. Results of PCR were visualized on a 1% agarose gel ran at 100volts for 30mins.

Once plants were between the 3 to 5 node stage, they were inoculated with BCTV using a cohort of 5 mixed stage viruliferous BLH, which were introduced to the youngest most developed leaf of the hemp using foam cages (36.5 x 25.4 x 9.5mm) (BioQuip) as to provide access to both the top and bottom side of the leaflet it was attached to. BLH were allowed to feed for 48hours in these small cages before being released into the larger cages for the remainder of the experiment.

Leaf tissue collection

Approximately 50mg of hemp leaf tissue was collected from the new growth 3 weeks post inoculation. Tissues were placed in ZYMO bead bashing tubes and immediately placed on ice. Scissors were cleaned with 10% bleach between each plant. Upon completion of collection, tissue was stored in -20°C for DNA extraction.

DNA extraction and qPCR quantification

DNA was extracted using the ZYMO Quick-DNA Plant/Seed Miniprep Kit following manufactures instructions. DNA was then diluted to 25ng/µl. qPCRs were performed using the Universal iQSYBR Green Supermix (Bio-Rad) using 20 µl reactions. Reaction conditions were as follows: 10µl of iQSYBR, 0.6µl of both forward and reverse primers, 7.8µl of molecular biology grade water, and 1µl of DNA. Samples were run in duplicate. Primers used: BCTV_ALL F: 'GGCTCCTTCAATGCCAAATTAC' BCTV_ALL R: 'CCTTCACGTCTTCATACTTCCC'. These primers target a 106bp region of the coat protein. This primer set was validated and optimized for this experiment. The following cycling conditions were used: hold stage at 94°C for 2 min, followed by 40 cycles of 95C for 15 sec and 60C for 1 min using the Quant Studio 3 Real-Time PCR Machine (Thermo Scientific). Cycle quantification (Cq) values were used to determine positive samples, all samples with a Cq value of <40 were considered positive. A seven-point standard curve was produced by creating a serial dilution of BCTV plasmid (from the V2 protein). This seven-point standard curve was performed on each plate as well as a positive control that remained consistent from plate to plate. The standard curves were averaged into one curve (Supplemental Figure 2). The standard curve equation $y = -3.4356x + 37.941$ was used to determine the primer efficiency and virus quantification. Copy number was determined in individual samples using the following equation: $10^{(Cq - \text{y-intercept})/\text{Slope}}$. The average viral load

was then determined for each hemp genotype. Samples that fell outside the range of the standard curve were confirmed positive or negative by analyzing the results of the qPCR on a 1% agarose gel.

BCTV impacts on cannabinoid analysis

Plant maintenance and tissue collection

To evaluate the effects of BCTV on cannabinoids, two distinct genotypes of hemp were analyzed – 4681 (low-cannabinoid) and 4394 (high-cannabinoid). For both genotypes, there was a total of 8 female plants, four of which were infected with BCTV as described above and the remaining four in each group were clean (control plants). The hemp plants were grown to flower maturity in a greenhouse. Once the end of female flowering was determined as described in Campbell et al. 2019, flower masses were collected, placed in 50mL conical tubes and stored at -80°C. At the time of harvest, the new growth was sampled from each plant to ensure BCTV infection. DNA was extracted and qPCR was performed as previously described to determine viral load within each plant. Additionally, the control plants from both cultivars were tested for absence of BCTV to confirm their clean status.

Ultra-Performance Liquid Chromatography Tandem Mass Spectromomy (UPLC-MS/MS)

Samples were transported from the -80°C freezer directly to be lyophilized (Labconco FreeZone 4.5 plus). Samples were lyophilized at -80°C, <0.02 mBar, for approximately 24 hours. After lyophilization, samples were homogenized for 5 min using a bead beater (Next Advance, Troy, NY, USA). After homogenization, about 40 mg of tissue per sample was weighed into 2-mL Eppendorf tubes. One mL of cold 80% methanol in water was added to each sample and samples were vortexed vigorously for 30 min at 4 °C. Samples were then sonicated in an ice bath

for 15 min and then were vortexed vigorously for 30 min at 4 °C. After extraction, Samples were centrifuged at 15,000 x g and 4 °C for 10 min. Supernatants were recovered and diluted 10 times and 100 times in cold 50% methanol. Then 100 µL of diluted sample was mixed with 20 µL of internal standard (IS) and stored at -20 °C until analysis. An aliquot (10 µL) was taken from each study sample to be pooled to generate a quality control (QC) sample. The authentic standards of cannabinoids and labelled standard THC-d3 were purchased from Cerilliant (TX, USA). Internal standard (IS), 100 ng/mL of THC-d3 were prepared in 50% methanol.

LC-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 Premier HSS T3 column (2.1 x 100 mm, 1.8 µm, Waters, MA, USA). Mobile phases were water with 0.1% formic acid (A) and acetonitrile (B). Samples were held at 6 °C in the autosampler, and the column was operated at 45 °C. Injection volume was 2 µL. The capillary voltage of MS detector was set to 0.7 kV in positive mode. Inter- channel delay was set to 3 msec. Source temperature was 150 °C and desolvation temperature 450 °C. Desolvation gas flow was 1000 L/h, cone gas flow (nitrogen) was 150 L/h, and collision gas flow (argon) was 0.15 mL/min. Nebulizers pressure (nitrogen) was set to 7 Bar. Autodwell feature was set for the collection of 12 points-across-peak. Cone voltage and collision energy (CE) of each MRM was optimized. High abundance compounds (CBDVA, CBGA, CBDA, THCVA, THCA, CBLA/CBCA) in the current sample set were analyzed using "de-optimized" cone and CE voltage. Samples were injected in a randomized order.

The raw data files were imported into the Skyline opensource software package (MacLean et al., 2010). Visual inspections were performed for each target analyte for retention time and peak area integration. Peak areas were extracted for target compounds detected in

biological samples and normalized to the peak area of the appropriate internal standard or surrogate in each sample. Absolute quantitation (µg/g) was calculated using the linear regression equation generated for each compound from the calibration curve.

Hop latent viroid screening

Plant source and maintenance

This pathogen was not identified within the 2021 or 2022 virome, but considering the emergence and importance of this pathogen, hemp genotypes were evaluated for resistance. Similarly to the BCTV screening, 14 genetically unique genotypes of hemp were obtained from New West Genetics (Fort Collins, CO) and utilized to evaluate for HLVd resistance. These hemp plants were grown and maintained as described above.

Viroid inoculation

HLVd infected hemp plants were maintained (cultivars Elite and Unicorn) to use for inoculum in mechanical inoculations. These plants were confirmed to be positive for HLVd before their use as inoculum by using RT-PCR. cDNA was synthesized using the Versco cDNA synthesis kit (Thermo Scientific) Primers used: HLVd-F: 'CCACCGGGTAGTTTCCAACT' HLVD-R: 'ATACAACTCTTGAGCGCCGA' These primers target the complete HLVd genome (256 bp) (Matoušek and Patzak, 2000). 20µl reactions using DreamTaq Green PCR Master Mix (Thermo Fisher) were ran for each sample. Reaction conditions were as follows: 10 µl DreamTaq, 1 µl of the forward and reverse primers, 7 µl molecular biology grade water, and 2 µl of diluted sample DNA. PCR thermocycling conditions were as follows: 94°C 2mins [94°C 45sec 58°C 45sec 72°C 45sec] x40 72°C 5min 4°C hold. Results of PCR were visualized on a 1% agarose gel ran at 100volts for 30mins.

Once plants were at the 5-7 node growth stage, they were mechanically inoculated with HLVd using a combination of two methods: a sap-to-sap method and a rough sponge method. For both methods an inoculation buffer was used as inoculum consisting of 1 gram of HLVd infected tissue obtained from stock plants and ground in 10mL of 20mM sodium phosphate buffer (pH 7) using a mortar and pestle on ice. For the sap-to-sap method, each plant had 1-3 meristems removed. Using a sterile cotton swab, the inoculation buffer was applied to each of these wounds for 30seconds. Additionally, each plant was mechanically inoculated using a rough sponge. The two youngest yet most developed leaves from each plant were inoculated by applying the inoculation buffer to the sponge and gently rubbing the top and bottom side of each leaf until water soaked lesions appeared.

Leaf tissue collection

Approximately 100mg of hemp leaf tissue was collected from the new growth 6 weeks post inoculation. Tissue was placed in a 2mL Eppendorf tube and flash froze in liquid nitrogen. Scissors were cleaned with 10% bleach between each plant and gloves changed. Upon completion of collection, tissue was stored at -80°C for RNA extraction.

RNA extraction and RT-qPCR quantification

RNA was extracted using a lithium chloride method adapted from Vennapusa et al. 2020 using the following steps: Tissue was ground in liquid nitrogen using a mortar and pestle; to the ground sample, 1mL of RNA extraction Buffer was added then mixed via vortex; the homogenate was then incubated at room temperature for 10 minutes; 100µl of 20% SDS was then added, vortexed and incubated at room temperature for 2 minutes; the homogenate was then centrifuged at 16,000 xg for 5mins at 4°C; 600µl of the upper aqueous phase was then collected and 600µl of phenol:cholorform:isoamyl alcohol (25:24:1) was added and then mixed via

vortexing; the homogenate was then centrifuged 16,000xg for 5mins at 4°C; 400µl of the upper aqueous phase was then collected; 200µl of cholorform:isoamyl alcohol was added then vortexed; the homogenate was then centrifuged at 16,000xg for 5mins at 4°C; 300µl of the upper aqueous phase was collected and to it 160µl of 8M LiCl was added, and microcentrifuge tube was inverted 6 times; 46µl of 3M sodium acetate was then added and microcentrifuge tube was inverted 6 times; samples were then incubated for 48 hours at -20°C; samples were removed from -20°C and centrifuged at 16,000xg for 14mins at 4°C; supernatant was discarded and pellet washed with 500µl of 2M LiCl by centrifuging at 16,000 xg for 15 mins at 4°C; supernatant was decanted and pellet was washed with chilled 80% ethanol and centrifuged at 16,000 xg for 5mins at 4°C; ethanol was then decanted and residual ethanol was allowed to evaporate in a biosafety cabinet for 15mins; RNA pellet was then dissolved in 22µl of molecular biology grade water; RNA was diluted to 30ng/µl.

RT-qPCRs were performed using the Taq-Path 1-Step Multiplex Master Mix (No ROX) (Applied Biosystems) using 20µl reactions. Reaction conditions were as follows: 5µl Taq-Path 1-Step Multiplex Master Mix, 1µl primer/probe mix, 12µl molecular biology grade water, and 2µl RNA. Samples were run in duplicate. Primers and probes were multiplexed and targeted a ~100bp region of the HLVd genome and the hemp genome. These primers and probes are proprietary and were provided by Dr. Tassa Salidi 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 2mins, 53°C hold for 10mins, 95°C hold for 2mins, 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 as well as a positive control that remained consistent from plate to plate. The standard curves were averaged into one curve (Supplemental Figure 3). The standard curve equation $y = -3.1091x + 47.488$ was used to determine primer efficiency and virus quantification. Copy number was determined in individual samples using the following equation $10^{(Cq - y\text{-}intercept)/Slope}$. The average viroid load was then determined for each hemp genotype.

Statistical analysis

All statistical analysis were performed in R version 4.3.0 (2023-04-21). An ANOVA was performed to evaluate the difference in means of BCTV between hemp genotypes. Due to non-parametric distribution of residuals, a Kruskal-Wallis rank sum test was performed to evaluate the difference in log copy number of HLVd between genotypes. Pearson's Chi-squared was performed to determine differences in percent disease index between genotypes in both the BCTV and HLVd assays. A T-Test was performed on normally distributed cannabinoids and a Wilcoxon test was performed for those that were non-parametric in order to determine cannabinoid concentration in response to BCTV infection.

RESULTS

Hemp virome summary statistics

Throughout this study, nine different hemp fields were sampled representing four hemp producing regions of Colorado. In 2021 the Northern and Western regions were sampled consisting of fields in Larimer, Morgan, Mesa and Montrose counties. In 2022 the Southwestern and Southern regions were sampled consisting of a single field from both Montezuma and Rio Grande counties. For both years, ten plants from two cultivars were sampled from each field three times throughout the growing season resulting and pooled accordingly (Figure 4). This sampling resulted in 36 samples for 2021 and 12 samples for 2022. Data sets contained an average of 4,318,835 sequences for 2021 and 5,198,784 sequences for 2022. After removal of adapter sequences and low quality reads there was an average of 4,110,561 sequences remaining per library for 2021 (95.18%) and 5,115,683 sequences remaining for 2022 (98.4%). Average length after trimming was 131.6 in 2021 and 143.88 in 2022. For both 2021 and 2022, all sequences were comprised of bases with an average phred score >25. Removal of host derived reads left an average of 41,148 reads per sample in 2021 (1%) and 29,728 reads per sample in 2022(0.58%) (Supplemental Table 1).

Summary of hemp viromes in 2021 and 2022

Overall, HTS analysis revealed seven viruses infecting hemp in Colorado across 2021 and 2022 (Figure 6). We were able to assemble complete or partial genomes of these viruses using the HTS data. In 2021 we visited several fields within Larimer, Morgan, Mesa and Montrose counties. Within these fields we identified five viruses: alfalfa mosaic virus (AMV),

beet curly top virus (BCTV), cannabis cryptic virus (CanCV), cannabis sativa mitovirus (CasaMV1), and grapevine line pattern virus (GLPV). In 2022 we visited two fields, one in Montezuma county and one in Rio Grande county. Between these two fields we identified six viruses: BCTV, CanCV, CasaMV1, GLPV, opuntia umbra like virus (OULV), and tomato bushy stunt virus (TBSV).

Virus diversity by plant phenology

Hemp fields were sampled systematically throughout the growing season at 3 different stages of plant phenology: early vegetative (2-4 weeks in age), advanced vegetative (7-10 weeks in age), and mature flowering (12-16 weeks in age). This systematic visitation provides insight as to what viruses are present when throughout the growing season. In both years we observed the same pattern, as the growing season progressed, virus incidence and diversity increased (Figure 7A and B).

Virus diversity by cultivar and field

Of the 9 fields visited, 8 were sampled at two different locations to target different cultivars of hemp from the same field. Virus count was analyzed separately for each individual cultivar (Figure 8). From this figure, it is observed that at some fields, such as ME3, viromes between cultivars are identical, consisting exclusively of CasaMV1. Whereas at other fields, such as MZ, the components of the viromes between cultivars differed vastly, despite their close spatial proximity.

Viruses by regions

Throughout the sample collection over 2021 and 2022, 4 regions of Colorado were visited: Northern, South Central, Southwest and Western. Virus diversity and count were evaluated at the regional level to illuminate differences between regional viromes. Indeed, each region has its own unique virome. The most prevalent viruses detected across all regions were BCTV, CasaMV1, and GLPV, all being detected in 3 out of the 4 regions (Figure 9).

Viral load within virome

In order to determine what viruses were dominating samples, and the virome as a whole, viral load was determined by calculating the log transformed transcripts per million data (TPM) (Eliash, N. et al., 2022). TPM is a read normalization method that allows comparison between reads associated with each of the viruses found between samples. Thus, giving an idea of viral load within each sample and allowing for direct comparisons between samples. Overall, the highest viral load belongs to CasaMV1 followed by GLPV and CanCV (Figure 10).

Genetic resistance for BCTV in hemp

To determine if hemp genotypes exhibit resistance to BCTV, we screened 13 genetically unique genotypes of hemp resulting from a bi-parental F2 population obtained from New West Genetics (Fort Collins, CO). There was a statistically significant difference in viral load (log copy number) between genotypes resulting from a one-way ANOVA (F=2.228; df=12; P=0.02) (Figure 11A). Observably, the two genotypes that harbored the highest viral load were 791 and 3584 while the two genotypes with the lowest viral load were 4587 and 4710. Percent disease index (PDI) was also evaluated, which is number of BCTV infected plants over the total number

of plants per genotype. There were two genotypes that stood out by having a statistically significantly lower PDIs, genotype 4587 (p-value 0.04) and genotype 4710 (p-value = 0.04) utilizing Pearson's Chi-Squared test (Supplemental Table 2). Interestingly, these were the same two genotypes that harbored the lowest BCTV viral load. Additionally, there was a strong correlation observed between average log copy number per genotype and PDI utilizing a Spearman's rank correlation rho (S=88.41, P=0.002, rho=0.75) (Figure 11B).

