THESIS

BEET CURLY TOP VIRUS-BEET LEAFHOPPER DYNAMICS IN HEMP IN COLORADO

Submitted by

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ABSTRACT

BEET CURLY TOP VIRUS-BEET LEAFHOPPER DYNAMICS IN HEMP IN COLORADO

Hemp (*Cannabis sativa* L.) production within North America has dramatically increased in recent years following legislative changes in the 2014 and 2018 Farm Bills that allowed legal paths for its production. However, due to previous restrictions on this crop it has been understudied in the U.S. since its production declined, and ultimately was eliminated. Restrictive laws largely prevented any research regarding management of this crop. Among the understudied issues were those associated with disease identification and management.

One newly described disease of the crop in Colorado beet curly top virus (BCTV). This viral pathogen is transmitted by an insect vector, the beet leafhopper. To better understand the diversity and prevalence of BCTV strains infecting hemp in Colorado, beet curly top virus (BCTV) was detected at high incidence (81%) in leaf samples from 12 counties in 2019. Two different strains of BCTV, BCTV-Worland and BCTV-Colorado were found present in single or mixed infection in hemp leaf samples. Phylogenetic analysis revealed BCTV sequences from hemp formed a distinct group along with BCTV-Colorado and BCTV-Worland strains. To determine other potential viral and viroid pathogens in hemp, shotgun metagenomic analysis was performed. Virome analysis revealed the presence of seven viruses and one viroid. Of these, cannabis cryptic virus, cannabis sativa mitovirus, citrus yellow vein associated, opuntia-like virus and hop latent viroid sequences that had high sequence similarity with their corresponding sequences in GenBank. In contrast, tobacco streak virus sequence was highly variable compared to sequences in GenBank suggesting a new genotype of this virus. The data presented here has

important implications for the epidemiology and management of the various diseases of hemp and will lead to the development of integrated pest management strategies designed to interrupt transmission cycles and facilitate efficient crop production.

Beet leafhopper abundance was monitored throughout the hemp season to understand timing of emergence and flight patterns in the north and western regions of Colorado as well as identify timing of population peaks. Virus incidence in hemp and weed species were assessed using PCR analysis. Beet curly top virus was detected earlier in western field sites of Colorado before being detected in northern survey sites. Of the 41 different weed species surveyed, the weeds that most often tested positive for BCTV, contributing to transmission prevalence were *Lactuca serriola* (prickly lettuce), *Taraxacum officinale* (dandelion), and *Cichorium intybus* (chicory).

Life history assays were conducted using viruliferous and non-viruliferous beet leafhoppers in both sugar beet and hemp plants to understand if there were any fitness advantages or costs associated with being a carrier of the virus. Viruliferous beet leafhoppers reared on sugar beet produced more offspring than non-viruliferous treatments. There was no difference between viruliferous, and non-viruliferous beet leafhoppers reared on hemp, suggesting that these virus mediated differences in life history are induced in host plant interactions with the vector. However, beet leafhoppers were able to survive 7-day periods and successfully oviposit and develop on hemp.

Understanding migration timing and patterns will result in a more thorough understanding of the pest ecology of the beet leafhopper, which will lead to targeted control strategies to incorporate into integrated pest management tactics to interrupt BCTV transmission cycles, in turn improving yield and farming efficiency.

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I would like to start by respectfully recognizing the original stewards of the land, Arapaho, Cheyenne, and Ute Nations and peoples. When we think of indigenous lives and landscapes, we see romantic lush environments. This is because of the deep relationship these groups have had for hundreds of years, that goes beyond the financial motivation that leads land management practices today. I want to recognize the violence and suffering associated with these lands being taken from these resilient individuals. The ultimate goal of acknowledging the original stewards is action, and we should strive to return stolen lands to these people.

I cannot continue without acknowledging the guidance and inspiration I have received from my advisor, Dr. Punya Nachappa. If this experience were any other way, it would have been entirely different. I have loved exploring this system and the encouragement to indulge my curiosity of vector-virus interactions. I am sad to leave, but that is one of the best ways to end a chapter. Thank you for your time, patience, and collaboration.

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Lastly, I want to acknowledge my partner, Teague Embrey, for making my life that much richer.

DEDICATION

For Hillary. Keep taking up space.

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CHAPTER 1 – Introduction

Beet Curly Top Disease

Beet curly top disease has been of economic significance and a major threat to agriculture since the early 1900's, impacting major crops including common bean, spinach, peppers, sugar beet, squash, tomato and now hemp (Bennet, 1971; Chen and Gilberston 2016; Giladi et al. 2020). *Beet curly top virus* belongs to the genus *Curtovirus*, from the *Geminiviridae* family. Geminiviruses are single stranded DNA viruses that are highly divergent and utilize insect vectors including whiteflies (*Bemisia tabaci*) and leafhoppers to effect transmission (Briddon et al., 1998). There are three species of Curtoviruses, which include Beet curly top virus (BCTV), Spinach severe curly top virus (SpSCTV) and Horseradish curly top virus (HrCTV). Curtovisuses are defined by sharing a 77% genome-wide pairwise identity and strains are classified by a shared 94% identity threshold. Curly top disease impacts over 300 different plant species from over 44 different families (Bennett, 1971). Beet curly top virus species consists of 11 distinguished strains: BCTV-California/Logan (CA/Logan), BCTV-Colorado, BCTV-Worland (Wor), BCTV-Mild (Mld), BCTV-Severe (Srv), BCTV-Severe pepper (SvrPep), BCTV-pepper curly top (PeCT), BCTV-Pepper yellow dwarf (PeYD) and BCTV-Spinach curly top (SpCT), BCTV-Kim1, and BCTV-LH71 (Varsani et al., 2014; Strausbaugh and Eujayl, 2017). These viruses and strains differ in their DNA sequence and the symptoms they cause in certain hosts (Soto and Gilbertson, 2003). These BCTV strains contain a monopartite genome of 2.9-3.0 kb and all curtoviruses are exclusively transmitted by leafhoppers in the genus *Circulifer* (=*Neoaliturus*) (Chen and Gilbertson, 2016). Curtoviruses arose the recombination of a begomovirus and matresvirus (Stanley, 1986, Hormuzdi and Bisaro, 1993). New strains of BCTV have evolved through recombination events, resulting in

different infection capacities between particular hosts, e.g. BCTV-Pepper yellow dwarf and BCTV-Spinach curly top (Chen and Gilbertson, 2011, Varsani et al., 2014). Different strains of BCTV can be host specific and cause certain symptom types in specific hosts, strains can also compete between one another for dominant infection in plant host (Peinado et al., 2018).

Vector Life History

The vector beet leafhopper (BLH) *C. tenellus* Baker is the only known mode of transmission of BCTV. Like most insects, the BLH's development is variable and is influenced by temperature, rainfall, and availability of host plants. Beet lefhoppers prefer an arid climate, with varied phenology by geographic region. In California, BLH overwinter as mated females on perennial plants in the foothills throughout the fall and begin oviposition in January and February on annual plants (Bennet, 1971). In New Mexico, BLH overwinter in southern regions on the host plant, London rocket, a cool-season annual in the Brassicaceae family (Creamer, 2020). This plant serves as a food and oviposition source for the BLH and as a reservoir for BCTV. Kochia, a weed species in the Chenopodiaceae family, can germinate at lower temperatures and has overlapping phenologies with London rocket serving as an intermediate host between weed and agricultural perennials (Creamer, 2020). In Arizona, there are two leafhopper migrations, in the spring (March-May) and fall (October-December) that have historically aligned with the biannual crop lettuce (Coudriet and Tuttle, 1963). Upon encountering host plants, female BLH oviposit their eggs into leaf and stem tissue, laying between 200-300 eggs. Eggs take anywhere from 5-40 days to hatch and the insect goes through five nymphal instars before developing fully into an adult (Chen and Gilbertson, 2016). BLH are hemimetabolous insects that feed through their stylet in all stages of development. The stylet is a piercing sucking mouthpart that allows the insect to acquire

phloem from the plant. Beet leafhoppers are polyphagous, meaning that they can feed on a wide range of host plants, and they can also oviposit on a variety of these plants (Cook, 1967). Both of these characteristics contribute to the success of this insect as an important vector on many crops.

Origin of BLH and BCTV

The beet leafhopper is native to the Mediterranean Basin (Bennet and Tanrisever, 1958). The first report of BCTV was made from BLH observed on sugar beet in Grand Junction, Colorado in 1895 (Chen and Gilbertson, 2016). It was originally thought that beet leafhoppers were native to the United States but mating experiments between BLH populations from California and Morocco were the same species of insect (Frietag et al., 1955). Transmission of BCTV by north African BLH populations was also evidence that both leafhopper were the same vector. Beet curly top virus and BLH are a classic example of unintentional introduction of pest by human movement. The vector and virus likely arrived with migrating communities who brought sugar beet during the gold rush periods of the western United States.

Beet curly top virus was determined to be associated with BLH in 1909 in North America (Ball, 1909). It wasn't until 1955 that a survey was performed revealing the wide distribution of BCTV in The Mediterranean Basin in countries like Turkey, and the Middle East (Iran), which established that the origin of this virus was from the Old World. Other *Circulifer* vectors of BCTV were also found in the Mediterranean Basin, including *C. haematoceps* and *C. opacipennis* (Yazdi et al., 2008). The genus *Curtovirus* arose from the recombination events between white fly transmitted *Begomovirus* and leafhopper transmitted *Mastrevirus* (Harrison and Robinson, 1999). Mastreviruses have a limited geographic distribution specific to the Old

World, supporting the clause that this virus evolved outside of the United States (Briddon et al., 1998).

Virus Transmission

Acquisition of BCTV occurs when a BLH feeds on the phloem of an infected plant. Within the leafhopper, the virus is transmitted in a circulative manner where virions circulate through the gut, traverse the gut and reach the salivary glands for transmission to occur; hence, it requires a greater time for acquisition and transmission (hours to days) when compared to non-circulative or stylet-borne viruses (Stahl and Carsner, 1916; Frietag, 1936; Chen and Gilbertson, 2016). Although circulative viruses remain transmissible in the insect's body over time, BCTV is non-propagative and does not replicate within the insect. These viruses are retained after molts and have a latent/incubation period between acquisition and transmission of 4 hours. The virus moves through the insect vector's body and once it reaches the salivary glands, the virus can be transmitted upon feeding (Bragard et al., 2013, Yadav & Rana, 2020). The movement of the virus through the body of adult BLH was studied, and found BCTV circulated through the vector's body first in the digestive tract after 1 hour, the hemolymph after 3 hours and in the salivary glands after 4 hours (Soto and Gilbertson, 2003). Beet leafhoppers can transmit the virus after the four-hour latent period. Several studies have investigated length of acquisition access period (AAP) and its impact on transmission, one of which demonstrated that AAPs of 1 min, 30 min, 1 h, and 4 h, resulted in infection rates of 3, 28, 44, and 76% (Bennett and Wallace, 1938). Longer period of AAP result in higher rates of transmission efficiency. Beet leafhoppers can acquire BCTV within a minute of feeding on an infected plant and transmit the virus four hours after an infected meal.

There is a degree of susceptibility to BCTV between different plant types. More susceptible cultivars will have a higher titer of BVTV virions, which will result in a higher rate of acquisition by BLH upon phloem feeding (Carsner, 1919, Bennett, 1962). Younger adult BLH tend to be more efficient vectors than older BLH, and adult males are more effective at BCTV transmission than females, likely because females are occupied with oviposition (Bennett, 1962). Younger plants are more susceptible to BCTV infection and tend to be more desired by BLH (Romney, 1943).

Persistent non-propagative transmission in BLH was demonstrated through the initial strategies of recovering a non-viruliferous colony. The strategy used was watching nymph emerge under the microscope and once fully emerged were immediately transferred to a BCTV non-viruliferous sugar beet plant for development (Frietag, 1936). The presence or absence of symptoms in host plants has been a historic strategy used prior to molecular confirmation of virus transmission, along with rearing BLH on other non-susceptible plants like *Chenopodium murale* and *Rumex crispus* (Carsner, 1926). Nymphs that had never taken a meal from viruliferous plants did not lead to any symptom development when transferred to a BCTV-free plant. Today, there are many molecular tools available for general Curtovirus and strain specific detection and quantification (Strausbaugh et al, 2008, Varsani et al., 2014, Peinado et al. 2018).

Beet leafhopper host preference and behavior

Several studies have designed host plant assays to observe transmission, settling and oviposition preferences. Several studies illustrate BLH preference for sugar beet as a host, demonstrated by extended settling, feeding, oviposition and successful nymph emergence (Thomas, 1972, Thomas and Boll, 1977, Munyaneza and Upton, 2005). These studies illustrate

the role of sugar beet in the life cycle of the beet leafhopper's development, and why assays will be performed on sugar beet hosts.

To date, there are few studies that focus on the impact of BCTV on the life history of its vector, and no evidence that BCTV has a negative impact on the fitness and fecundity of the BLH (Chen and Gilbertson, 2016). What is known about the effects of BCTV on *C. tenellus* is that it delays development of nymphs into adults, requiring 1-10 additional days in both male and female nymphs reared on BCTV infected sugar beets compared to virus free plants and nymphs (Severin, 1946).

Management Efforts

Management efforts for BCTV began in the early to mid-1900's, but it remains a difficult disease to control because of the complex host range and migratory behavior of BLH (reviewed in Chen and Gilbertson, 2016). Management efforts have included cultural practices, planting of resistant cultivars, removal of surrounding weeds, and vector management using pesticides. Cultural practices such as planting sugar beets earlier in in the season so that they have a higher tolerance for the virus when it becomes present. Sugar beets with 4 leaves and up have demonstrated a reduction in yield loss and symptoms (Wintermantel and Kaffka, 2006).

Neonicotinoids such as imidacloprid and thiamethoxam have provided sufficient control of the vector (Strausbaugh et al. 2008; Strausbaugh et al. 2006). In these management programs, pesticides were applied to agricultural and overwintering perennial habitat to try and reduce BLH populations; these strategies were successful at reducing spring populations but not sustainable as a long-term control method. Furthermore, developing regulations will likely prohibit the use of neonicotinoids.

Many efforts have been put into the development of resistant cultivars through breeding programs. Sugar beet cultivars that have been evaluated and approved for BCTV resistance which is determined by selecting lines that exhibit the lowest symptom severity after BCTV inoculation include: 32297-91, 32301-91, 32303-91, 32306-91, 32307-91, 32309-91, 32311-91, 32313-91, 32322-91 and O.T-201 (Montazeri et al 2016). Although BCTV resistant cultivars do not yield as well as susceptible in the absence of BCTV (Kaffka et al. 2002), this control strategy has been effective in reducing yield loss in years of high BCTV incidence (Montazeri et al. 2016).

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CHAPTER 2 – First insights into the virus and viroid communities in hemp (*Cannabis sativa*)[1]

Introduction

Hemp is a multifaceted crop, sourcing communities with food, fiber and medicinal properties (Schluttenhofer and Yuan, 2017). With the passage of the 2014 Farm Bill, the initial experimental production of hemp began. The 2018 Farm Bill re-evaluated hemp to no longer be considered a controlled substance and is now a legal agricultural crop in the U.S. Within the bill, Section 7606 (Legitimacy of Industrial Hemp Research) provided a formal definition of the crop as "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% on a dry weight basis." Today, the United States is recognized worldwide as the top producer of cannabis (Punja, 2021). Currently, 46 U.S. states have passed laws to define the crop and remove barriers to its production. In 2020, hemp was produced on 336,655 acres with 13,475 grower licenses across 34 states, and 46 states with active hemp program and 41 tribes with approved USDA plans, according to "U.S. Hemp Report," by the organization Vote Hemp (www.votehemp.com). This is a more than 300% increase since 2018 licensed acreage. Colorado, the leading state in hemp production in the new era, increased production from 4,873 licensed acres in 2017 to 36,225 licensed acres in 2020. With the current resurgence of hemp as a crop to be produced within the U.S. there are many challenges with associated pests and diseases that are essentially undescribed, as are the management strategies. Disease identification and management is an increasing challenge for hemp farmers across the country.

[1] In review to *Frontiers in Agronomy* with Kaitlyn Langemeier, Tessa Albrecht, Jacob MacWilliams, Whitney Cranshaw, Ana Cristina Fulladolsa Palma, Marylee L. Kapuscinski, Mark Stenglein and Punya Nachappa.

As production increases, the crop diversifies, and in turn legitimizes, the emergence of viral diseases and their spread is imminent (Fike, 2016).

There are over 100 pathogens that affect hemp with potential to cause economic damage (McPartland, 1994;1996;McPartland and Cubeta, 1997;McPartland, 1999;McPartland et al., 2000). These include fungal, bacterial, viral, and nematode species that affect hemp during production. A recent review summarizes important diseases affecting the cannabis and industrial hemp production in North America (Punja, 2021). In contrast to the published reports on bacteria, fungi and molds affecting hemp, there is paucity of information on viruses and viroids. The earliest reports of plant viruses affecting hemp were hemp streak virus (HRS) described in 1941 (Röder, 1941) and hemp mosaic virus (HMV) described in 1958 (Ceapoiu, 1958); however, the causal agents of these diseases have yet to be isolated and characterized. Other viruses that are known to infect hemp are alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), potato virus X (PVX), tomato ringspot virus (TomRSV), potato virus Y (PVY), broad bean wilt virus (BBWV), arabis mosaic virus (ArMV), and raspberry ringspot virus (RpRSV) (Kegler and Spaar, 1997). More recently, cannabis cryptic virus (CanCV) was isolated from hemp plants (Ziegler et al., 2012;Righetti et al., 2018). In addition, hemp was found to be infected with hop latent viroid (HLVd) in California (Bektaş et al., 2019;Warren et al., 2019). A survey of cannabis farms in Israel detected the presence of lettuce chlorosis virus (LCV) in plants showing leaf yellowing, interveinal chlorosis, that are typically associated with general nutrient deficiency (Hadad et al., 2019). Diagnosis of hemp viruses is challenging due to the lack of research that characterizes symptomology and transmission mechanisms of known and novel viruses. Metagenomic next generation sequencing (NGS) technologies circumvent many of these problems and allows for the characterization of complete genomes

from known or novel viruses (Nachappa et al., 2020). Indeed a diversity of virus and viroid communities were identified in hemp that were previously unknown using metagenomic sequencing (Nachappa et al., 2020).

In 2019, beet curly top virus (BCTV) was found infecting hemp plants in Colorado (Giladi et al., 2020) and the virus was also detected in Arizona (Hu et al., 2021). *Beet curly top virus* is a type member of the genus *Curtovirus* in the family *Geminiviridae* (Chen and Gilbertson, 2016). Curly top disease is one of the most economically-important disease for sugar beet production in western United States; in addition, BCTV has resulted in yield losses in vegetable crops such as tomato, pepper, spinach, cucurbits and common bean (Chen and Gilbertson, 2016). The virus is transmitted in a circulative non-propagative manner by the leafhopper, *Circulifer tenellus* Baker (Hemiptera: Cicadellidae) that can infect over 300 plant species (Bennett, 1971). There are several different strains of BCTV including, BCTV-California/Logan (CA/Logan), BCTV-Colorado, BCTV-Worland (Wor), BCTV-Mild (Mld), BCTV-Severe (Srv), BCTV-Pepper curly top (PeCT), BCTV-Pepper yellow dwarf (PeYD) and BCTV-Spinach curly top (SpCT) [Reviewed in (Chen and Gilbertson, 2016)]. The occurrence of the various strains of BCTV varies over time by geographic region and are often observed as co-infections (Creamer, 2020a).

The objectives of this study were to better understand the diversity, symptomology and distribution of BCTV, and to analyze the presence of established and emerging virus/viroid communities in hemp in Colorado using shotgun metagenomic analysis. The information obtained in this study will aid in the development of accurate detection methods and effective virus and vector management strategies to minimize disease incidence and spread.

Materials and Methods

Hemp leaf tissue and insect sample collection. Symptomatic hemp leaf tissue samples were harvested from individual hemp plants from hemp grower fields in Delta County, Colorado throughout the 2019 grow season between July and September. In addition, symptomatic leaf tissues were obtained from samples sent to the Plant Diagnostic Clinic at Colorado State University for diagnosis by hemp growers from 10 counties. A total of 100 mg of leaf tissue was harvested and stored in a 2 mL microcentrifuge tube and placed in -20°C until DNA extraction. To test potential insect vectors of BCTV, leafhopper species were collected in Delta County, Colorado from weeds and surrounding vegetation of several BCTV-infected hemp fields. Insects were stored in 90% alcohol and submitted to Dr. Chris Dietrich at the University of Illinois Urbana-Champaign for species identification. Additionally, samples of 3-5 leafhoppers of each species were stored in a 2 mL microcentrifuge tube and placed in -20°C until DNA extraction.

DNA extraction and quantitative PCR analysis. Plant DNA extraction was performed following the manufacturer's recommendations using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). All samples were eluted in 100 µL purified water for subsequent PCR analysis. Insect DNA extraction was performed following the manufacturer's protocol using the DNeasy Blood & Tissue Kit (Qiagen). Leafhoppers were separated by species with 3-5 insects per tube and ground using Tissuelyser (Qiagen). All samples were eluted in 100 µL purified water for subsequent PCR analysis. The quantity of leaf and insect DNA was determined using a NanoDrop One spectrophotometer (Thermo Fisher, Waltham, MA) and stored at -20°C until virus detection.