Beet curly top virus impact on cannabinoids

To assess if BCTV has an impact on cannabinoids, we analyzed the cannabinoids within mature inflorescences between BCTV infected and control (healthy) plants for a high cannabinoid genotype (4394) and a low cannabinoid genotype (4681). A total of 16 cannabinoids were analyzed. There was no impact of BCTV infection on cannabinoid concentrations in both the high and low cannabinoid genotypes (Table 4).

Genetic resistance for HLVd in hemp

Although not found within the 2021 or 2022 hemp virome, we determined this viroid an important threat to the cannabis industry and decided to screen for resistance to it. This was performed utilizing 14 genetically unique genotypes of hemp obtained from New West Genetics (Fort Collins, CO). Due to space restrictions within the greenhouse, not all genotypes could be screened simultaneously. This resulted in a group effect based on when a given genotype was screened, groups were statistically analyzed separately. No statistical significance was determined for the difference in viroid load (log copy number) between genotypes resulting from a Kruskal-Wallis rank sum test (Group 1: Kruskal-Wallis chi-squared = 12.112, df = 8, p-value =

0.1463) (Group 2: Kruskal-Wallis chi-squared = 1.1701, df = 4, p-value = 0.883) (Figure 12A). Percent disease index (PDI) was again evaluated as previously described. There was one statistically significant difference observed in Group 1, genotype 517. This genotype had a significantly lower PDI than the other genotypes in that group (Pearson's Chi-squared test p-value = 0.04) (Supplemental Table 3). Anecdotally this genotype had the lowest viroid load within Group 1. Again, there was a correlation observed between average log coy number per genotype and PDI utilizing a Spearman's rank correlation rho [Group 1 (S = 1.004, p-value = 1.75×10^{-7}, rho = 0.99)] [Group 2 (S= 0.51, p-value = 0.004, rho = 0.97)] (Figure 12B).

DISCUSSION

Overall, the objectives of this study were to describe the diversity and distribution of viruses associated with hemp in Colorado and to determine if there is genetic resistance to pathogens in hemp. We were able to identify 7 viruses infecting hemp across Colorado, 5 in 2021 and 6 in 2022, thus determining the viral threats in field grown hemp in this area of major production. Due to the various phenological timepoints that were sampled, it was observed that virus diversity and incidence increases as the growing season progresses, likely due to pathogen dissemination events such as vector introduction. Indeed, arrival of known insect-vectored viruses such as BCTV and AMV were observed later in the growing season. However, viruses that were reported or thought to be seed transmissible such as CanCV, CasaMV1 and GLPV are present from the beginning of the growing season, suggesting that these pathogens were present in the seed of these hemp plants.

One of the most predominant viruses in the virome was CasaMV1. This virus was by far the most prevalent virus detected in 2021, nearly all samples were infected with this virus, however it was detected less frequently in 2022. CasaMV1 also had the overall highest viral load within the 2021 virome and was found at moderate levels in 2022. Interestingly, despite its vast distribution within this study, it was not detected in the South Central region, Rio Grande county. CasaMV1 has been reported in cannabis globally (Nibert et al., 2018; Chiginsky et al., 2021; Punja et al., 2023a; Miotti et al., 2023b; Lopez-Jimenez et al., 2023). CasaMV1 is a *Mitovirus* in the *Mitoviridae* family. Mitoviruses replicate in their host's mitochondria, their genome encodes only an RNA-dependent RNA-polymerase, thus they do not encode for capsid proteins and cannot form virions (Hillman & Cai, 2013; Shahi et al., 2019). No vectors have been detected for

CasaMV1 to date. It can be hypothesized that CasaMV1 is spread via seed and asexual propagation. Supporting this, CasaMV1 was detected in the seeds of cannabis (Punja et al., 2023a). Previously, mitoviruses have only been reported in fungi, but an analysis of publicly available transcriptome data revealed that mitoviruses are indeed present in plants (Nibert et al, 2018). CasaMV1 has been isolated from symptomatic tissue in cannabis (Miotti et al., 2023b; Punja, et al., 2023a), but the symptomology has yet to be attributed to the pathogen. Due to the spread of CasaMV1 in cannabis, potential impacts on yield should be studied.

GLPV was the next most predominant virus identified. It had both a high viral load as well as identified in numerous samples. This virus has been previously identified in cannabis in Colorado and Italy (Chiginsky et al., 2021; Miotti et al., 2023b). It is no surprise that we again identified this virus in Colorado in both 2021 and 2022. GLPV was detected in all regions except for South Central. GLPV was identified at all timepoints throughout the study indicating the possibility of its presence in seed. In some cultivars, we can see the introduction of GLPV later in the growing season, indicating horizontal transmission of this virus. GLPV is a *Ilarvirus* in the *Bromovirdae* family, it was originally identified in grapevines in Hungary in the 1980's (Lehoczky et al., 1987). Typical symptomology in grapes include bright yellow discolorations on the leaves in various patterns including blotches, rings, or "maple-leaf" line pattern [Reviewed in (Martelli et al., 2017)]. GLPV has been proven to be both seed and mechanically transmissible (Lehoczky et al., 1989; Lehoczky et al., 1992). In cannabis it was isolated from symptomatic tissue in Italy, but causation could not be attributed to the pathogen without further testing (Miotti et al., 2023b).

Similar to CasaMV1, CanCV has been reported in cannabis globally (Ziegler et al., 2012; Righetti et al., 2018; Chiginsky et al., 2021; Miotti et al., 2023b). We detected CanCV in both

2021 and 2022, but it was only found in the Northern and South Central regions. CanCV is a *Betapartitivirus* in the *Partitiviridae* family. It was first officially described and identified in cannabis by Ziegler et al. in 2012 where it was detected in 5 different varieties as well as progeny from seed of infected plants, indicating the virus may be widespread in cannabis and that it is seed transmissible (Ziegler et al., 2012) While investigating the causal agent for Hemp Streak syndrome, Righetti et al. found CanCV in plants displaying the typical symptoms for the syndrome, however, they also isolated CanCV from asymptomatic plants thus no association between CanCV and symptoms could be drawn (Righetti et al., 2018). Similarly, Miotti et al. did not find any associations between CanCV infection with specific symptomology (Miotti et al., 2023b).

The next most predominant virus was BCTV, which was detected at a more moderate viral load in the virome. This makes sense as it was not found in nearly as many samples as the previously described viruses. It was detected in both 2021 and 2022 in several counties spanning South Central, Southwest and Western Colorado. BCTV has been widely documented in hemp across the western United States (Giladi et al., 2020; Hu et al., 2021; Chiginsky et al., 2021; Melgarejo et al, 2022; Rivedal et al., 2022; Schoener & Wang, 2023; Jarugula et al., 2023). BCTV is a *Curtovirus* in the *Geminivirdae* family. It is a major pathogen of sugar beets, but its host range includes various crops, weeds and ornamentals. Transmission of BCTV is limited to being vectored by the beet leafhopper (*C. tenellus*) (Chen and Gilbertson 2016; Creamer 2020). Supporting this, we did not identify BCTV at any of the early vegetative timepoints, showing that BCTV relies on its vector, the beet leafhopper for introduction. BCTV is commonly reported to cause notable symptomology in hemp, including chlorotic leaves, mosaic mottling of leaves, shortened internode length, up-curled thickened and serrate leaves, and curling and twisting of

new leaves. Ultimately, BCTV can lead to overall plant decline and potentially impacts yield of the fiber or flower production (e.g.: Hu et al., 2021; Melgarejo et al., 2022). In 2022, we documented similar symptoms in Southwestern and South Central Colorado. Again, due to the pooling nature of this study, we cannot attribute individual plant symptomology to disease incidence.

Due to BCTV's prevalence throughout the western United States, as well as the potential losses associated with this pathogen, we decided to screen hemp germplasm obtained from New West Genetics (Fort Collins, CO) for resistance to BCTV. Through this study we found that viral load indeed varies between the genotypes of hemp. This indicates that the individual genotypes of hemp support BCTV infection differently, the infection level (viral load) is not the same between genotypes which could confer resistance. Additionally, when we looked at the PDI of the individual genotypes, two stood out by having a lower number of infected plants: 4587 and 4710. Despite being inoculated with viruliferous BLH, these genotypes managed to evade infection at a higher rate than other genotypes within this study. Additionally, we detected a correlation between PDI and average log copy number. These results provide first evidence of genetic resistance to BCTV in hemp and can aid in the development and breeding of BCTV-resistant hemp cultivars.

Since BCTV is an emerging pathogen in hemp, we wanted to take a closer look at the possible effects this pathogen. It is known that BCTV infection can produce dramatic symptomology in hemp plants, that may ultimately result in reduction of plant biomass and overall crop loss thus reducing yield (Hu et al., 2021). To the best of our knowledge, no work has been done to assess the impacts of BCTV on cannabinoids. Plants, being sessile organisms, rely on secondary metabolites to respond to various biotic and abiotic cues including pathogen

infection. Volatile organic compounds, a type of secondary metabolite, are often emitted in response to herbivore presence or pathogen infection (e.g. Kessler and Kalske, 2018). As secondary metabolites, cannabinoids have the hypothesized role to defend plants against various stressors including: UV radiation, water stress, herbivores, and pathogens (Gorelick and Bernstein, 2017; Tanney et al., 2021). In our study, we found no response on cannabinoid levels in response to BCTV infection. This contradicts other literature with documented response of cannabinoids to herbivores (Kostanda and Khatib, 2022; Park et al., 2022) and HLVd (Punja et al., 2023b). It is possible that the treatment plants initially mounted a defense that would have been represented in the cannabinoid profile to the BCTV infection, but since tissue sampling took place approximately 3 months after infection the response was lost.

Similar to BCTV, AMV was detected exclusively later in the growing season, reflecting the occurrence of a transmission event, likely by one of AMV's many aphid vectors. AMV was found at a moderate viral load in the 2021 virome, considering it was only detected in a single sample. AMV has previously been identified as a pathogen of hemp, even being found in fields in Germany in the late 1960's, but has yet to be identified in fields since then (Kegler & Spaar, 1997; Schmidt & Karl, 1970). In our study, we detected AMV exclusively in 2021 in Larimer County (Northern Colorado). AMV is an *Alfamovirus* virus in family *Bromovirdae*. It has a broad host range, affecting numerous economically important crops and is commonly transmitted via aphids, but can also be transmitted mechanically and through seed (e.g. Hull, 1969; Jones & Pathipanawat, 1989; Swenson, K. G., 1952). In hemp AMV has been reported to cause light green spotting, yellow streaking, yellowing of the veins and slight curling of the new leaves (Kegler & Spaar, 1997; Schmidt & Karl, 1970). Due to the pooling of samples in this study,

virus symptomology cannot be attributed to an individual plant. However, anecdotally, there was very little symptomology exhibited across all plants sampled in 2021.

OULV was detected at low viral load within the virome and was only reported at a single field in both cultivars at the final timepoint. OULV was previously described in Colorado hemp in 2019 (Chiginsky et al., 2021). We again detected this virus in 2022 in Southwestern Colorado, Montezuma County. OULV is an unclassified *Umbravirus* in *Tombusviridae*. Umbraviruses are known to be capsidless viruses that rely on a helper virus, typically a member of *Luteoviridae* for encapsulation and transmission via insect (International Committee on Taxonomy of Viruses, 2024). Interestingly, no viruses in the *Luteoviridae* family were recovered in these samples, however evidence for horizontal transmission is evident as it was only detected at the mature flowering phenological stage. Symptomology has yet to be attributed to this virus in hemp.

TBSV has yet to be reported in cannabis. We found this virus in 2022 in the South Central region of Colorado in Rio Grande county. This virus was the least prevalent identified in this study, it was only found in one sample and had the overall lowest viral load. Additionally, it was identified only at the mature flowering timepoint, indicating the introduction of this virus via some form of horizontal transmission. TBSV is a *Tombusvirus* in *Tombusviridae*. It has a broad host range and is commonly used as a model organism for plant virology (e.g. Yamamura & Scholthof, 2005). Vegetatively, in tomatoes, TBSV is reported to cause bushy growth, stunting, purpling of leaves, necrosis, as well as yellow spotting and malformation on the younger leaves, in the fruit it can reduce yield, produce chlorotic rings, blotching, and lines as well as reduced fruit size [Reviewed in (Martelli et al., 1988)]; (Luis-Arteaga et al., 1996). Transmission of TBSV has been reported mechanically, via soil, and vertically by seed (e.g. Martelli et al., 1988). Again, while there were symptomatic plants in the pool that TBSV was detected in, we cannot

attribute these symptoms to the disease. To our knowledge, this is the first report of TBSV in cannabis.

We observed that some cultivars from the same field had vastly different viromes, while others had identical viromes. For example, from field ME3, cultivars 'White' and "Abbey Rd' were sampled, in both cultivars, only CasaMV1 was detected. Whereas field MZ cultivars 'Cherry Wine' and 'Matterhorn' were sampled, the viromes between these two cultivars were vastly different. Despite their close proximity to each other, 'Cherry Wine' was infected with OULV, CasaMV1, and BCTV whereas 'Matterhorn' was infected with OULV and GLPV. This suggests that there may be varying susceptibilities to viruses between cultivars of hemp.

In addition to known viruses, this study also identified novel viruses. These novel viruses were identified in the BLAST but have low percent identity to their closest hit (Supplemental Table 4). Typically, to differentiate between individual viral species, each viral genus has its own percent nucleotide identity cut off, where anything below that threshold is considered a different species. This percent nucleotide identity ranges depending on the viral genus of interest, and some genera do not rely on nucleotide identity to differentiate between viral species. Although the goal of this study was not to characterize new viruses, it is important to report these novel viruses to aide in future research.

Although HLVd was not found in the 2021 or 2022 Colorado hemp virome, this pathogen has become an emerging threat to the cannabis industry. It has been reported throughout North America (Bektaş et al., 2019; Warren et al., 2019; Chiginsky et al., 2021; Rivedal et al., 2022; Jarugula et al., 2023; Punja et al., 2023b), but potentially worldwide as it is found globally in hops (e.g. Barbra et al., 1990; Liu et al., 2008; De Jonghe et al., 2016; Eiras et al., 2023). Both hops and cannabis belong to the same family, *Cannabaceae*. Recently, HLVd has been proven to

reduce the cannabinoid yield in inflorescences of mature female cannabis plants (Punja et al., 2023b). This is of huge concern for producers as one of the major reasons for hemp production in the US is for the harvest of CBD and other phytocannabinoids from the flowers (U.S. Department of Agriculture, 2020). This pathogen has the potential to be economically devastating to the industry. For this reason, we decided to include HLVd in our resistance screening. Our results show no significant variation of HLVd load between hemp genotypes. However, there was one genotype that exhibited a significantly lower proportion of diseased plants than the other genotypes, 517, with a PDI of 42.86%. This could indicate resistance within this genotype to HLVd. Variation in disease prevalence was previously observed in cannabis in Canada, with several genotypes having lower frequency of diseased plants (Punja et al., 2023b). Thus, supporting the finding of this study, there does appear to be genetic resistance to HLVd in hemp. A group effect was observed based on when genotypes were screened. This could be due to varying viroid levels in the inoculum used to inoculate the plants. All source plant material was confirmed HLVd positive via end point PCR, but viroidal load was not quantified. In future screenings, source plants should be tested via qPCR and should fall within a range of acceptable viroidal loads to be used as inoculum.

To conclude, this research has systematically documented the Colorado hemp virome and has shown the potential for resistance to pathogens in hemp. These findings will serve as a useful resource to further integrated pest management strategies in hemp cultivation as well as future research regarding hemp-pathogen interactions.

FUTURE DIRECTIONS

Regarding the virome, there are several exciting avenues for future research. The first of which being further characterization of the novel viruses detected. Further phylogenetic and molecular analyses are required to fully characterize these viruses. Future work will utilize the sequences obtained from HTS, in addition to the original tissue to be used for re-extraction, to aide in constructing complete genomes from these viruses. Research into the symptomology, yield loss, and other fitness effects of the viruses identified within the virome are also essential points of future research. For instance, we know that GLPV is globally distributed in cannabis, yet we still have no evidence on what the effects of this pathogen may be. Future studies that do not rely on pooling tissue will be necessary to tease apart the symptomology observed, as well as controlled studies to document the progression of the disease associated with this pathogen as well as fitness costs associated with infection.

However, most importantly, the results of the virome analysis will be used to aide in the development of molecular diagnostic tools for the rapid and accurate detection of these pathogens. RNA obtained from this study has already been shared with collaborators who are working on developing these very tools. These tools will be an invaluable resource for researchers, breeders and most of all growers.

The genotypes screened for resistance to both BCTV and HLVd should undergo further testing. This testing should include a larger sample size in a field setting, mimicking how this crop is intended to be grown. However, these future studies could focus in on the genotypes of interest identified within this study, or the genotypes that harbored a lower viral load. Additionally, the genotypes of interest should be evaluated further, potentially through RNA

sequencing, to identify domains associated with resistance. Furthermore, it would be interesting to evaluate this panel of hemp genetics for tolerance to pathogens. Tolerance to pathogens is defined as the ability to harbor a normal pathogen load within the plant, all while reducing symptomology and/or having little to no fitness costs associated with the infection (Cooper & Jones, 1982). Host plant tolerance, if identified, would be another sustainable way to manage these diseases. Ultimately, this research is a small contribution to the emerging area of research surrounding the sustainable management of diseases and pests of hemp. This research will serve as a resource for others studying management strategies which will directly benefit the growers and therefore help to promote sustainable agriculture.

FIGURES AND TABLES

Table 1: Summary of viruses and viroids known to infect hemp.

Family	Virus/Viroid	Detected in Field Grown Hemp?	First Reported by
Bromoviridae	Alfalfa mosaic virus	Yes	Schmidt & Karl 1970
	Cucumber mosaic virus	Yes	Schmidt & Karl 1970
	Grapevine line pattern virus	Yes	Chiginsky et al., 2021
	Tobacco streak virus	Yes	Chiginsky et al., 2021
Secoviridae	Arabis mosaic virus	No	Kegler & Spaar 1997
	Raspberry ringspot virus	No	Kegler & Spaar 1997
	Broad bean wilt virus	No	Kegler & Spaar 1997
Tospoviridae	Tomato spotted wilt virus	No	Kegler & Spaar 1997
Alphaflexiviridae	Potato virus X	No	Kegler & Spaar 1997
Potyviridae	Potato virus Y	No	Kegler & Spaar 1997
Partitivirdae	Cannabis cryptic virus	Yes*	Ziegler et al., 2012
Mitoviridae	Cannabis sativa mitovirus 1	Yes*	Nibert et al., 2018
Closteroviridae	Lettuce chlorosis virus**	Yes	Hadad et al., 2019
Pospiviroidae	Hop latent viroid	Yes	Bektaş et al 2019 and Warren et al 2019
Geminiviridae	Beet curly top virus	Yes	Giladi et al., 2020
Tombusviridae	Opuntia umbra-like virus	Yes	Chiginsky et al., 2021
Unclassified?	Citrus yellow vein-associated virus	Yes	Chiginsky et al., 2021
Unclassified – *Partitivirdae*	Cannabis associated partiti like virus	Yes	Miotti et al., 2023b
Closteroviridae	Cucurbit chlorotic yellows virus**	Yes	Gezovitch et al., 2023

*Original study was not field based or unknown, but has since been found in field grown hemp
**First identified in cannabis, not specifically hemp

Table 2: County, field site and cultivar information for 2021 2022 hemp virome.

Year	County	Field Name	Cultivar
2021	Larimer	AR	Unicorn
		SE	2730
			4113
		OL	2624
			4788
	Morgan	PC	2463
			4000
	Mesa	ME3	White
			Abbey Rd
	Montrose	MO3	TS13*
			TS13.2*
		MO4	TS13.3*
			TS14
2022	Rio Grande	SLV	Hhlukhiv's Ki 51*
			Hhlukhiv's Ki 51.2*
	Montezuma	MZ	Matterhorn
			Cherry Wine

*Cultivars TS13 and Hhlukhiv's Ki 51 were obtained from several locations or twice from the same field. To account for these multiple samplings, a decimal was added to distinguish replicates.

Table 3: BLAST results for the representative viruses found in the hemp virome.

Virus	Nearest GenBank Sequences	%nt identity	% coverage	Genome Coverage	E-value	Accession *	Contig Length (bp)
Alfalfa mosaic virus RNA 1	MH332897	99.63	95	Complete	0	/	3491
Alfalfa mosaic virus RNA 2	MK607975	99.23	74	Complete	0	/	1957
Alfalfa mosaic virus RNA 3	MK648426	99.48	93	Complete	0	/	1907
Beet curly top virus	KX867057	99.04	17	Partial	0	/	519
Cannabis cryptic virus RNA 1	MT893743	99.82	94	Complete	0	/	2202
Cannabis cryptic virus RNA 2	KX709965	99.62	92	Complete	0	/	2099
Cannabis sativa mitovirus	MT878083	98.53	92	Complete	0	/	2919
Grape vine line pattern virus RNA 1	MW888424	98.53	92	Complete	0	/	2919
Grape vine line pattern virus RNA 2	MW888423	98.8	94	Complete	0	/	2246
Grape vine line pattern virus RNA 3	MW888422	99.68	99	Complete	0	/	2506
Tomato bushy stunt virus	MZ202331	98.1	8	Partial	0	/	489
Opuntia umbra-like virus	MT909563	97.84	41	Partial	0	/	1252

*Currently not uploaded to NCBI GenBank, will be uploaded before publication.

Table 4: Analysis of impacts of BCTV on cannabinoid levels in high and low cannabinoid cultivars.

Cultivar	Cannabinoid	Treatment	Mean (μ/g)	SE	t-test **	df	*P*-value
4681 (high cannabinoid)	CBDV	clean	25.28	10.93	-0.87	5.77	0.42
		BCTV	12.95	8.96			
	THCV	clean	2.50	0.87	-1.37	5.31	0.23
		BCTV	1.05	0.60			
	CBD*	clean	833.85	381.35	5.00	/	0.49
		BCTV	331.91	236.00			
	CBG*	clean	13.10	2.15	4.00	/	0.34
		BCTV	9.83	5.86			
	CBGA	clean	245.61	109.60	-0.40	5.93	0.70
		BCTV	186.15	98.01			
	CBCV	clean	2.36	0.84	-1.26	5.24	0.26
		BCTV	1.10	0.56			
	THCVA*	clean	62.09	43.92	7.00	/	0.89
		BCTV	42.18	19.57			
	CBN	clean	0.35	0.14	-1.54	4.35	0.19
		BCTV	0.11	0.07			
	CBDVA*	clean	1459.33	994.46	6.00	/	0.69
		BCTV	1474.46	838.47			
	delta9THC	clean	55.75	22.29	-1.46	4.27	0.21
		BCTV	19.76	10.52			
	CBNA*	clean	1.44	0.43	6.00	/	0.69
		BCTV	1.02	0.42			
	CBC	clean	39.00	15.73	-1.20	4.75	0.28
		BCTV	17.21	8.92			
	THCA	clean	353.23	30.35	0.88	3.11	0.44
		BCTV	553.70	224.91			
	CBT	clean	0.34	0.10	-0.30	5.19	0.78
		BCTV	0.29	0.15			
	CBLA.CBCA	clean	1038.94	88.82	0.87	3.12	0.45
		BCTV	1601.67	639.91			
	CBDA	clean	22265.50	3688.95	0.45	3.79	0.68
		BCTV	27100.03	10099.13			

4394 (low cannabinoid)	CBDV*	clean BCTV	4.66 6.14	2.90 4.74	7.00	/	0.89
	THCV*	clean BCTV	0.59 0.79	0.33 0.54	8.00	/	1.00
	CBD*	clean BCTV	749.51 677.32	562.18 252.86	11.00	/	0.49
	CBG	clean BCTV	23.60 22.49	4.92 8.65	-0.11	4.76	0.92
	CBGA	clean BCTV	2826.07 5765.65	451.08 3578.94	0.81	3.10	0.47
	CBCV*	clean BCTV	0.79 0.54	0.31 0.31	4.00	/	0.34
	THCVA	clean BCTV	23.62 22.38	6.43 10.35	-0.10	5.02	0.92
	CBN	clean BCTV	0.16 0.21	0.06 0.05	0.55	5.48	0.60
	CBDVA	clean BCTV	754.22 946.22	234.11 382.79	0.43	4.97	0.69
	delta9THC*	clean BCTV	37.92 38.32	25.10 16.30	10.00	/	0.69
	CBNA	clean BCTV	2.39 2.62	0.20 0.56	0.38	3.75	0.72
	CBC	clean BCTV	38.57 21.09	15.59 7.38	-1.01	4.28	0.36
	THCA	clean BCTV	777.49 981.31	77.61 267.80	0.73	3.50	0.51
	CBT*	clean BCTV	0.56 0.24	0.29 0.06	5.00	/	0.49
	CBLA.CBCA	clean BCTV	2230.66 2869.15	218.23 789.93	0.78	3.46	0.49
	CBDA	clean BCTV	52732.40 74034.66	6227.65 21496.71	0.95	3.50	0.40

*Non-parametric, Wilcoxon test ran
** For non-parametric, Wilcoxon test statistic “W” reported

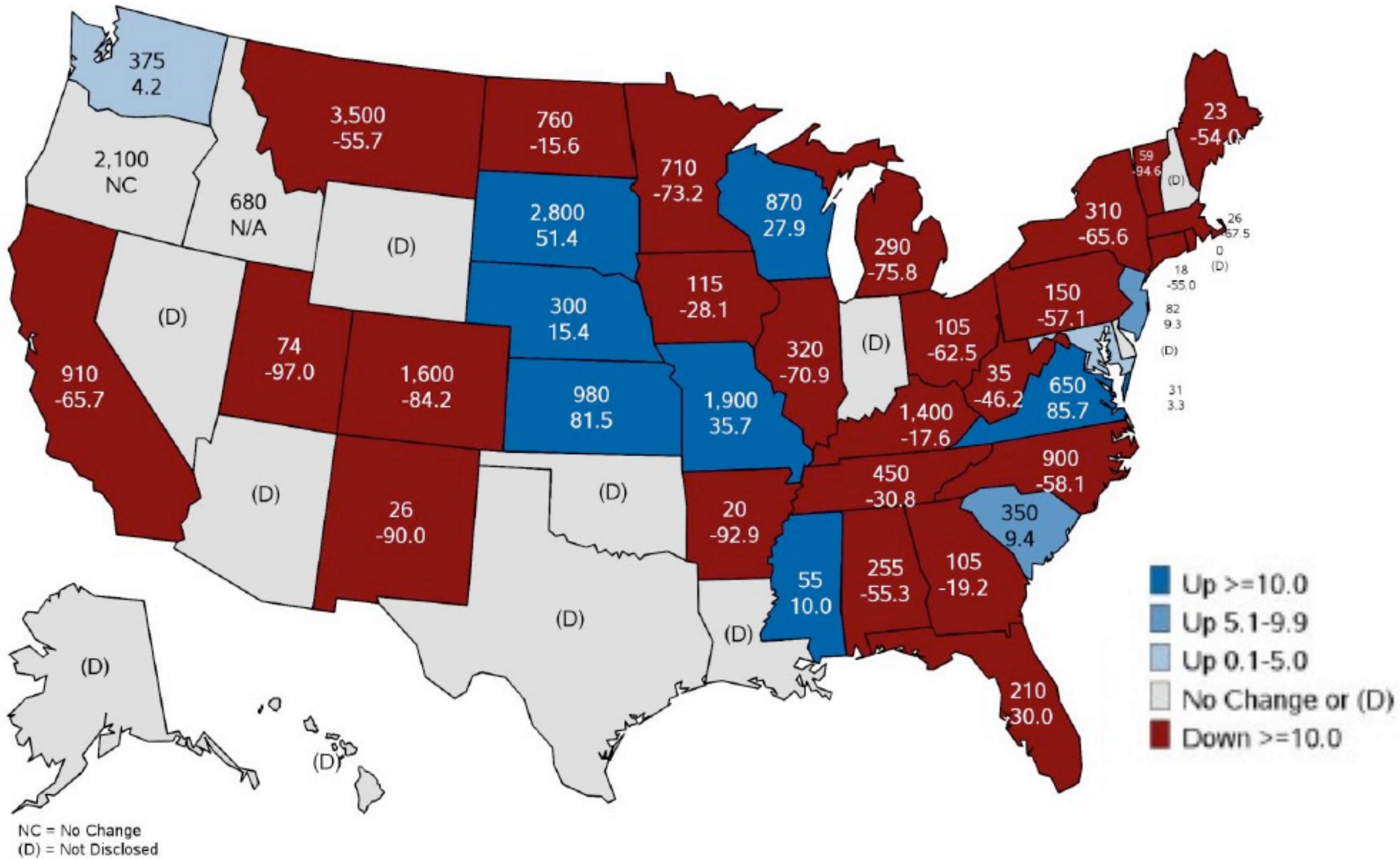


Figure 1: USDA-NASS 2022 hemp report showing current hemp acreage planted and changes in acreage planted from 2021 (USDA, NASS, 2023)

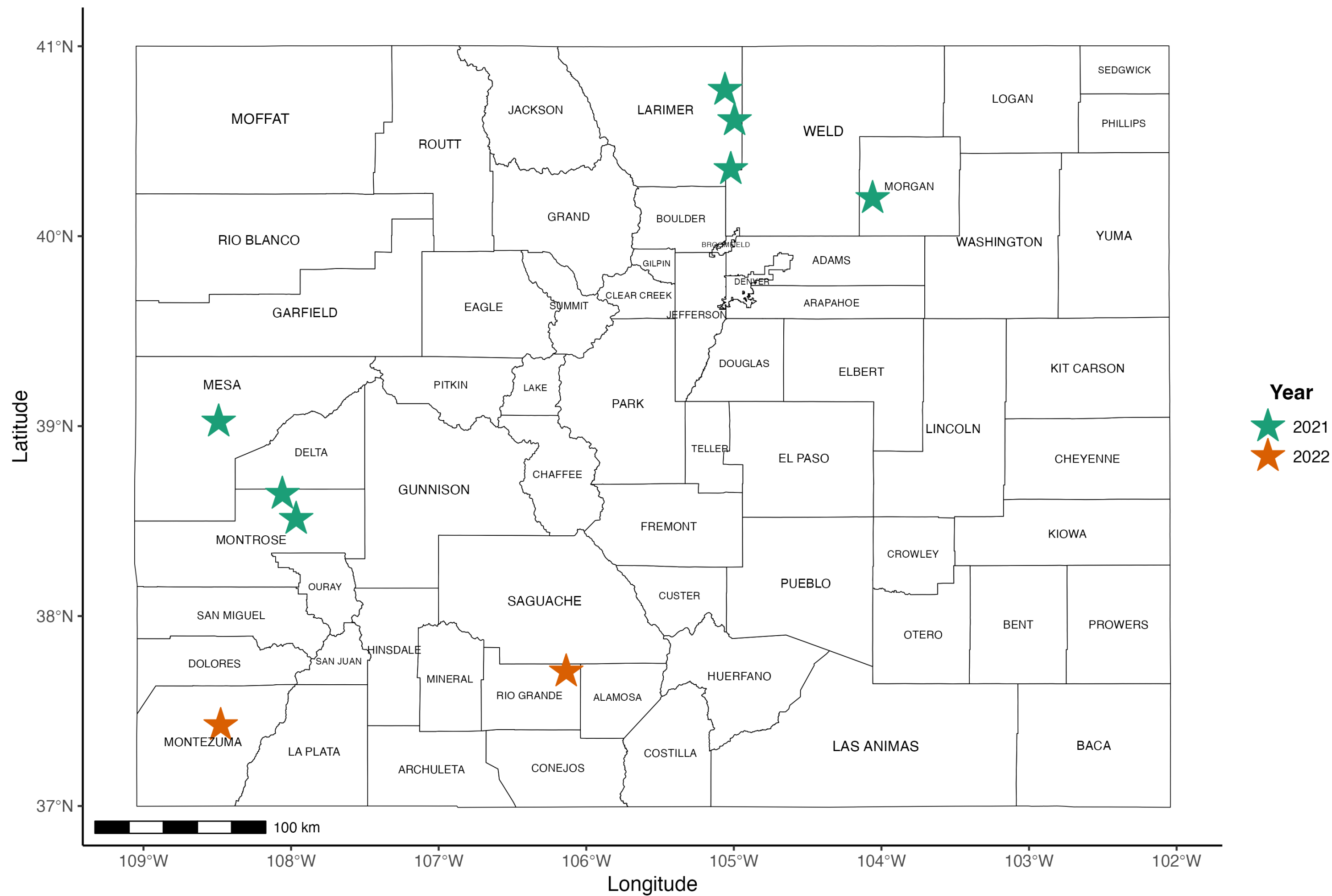


Figure 2: Locations of hemp fields sampled in 2021 and 2022.