To detect BCTV in hemp leaf tissues and leafhoppers, samples were analyzed by PCR using GoTaq® Flexi DNA polymerase (Promega, Madison, WI). The amplification cycle consisted of 94°C initial denature for 5 minutes, 25 cycles of denaturation at 94°C for 1 minute, 58°C annealing for 2 minutes, and 72°C extension for 2 minutes, followed by a 10-minute final extension. All PCR products were visualized on 1% agarose gel. Three different primer pairs targeting BCTV-Wor, BCTV-Colorado and BCTV-Svr were used and are listed in Table 2.1. The PCR products were excised from the agarose gels and purified using DNA Clean & Concentrator™-5 (Zymo Research, Irvine, CA). One-two PCR products from each location were randomly selected and submitted for Sanger sequencing at Genewiz Inc. to confirm the virus identity using strain-specific primers (Table 2.1). The sequences for each BCTV strain were checked for identity against the non-redundant (nr) database using blastn in the NCBI database.

Virome analysis. Total RNA was extracted from a composite of 5 leaves that previously tested positive for BCTV from several locations in Colorado. The samples originated from outdoor hemp production in Delta, Pueblo, Boulder, Rio Blanco and Conejos counties and one indoor production in Larimer County in Colorado. Total RNA was extracted as described above and checked for quality using a Nanodrop One spectrophotometer (ThermoFisher Scientific) and quantity using a Qubit 3.0 fluorometer (ThermoFisher Scientific). Approximately 2 µg of RNA was submitted to the CSU Next Generation Sequencing Facility, where library preparation, quality measurements, and sequencing was performed. Briefly, RNA quality was confirmed using an Aligent Tapestation instrument. Shotgun RNA libraries were constructed using the Kapa Biosystems RNA HyperPrep kit (Roche, IN, USA) according to the manufacturer's

instructions. Pooled libraries were sequenced on an Illumina NextSeq 500 instrument to produce single-end 150 nucleotide (nt) reads.

Bioinformatic Analyses. Virus and virus-like sequences were identified as previously described (Cross et al., 2018). Analysis scripts are available at https://github.com/stenglein-lab/taxonomy_pipeline/. Low quality and adapter sequences were removed using cutadapt software (Martin, 2011) and duplicate reads were collapsed with cd-hit (Li and Godzik, 2006). Host (hemp)-derived reads were removed by bowtie2 alignment (Langmead and Salzberg, 2012) to the hemp reference genome (assembly accession GCF_900626175.1). Remaining non-host reads were assembled into contigs using the Spades assembler (Bankevich et al., 2012). Contigs and non-assembling reads were taxonomically categorized first by nucleotide-level alignment to the NCBI nucleotide (nt) database using BLASTN, and then by protein-level alignment to the NCBI protein (nr) database using the diamond aligner (Altschul et al., 1990;Buchfink et al., 2015). This produced a comprehensive metagenomic classification of all non-host reads. Although we focused on viruses, this also constitute a valuable dataset about the entire hemp-associated microbiota (bacteria, fungi, etc.) for future use by us and others. Candidate virus sequences were manually validated by aligning reads to draft genome sequences using bowtie2. Lastly, the raw sequence data was deposited in the NCBI Sequence Read Archive (SRA) repository under NCBI BioProject accession PRJNA762365.

Phylogenetic Analysis. The BCTV sequences from this study were aligned with sequences of all 11 BCTV strains from other host plants categorized by Strausbaugh et al. (2017) and BCTV sequences from hemp deposited in the GenBank database using Muscle version 2.0 (Edgar, 2004). The BCTV phylogeny includes aligned BCTV detected from hemp from Colorado and

analyzed relationships between sequences from BCTV-Worland (Wor), BCTV-Colorado (CO), BCTV-Kimberly1 (Kim1), BCTV-Leafhopper71 (LH71), BCTV-Severe (Svr), BCTV-CA/Logan (CA/Logan), BCTV-Mild (Mld), BCTV-Severe pepper (SvrPep), BCTV-Spinach curly top (SpCT), and BCTV-Pepper curly top (PeCT). The two previously published BCTV sequences from hemp from other studies were included and the BCTV phylogeny was rooted to BCTV-Pepper yellow dwarf (BCTV-PeYD) strain. All phylogenetic analyses were performed using sequence alignments generated using Muscle in MEGA X: Molecular Evolutionary Genetics Analysis (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model with 1000 pseudo-replicates (Tamura and Nei, 1993). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Phylogenetic analysis was also performed on Tobacco streak virus (TSV), using all three TSV RNA 1-3 detected from shotgun sequencing performed in this study and analyzed with sequences from *Tobacco streak virus* from previous studies, *Papaya cytorhabdovirus* (PpVE), *Bean-associated cytorhabdovirus* (BaCV), *Privet ringspot virus* (PrRSV), *Strawberry necrotic shock virus* (SNSV), *Blackberry chlorotic ringspot virus* (BCRV), and *Parietaria mottle virus* (PMoV). All three TSV phylogenies were rooted to corresponding *Elm mottle virus* (EMoV) RNA1-3 as an outgroup. Phylogenetic analysis of RNA-dependent RNA polymerase gene sequences of citrus yellow vein-associated virus (CYVaV) and opuntia umbra-like virus (OULV) obtained from hemp samples collected during the 2019 field season in Colorado. Sequences were aligned and analyzed along with top sequence matches from GenBank and other closely related viruses available in GenBank. Analysis of CYVaV and OULV included viruses: *Fig umbra-like virus* (FULV), *Fig luteovirus* (CRLV), *Ethiopian maize-associated virus* (EMaV), *Sugarcane umbra-like virus* (SULV),

Papaya virus Q (PpVQ), and *Babaco virus Q* (BVQ).The CYVaV/OULV phylogram was rooted to outgroup *Papaya meleira virus* (PMeV2).

Results

Beet curly top virus diversity and distribution in hemp

In 2019, we analyzed 135 symptomatic hemp tissue samples from different stages (vegetative and reproductive) of hemp plants from outdoor productions in 12 different counties spanning the predominant hemp-growing regions in Colorado (Fig. 2.1). PCR analyses confirmed BCTV in most hemp leaf samples with 81% (109/135) incidence. Among the samples that tested positive for BCTV, incidence of BCTV-CO was 11% (15/135) followed by BCTV-Wor 5% (7/135) and 64% (86/135) coinfection of BCTV-Wor and BCTV-CO. In contrast, we did not detect the BCTV-Svr in any of the samples (Table 2.2). The BCTV-positive plants exhibited various virus symptoms in hemp including yellowing, mottling, curled leaves and stunting (Nachappa et al., 2020). All symptoms observed in hemp were found to be associated with BCTV infection (Supplementary Table 2.1). Other symptomatic hemp tested negative for the presence of BCTV likely had some other infection type influencing symptom expression.

Phylogenetic analysis based on a fragment of the BCTV coat protein (CP) sequences from 20 hemp samples from various locations in Colorado (accessions: MW604759-MW604777) had nt identity to one another between 98.99-98.24%. Samples from this study showed 97.28-99.13% nt sequence similarity with BCTV sequences from cannabis (BCTV-Can) from Colorado (MK803280.1), and 97.21-99.78% nt similarity with BCTV-Can from Arizona (MW182244.1). The BCTV sequences from the current study showed high nt similarity of 97.27-99.35% with BCTV-Wor and 92.84-94.99% nt match to BCTV-CO

sequences available in GenBank. Phylogenetic analysis revealed that BCTV sequences from hemp form a distinct group including both BCTV-Wor and BCTV-CO sequences (Fig. 2.1).

Leafhopper species that were collected from vegetation around hemp fields in Delta County in Colorado included: *C. tenellus* Baker*, Empoasca sp., Balclutha neglecta* DeLong & Davidson*, Macrosteles quadrilineatus* Forbes*, Ceratagallia uhleri* Van Duzee*,* and *Exitianus exitiosus* Uhler. Of the six species collected, *C. tenellus* was the only species from which BCTV was detected. All *C. tenellus* 100% tested positive for the presence of BCTV; 56 % (5/9) tested positive for BCTV-CO, 0% (0/9) tested positive for a single infection of BCTV-Wor, and 44% (4/9) insect samples tested positive for coinfection of BCTV-Wor and BCTV-CO strains.

Hemp virome analysis

The description of the hemp virome was conducted using shotgun metagenomic sequencing of total RNA from leaf samples. Samples were collected in 2019 from field sites in Boulder, Conejos, Delta, Pueblo and Rio Blanco, and leaf samples from an indoor hemp cultivation in Larimer County. Datasets contained an average of 12.4 $x10^6$ reads. After removal of low quality and adapter sequences, there was an average of $11.6x10^6$ sequences remaining per library (94%). Duplicate reads were collapsed leaving an average of $1.4x10^6$ unique reads per dataset (12%). Removal of host-derived reads left an average of $7.8x10^4$ reads per sample (0.6%) (Supplementary Table 2.2). Shotgun metagenomics analysis revealed the presence of 7 viruses and one viroid in hemp samples from Colorado (Table 2.3). We assembled complete or nearly complete genomes of several of the viruses using NGS data (Table 2.3).

Cannabis sativa mitovirus 1 (CasaMV1) was detected in hemp from all counties with the exception of Larimer County (Table 2.3). Alignment of the complete RNA-dependent RNA polymerase (RdRp) gene of CasaMV1 sequences from four datasets (MT878080-MT878083) showed high nt similarity with each other (99.89-100%) and exhibited 88% nt identity to CasaMV1 from *C. sativa* (BK010438.1). The fifth sequence, from hemp from Boulder County, had a 99.72% identity match to a different mitovirus recovered from *C. sativa* (BK010437.1) (Table 2.3).

Four of the six counties showed the presence of BCTV specifically BCTV-CO and BCTV-Wor (MT878075- MT878078). These sequences shared nt identity between 96.89-98.54%. The sample from Conejos County (MT878078) had a top match with BCTV-Can-AZ (MW182244.1) with an identity match of 98.22%. The sequences from Delta and Rio Blanco counties were similar to BCTV-CO with nt identity of 96.95-98.66% and the sequences from Pueblo matched BCTV-Wor with nt identity of 99% (Table 2.3). There was insufficient coverage to recover a coding complete assembly for the BCTV-Wor sequences from Pueblo.

Citrus yellow vein-associated virus (CYVaV) was detected in hemp from two counties (Delta and Rio Blanco) and these two sequences (MT893740 and MT893741) were partial sequences of the RdRP of CYVaV gene and shared of 97.51% nt identity with each other. The sequences from this study had an 89.72% nt identity with CYVaV identified from citrus (NC_040311) (Fig. 2.3). Opuntia umbra-like viruses (OULV) were also detected in these two locations (MT909563 and MT909562), sharing nt identity of 98.46% with each other, and a top BLASTN hit of OULV detected in the barberry fig with 97.96% nt identity (MH579715). OULV and CYVaV sequences share a 76.80-76.83% nt identity, coming up in one another's NCBI blast searches with shared similarity of the RdRp gene. CYVaV and OULV sequences

were combined top matched from NCBI GenBank for phylogenetic analysis. These sequences share similarity with the RdRp gene. CYVaV and OULV formed separate groups in phylogenetic analysis (Fig. 2.3).