Figure 3: Various symptoms observed in 2022 growing season. (A) stunted and yellowing new growth observed in advanced vegetative stage in Montezuma county, **(B)** curled leaves observed in the advanced vegetative stage in Rio Grande county, **(C)** stunted and yellowing curled growth observed in mature flowering stage in Montezuma county, **(D)** malformed growth observed in mature flowering stage in Rio Grande county.

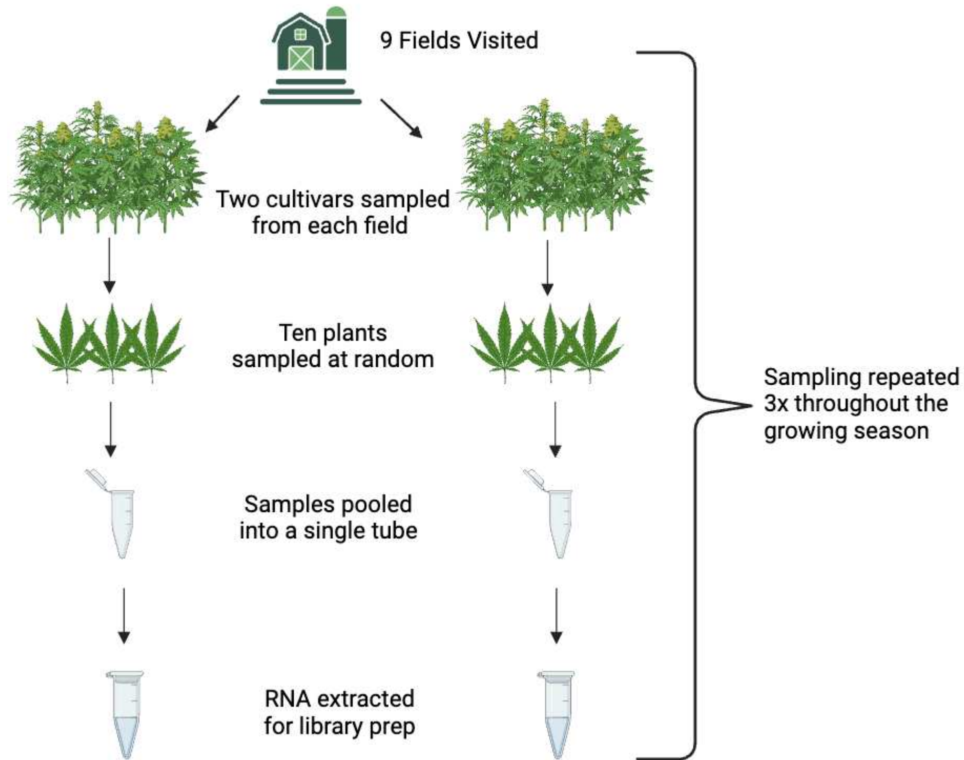


Figure 4: Virome sampling and pooling methodology. In total 9 fields were visited, 7 in 2021 and 2 in 2022. From each field 2 cultivars were sampled, taking tissue samples from the new growth of 10 different plants within each cultivar. Tissue from these 10 plants were then pooled into a single tube and RNA was extracted. This sampling occurred 3 times throughout the growing season to represent different stages of plant phenology. This sampling and pooling methodology resulted in a total of 48 pools. Of those pools, 36 were from 2021, and 12 were from 2022.

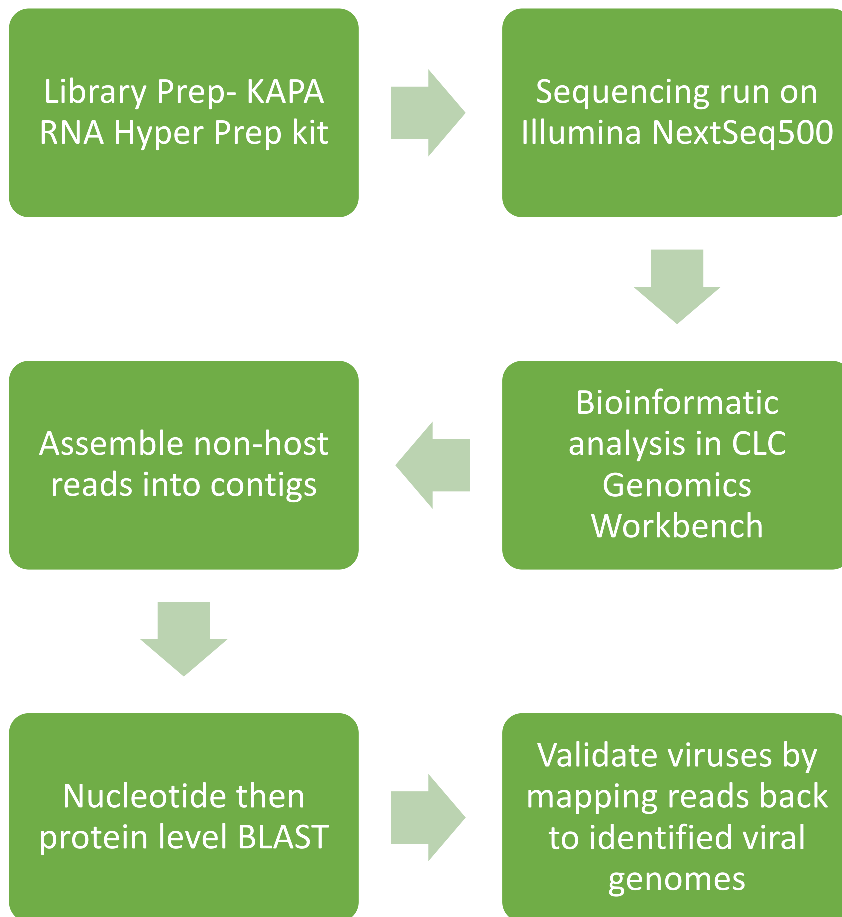


Figure 5: Workflow for virome analysis. Library preparation and bioinformatic analysis pipeline performed on samples.

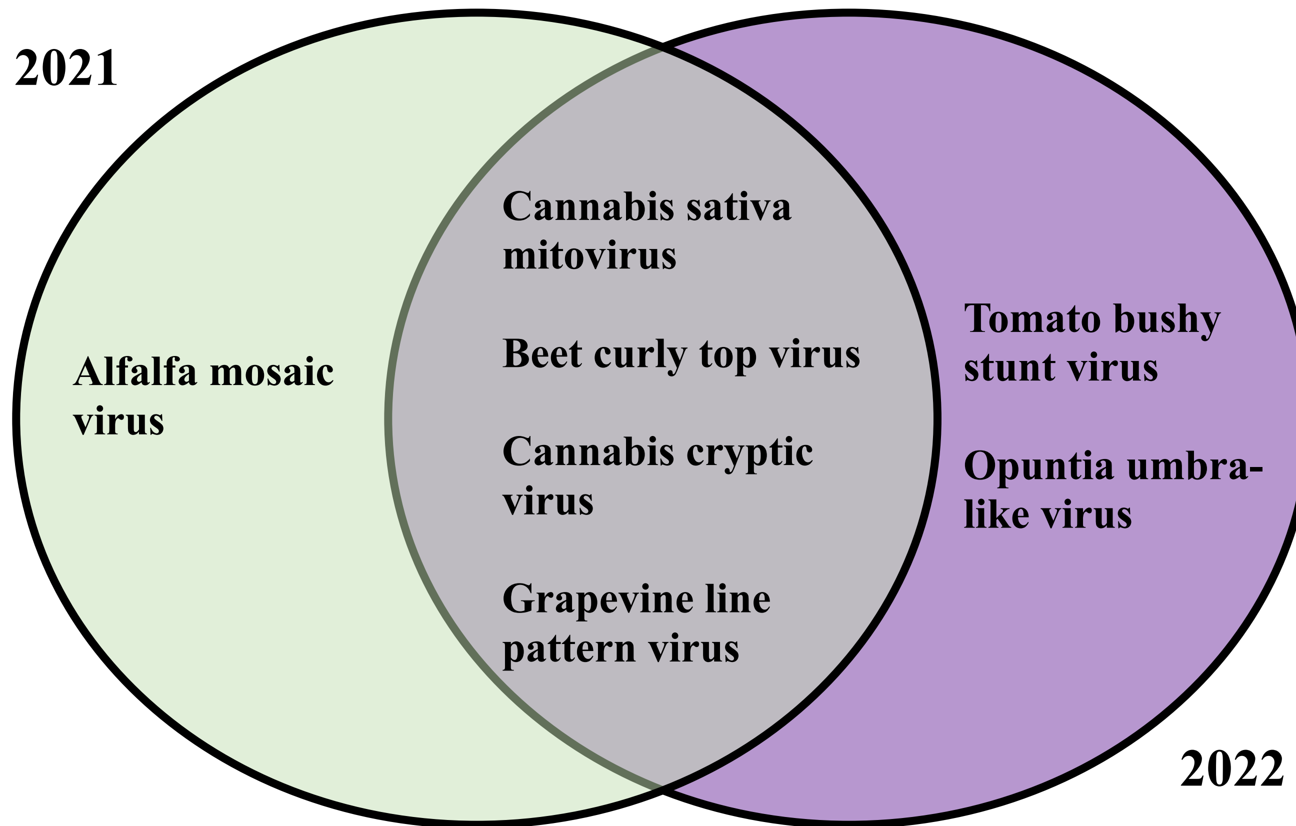


Figure 6: Viruses found in hemp virome for each year. Venn diagram depicting viral species detected through high throughput sequencing in the hemp virome from 2021 and 2022.

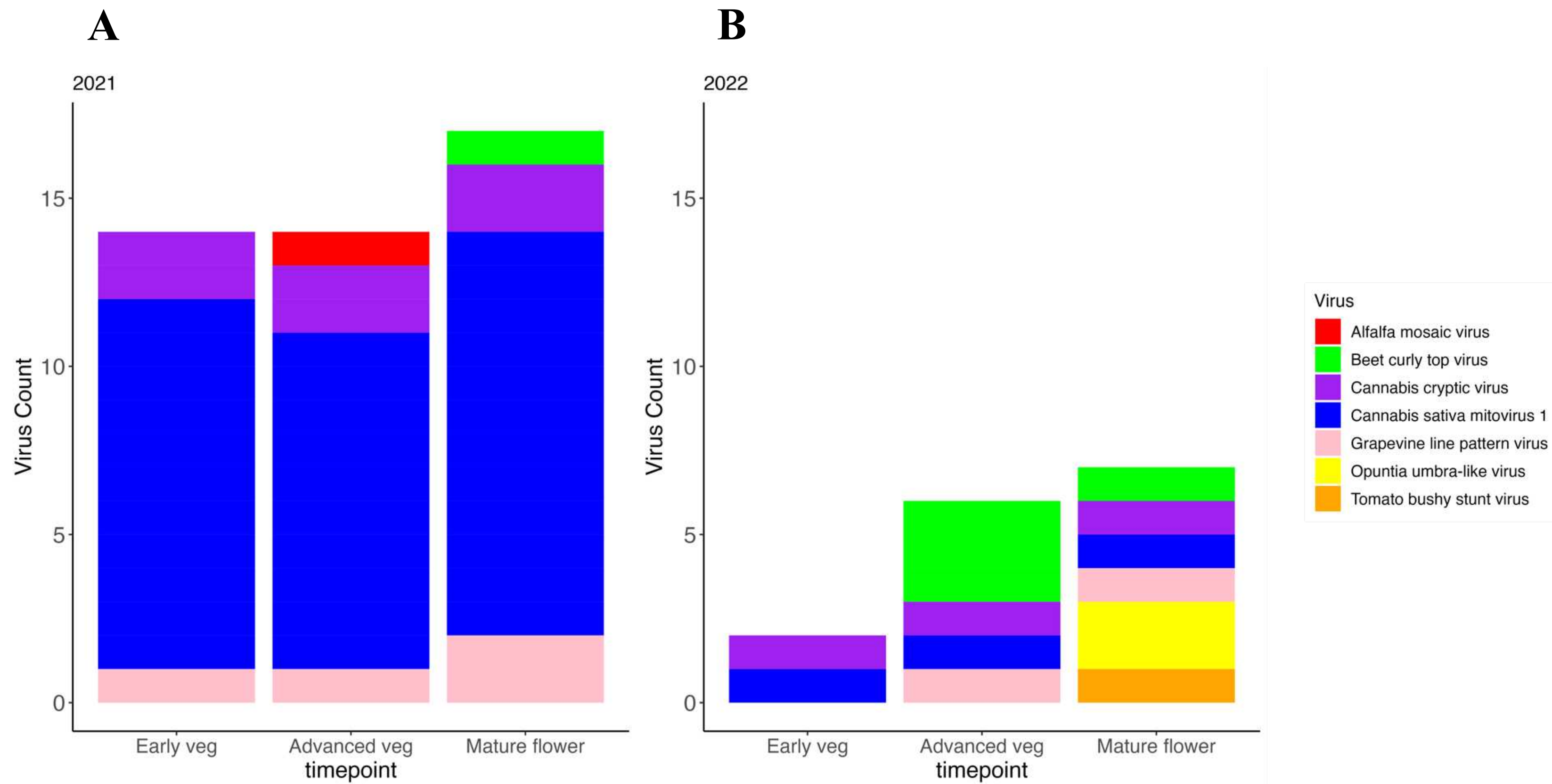


Figure 7: Viral diversity and distribution by phenological timepoint. Split bar graphs illustrating the diversity and prevalence of viruses at each timepoint for both years. **(A)** Virus count and diversity observed in 2021 at three distinct phenological stages, sample size n = 7 fields **(B)** Virus count and diversity observed in 2022 at three distinct phenological stages, sample size n = 2 fields.