Tobacco streak virus (TSV) was detected in hemp virome dataset from Conejos County encoding all three RNAs (MT893737-MT893739). The TSV RNA 1 sequence from the current study (MT893739) had 82.32% nt identity to a fragment of the replicase gene of TSV sequences from soybean (MT602534). The TSV RNA 2 sequence (MT893738), had 82.58% nt identity match with the gp1 putative viral polymerase in TSV from *Dahlia pinnata* (KR017709.1). Lastly, TSV RNA 3 (MT893737) had an 80.56% identity match to TSV RNA 3 from soybean from Brazil (MT360269.1) which encodes the movement and coat protein genes. These TSV RNA sequences from the current study range from 81-83% similar to previously observed TSV detected from various host plants, indicating this as a novel genotype of TSV (Fig. 2.4).

Cannabis cryptic virus (CanCV) corresponding to RNA segments 1 and 2 were assembled (MT893742 and MT893743) from the Larimer County sample. The CanCV RNA 1 (MT893742) sequence matched a partial region of the RdRP gene and shared a 99.57% and 99.31% nt identity with CanCV RdRp sequences found in *C. sativa* from Italy and Germany (KX709964 and JN196536). The CanCV RNA 2 segment (MT893743) matched the partial sequence of the CP gene and shared 99.56 and 95.98% nt identity with sequences corresponding to MT893742 and MT893743 sequences from this study.

Grape line pattern virus (GLPV) was detected from Larimer County in three RNA segments. GLPV RNA1 segment (MW888424) had the highest nt identity of 97.16% to the methyltransferase from a GLPV isolate from grapevine in Hungary (MT319109), followed by

the replication protein of Hop yellow virus with a 95% nt match from hops in China (MG727388). GLVP RNA2 segment (MW888423) matched the of GLPV with a nt match of 98.23% (MT319110) coding for the RdRp gene. GLVP RNA3 (MW888422) had a nt match of 99.16% to the movement protein of GLPV (MT319111).

Two complete genomes of Hop latent viroid (HLVd) were assembled from Boulder and Delta counties (MZ090889 and MZ090890). The HLVd detected from Delta County (MZ090890) had a100% shared nt identity to X07397.1, a sequence submitted to GenBank from hops. The second HLVd sequence (MZ090889) from Boulder County, shared a 100% nt identity with HLVd collected from hops growing in a commercial garden in China (EF613183.1).

Discussion

One of biggest challenges facing the hemp industry is detection and management of plant viral diseases (Nachappa et al., 2020;Punja, 2021). As hemp production areas expand across the U.S., it is highly likely that there are additional undiscovered viruses and viroids in hemp. These may potentially impact the hemp crop, but hemp may also serve as a reservoir for some viruses that may then spread to other economically important crops in the vicinity. In the current study, we assessed the diversity and distribution of BCTV affecting hemp in Colorado using PCR analysis.

A survey of symptomatic hemp tissue samples from different stages from outdoor productions across 12 counties revealed high incidence (81%) of BCTV in Colorado. Symptoms observed on collected samples were overall stunting, yellowing, folding of lateral leaves, flattening of the stem, and tightly twisted leaves in the center of the plant with asymptomatic shoots growing from lateral branches. Growers reported disease incidence levels

above 90% in some cases and disease severity reaching greater than 50% of leaf area in infected plants. This led to diminished crop yield, including low quality of the flower and overall stunted growth (John House, hemp grower personal communication). The symptoms observed were variable among plants, and disease progression differed across farms (Nachappa et al., 2020). Given the broad host range of the virus and the vector [Reviewed in (Chen and Gilbertson, 2016)], it is possible that curly top disease may become one of the most serious disease affecting hemp production. There are 11 BCTV strains and the presence of the strains in a particular location may change over time and are often found as mixed infections (Strausbaugh et al., 2017;Creamer, 2020b). For instance, a survey of BCTV strains from Idaho and Oregon showed a shift from BCTV-Svr as the predominant strain in 2006 and 2007 to CO and Wor (mild strains) becoming dominant in 2012-2015 (Strausbaugh et al., 2017). One reason for the shift in strains may be due to increasing acreage of strain-specific commercial resistant cultivars that caused selection pressures for mild strains. In addition, new strains can emerge likely due to recombination among strains during mixed infections (Strausbaugh et al., 2008;Chen et al., 2010;Bach and Jeske, 2014).

We identified the mild strains, BCTV-CO and BCTV-Wor present as a single or mixed infection in hemp leaf samples in Colorado using strain-specific primers. In contrast, we did not detect the BCTV-Svr in any of the samples. The DNA fragments from Sanger sequencing and whole genomes from NGS revealed high nt similarity to BCTV genotype previously from Colorado (Giladi et al., 2020) and Arizona (Hu et al., 2021). Reports suggest that mild strains (strains that produce mild symptoms such as slight leaf curling, stunting, and vein thickening) are more effective in infecting alternative and weed hosts than severe strains (Chen and Gilbertson, 2009). All the beet leafhoppers collected in the current study also carried BCTV-

Wor and CO strains. This complements the findings that CO and Wor are the predominant strains in hemp. The beet leafhopper transmits BCTV in a circulative manner (Bennett, 1971). The leafhopper acquires the virus in as little as 1-2 min, but maximum accumulation occurs after acquisition access period (AAP) of 24-48h (Soto and Gilbertson, 2003). There is a 4-hour latent period before the insect can transmit the virus. The longer the AAP the higher the rate of transmission. The virus does not replicate in the insects (Soto and Gilbertson, 2003). Leafhoppers can transmit the virus by feeding for 15 minutes and the virus is retained for days to weeks, but there is no transstadial transmission (Bennett, 1971).

There is limited information about curly top epidemiology in Colorado. Beet leafhoppers are most commonly found in the Western Slope where outbreaks of this disease have been reported on many crops such as tomato, bean, squash, sugar beets, spinach. It is thought that leafhoppers migrate from their overwintering sites in southern states including Arizona and New Mexico to Colorado in spring. The insect survives the winter on various kinds of weedy plants, particularly mustard-family (Bennett, 1971;Creamer, 2020b). Hence, the abundance of winter host plants in the southern breeding areas could be an important factor in the number of beet leafhoppers that appear in Colorado in spring. We hypothesize that in winter/spring 2019 moisture conditions were favorable in southern breeding areas to support the large population of overwintering plants on which beet leafhoppers could develop. Subsequently there was a large number of BLH migrants that moved into Colorado causing a BCTV outbreak in western Colorado on several crops, including hemp, which is one of the most widely grown crops in Colorado in 2019.

Virome analysis revealed a diversity of viruses and one viroid pathogen infecting hemp in Colorado. The number of identified viruses and viroid in each location ranged from 2-5.

Beet curly top virus, CasaMV1, and HLVd were commonly present in several locations, whereas other viruses were unique to specific locations Cannabis sativa mitovirus 1 was detected in hemp from all counties sampled with the exception of Larimer County, where tissues were collected from indoor hemp production. Mitoviruses are capsidless viruses known for their ability to infect eukaryotic mitochondria (Shahi et al., 2019). Several mitovirus sequences have been recovered during the transcriptome analysis of a variety of invertebrates across different phyla (Shi et al., 2016). Complete plant mitovirus genomes were recovered from publicly available transcriptome data of 10 different plant species including hemp, hops and sugar beet (Nibert et al., 2018). These viruses are generally considered to be cryptic viruses and there is no information on the impact on plant hosts.

A new genotype of TSV which is only 81-83% identical to the closest TSV sequence in GenBank was identified from hemp samples from Conejos County. Tobacco streak virus has a wide host range (Brunt et al., 1996) and is transmitted by thrips and pollen (Sdoodee and Teakle, 1988;Sharman et al., 2015) and by seed transmission (Sharman et al., 2009). The virus was first reported to infect hemp in 1971 and had described symptoms of stunting and mosaic patterning (Hartowicz et al., 1971). TSV is a nonenveloped, quasi-spherical virion with tripartite (RNA1, RNA2, and RNA3) segmented linear (+) sense RNA genome and all 3 segments were retrieved in the hemp virome dataset. Further characterization of these sequences is challenging because the leaf samples from number of plants were pooled and/or mixed infection of viruses in these plants.

Cannabis cryptic virus (CanCV) was only detected in the indoor hemp sample from Larimer County and had high nt sequence similarity (99%) to the RdRP gene of previously reported CanCV sequence (Righetti et al., 2018). Historically, interveinal chlorosis and leaf

margin wrinkling in hemp was attributed to the so-called hemp streak virus (HSV) (Röder, 1941). In 2012, Ziegler and colleagues (Ziegler et al., 2012) first identified CanCV accidently while using hemp as a host for a hop latent virus (*Carlavirus*) and found that the virus was seed-transmissible. More recently, Righetti et al. (2018) tested hemp samples with typical hemp streak syndrome and identified CanCV in all tested samples irrespective of presence and severity of symptoms. This suggests calls into question the role of CanCV in symptomology.

Citrus yellow vein-associated virus (CYVaV) and opuntia umbra-like virus were identified from Delta and Rio Blanco counties. Citrus yellow-vein disease (CYVD) was first reported Dr. L. G. Weathers in California in 1957 (Weathers, 1957) resulting in typical yellow vein symptoms. Recently, Kwon and colleagues (2021) demonstrated that CYVD is associated with a virus-like agent, tentatively named CYVaV and is transmitted via grafting to virtually all citrus varieties. The virus appears to be closely related to unclassified virus-like RNAs in the family *Tombusviridae* specifically opuntia umbra-like virus (OULV) (Kwon et al., 2021). Indeed, phylogenetic analysis of the RdRp and ORF1-3genes placed the CYVaV and OULV from the current study in a well-supported cluster. Umbraviruses lack a capsid protein (CP) gene, which makes them dependent on a 'helper' virus, usually from the Luteoviridae, for replication, encapsidation and cell-to-cell movement (Syller, 2003). However, no helper virus was identified using NGS in this dataset. It is possible that a helper virus too distant to be recognized was missed by the analysis. Indeed, the first report of OULV from *Opuntia ficus indica* fruit cactus plants with symptoms of pad swelling disease was not associated with Luteoviridae (Felker et al., 2019). The authors hypothesized that as Umbraviruses occur throughout the plant, but Luteoviruses only occur in the phloem, low concentrations of

Luteovirus can be expected in the sample. To our knowledge, this is the first report of CYVaV and OULV in hemp.

Hop latent viroid has been previously identified in hemp from symptomatic and asymptomatic plants (Bektaş et al., 2019) and from symptomatic plants (Warren et al., 2019). Viroids are small non-encapsulated infectious pathogens, comprised of closed single stranded RNA molecules and biological resources to drive host specificity (Flores et al., 2005). There was high nt sequence similarity (99%-100%) between the HLVd genomes retrieved from hemp. Typical symptoms of HLVd include stunting, malformation or chlorosis of leaves, brittle stems, and reduction in yields. Indeed, some of the samples collected from Delta County demonstrated typical HLVd symptoms. In addition to mechanical transmission, transmission by aphid vectors at low efficiency has also been reported (Adams and Barbara, 1982).

Conclusions and future perspectives

Our study identified a diversity of viruses and viroids in hemp in Colorado using shotgun metagenomics. The number of identified viruses and viroid in each location ranged from 2-5. Cannabis sativa mitovirus, BCTV and HLVd were commonly present in several locations, whereas other viruses were unique to specific locations. We identified a divergent virus with 81% sequence identity to TSV. Future research should focus on surveying hemp viruses across multiple hemp genotypes, locations, and stages of the crop. Overall, outcomes of this study and future research will result in the identification of unique target sequences for the development of rapid and accurate nucleic acid-based detection tools for viruses and viroids and a rich metagenomic characterization of hemp-associated pathogens. The viruses/viroids identified in the study were mechanically-transmitted, seed-transmitted or insect-transmitted making

management of these viruses challenging. Planting certified disease-free materials will be critical to minimize disease spread. Future investigations should explore vector metagenomics to determine potential insect vectors of viruses/viroids associated with hemp with the goal of identifying anticipated threats and developing early prevention tactics. Once the insect vector community is identified, a deeper exploration of timing of insect flights, reproduction and feeding behaviors can be targeted for pest management and interruption of transmission cycles. Lastly, identifying potential sources of resistance to insect pests and viruses is an especially important disease control strategy, as commercial insecticides that are effective against insect vectors in other crops are restricted on hemp.

Tables and Figures

Table 2.1 Primers used to detect beet curly top virus (BCTV) strains in hemp (*C. sativa*). BCTV-Universal primers were used for initial detection and samples that tested positive were tested using BCTV-Worland, BCTV-Severe, and BCTV-CO for strain specificity using PCR.

Target	Sequence (5' – 3')	Product	Reference
BCTV-Worland	TGATCGAGGCATGGTT/ CAACTGGTCGATACTGCTAG	506 bp	Chen et al. 2010
BCTV-Severe	GCTGGTACTTCGATGTTG/ CAACTGGTCGATACTGCTAG	720 bp	Chen et al. 2010
BCTV-Universal	GCTTGGTCAAGAGAAGT/ CAACTGGTCGATACTGCTAG	496 bp	Strausbaugh et al. 2008
BCTV-Colorado	TGCGAGGACGCTTCTTGATT/ GGGCCGACTCTTATTTTCGG	463 bp	This study

Table 2.2 Incidence of BCTV strains associated with hemp in Colorado. Leaf samples that tested positive for the presence of BCTV were tested using BCTV-Wor and BCTV-CO strain-specific primers using PCR.

Location (county)	BCTV-strain identification (%)		
	BCTV-CO	BCTV-Wor	Co-infection
Delta	13% (10/78)	3% (2/78)	81% (63/78)
Pueblo	0% (0/1)	0% (0/1)	100% (1/1)
Montezuma	0% (0/3)	66% (2/3)	33% (1/3)
Larimer	50% (2/4)	0% (0/4)	50% (2/4)
Montrose	0% (0/2)	0% (0/2)	100% (2/2)
Garfield	0% (0/6)	0% (0/6)	83% (5/6)
El Paso	0% (0/3)	66% (2/3)	33% (1/3)
Weld	0% (0/1)	0% (0/1)	100% (1/1)
Rio Blanco	20% (1/5)	0% (0/5)	80% (4/5)
Mesa	0% (0/23)	0% (0/23)	22% (5/23)
Conejos	0% (0/1)	100% (1/1)	0% (1/1)
Otero	29% (2/7)	0% (0/7)	14% (1/7)

[a]Number in parenthesis is samples tested positive /total number of samples.

Table 2.3 Summary of hemp viromes from Colorado.

Sample	Viruses/viroids	Nearest GenBank Sequences	% nt identity	Coding Sequence	Avg. fold coverage	Accession number	Length (bp)
Boulder	Cannabis sativa mitovirus 1	BK010438.1	88%	Complete	125	MT878083	2817
	Cannabis sativa mitovirus 1	BK010437.1	99%	Complete	120	MT878084	2821
	Hop latent viroid	EF613183.1	100%	Complete	118	MZ090889	236
Conejos	Cannabis sativa mitovirus 1	BK010438.1	88%	Complete	158	MT878082	2819
	Tobacco Streak virus - RNA1	MT602534.1	82%	Complete	23	MT893739	3420
	Tobacco Streak virus - RNA2	KR017709.1	83%	Complete	25	MT893738	2851
	Tobacco Streak virus - RNA3	MT360269.1	81%	Complete	215	MT893737	2173
	Beet curly top virus BCTV-Can-AZ	MW182244.1	98%	Partial	14	MT878078	1294
Larimer	Grapevine line pattern virus - RNA1	MT319109.1	97%	Complete	26	MW888424	2374
	Grapevine line pattern virus - RNA2	MT319110.1	98%	Partial	32	MW888423	3136
	Grapevine line pattern virus - RNA3	MT319111.1	99%	Complete	76	MW888422	2511
	Cannabis cryptic virus - RNA 1	KX709965.1	99%	Complete	8	MT893742	455
	Cannabis cryptic virus - RNA 2	KX709964.1	99%	Partial	Low	MT893743	2322
Delta	Opuntia umbra-like virus	MH579715.1	97%	Complete	338	MT909563	2988
	Hop latent viroid	X07397.1	100%	Partial	7	MZ090890	256
	Cannabis sativa mitovirus 1	BK010438.1	88%	Complete	320	MT878081	2815
	Citrus yellow vein-associated virus	NC_040311.1	90%	Complete	775	MT893741	2854
	Beet curly top virus-Colorado	KX867022.1	97%	Partial	13	MT878076	1804
	Beet curly top virus-Colorado	KX867015.1	99%	Partial	13	MT878077	968
Pueblo	Beet curly top virus-Worland	KX867017.1	99%	Partial	5	-[a]	-[a]
	Beet curly top virus-Worland	AY134867.1	99%	Partial	5	-[a]	-[a]
	Cannabis sativa mitovirus 1	BK010438.1	88%	Complete	400	-[a]	-[a]
Rio Blanco	Citrus yellow vein-associated virus	NC_040311.1	90%	Complete	190	MT893740	2932
	Cannabis sativa mitovirus 1	BK010438.1	88%	Complete	180	MT878080	2818
	Beet curly top virus-Colorado	KX867022.1	98%	Partial	40	MT878075	2732
	Opuntia umbra-like virus	MH579715.1	98%	Complete	491	MT909562	2913

[a]There was insufficient coverage to obtain a contig for BCTV-Wor from Pueblo county; hence we did not submit sequence to GenBank

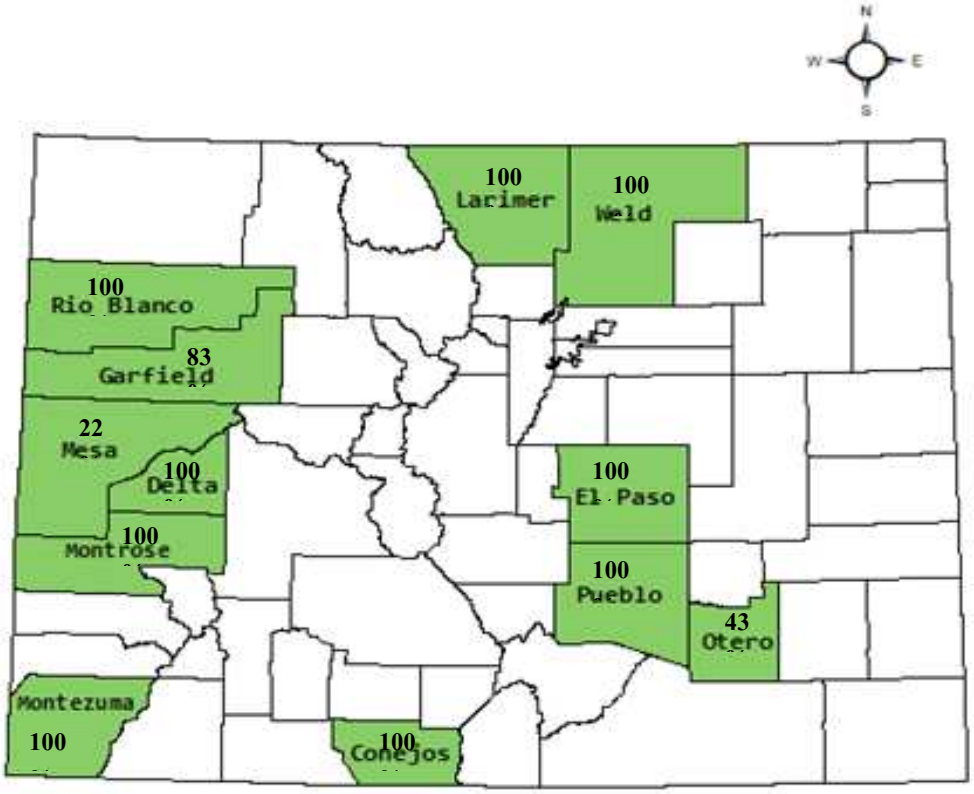

Figure 2.1 Incidence of beet curly top virus (BCTV) in hemp leaf samples collected during the 2019 field season in Colorado as determined by PCR analysis. Incidence was determined by the number of samples that tested positive for BCTV over total number of samples tested from that county.