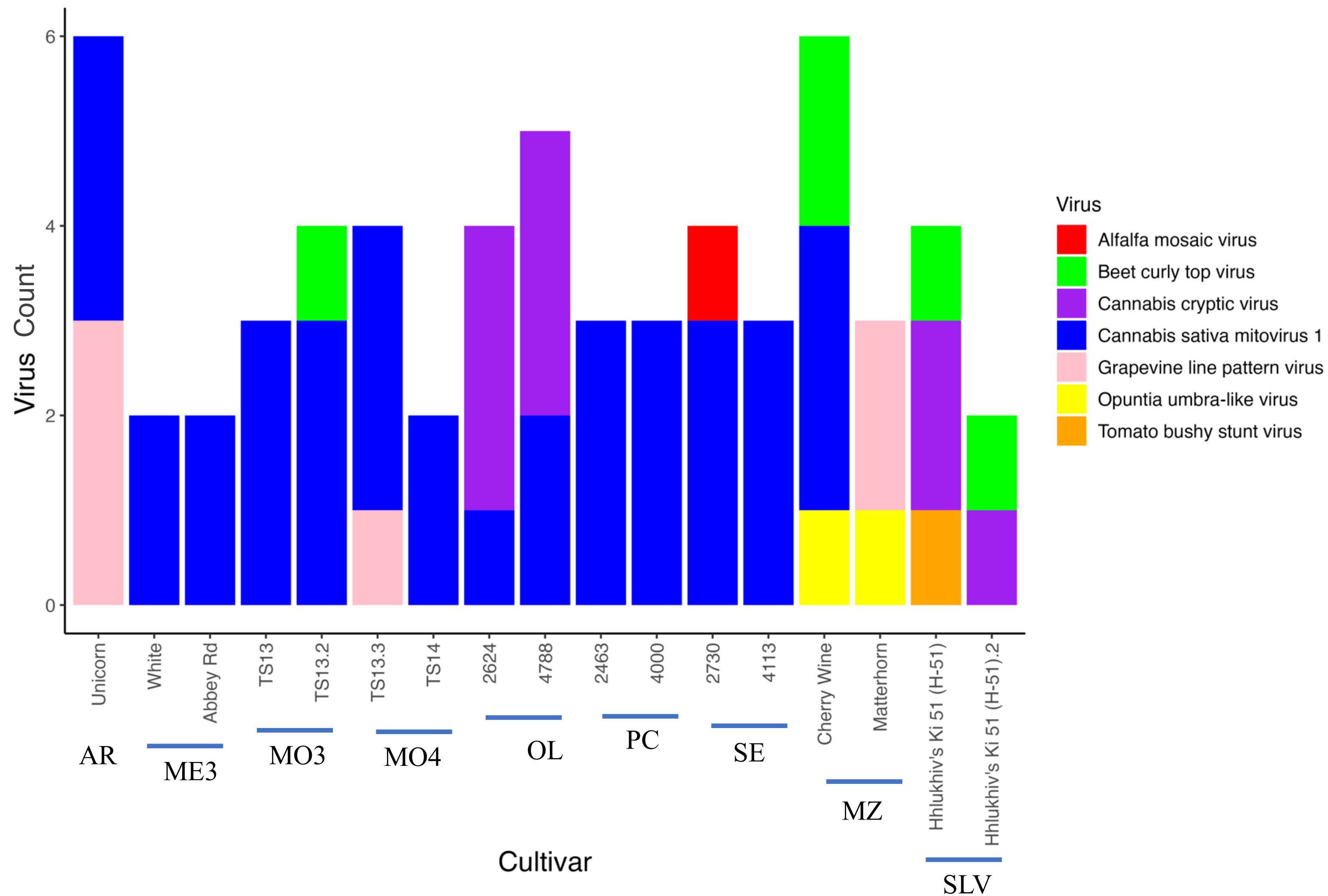


Figure 8: Individual viromes of hemp cultivars. Virus count separated by individual cultivars sampled from throughout this survey. Blue bars connect cultivars that belong to the same field to highlight viral diversity between cultivars in the same location.

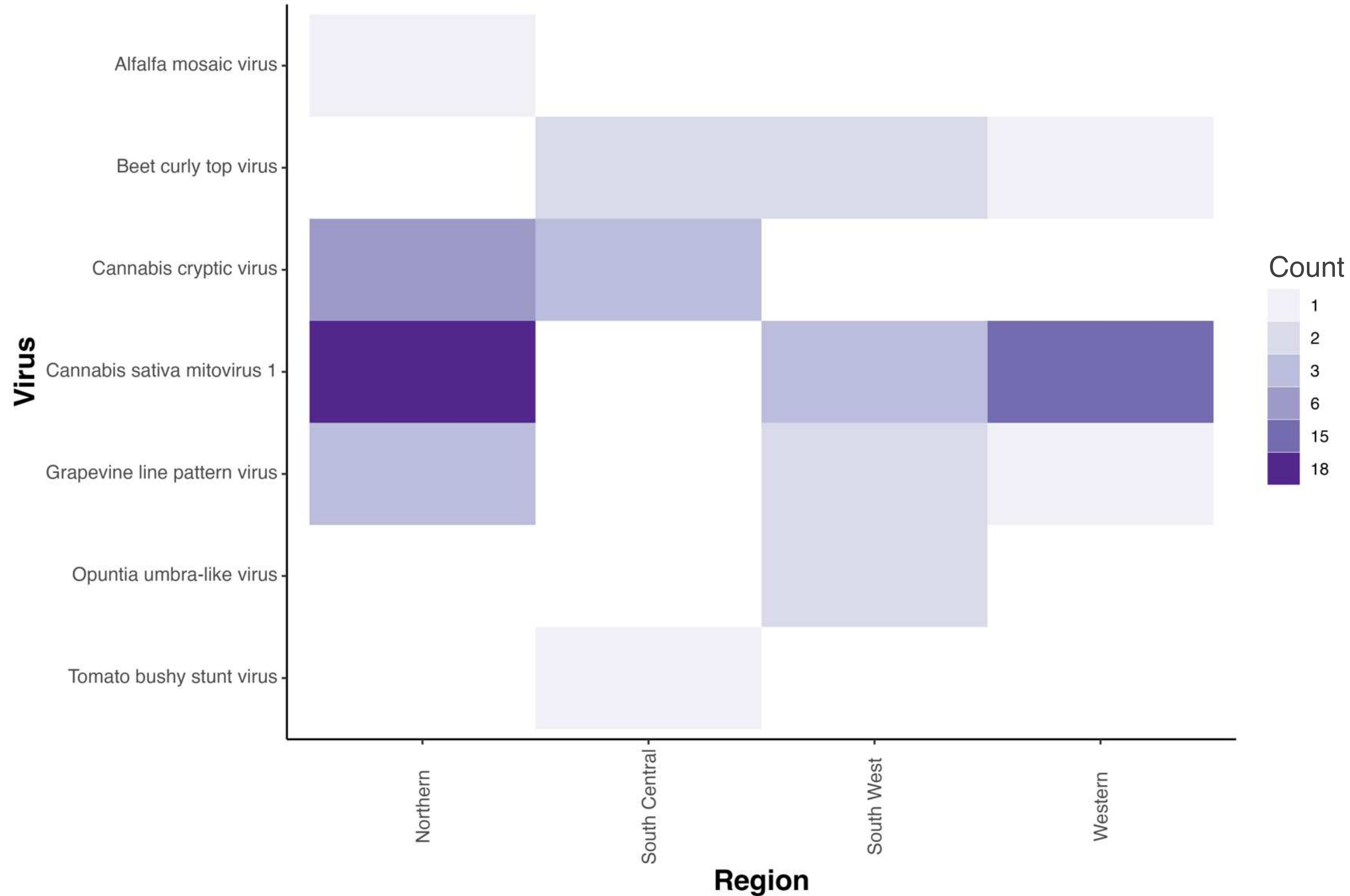


Figure 9: Viral count by region. Heatmap illustrating total virus count from each region of Colorado that was sampled throughout the growing season in both 2021 and 2022. Heatmap was generated by counting each time a virus was observed in each region. The darker the purple, the more virus was identified in the given region.

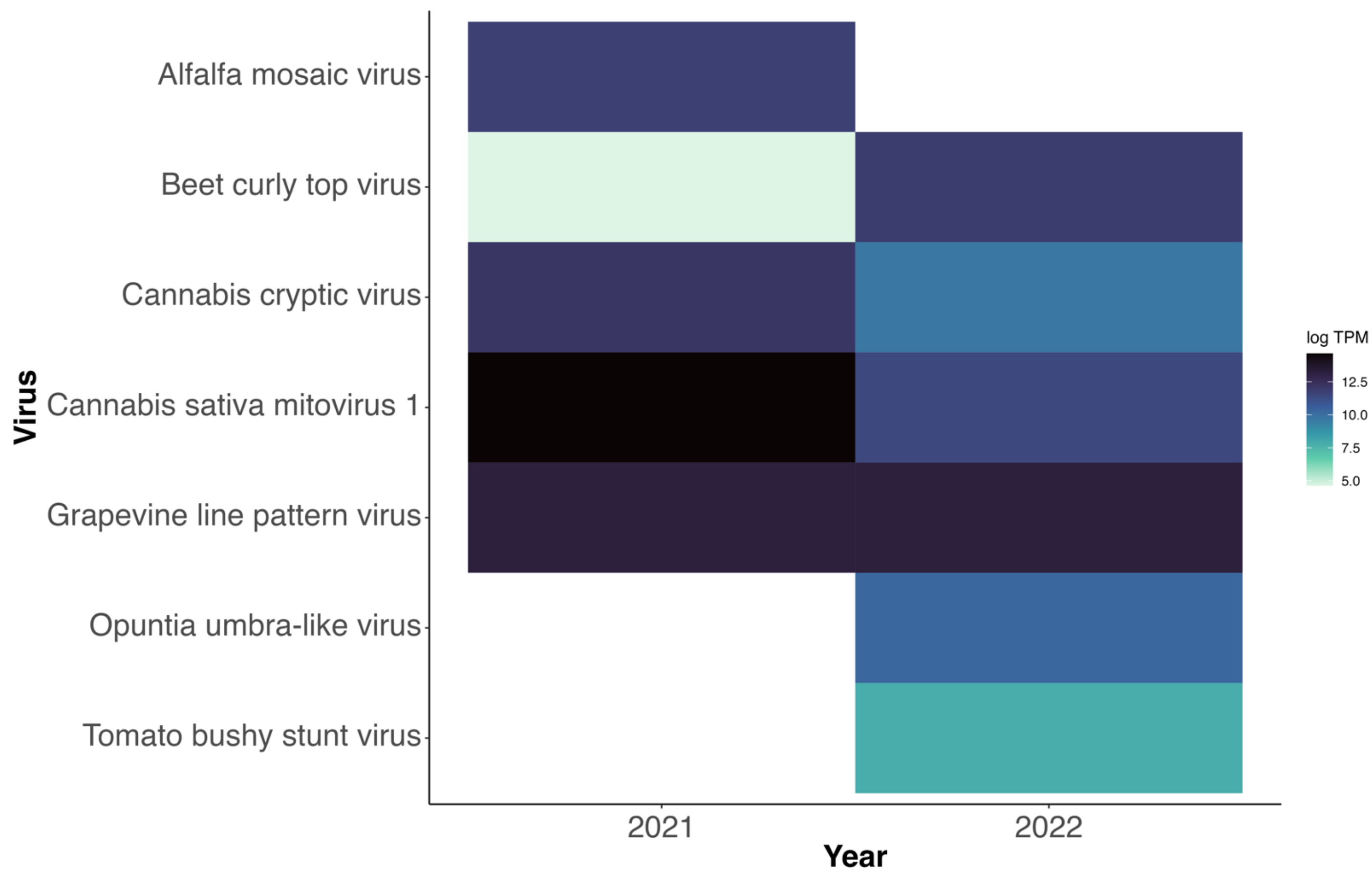


Figure 10: Viral load in hemp leaf samples collected in 2021 and 2022. Viral load is displayed as log transformed transcript per million (TPM) data from viral reads for each year.

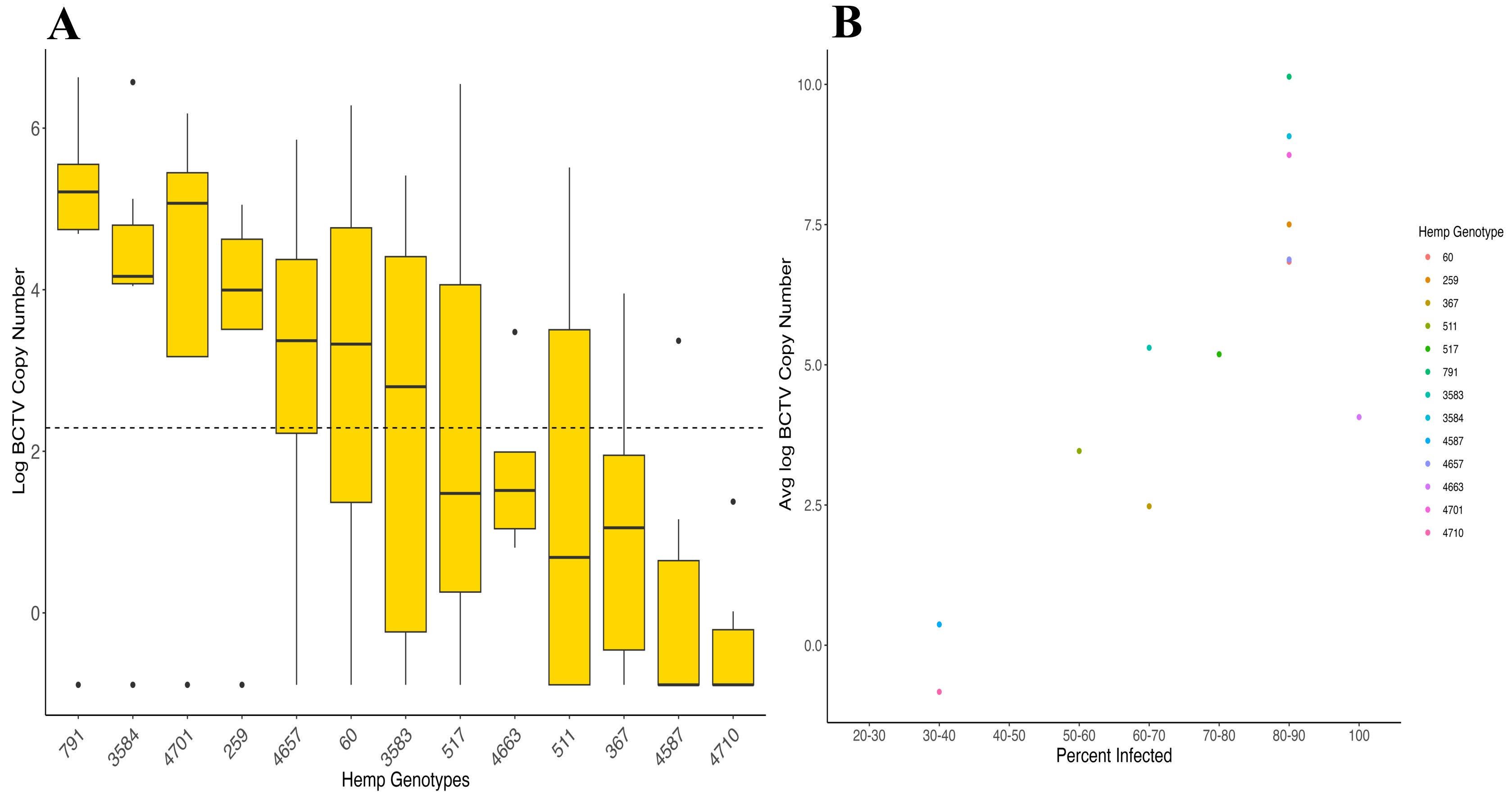


Figure 11: Screening for BCTV resistance in diverse panel of hemp genotypes. (A) Average log BCTV copy number shown for each genotype, n = 5-7 per genotype. One-way ANOVA revealed variation in means ($P = 0.02$). The bars across the boxes represent the mean, top and bottom of the boxes represent the lower and upper quartiles, ends of the "whiskers" represent the highest and lowest data points observed, black dots represent outliers (**B)** Percent disease index of hemp genotypes by average log BCTV copy number, hemp genotypes were grouped by their percent disease index as shown on the x axis. Statistical analysis revealed a correlation between percent of plants infected and the average log BCTV copy number ($P = 0.002$).