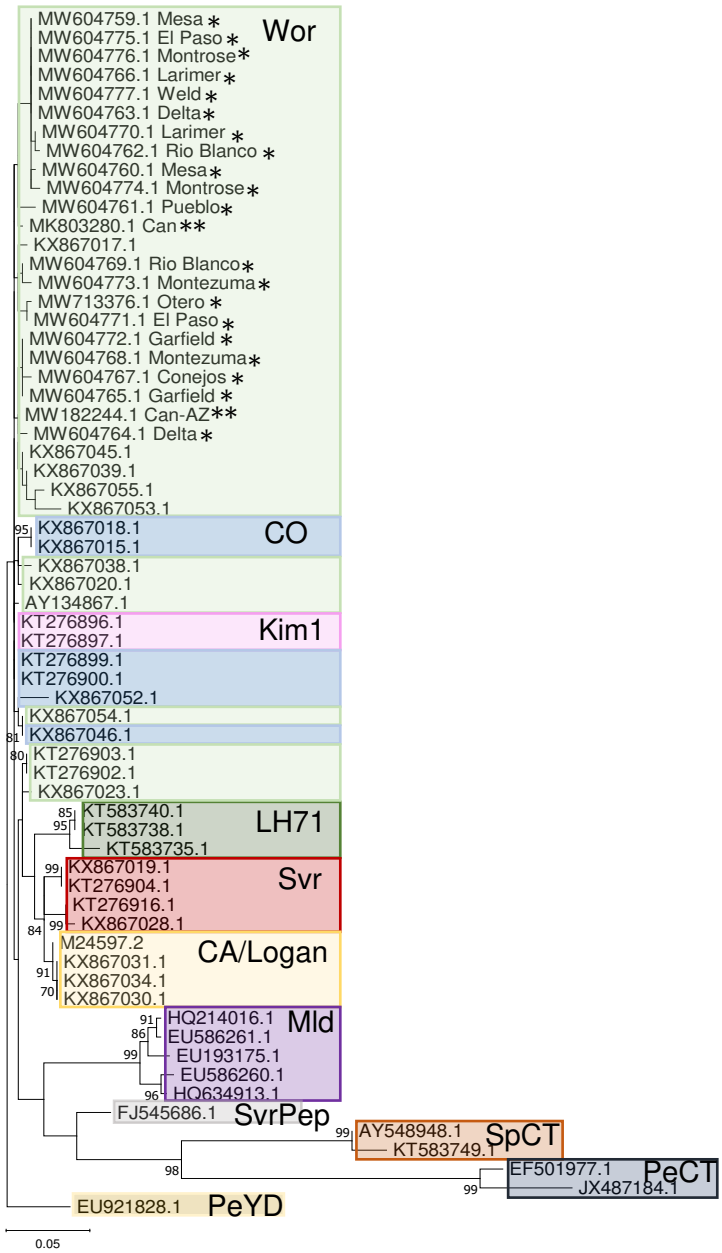

Figure 2.2 Phylogenetic analysis of partial coat protein sequences of beet curly top virus (BCTV) obtained from hemp samples collected during the 2019 field season in Colorado aligned with representative sequences of all 11 strains of BCTV. Pepper yellow dwarf virus (PeYD) strain is used as an outgroup. Other strains included are BCTV-Worland (Wor), BCTV-Colorado (CO), BCTV-Kimberly1 (Kim1), BCTV-Leafhoppe71r (LH71), BCTV-Severe (Svr), BCTV-CA/Logan (CA/Logan), BCTV-Mild (Mld), BCTV-Severe pepper (SvrPep), BCTV-Spinach curly top (SpCT), BCTV-Pepper curly top (PeCT). Scale bar indicates number of substitutions per site. Phylogenetic analysis by maximum likelihood method was done using Muscle in MEGAX. Asterisks indicate genotypes from Colorado from this study, double asterisk indicate accessions from previously published BCTV-Cannabis genotypes. Bootstrap values are indicated on the nodes. Bootstrap values less than 70% out of 1000 replicates are not shown.

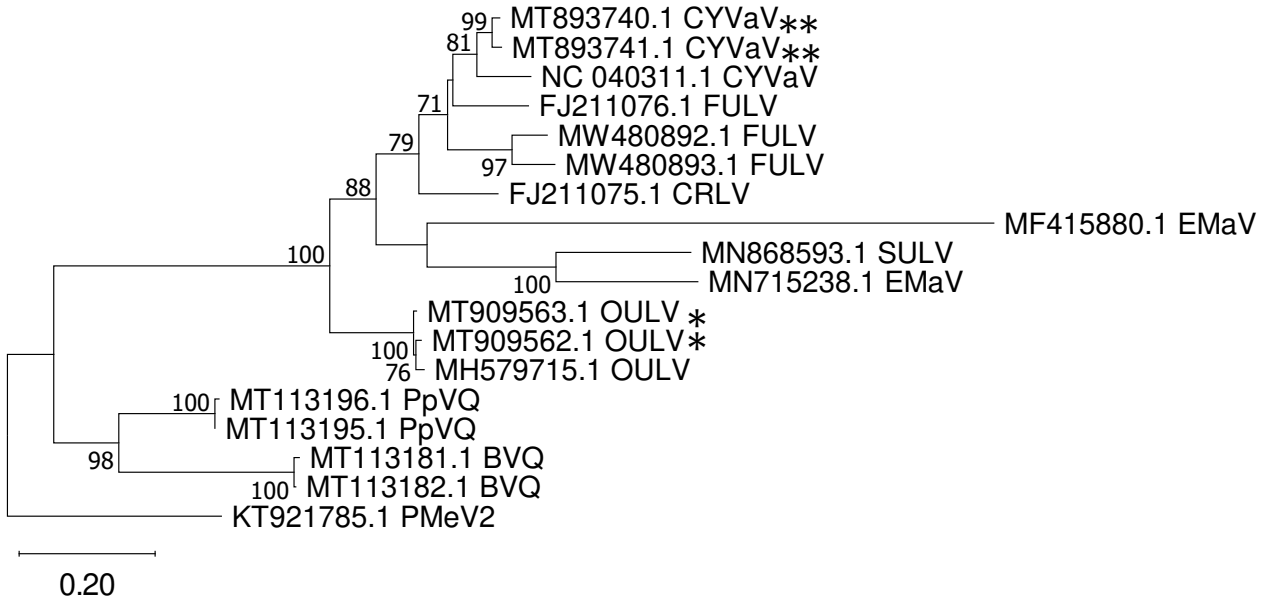

Figure 2.3 Phylogenetic analysis of RNA-dependent RNA polymerase gene sequences of citrus yellow vein-associated virus (CYVaV) and opuntia umbra-like virus (OULV) obtained from hemp samples collected during the 2019 field season in Colorado. Sequences were aligned and analyzed along with top sequence matches and closely related viruses of both detected viruses available in GenBank. The phylogram is rooted to *Papaya meleira virus* (PMeV2). Other viruses in phylogeny include: *Fig umbra-like virus* (FULV), *Fig luteovirus* (CRLV), *Ethiopian maize-associated virus* (EMaV), *Sugarcane umbra-like virus* (SULV), *Papaya virus Q* (PpVQ), and *Babaco virus Q* (BVQ). Scale bar indicates number of substitutions per site. Phylogenetic analysis by maximum likelihood method was done using ClustalW in MEGAX. Double asterisks indicate CYVaV and single asterisk indicate OULV genotypes from Colorado. Bootstrap values are indicated on the nodes.

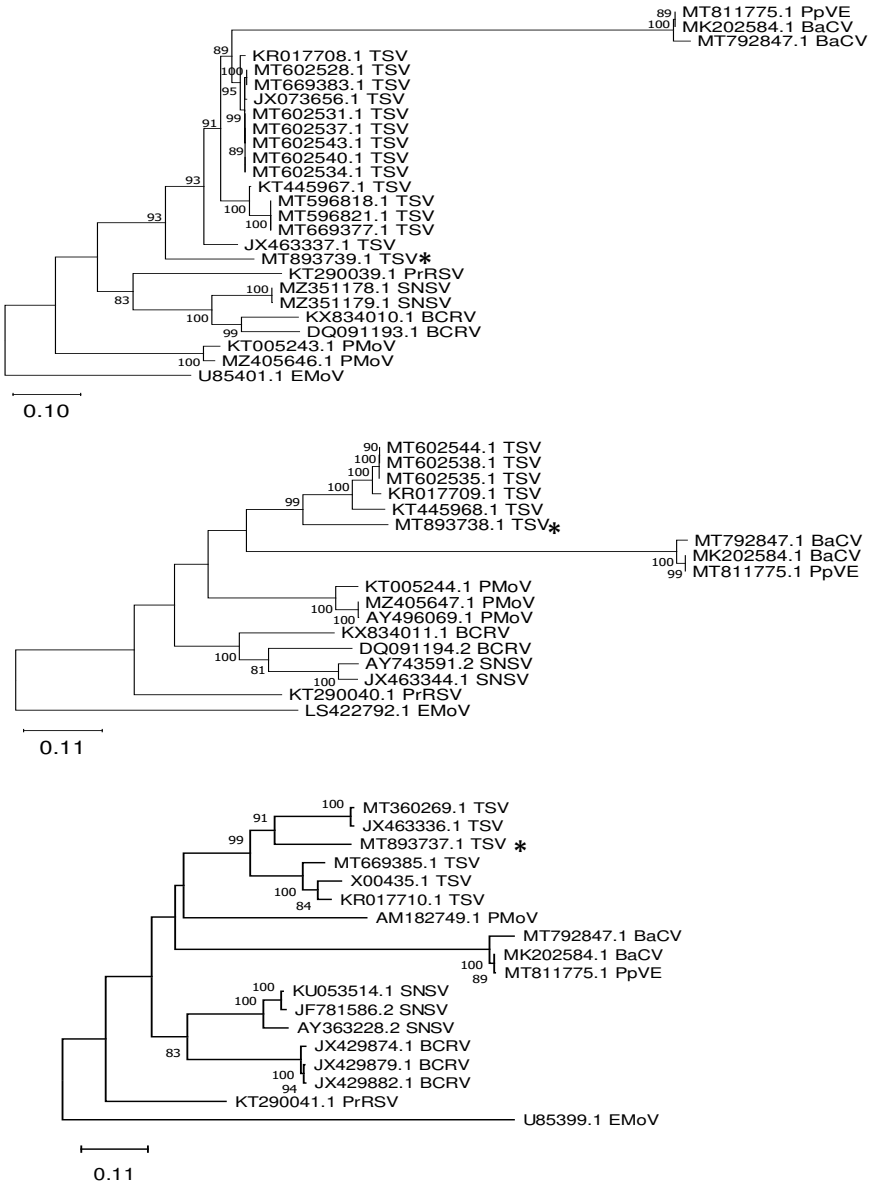

Figure 2.4 Phylogenetic analysis of tobacco streak virus (TSV) RNA 1-3 obtained from hemp samples collected during the 2019 field season in Colorado aligned with other TSV sequences and closely related viruses available in GenBank. (A) TSV RNA 1 encodes the replicase protein gene, (B) TSV RNA 2 encodes RNA-dependent RNA polymerase gene, and (C) TSV RNA3 encodes the movement protein. All three TSV RNA 1-3 trees were rooted to *Elm mottle virus* (EMoV) RNA1-3. Other viruses in trees include: *Papaya cytorhabdovirus* (PpVE), *Bean-associated cytorhabdovirus* (BaCV), *Privet ringspot virus* (PrRSV), *Strawberry necrotic shock virus* (SNSV), *Blackberry chlorotic ringspot virus* (BCRV), and *Parietaria mottle virus* (PMoV). Scale bar indicates number of substitutions per site. Phylogenetic analysis by maximum likelihood method was done using ClustalW in MEGAX. Asterisks indicate genotypes from Colorado from this study. Bootstrap values are indicated on the nodes. Bootstrap values less than 70% out of 1000 replicates are not shown.