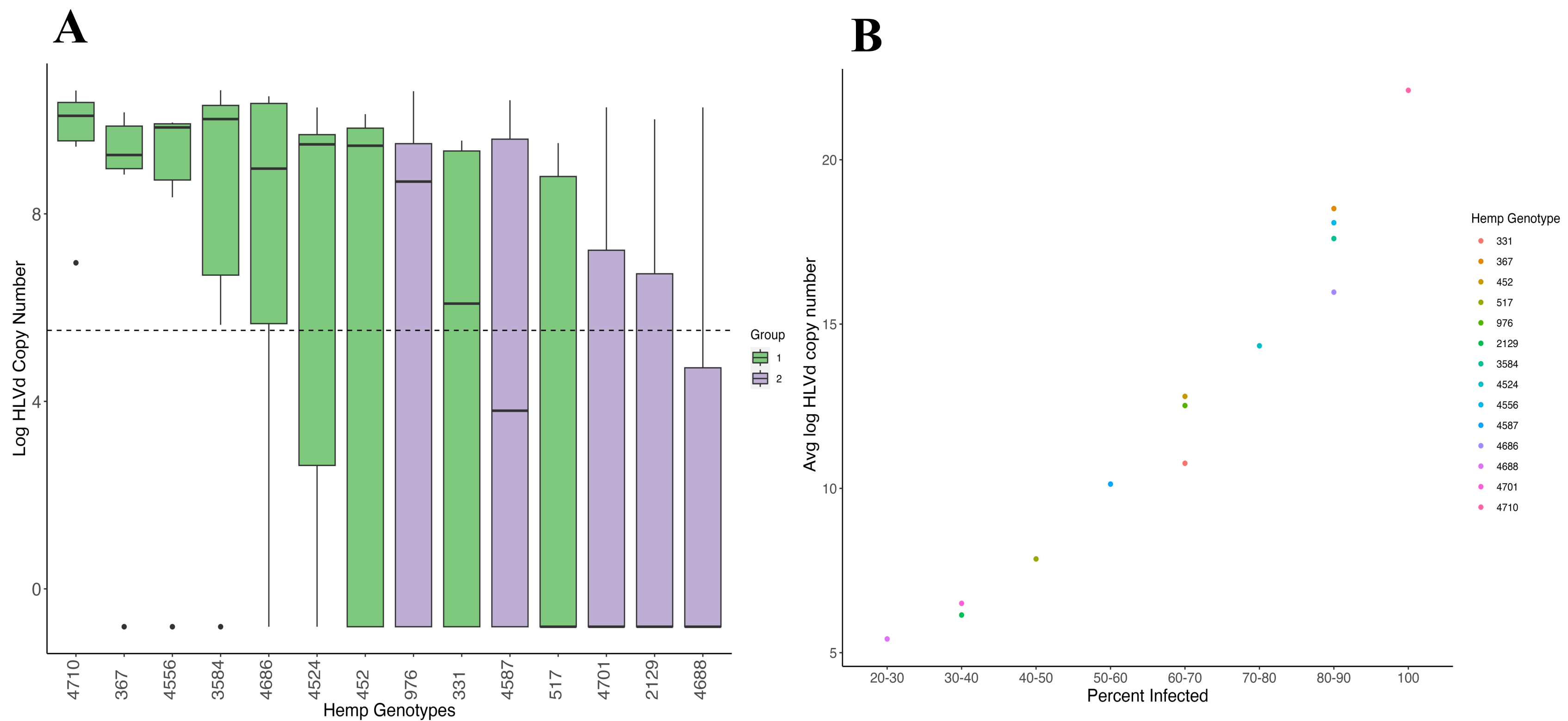


Figure 12: Screening for HLVd resistance in diverse panel of hemp genotypes. (A) Average log HLVd copy number shown for each genotype, n = 5-7 per genotype. Kruskal-Wallis rank sum test revealed no statistically significant variation in means (Group 1 P = 0.15, Group 2 P = 0.88). The bars across the boxes represent the mean, top and bottom of the boxes represent the lower and upper quartiles, ends of the "whiskers" represent the highest and lowest data points observed, black dots represent outliers **(B)** Percent disease index of hemp genotypes by average log HLVd copy number, hemp genotypes were grouped by their percent disease index as shown on the x axis. Statistical analysis revealed a correlation using Spearman's rank correlation rho between percent of plants infected and the average log HLVd copy number (Group 1 P = 1.75 e-07, Group 2 P = 0.004).

REFERENCES

Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M., & Boonham, N. 2009. Next-generation sequencing and metagenomic analysis: A universal diagnostic tool in plant virology. Molecular Plant Pathology, 10(4), 537–545.

Agricultural Act of 2014

Agricultural Improvement Act of 2018

Al Rwahnih, M., Daubert, S., Golino, D., & Rowhani, A. 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology, 387(2), 395–401.

Albrecht, T., White, S., Layton, M., Stenglein, M., Haley, S., & Nachappa, P. 2022. Occurrence of Wheat Curl Mite and Mite-Vectored Viruses of Wheat in Colorado and Insights into the Wheat Virome. Plant Disease, 106(10), 2678–2688.

Baker, B. P., Green, T. A., & Loker, A. J. 2020. Biological control and integrated pest management in organic and conventional systems. Biological Control, 140, 104095.

Barbara, D. J., Morton, A., Adams, A. N., & P.green, C. 1990. Some effects of hop latent viroid on two cultivars of hop (Humulus lupulus) in the UK. Annals of Applied Biology, 117(2), 359–366.

Beckerman, J., Stone, J., Ruhl, G., & Creswell, T. 2018. First Report of Pythium ultimum Crown and Root Rot of Industrial Hemp in the United States. Plant Disease, 102(10), 2045–2045.

Bektaş, A., Hardwick, K. M., Waterman, K., & Kristof, J. 2019. Occurrence of Hop Latent Viroid in Cannabis sativa with Symptoms of Cannabis Stunting Disease in California. Plant Disease, 103(10), 2699.

Burger, J. T., & Maree, H. J. 2015. Metagenomic Next-Generation Sequencing of Viruses Infecting Grapevines. In C. Lacomme (Ed.), Plant Pathology: Techniques and Protocols (pp. 315–330). Springer.

Campbell B. J., Berrada, A. F., Hudalla, C., Amaducci, S., McKay, J. K. 2019. Genotype x Environment Interactions of Industrial Hemp Cultivars Highlight Diverse Responses to Environmental Factors. Agrosystems, Geosciences & Environment 2:180057

Chen, L., and Gilbertson, R. L. 2016. Chapter 17: Transmission of Curtoviruses (*Beet curly top virus*) by the beet leafhopper (*Circulifer tenellus*). Pages 243-262 in: J. K. Brown (ed) Vector-Mediated Transmission of Plant Pathogens, The American Phytopathological Society.

Chiginsky, J., Langemeier, K., MacWilliams, J., Albrecht, T., Cranshaw, C., Fulladolsa, A. C., Kapuscinski, M., Stenglein, M., Nachappa, P. 2021. First Insights Into the Virus and Viroid Communities in Hemp (*Cannabis Sativa*). Frontiers in Agronomy 3:778432

Cooper, J. I. & Jones, A. T. 1983. Responses of Plants to Viruses: Proposals for the Use of Terms. Phytopathology, 73(2), 127.

Creamer, R. 2020. Beet curly top virus transmission, epidemiology, and management. In: Applied Plant Virology. Edited by Awasthi, L. P. Chapter 37 pp. 521-527. Academic Press.

Czotter, N., Molnar, J., Szabó, E., Demian, E., Kontra, L., Baksa, I., Szittya, G., Kocsis, L., Deak, T., Bisztray, G., Tusnady, G. E., Burgyan, J., & Varallyay, E. 2018. NGS of Virus-Derived Small RNAs as a Diagnostic Method Used to Determine Viromes of Hungarian Vineyards. Frontiers in Microbiology, 9.

De Jonghe, K., Van Bogaert, N., Vandierendonck, S., Smagghe, G., & Maes, M. 2016. First Report of Hop latent viroid in Belgian Hops. Plant Disease, 100(9), 1956–1956.

Dias, N. P., Hu, R., Hensley, D. D., Hansen, Z. R., Domier, L. L., & Hajimorad, M. R. 2022. A Survey for Viruses and Viroids of Peach in Tennessee Orchards by RNA Sequencing. Plant Health Progress, 23(3), 265–268.

Donaire, L., Wang, Y., Gonzalez-Ibeas, D., Mayer, K. F., Aranda, M. A., & Llave, C. 2009. Deep-sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes. Virology, 392(2), 203–214.

Eiras, M., de Oliveira, A. M., de Fátima Ramos, A., Harakava, R., & Daròs, J.-A. 2023. First report of citrus bark cracking viroid and hop latent viroid infecting hop in commercial yards in Brazil. Journal of Plant Pathology, 105(2), 603–603.

Eliash, N., Suenaga, M., & Mikheyev, A. S. 2022. Vector-virus interaction affects viral loads and co-occurrence. BMC Biology, 20(1), 284.

Elwan, E. A., Rabie, M., Aleem, E. E. A., Fattouh, F. A., Kagda, M. S., & Zaghloul, H. A. H. 2023. Exploring virus presence in field-collected potato leaf samples using RNA sequencing. Journal of Genetic Engineering and Biotechnology, 21(1), 106.

Fike, J. 2016. Industrial Hemp: Renewed Opportunities for an Ancient Crop. Critical Reviews in Plant Sciences, 35(5–6), 406–424.

Fike, J. H., Darby, H., Johnson, B. L., Smart, L., & Williams, D. W. 2020. Industrial Hemp in the USA: A Brief Synopsis. In G. Crini & E. Lichtfouse (Eds.), Sustainable Agriculture Reviews 42: Hemp Production and Applications (pp. 89–109). Springer International Publishing.

Gezovitch, O., Luria, N., Lachman, O., Sela, N., Smith, E., & Dombrovsky, A. 2024. Cucurbit chlorotic yellows virus, a crinivirus infecting Cannabis sativa plants. Plant Pathology, 73(1), 47–56.

Giladi, Y., Hadad, L., Luria, N., Cranshaw, W., Lachman, O., & Dombrovsky, A. 2020. First Report of Beet Curly Top Virus Infecting Cannabis sativa in Western Colorado. Plant Disease, 104(3)

Gorelick, J. and Bernstein, N. 2017. Chemical and Physical Elicitation for Enhanced Cannabinoid Production in Cannabis. In: Chandra, S., Lata, H., ElSohly, M. (eds) Cannabis sativa L. Botany and Biotechnology pp 439-456

Grunwald, D., Wijesinghege, C., Gordon, T., Stansell, Z., & Ellison, S. 2023. First Report of Tobacco Streak Virus in *Cannabis sativa* in New York. Plant Disease. 0 0:ja

Gu, Y.-H., Tao, X., Lai, X.-J., Wang, H.-Y., & Zhang, Y.-Z. 2014. Exploring the Polyadenylated RNA Virome of Sweet Potato through High-Throughput Sequencing. PLoS ONE, 9(6), e98884.

Hadad, L., Luria, N., Smith, E., Sela, N., Lachman, O., & Dombrovsky, A. 2019. Lettuce Chlorosis Virus Disease: A New Threat to Cannabis Production. Viruses, 11(9), Article 9.

Hilje, L., Costa, H. S., & Stansly, P. A. 2001. Cultural practices for managing Bemisia tabaci and associated viral diseases. Crop Protection, 20(9), 801–812.

Hillman, B. I., & Cai, G. 2013. Chapter Six - The Family Narnaviridae: Simplest of RNA Viruses. In S. A. Ghabrial (Ed.), Advances in Virus Research (Vol. 86, pp. 149–176). Academic Press.

Hily, J.-M., Candresse, T., Garcia, S., Vigne, E., Tannière, M., Komar, V., Barnabé, G., Alliaume, A., Gilg, S., Hommay, G., Beuve, M., Marais, A., & Lemaire, O. 2018. High-Throughput Sequencing and the Viromic Study of Grapevine Leaves: From the Detection of Grapevine-Infecting Viruses to the Description of a New Environmental Tymovirales Member. Frontiers in Microbiology, 9.

International Committee on Taxonomy of Viruses (ICTV) 2024: https://ictv.global/report_9th/RNApos/Umbravirus

Hu, J., Masson, R., and Dickey, L. 2021. First report of Beet curly top virus infecting industrial hemp (*Cannabis sativa*) in Arizona. Plant Disease. 105(4): 1233-1233.

Hull, R. 1969. Alfalfa mosaic virus. Advances in Virus Research. 15, 365-433

Jarugula, S., Wagstaff, C., Mitra, A., Crowder, D. W., Gang, D. R., & Naidu, R. A. (2023). First Reports of Beet Curly Top Virus, Citrus Yellow Vein-Associated Virus, and Hop Latent Viroid in Industrial Hemp (Cannabis sativa) in Washington State. Plant Disease, 107(9), PDIS-12-22-2981-PDN. https://doi.org/10.1094/PDIS-12-22-2981-PDN

Jo, Y., Choi, H., Kim, S.-M., Kim, S.-L., Lee, B. C., & Cho, W. K. 2017. The pepper virome: Natural co-infection of diverse viruses and their quasispecies. BMC Genomics, 18(1), 453.

Jo, Y., Choi, H., Lee, J. H., Moh, S. H., & Cho, W. K. 2022. Viromes of 15 Pepper (Capsicum annuum L.) Cultivars. International Journal of Molecular Sciences, 23(18), Article 18.

Jo, Y., Kim, S.-M., Choi, H., Yang, J. W., Lee, B. C., & Cho, W. K. 2020. Sweet potato viromes in eight different geographical regions in Korea and two different cultivars. Scientific Reports, 10(1), Article 1.

Jo, Y., Lian, S., Chu, H., Cho, J. K., Yoo, S.-H., Choi, H., Yoon, J.-Y., Choi, S.-K., Lee, B. C., & Cho, W. K. 2018. Peach RNA viromes in six different peach cultivars. Scientific Reports, 8(1), 1844.

Johansen, E., Edwards, M. C., & Hampton, R. O. 1994. SEED TRANSMISSION OF VIRUSES: Current Perspectives. Annual Review of Phytopathology, 32(1), 363–386.

Johnson, P. 1999. Industrial hemp: A critical review of claimed potentials for Cannabis sativa. TAPPI J 82:113-123.

Johnson, R. 2018. Hemp as an agricultural commodity. CRS Report for Congressional Research Service, Washington DC.

Jones, R. A. C. 2004. Using epidemiological information to develop effective integrated virus disease management strategies. Virus Research, 100(1), 5–30.

Jones, R. A. C., & Pathipanawat, W. 1989. Seed-borne alfalfa mosaic virus infecting annual medics (Medicago spp.) in Western Australia. Annals of Applied Biology, 115(2), 263–277.

Kegler, H. & Spaar, D. 1997. Zur virusanfälligkeit von hanfsorten (Cannabis sativa L.). Archives of Phytopathology & Plant Protection, 30:5, 457-464.

Kessler, A. and Kalske, A. 2018. Plant Secondary Metabolite Diversity and Species Interactions Annual Review of Ecology, Evolution, and Systematics 49:115-38.

Kostanda, E., and Khatib, S. 2022. Biotic stress caused by *Tetranychus urticae* mites elevates the quantity of secondary metabolites, cannabinoids and terpenes, in *Cannabis sativa* L. Ind. Crops Prod. 176, 114331.

Kreuze, J. F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., & Simon, R. 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. Virology, 388(1), 1–7.

Lai, X., Wang, H., Wu, C., Zheng, W., Leng, J., Zhang, Y., & Yan, L. 2022. Comparison of Potato Viromes Between Introduced and Indigenous Varieties. Frontiers in Microbiology, 13.

Lee, H.-J., Kim, S.-M., & Jeong, R.-D. 2023. Analysis of Wheat Virome in Korea Using Illumina and Oxford Nanopore Sequencing Platforms. Plants, 12(12), Article 12.

Lehoczky, J., D. Boscia, G.P. Martelli, J. Burgyan, M.A. Castellano, L. Beczner, and G. Farkas. 1987. Occurrence of line pattern a hitherto unknown virus disease of grapevine in Hungary. Kertgazdasag 19:61-79.

Lehoczky, J., D. Boscia, J. Burgyan, M.A. Castellano, L. Beczner, and G. Farkas. 1989. Line pattern, a novel virus disease of grapevine in Hungary. In Proceedings 9th meeting of ICVG, Kiryat Anavim, Israel, 23–30.

Lehoczky, J., G.P. Martelli, and J. Lazar. 1992. Seed transmission of grapevine line pattern virus. Phytopathologia Mediterranea 31: 115–116.

Liu, S., Li, S., Zhu, J., Xiang, B., Cao, L. 2008. First report of Hop latent viroid (HLVd) in China. Plant Pathology 57, 400.

Lopez-Jimenez, J., Herrera, J., & Alzate, J. F. 2023. Expanding the knowledge frontier of mitoviruses in Cannabis sativa. Infection, Genetics and Evolution, 116, 105523.