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CHAPTER 3 – Dynamics of beet leafhopper (*Circulifer tenellus*) and beet curly top virus in Colorado hemp

Introduction

Curly top disease is caused by *Beet curly top virus* (BCTV) a member of the *Curtovirus* genus in the family *Geminiviridae* (Bennett, 1971). Over 300 different plant species are susceptible to BCTV, including agriculturally significant crops, and weeds which facilitates the transmission cycle of the virus through providing overwintering food and oviposition source for the beet leafhopper vector and virus reservoir. There are three species of Curtoviruses, which include Beet curly top virus (BCTV), Spinach severe surly top virus (SpSCTV) and Horseradish curly top virus (HrCTV). Beet curly top virus consist of nine distinguished strains: BCTV-California/Logan (CA/Logan), BCTV-Colorado, BCTV-Worland (Wor), BCTV-Mild (Mld), BCTV-Severe (Srv), BCTV-Severe pepper (SvrPep), BCTV- curly top (PeCT), BCTV-Pepper yellow dwarf (PeYD) and BCTV-Spinach curly top (SpCT) (Varsani et al., 2014). The different BCTV strains differ in their ability to infect different host plants, their ability to be acquired and transmitted by beet leafhoppers between hosts, the geographic range in which they occur and symptom types they cause (Chen et al., 2009, Chen and Gilbertson, 2016). BCTV strains are commonly found in different combinations of co-infection, in which they can compete with one another for dominant infection (Peinado et al., 2018, Creamer, 2020). Curtoviruses are present throughout the western United States, and can be found in California, Iowa, Idaho, Colorado, New Mexico Illinois, Maryland, Michigan, Minnesota, Nebraska, North Carolina, Ohio, South Dakota, Texas, Virginia, Washington, Wisconsin, and also in southwestern Canada and Mexico (Bennett, 1971, CABBI and EPPO, Strausbaugh et al., 2008, Lam et al., 2009,)

Beet curly top virus is transmitted in a circulative manner by the beet leafhopper (BLH), *Circulifer* (=*Neoaliturus*) *tenellus* Baker (Hemiptera: Cicadelidae), a polyphagous insect that feeds on fluids of the plant phloem (Cook, 1967). Beet leafhoppers can acquire the virus in as little as one minute of feeding on an infected plant and can begin transmitting the virus to other plants after the virus has fully circulated through the insects' gut and into the salivary glands for transmission, taking as little as four hours (Bennett and Wallace, 1938, Soto and Gilbertson, 2003). Once the virus has been acquired by the insect, it can persist within BLH for a maximum of eight to ten weeks, gradually decreasing over time. This was observed on insects given and extended AAP to maximize viral titer and then were help on resistant crops (sweet corn) to observe their ability to maintain virus without the ability to recharge (Severin, 1924, Severin, 1934, Bennett and Wallace, 1938.

There are several factors that influence the titer of BCTV transmission by BLH into a plant, and the titer acquired by the insect between types of plant. A higher density of viruliferous insects on a plant and extended length of feeding time are two elements that will increase the quantity of virus transmitted between insects to the plant (Soto and Gilbertson, 2003). There is a sex and age effect that impacts rate of transmission by BLH. Male BLH are more efficient than females, young adults are more efficient than older adults and first instar nymphs are more efficient than later instar nymphs at BCTV transmission (Bennett, 1962). Younger plants are also more susceptible to BCTV infection than older plants (Giddings, 1942, Duffus and Skoyen, 1977). Feeding time of a BLH on an plant influenced the quantity of virus acquisition by the insect, host plants and preference increase the amount of time a BLH will spend time feeding (Bennett, 1962). When evaluating tomato (a non-host) and sugar beet, both of which are highly susceptible to BCTV, BLH will acquire higher BCTV viral titers to sugar beet because they have

a higher feeding preference and ability to be hosted (Chen and Gilbertson 2009). They will more likely be observed to settle, feed and oviposit on sugar beet, in contrast to tomato where they will be observed feeding for shorter periods and moving around more frequently. Longer acquisition access (AAP) and inoculation periods (IAP) will result in higher rates of transmission by BLH (Bennett and Wallace, 1938). Virus strain also contributes to the successful inoculation of virus. In a BLH transmission study, BCTV-Severe, which is associated with BCTV-Severe strain, acquired from sugar beet had a higher acquisition by the BLH and transmission rate than observed with BCTV-Wor (Chen and Gilbertson, 2009). Understanding the migratory patterns of BLH is a critical step to disrupt transmission cycles. Younger plants tend to be more susceptible and sensitive to BCTV infection, timing early planting to secure older plants before BLH flights can be beneficial for crop yield (Wang et al., 1999).

In California, BLH overwinter in the coastal range west of the San Joaquin valley, where they oviposit and survive on annual weeds, which also serve as a reservoir for BCTV. In mid to late spring, as the temperature begins to warm up and rain initiates germination of new weeds, nymphs emerge acquiring BCTV from the infected plants they emerged and migrate from drying weeds and migrate between crops and weeds into the valley as different hosts become available (Hallock and Douglass, 1956, and Stahl, 1920). Eventually BLH travel into agricultural crops, transmitting BCTV as they travel and reproduce (Cook, 1967). BLH return to overwintering sites in the late fall as temperatures cool and crops are harvested. In Washington and Oregon, the Colombia basin is an overwintering and breeding site for BLH, with emergence as early as April running through May (Hills, 1937, Cook, 1942, Munyaneza et al., 2005). Populations peaks vary by year, generally with an initial peak in May, with a general increase in density in warm summer months of June through August and an end-of-season peak in September through

October that source overwintering BLH populations (Murphy et al., 2012). In New Mexico, historically the Rio Grande valley is a prominent spring and winter breeding site for BLH, from which they migrate north and east throughout northern New Mexico, west Texas, Oklahoma, Kansas and Colorado (Romney, 1939). A 2001-2002 survey of southern New Mexico chili fields found BLH at all sites, beginning in March and April, with population speaking in June and August. BCTV infected weeds were detected at these fields June through September, corresponding with the timing of peak density BLH (Creamer at al., 2003). Migration timing also impacts agriculturally significant crops by region, such as tomato in California, common bean in Washington and chili pepper in New Mexico (Carsner, 1926, Goldberg, 1999, and Chen et al., 2010). Disease incidence and severity varies by crop and year and is difficult to predict.

The migration BLH was studied between the years of 1931 through 1938 to understand the timing of leafhopper abundance between eastern Utah and Western Colorado. BLH were observed as early as May and April, depending on the temperature of the winter and were hosted on early germinated Russian thistle, *Kali tragus* (Cook, 1967). BLH overwinter utilizing hosts like *Atriplex corrugata* and transition to perennial weeds as they become available in the warmer months. There is a territory south of grand Junction Colorado that runs across the Western Slope of Colorado through Utah and Nevada that is essentially free of winter BLH (Cook, 1967). Overwintering sites that drive Colorado BLH populations are from northern Utah near the Salt Lake Basin, sitting at an elevation around 4,000 ft, and southern Arizona (Knowlton, 1932, Douglass, 1954). These areas are populated as early as April and May depending on host plant availability and temperature. Regardless of region, a common lifestyle strategy of the BLH is to utilize weed hosts to facilitate migration as they seek suitable hosts for oviposition, including mustards *Sisymbrium irio* and *Descurainia sophia* (London rocket and flixweed), *Amaranthus*

retroflexus (pigweed), *Anoda cristata*, *Kochia* sp., and *Chenopodium* sp., *Amaranthus* sp., *Anoda cristata*, *Datura* sp., *Salsola iberica*, *Physalis wrightii*, *Solanum elaeagnifolium*, *Malva parviflora*, *Sphaeralcea ambigua* and *Descurainia sophia* (Cook, 1967, Bennett, 1971, Lam et al., 2009). Knowing that BLH use incidental weed species provides an opportunity to perform vector-virus surveillance in association with profitable crops such as hemp in Colorado.

Beet curly top virus was discovered to infect hemp in 2018 (Giladi et al., 2020). It is not yet well understood the relationship between BLH and its ability to survive and reproduce on hemp, which this study addresses. Hosts of BLH can support the survival and reproduction of the vector, non-hosts can be susceptible to the BCTV, however high mortality or lack of oviposition of the insect is observed in association with these plants. This has been observed in bean, tomato and chili pepper (Munyaneza and Upton, 2005, and Senado et al., 2012). Understanding the interaction between BLH and various crops is valuable in gaining insight on BCTV transmission cycles. For instance, BLH typically die after feeding on chili yet BCTV remains an agricultural threat in chili cultivation.

Life history assays have been performed to analyze survival, settling behavior and development of leafhopper in various crops and BCTV infected versus non-infected sugar beet (Severin, 1946, Munyaneza and Upton, 2005). Crops like bean and tomato are both susceptible to BCTV and serve as poor hosts to the BLH. High mortality and low reproductive capacity of BLH has been observed on these crops (Munyaneza and Upton, 2005). When evaluating the influence of BCTV on the development time of BLH, it was found that BLH reared on BCTV infected sugar beet required up to 10 days longer to develop fully into the adult stage. Vector transmitted pathogens can have relationships that manipulate the insect vector or plant physiology to favor the persistence and encourage continued feeding or development by the

insect to encourage transmission (Kennedy, 1951, Miller and Coon, 1964, Keough et al., 2016, Chesnais et al., 2019). An example of these types of advantages are observed by *Frankliniella occidentalis* Pergrande (western flower thrips) where nymphal development and rate of survival is improved in *Tomato spotted wilt virus* infected plants compared to virus free plants (Belliure, 2005). It is this existing relationship where vectors benefit from virus-infected plants that we are interested in exploring these dynamics in the BLH-BCTV system.

This goal of this study was to assess the ecology and epidemiology of BLH and BCTV in association with Colorado's changing agricultural landscape, particularly in association with hemp. Vector-virus surveillance was performed to study the migratory patterns of BLH and the timing in which BCTV is observed in hemp, sugar beet and weed reservoirs in the north (Front Range) and western regions (Western Slope) of Colorado. Further, life history assays were performed to evaluate if any differences exist in the development and life history characteristics of viruliferous BLH in comparison to non-viruliferous BLH in both sugar beet and hemp crops. We hypothesize that BLH abundance will vary with time (between field seasons) and region (northern and western sites). Beet curly top will be detected in hemp, sugar beet, or weeds following peaks in BLH abundance. We hypothesize BCTV will positively impact BLH life history traits, due to the relationship between BLH and BCTV which evolved before the early 1900's and remains to be the only mode of transmission.

Materials and Methods

Field sites. This study surveyed four sugar beet fields in northern Colorado (Front Range) and two hemp fields in western Colorado (Western slope) in 2020 (Fig. 3.1A), and 11 hemp fields in northern and western Colorado in 2021 (Fig. 3.1B). Maps were designed using the geoprocessing

software, ArcGIS Pro version 2.6.0 (Esri Inc.). Field sites included in 2020 included two Montrose county hemp fields at: 38°38'34.7"N 108°03'31.8"W and 38°39'01.6"N 108°01'54.9"W, and four sugar beet fields in Larimer and Weld county at: 40°64'37.6"N 104°99'56.2"W, 40°43'05.3"N 104°92'60.3"W, 40°28'77.5"N 104°79'19.9"W, and 40°30'46.7"N 104°76'84.2"W. Hemp fields surveyed in 2021 included five fields in Larimer and Morgan county: 40.352444"N 105.020444"W, 40.2578889"N 104.060722"W, 40.770056"N 105.059889"W, 40.199972"N 104.059639"W, 40.61115"N 104.996896"W, and six fields in Mesa and Montrose county: 38°38'34.7"N 108°03'31.8"W, 39.112855"N 108.371084"W, 39.022759"N 108.490561"W, 38.60994"N 108.07339"W, 38.5605"N 107.96069"W, 38.51111"N 107.965528"W. Fields located in Larimer, Weld and Morgan county are referred to in this study as the northern region, which is also east of the Rocky Mountains. Fields located in Montrose and Mesa County are referred to as the western region and are located in the western slope of Colorado.