Luis-Arteaga, M., Rodríguez-Cerezo, E., Fraile, A., Sáez, E., & García-Arenal, F. 1996. Different tomato bushy stunt virus strains that cause disease outbreaks in solanaceous crops in Spain. Phytopathology, 86(5), 535-542.

Luria, N., Smith, E., Sela, N., Koren, A., Lachman, O., & Dombrovsky, A. 2019. Insights Into a Watermelon Virome Contribute to Monitoring Distribution of Whitefly-Borne Viruses. Phytobiomes Journal, 3(1), 61–70.

Ma, Y., Marais, A., Lefebvre, M., Faure, C., & Candresse, T. 2020. Metagenomic analysis of virome cross-talk between cultivated Solanum lycopersicum and wild Solanum nigrum. Virology, 540, 38–44.

MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. 2010. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments.

Mark, T., Shepherd, J., Olson D., Snell W., Proper S., & Thornsbury S. 2020. Economic Viability of Industrial Hemp in the United States: A Review of State Pilot Programs, EIB-217, U.S. Department of Agriculture, Economic Research Service.

Martelli, G. P., Golino, D. A., & Katis, N. I. 2017. Other Grapevine Viruses of Lesser Economic Importance. In B. Meng, G. P. Martelli, D. A. Golino, & M. Fuchs (Eds.), Grapevine Viruses: Molecular Biology, Diagnostics and Management (pp. 365–371). Springer International Publishing.

Martelli, G.P., Gallitelli, D., Russo, M. 1988. Tombusviruses. In: Koenig, R. (eds) The Plant Viruses. The Viruses. Springer, Boston, MA.

Matoušek, J. and Patzak, J. 2000. A low transmissibility of hop latent viroid through a generative phase of *Humulus lupulus* L. Biologia Plantarum 43 (1): 145-148.

McLean, G. D., Burt, J. R., Thomas, D. W., & Sproul, A. N. 1982. The use of reflective mulch to reduce the incidence of watermelon mosaic virus in Western Australia. Crop Protection, 1(4), 491–496.

McPartland J.M., Clarke R.C., Watson D.P. 2000. Hemp diseases and pests: management and biological control: an advanced treatise. CABI, Oxford, UK

McPartland, J. M., Hegman, W., & Long, T. 2019. *Cannabis* in Asia: its center of origin and early cultivation, based on a synthesis of subfossil pollen and archaeobotanical studies. Vegetation History and Archaeobotany, 28(6), 691–702.

Melgarejo, T. A., Chen, L.-F., Rojas, M. R., Schilder, A., & Gilbertson, R. L. 2022. Curly Top Disease of Hemp (Cannabis sativa) in California Is Caused by Mild-Type Strains of Beet curly top virus Often in Mixed Infection. Plant Disease, 106(12), 3022–3026.

Miljanić, V., Jakše, J., Kunej, U., Rusjan, D., Škvarč, A., & Štajner, N. 2022. Virome Status of Preclonal Candidates of Grapevine Varieties (Vitis vinifera L.) From the Slovenian Wine-Growing Region Primorska as Determined by High-Throughput Sequencing. Frontiers in Microbiology, 13.

Miotti, N., Passera, A., Ratti, C., Dall'Ara, M., & Casati, P. 2023a. A Guide to Cannabis Virology: From the Virome Investigation to the Development of Viral Biotechnological Tools. Viruses, 15(7), Article 7.

Miotti, N., Sukhikh, N., Laboureau, N., Casati, P., & Pooggin, M. M. 2023b. Cannabis Virome Reconstruction and Antiviral RNAi Characterization through Small RNA Sequencing. Plants, 12(23), 3925.

National Hemp Report, NASS

Nibert, M. L., Vong, M., Fugate, K. K., & Debat, H. J. 2018. Evidence for contemporary plant mitoviruses. Virology, 518, 14–24.

Nuzzo, F., Moine, A., Nerva, L., Pagliarani, C., Perrone, I., Boccacci, P., Gribaudo, I., Chitarra, W., & Gambino, G. 2022. Grapevine virome and production of healthy plants by somatic embryogenesis. Microbial Biotechnology, 15(5), 1357–1373.

Orfanidou, C. G., Efthimiou, K., Katis, N. I., & Maliogka, V. I. 2022. Elucidating the sweet potato virome in Greece with the aid of high-throughput sequencing technology. Plant Pathology, 71(9), 1880–1891.

Park, S. H., Pauli, C. S., Gostin, E. L., Staples, S. K., Seifried, D., Kinney, C., Vanden Heuvel, B., D. 2022. Effects of short-term environmental stresses on the onset of cannabinoid production in young immature flowers of industrial hemp (*Cannabis sativa* L.). Journal of Cannabis Research 4:1

Perring, T. M., Gruenhagen, N. M., & Farrar, C. A. 1999. MANAGEMENT OF PLANT VIRAL DISEASES THROUGH CHEMICAL CONTROL OF INSECT VECTORS. Annual Review of Entomology, 44(1), 457–481.

Punja, Z. K. 2021. Emerging diseases of Cannabis sativa and sustainable management. Pest Management Science, 77(9), 3857–3870.

Punja, Z. K., Kahl, D., Reade, R., Xiang, Y., Munz, J., & Nachappa, P. 2023a. Challenges to Cannabis sativa Production from Pathogens and Microbes—The Role of Molecular Diagnostics and Bioinformatics. International Journal of Molecular Sciences, 25(1), 14.

Punja, Z., K., Wang, K., Lung, S., Buirs, L. 2023b. Symptomology, prevalence, and impact of *Hop latent viroid* on greenhouse-grown cannabis (*Cannabis sativa* L.) plants in Canada. Canadian Journal of Plant Pathology

Redila, C. D., Prakash, V., & Nouri, S. 2021. Metagenomics Analysis of the Wheat Virome Identifies Novel Plant and Fungal-Associated Viral Sequences. Viruses, 13(12), Article 12.

Righetti, L., Paris, R., Ratti, C., Calassanzio, M., Onofri, C., Calzolari, D., Menzel, W., Knierim, D., Magagnini, G., Pacifico, D., & Grassi, G. 2018. Not the one, but the only one: About Cannabis cryptic virus in plants showing ‘hemp streak’ disease symptoms. European Journal of Plant Pathology, 150(3), 575–588.

Rivarez, M. P. S., Pecman, A., Bačnik, K., Maksimović, O., Vučurović, A., Seljak, G., Mehle, N., Gutiérrez-Aguirre, I., Ravnikar, M., & Kutnjak, D. 2023. In-depth study of tomato

and weed viromes reveals undiscovered plant virus diversity in an agroecosystem. Microbiome, 11(1), 60.

Rivedal, H. M., Funke, C. N., & Frost, K. E. 2022. An Overview of Pathogens Associated with Biotic Stresses in Hemp Crops in Oregon, 2019 to 2020. Plant Disease, 106(5), 1334–1340.

Schappe, T., Ritchie, D. F., & Thiessen, L. D. 2020. First Report of Serratia marcescens Causing a Leaf Spot Disease on Industrial Hemp (Cannabis sativa). Plant Disease, 104(4), 1248.

Schmidt H. E. & Karl E. 1970. EinBeitrag zur Analyse der Virosen des Ranfes (Cannabis sativa L.) unter Beriicksichtigung der Ranfblattlaus (Phorodon cannabis Pass.) als Virusvcktor. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. 125(1), 16-22

Schoener, J. L., & Wang, S. 2023. Hemp Abnormal Growth Is Attributed to Mono-, Di-, or Tri-Infections of Spiroplasma citri, 'Candidatus Phytoplasma trifolii', and Beet Curly Top Virus. PhytoFrontiers, 3:780-784

Shahi, S., Eusebio-Cope, A., Kondo, H., Hillman, B. I., & Suzuki, N. 2019. Investigation of Host Range of and Host Defense against a Mitochondrially Replicating Mitovirus. Journal of Virology, 93(6), 10.1128/jvi.01503-18

Singh, K., Jarošová, J., Fousek, J., Chen, H., & Kundu, J. K. 2020. Virome identification in wheat in the Czech Republic using small RNA deep sequencing. Journal of Integrative Agriculture, 19(7), 1825–1833.

Singh, R., & Srivastava, A. 2020. Chapter 41—Prevention and control of viral diseases of crops. In L. P. Awasthi (Ed.), Applied Plant Virology (pp. 593–599). Academic Press.

Strausbaugh, C. A., Eujayl, I. A., and Wintermantel, W. M. 2017. Beet curly top virus strains associated with sugar beet in Idaho, Oregon, and a Western U.S. collection. Plant Disease 101:1373-1382

Swenson, K. G. 1956. Aphid transmission of a strain of Alfalfa mosaic virus. Phytopathology, 42(5)261-262

Szarka, D., Tymon, L., Amsden, B., Dixon, E., Judy, J., & Gauthier, N. 2019. First Report of Powdery Mildew Caused by Golovinomyces spadiceus on Industrial Hemp (Cannabis sativa) in Kentucky. Plant Disease, 103(7), 1773–1773. https://doi.org/10.1094/PDIS-01-19-0049-PDN

Tanney, C. A. S., Backer, R., Geitmann, A., Smith, D. L. 2021. Cannabis Glandular Trichomes: A Cellular Metabolite Factory. Frontiers in Plant Science 12:721986

Thomas, T., Gilbert, J., & Meyer, F. (2012). Metagenomics—A guide from sampling to data analysis. Microbial Informatics and Experimentation, 2(1), 3.

Turco, S., Golyaev, V., Seguin, J., Gilli, C., Farinelli, L., Boller, T., Schumpp, O., & Pooggin, M. M. 2018. Small RNA-Omics for Virome Reconstruction and Antiviral Defense Characterization in Mixed Infections of Cultivated Solanum Plants. Molecular Plant-Microbe Interactions, 31(7), 707–723.

U.S. Department of Agriculture. 2020. Hemp and Eligibility for USDA Programs. Farmers.Gov. https://www.farmers.gov/your-business/row-crops/hemp

United States Department of Agriculture (USDA), National Agricultural Statistics Service (NASS), Agricultural Statistics Board National Hemp Report. 2023. https://www.nass.usda.gov/Newsroom/Executive_Briefings/2023/04-19-2023.pdf

Varma, A. 1993. Integrated Management of Plant Viral Diseases. In Ciba Foundation Symposium 177—Crop Protection and Sustainable Agriculture (pp. 140–157). John Wiley & Sons, Ltd.

Vennapusa A. R., Somayanda, I. M., Doherty, C. J., Jagadish, S. V. K. 2020. A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber. Scientific Reports 10:16887

Villamor, D. E. V., Ho, T., Al Rwahnih, M., Martin, R. R., & Tzanetakis, I. E. 2019. High Throughput Sequencing For Plant Virus Detection and Discovery. Phytopathology, 109(5), 716–725.

Warren, J. G., Mercado, J., & Grace, D. 2019. Occurrence of Hop Latent Viroid Causing Disease in Cannabis sativa in California. Plant Disease, 103(10), 2699–2699.

Whitfield, A. E., Falk, B. W., & Rotenberg, D. 2015. Insect vector-mediated transmission of plant viruses. Virology, 479–480, 278–289.

Wu, X., Valli, A., García, J. A., Zhou, X., & Cheng, X. 2019. The Tug-of-War between Plants and Viruses: Great Progress and Many Remaining Questions. Viruses, 11(3), Article 3.

Xu, C., Sun, X., Taylor, A., Jiao, C., Xu, Y., Cai, X., Wang, X., Ge, C., Pan, G., Wang, Q., Fei, Z., & Wang, Q. 2017. Diversity, Distribution, and Evolution of Tomato Viruses in China Uncovered by Small RNA Sequencing. Journal of Virology, 91(11), 10.1128/jvi.00173-17.

Yamaura, Y. & Scholthof, H. B. 2005. Tomato bushy stunt virus: a resilient model system to study virus-plant interactions. Molecular Plant Pathology 6(5), 491-502.

Yao, Q., Peng, Z., Tong, H., Yang, F., Xing, G., Wang, L., Zheng, J., Zhang, Y., & Su, Q. 2019. Tomato Plant Flavonoids Increase Whitefly Resistance and Reduce Spread of Tomato yellow leaf curl virus. Journal of Economic Entomology, toz199.

Ziegler, A., Matoušek, J., Steger, G., & Schubert, J. 2012. Complete sequence of a cryptic virus from hemp (Cannabis sativa). Archives of Virology, 157(2), 383–385.

APPENDICIES

Supplemental Table 1: Summary statistics and quality control of high throughput sequencing data. Sample names begin with the field identifier followed by the sampling date.

Sample	Number of Sequences	Trimmed Sequences	Avg. length after trim	Phred Score >25	Filtered Host Reads	Non-host Reads
2021						
AR_1_6_16	4,461,962	4,242,964	130.74	4242964	4,216,900	26,064
AR_1_7_19	4,036,551	3,838,127	131.76	3838127	3,815,247	22,800
AR_1_8_26	4,017,758	3,834,958	132.64	3834958	3,790,091	44,867
ME3_2_6_30	4,405,719	4,178,664	129.93	4178664	4,129,470	49,194
ME3_2_8_29	4,804,172	4,558,806	131.09	4558806	4,544,078	14,728
ME3_4_6_30	3,824,255	3,649,779	132.35	3649779	3,636,674	13,105
ME3_4_8_29	5,017,574	4,779,267	131.66	4779267	4,516,321	262,946
MO3_1_6_30	5,050,305	4,814,461	131.96	4814461	4,765,274	49,187
MO3_1_7_20	4,412,317	4,201,501	132.42	4201501	4,175,583	25,918
MO3_1_8_28	3,949,473	3,764,239	131.76	3764239	3,754,593	9,646
MO3_2_6_30	4,099,150	3,916,411	133.2	3916411	3,876,209	40,202
MO3_2_7_20	3,875,774	3,687,261	131.98	3687261	3,674,040	13,221
MO3_2_8_28	4,311,951	4,115,731	132.61	4115731	4,027,754	87,977
MO4_1_6_30	4,278,142	4,073,407	132.49	4073407	4,055,060	18,347
MO4_1_7_20	4,127,415	3,933,504	132.18	3933594	3,922,349	11,245
MO4_1_8_28	4,199,806	3,995,468	131.73	3995468	3,982,324	13,144
MO4_2_7_20	4,912,971	4,680,071	131.94	4680071	4,668,107	11,964
MO4_2_8_28	4,260,352	4,043,757	128.54	4043757	3,862,596	181,161
OL_1_6_28	4,520,266	4,304,434	131.53	4304434	4,291,218	13,216
OL_1_8_26	2,985,600	2,844,194	132.21	2844194	2,832,696	11,498
OL_1_8_4	4,418,950	4,188,167	130.64	4188167	4,158,600	29,567
OL_2_6_28	5,193,491	4,954,391	132.31	4954391	4,924,131	30,260
OL_2_8_26	3,152,437	3,005,108	132.27	3005108	2,986,261	18,847
OL_2_8_4	4,532,904	4,316,341	131.38	4316341	4,270,779	45,562
PC_1_6_28	4,747,092	4,514,601	131.31	4514601	4,495,009	19,592
PC_1_8_26	4,303,397	4,089,389	131.13	4089389	4,057,705	31,684
PC_1_8_4	4,634,639	4,404,116	131.42	4404116	4,388,138	15,978
PC_2_6_28	3,827,592	3,642,531	132.22	3642531	3,629,880	12,651
PC_2_8_26	3,521,075	3,334,521	129.84	3334521	3,314,485	20,036
PC_2_8_4	4,516,434	4,307,397	131.78	4307397	4,202,151	105,246
SE_1_6_16	3,998,584	3,793,560	130.94	3793560	3,783,148	10,412
SE_1_7_19	4,857,041	4,621,035	130.96	4621035	4,552,076	68,959

SE_1_8_26	4,457,852	4,236,264	130.81	4236264	4,180,868	55,396
SE_2_6_16	5,305,330	5,057,414	132.76	5057414	5,020,159	37,255
SE_2_7_19	4,103,392	3,906,963	131.27	3906963	3,865,494	41,469
SE_2_8_26	4,356,321	4,151,379	131.85	4307397	4,133,384	17,995
average	**4,318,835**	**4,110,561**	**131.6**	**4114897**	**4,069,413**	**41,148**
2022						
MZ_1_7_11	4,833,617	4,760,087	144.01	4760087	4,742,880	17,207
MZ_1_8_16	3,490,272	3,435,357	143.8	3435357	3,418,252	17,105
MZ_1_9_26	9,583,673	9,426,708	143.33	9426708	9,330,808	95,900
MZ_2_7_11	4,620,670	4,550,095	144.03	4550095	4,538,626	11,469
MZ_2_8_16	6,035,442	5,939,131	144	5939131	5,914,941	24,190
MZ_2_9_26	2,122,119	2,088,098	144.12	2088098	2,077,266	10,832
SLV_1_6_24	4,613,837	4,543,299	144.07	4543299	4,525,662	17,637
SLV_1_7_28	5,290,083	5,204,001	144.09	5204001	5,184,441	19,560
SLV_1_9_9	7,206,810	7,093,382	143.92	7093382	7,035,625	57,757
SLV_2_6_24	5,103,850	5,018,118	143.78	5018118	4,997,313	20,805
SLV_2_7_28	4,585,571	4,512,170	143.9	4512170	4,493,657	18,513
SLV_2_9_9	4,899,464	4,817,755	143.47	4817755	4,771,993	45,762
average	**5,198,784**	**5,115,683**	**143.877**	**5115683**	**5,085,955**	**29,728**

Supplemental Table 2: Percent disease index for hemp genotypes inoculated with BCTV. X^2 value and P-value obtained from Pearson's Chi-squared test shown. Bold values indicate statistical significance.

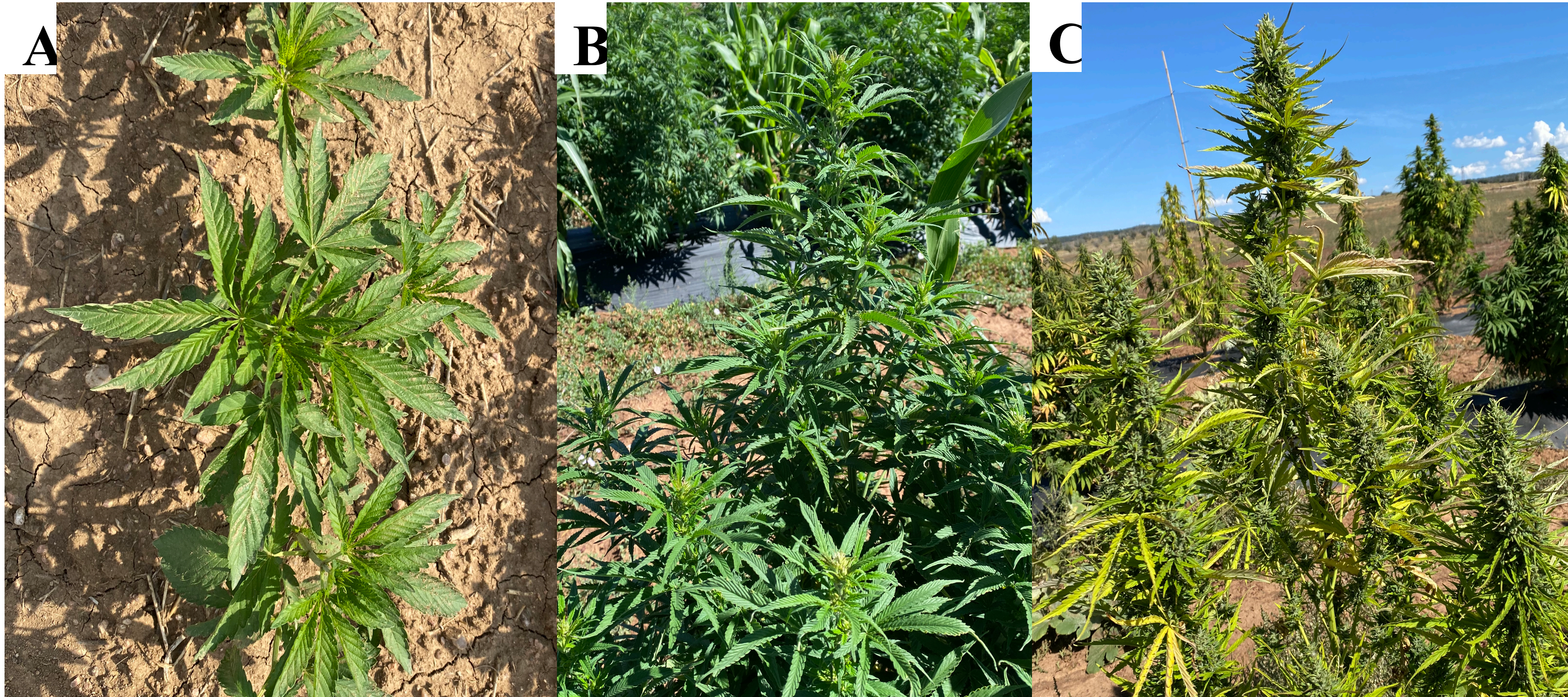
				Pearson's Chi-squared test	
Hemp Genotype	**Infected Plants**	**Uninfected Plants**	**Percent Disease Index**	**X^2**	***P* value**
60	4	1	80%	0.28667	0.5924
259	4	1	80%	0.28667	0.5924
367	4	2	66.66%	0.021812	0.8826
511	3	3	50%	1.1465	0.2843
517	5	2	71.42%	0.015941	0.8995
791	5	1	83.33%	0.60119	0.4381
3583	4	2	66.66%	0.021812	0.8826
3584	6	1	85.70%	0.97436	0.3236
4587	2	4	33.33%	3.9752	**0.04617**
4657	4	1	80%	0.28667	0.5924
4663	5	0	100%	2.3695	0.1237
4701	4	1	80%	0.28667	0.5924
4710	2	4	33.33%	3.9752	**0.04617**

Supplemental Table 3: Percent disease index for hemp genotypes inoculated with HLVd. X^2 value and P-value obtained from Pearson's Chi-squared test shown. Group 1 and Group 2 analyzed separately. Bold values indicate statistical significance.

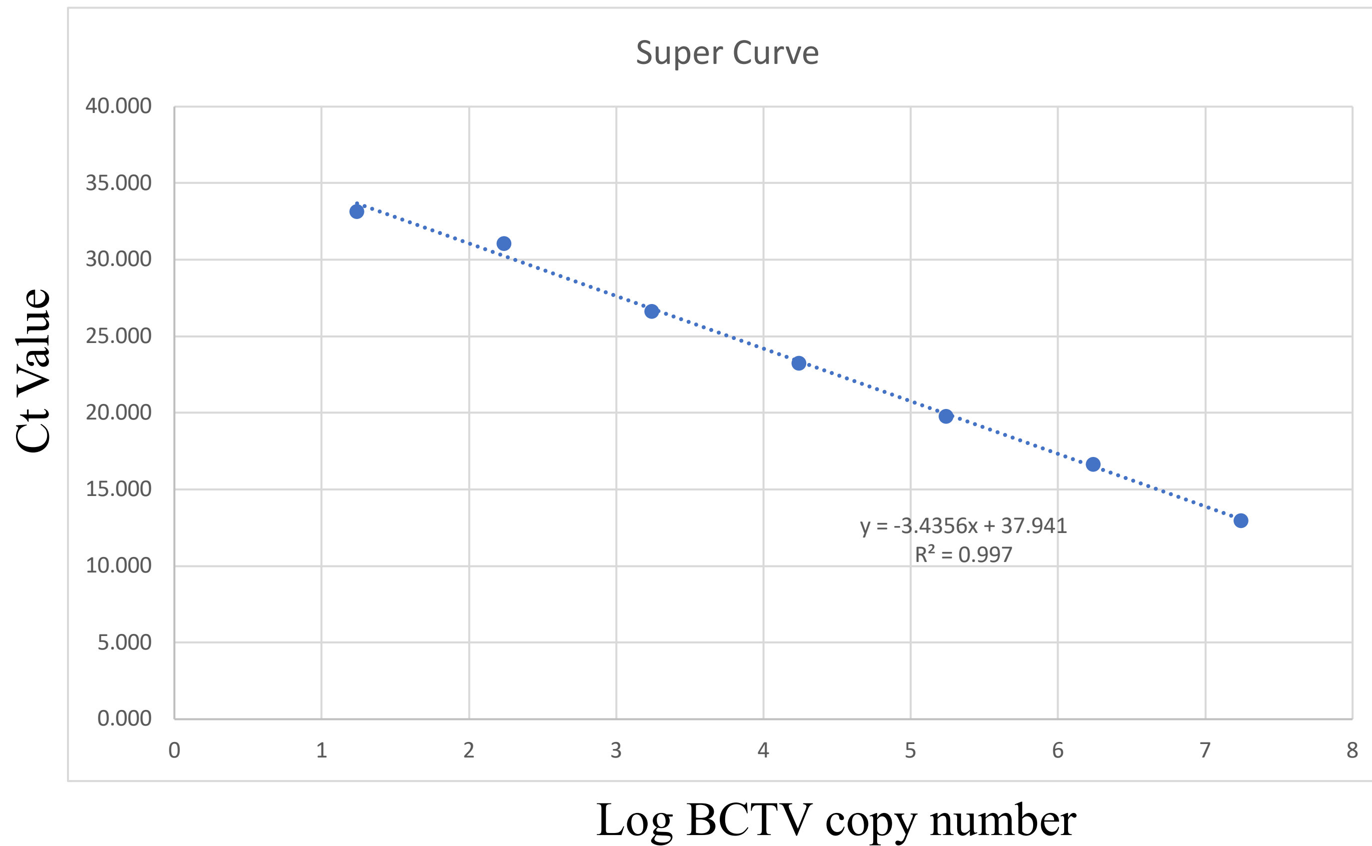
				Pearson's Chi-squared test		
Hemp Genotype	**Infected Plants**	**Uninfected Plants**	**Percent Disease Index**	**X^2**	**P value***	**Group**
331	3	2	60	0.56834	0.4509	1
452	3	2	60	0.56834	0.4509	1
4710	6	0	100	2.3625	0.1243	1
4556	5	1	83.33	0.30134	0.583	1
367	6	1	85.71	0.56743	0.413	1
517	3	4	42.86	4.0811	**0.04337**	1
3584	5	1	83.33	0.30134	0.583	1
4524	5	2	71.43	0.02931	0.8641	1
4686	4	1	80	0.10076	0.7509	1
976	3	2	60	1	0.3173	2
4688	2	5	28.57	0.49689	0.4809	2
4587	3	3	50	0.3125	0.5762	2
4701	2	4	33.33	0.13889	0.7094	2
2129	2	4	33.33	0.13889	0.7094	2

Supplemental Table 4: BLAST results for potential novel viruses identified in 2021 and 2022 virome.

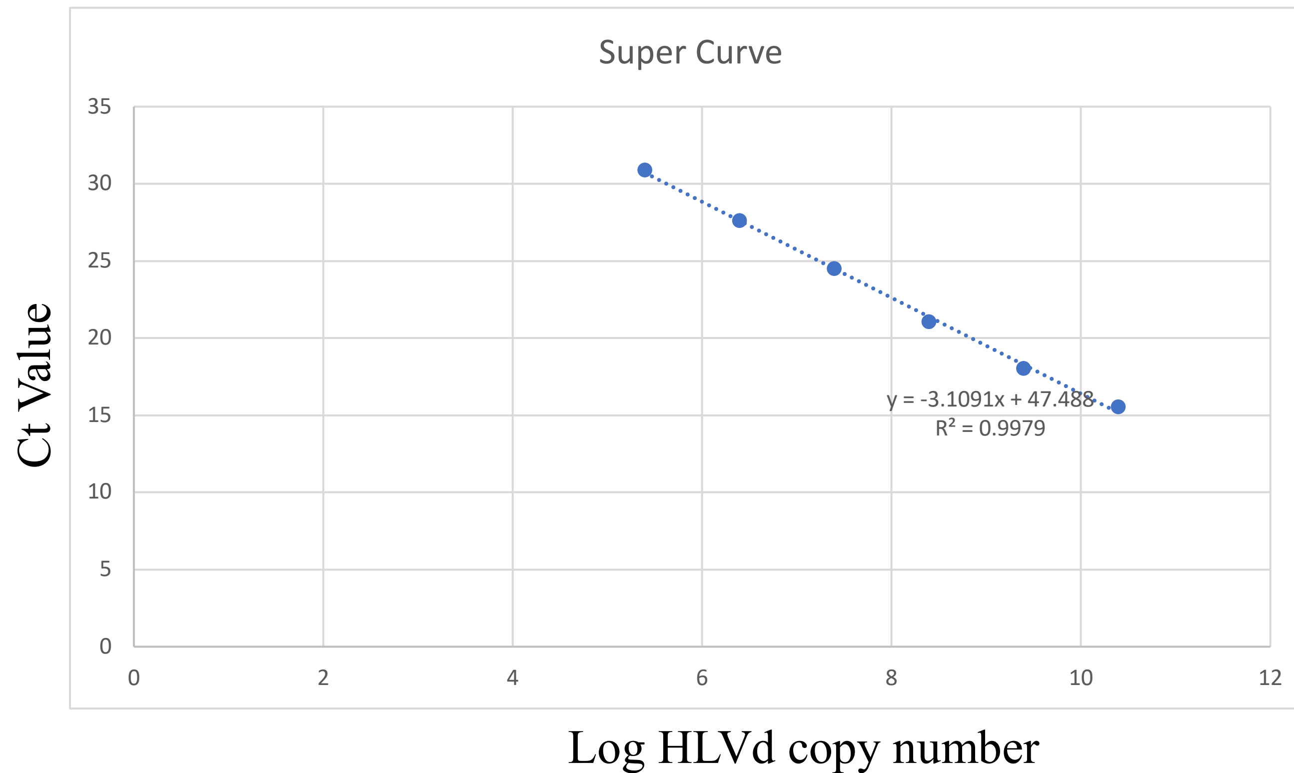
Virus	Nearest GenBank Sequences	%nt identity	% coverage	E-value
Maize-associated partiti like virus	MW063112	74.91	96.25	1.5834E-41
Potato virus T	WBG54358	50.44	98	1.7149E-23
Red clover vein mosaic virus	YP_002647021	60.32	54	2.1174E-17
Shallot virus X	UTU35106	54.02	95	2.7692E-25
Gymnadenia betaflexivirus 1	QQG34593	61.9	58	2.1683E-07
Olive virus T	UXN85468	46	94	6.69E-18
Phaseolus vulgaris alphaendornavirus 2	QDF44098	46.21	85	5.2905E-34
Pterostylis alphaendornavirus	ULN99160	68.35	88	7.8311E-28
Alphaendornavirus sp.	QII57747	44	71	4.6541E-13
Plant associated alphaendornavirus 1	UTQ50501	47.04	89	3.1117E-69
Triticeae associated alphaendornavirus	QPF20438	31.58	93	3.0643E-31
Hot pepper alphaendornavirus	BDU96795	46.46	98	6.6222E-25
Bell pepper alphaendornavirus	AYA43754	44.76	97	2.1714E-19



Supplemental Figure 1: Representative plant sizes at the various phenological timepoints of tissue sampling. (A) early vegetative **(B)** advanced vegetative **(C)** mature flowering.



Supplemental Figure 2: Standard curve utilized for BCTV copy number quantification. Standard curve was generated from the mean Ct values obtained from a ten fold dilution series of BCTV plasmid with copy numbers ranging from 17 to 1.74×10^7. Primer efficiency was determined to be 94.73%. Super curve was generated by averaging the standards from each plate ran. This standard curve was used to calculate the average number of BCTV coat protein copies in each genotype.



Supplemental Figure 3: Standard curve utilized for HLVd copy number quantification. Standard curve was generated from the mean Ct values obtained from a ten fold dilution series of a plasmid containing the HLVd genome with copy numbers ranging from 2.48×10^{10} to 2.48×10^{5}.Primer efficiency was determined to be 109.72%. Super curve was generated by averaging the standards from each plate ran. This standard curve was used to calculate the average number of HLVd genome copies in each genotype.