Leaf tissue collection. Leaf tissue samples from hemp were collected in a zig zag pattern across each field location, consisting of three leaves from 20 individual plants per field. Tissue from sugar beet leaves were collected similarly to hemp, in a zig zag pattern across fields. However, one leaf from 20 individual plants were collected for testing each field visit. Hemp and sugar beet leaf tissue was pooled by field and date collected into 500 mg samples, for detection of BCTV by ELISA testing. A total of 500 mg of plant tissue was used to perform each BCTV ELISA test. Hemp and sugar beet leaf tissues were collected bi-weekly from each field beginning the end of June through the timing of hemp harvest in the middle of September.

Survey of beet leafhoppers. Sweep sampling of BL was performed in and around the surrounding areas of designated hemp fields. Sweeps targeted the weeds surrounding the perimeter of the hemp fields. Approximately 0.5 acres were surveyed for BLH, at each field bi-weekly, and included a total of 400 sweeps with a 38cm diameter canvas net. The same net was used at all fields at each date, and all insect sampling was performed by the same person. Insects were aspirated from the sweep net and stored in Ziplock bags on ice while being transported to the Colorado State University laboratory for identification by dissecting scope. Identification of BLH was performed through morphological characteristics observed by dissecting scope. Dr. Chris Dietrich, from the University of Illinois was consulted for his expertise in leafhopper identification, described a key characteristics for the identification of beet leafhoppers is the square shape of the male subgenital plate (Fig. 3.2) (Young and Frazier, 1954, Dietrich, 2005). Other leafhopper species, *Empoasca sp., Balclutha neglecta* DeLong & Davidson*, Macrosteles quadrilineatus* Forbes*, Ceratagallia uhleri* Van Duzee*,* and *Exitianus exitiosus* Uhler, were frequently collected in sweep samples and were identified by collaborator Dr. Dietrich. These leafhopper species were tested for presence of BCTV, to evaluate potential vectors other than BLH. No BCTV was detected in previously mentioned leafhopper species.

Collection of weed samples. Weeds were collected around the perimeter of each of the 11 hemp fields surveyed. Weeds were collected by taking the entire weed or an approximate 1 g from a section of the plant. Beginning in April of 2021, 1-15 samples of present weed species were collected from each field every two weeks and organized by species. Weeds were identified by collaborators at Colorado State University including Melissa Franklin and Janet Hardin. Survey collaborators who assisted field visits included Kaitlyn Langemeier, Shanthini Ode, Laine

Hackenberg and Jordan Withycombe. Some variation in weed sampling existed due to phenology and occurrence between sites and regions. Samples were collected by randomly selecting Tissue samples were taken from 42 weed species, including: *Chorispora tenella* Pallas (blue mustard), *Kochia scoparia* Schrad, *Sinapis alba* Linnaeus (L.) (yellow mustard), *Malva parviflora* L. (common mallow), *Conyza canadensis* L. Cronq (horseweed), *Amaranthus retrofelxus* L. (red root pigweed), *Asteracea* sp., *Lepidium draba* L. (white top), *Cichorium intybus* L. (chicory), *Lactuca serriola* L. (prickly lettuce), *Plantago lanceolata* L. (narrow leaf plantain), *Helianthus* sp., *Convolvulus arvensis* L. (field bindweed), *Medicago sativa* L. (alfalfa), *Cannabis sativa* L. (volunteer hemp), *Rumex crispus* L. (curly dock), *Portulaca oleracea* L. (purselane), *Taraxacum officinale* Weber (dandelion), *Trifolium* sp. (clover), *Chenopodium album* L. (lambsquarter), *Erodium cicutarium* L. (redstem filaree), *Kali tragus* Mosyakin (Russian thistle), *Lactuca virosa* L. (wild lettuce), *Rhaponticum* spp. (Russian knapweed), *Sphaeralcea ambigua* Gray (globe mallow), *Mentha* sp. (Mint), *Brassica napus* L. (canola), *Cirsium arvense* L. (Canadian thistle), *Polygonum* sp. (knotweed), Pisum sp, (pea), *Ambrosia* sp., *Balsaminaceae* sp. (snapweed), *Sisymbrium irio* L. (London rocket), *Physalis wrightii* Gray (wrights ground cherry), *Hibiscus trionum* L. (venice mallow), *Tribulus terrestris* L. (puncture vine), and *Solanum rostratum* Dunal (buffalo bur), *Metzelia* sp., *Argemone albiflora* L. (prickly poppy), *Heterotheca villosa* Pursh (hairy goldenaster), *Salvia reflexa* Hornem (Rocky Mountain sage), and *Capsella bursa-pastoris* L. (shepherd's purse).

Detection of BCTV in hemp, sugar beet and weeds. Hemp tissue samples consisted of 20 pooled individual hemp plants, by field and date collected into microcentrifuge tubes with 500 mg of hemp leaf tissue and placed in 1.5 ml microcentrifuge tubes. Sugar beet leaf tissues were

processed identical to hemp. Following identification of weed species, 500 mg of leaf tissue was pooled from 1-15 plants of the same species by date and field collected and stored in 1.5 ml microcentrifuge tubes. For BCTV detection, hemp, sugar beet and weed tissue samples were homogenized using two Tungsten carbide beads (3 mm) using a Qiagen TissueLyser II bead mill for 60 seconds. Samples were tested for the presence of BCTV using an Enzyme-linked immunosorbent assays (ELISAs) by Creative Diagnostics®. Each plate contained a BCTV positive and negative as controls, and all tests were run in duplicates. Positives were determined through spectrophotometric quantification performed by an ELx800™ Universal Micro Plate Reader by Biotek instruments, inc. Test absorbance values that were double or greater than the score of negative controls were determined to be positive. Beet curly top incidence across fields was calculated by the percentage of fields where BCTV was detected by the total number of fields surveyed by region. Individual plant samples are currently being processed to determine BCTV incidence per plant.

Life history assays. BLH used in life history assays were originally collected in Idaho (Dr. Oliver Neher, Kimberly, ID, USA) and maintained on BCTV-Severe infected sugar beets. history assays were performed on both sugar beet (BPA90000) and hemp (Elite). Sugar beet plants that were six weeks old at the 13-15 leaf stage were used, and hemp were used at two weeks old at the two true leaf stage. Life history assays were performed using both crops, with BCTV viruliferous and non-viruliferous BLH. Non-viruliferous leafhopper were acquired through two methods, the first was by watching nymphs emerge from under a dissecting microscope from plant tissue and removing them with a fine paintbrush and relocating them to a non-infected sugar beet plant prior to exposure and acquisition of BCTV by infected meal. The

second method used was by rearing BCTV-viruliferous leafhopper on corn and removing nymphs as they emerge every few days and relocating them to sugar beet to build up a sustainable non-viruliferous population. Late instar BLH nymphs were aspirated and stored in a holding cage where their development was observed and timed out to be placed in male/female pairs on sugar beet plants once they were three days old. Viruliferous BLH infected the host plant they were reared on (hemp or sugar beet), this measured the fecundity of viruliferous BLH and how nymphs developed on BCTV infected hosts. The preoviposition period of BLH found in the field in July at a mean temperature of 80˚F was recorded to be three days (Severin, 1930). BLH were given a 7-day period for oviposition and were removed on the seventh day. Assays were observed every other day for the first emergence of nymphs from eggs until all nymphs were developed into adults. Sugar beet life history assays were conducted in a growth chamber with a 16:8 light:dark photoperiod at 32-34°C. The same process was repeated using leafhopper in hemp, except experiments took place in a greenhouse due to size difference of plant. Assays were also set using a total of 6 adult leafhopper, 2 males and 4 females per assay.

Statistical analysis. All data analysis was conducted using R statistical software (R-Core Team 2021, Version 4.0.2. Field incidence of BCTV was analyzed using a Fisher's Exact Test for Count Data to determine if differences in the percentage of fields that tested positive for BCTV between years and region exist. This test was performed between hemp and sugar beet from the 2021 season, between northern and western field sites from the 2020 season, hemp from the western sloped in 2020 and 2021 and overall BCTV field incidence between 2020 and 2021. Weed data was analyzed by using a Fisher’s Exact Test to evaluate the proportions of pooled weeds that tested positive for BCTV in relation to pooled weeds that did not and determine if

there is a statistically supported difference in frequency. This test was performed between weed species that detected BCTV. Weed incidence between northern and western survey regions was tested using a Pearson's chi-squared test. Tests were performed to determine if differences in incidence exist with support by a p-value $\alpha < 0.005$. Life history data was analyzed using a Mann-Whitney U Test to determine if there were any differences in means supported by a p-value $\alpha < 0.05$.

Results

BCTV incidence in hemp. In 2020, there was higher BCTV field incidence 100% (2/2) in hemp than in 2021 (9%, 1/11). In 2020, there was higher BCTV field incidence in hemp100% (2/2) than in sugar beet 25% (1/4). In the western survey region, 2020 experienced a higher level of field incidence of BCTV 100% (2/2) than in the 2021 season 17% (1/6). In 2021, there was higher BCTV incidence in hemp from fields surveyed in the western region 17% (1/6) than the northern region 0% (0/5) (Fig. 3.3A & 3.3B). Beet curly top virus incidence varied by region and year in Colorado.

BLH abundance. The first BLH observed in the western region hemp, on May 5th (Fig. 3.3A). Beet leafhopper activity was detected for the first time in the northern region on June 14th (Fig. 3.3B). There was one major peak in BLH abundance in the western region hemp on July 21st, two months after BLH activity was first detected. There were two peaks observed in BLH abundance in northern hemp sites, first on July 14th and then a gradual peak that reached a maximum abundance on September 10th. In both regions, BLH populations peaks took a month

to build after initial detection. There was an overall greater abundance of BLH in the northern region than in the western survey region.

BCTV incidence in weed species. Weed survey results detected BCTV from nine different weed species 21% (9/42), and a total of 47 pooled samples (Table 3.1). The total number of weed samples tested were 1,090, of which 4% (47/1090) of them tested positive for BCTV. Beet curly top virus was detected at the highest incidence from: horseweed 50% (1/2), dandelion 44% (16/36) prickly lettuce 32% (18/56), and chicory 27% (3/11). Statistical evidence by the Fisher's Exact Test supports higher proportion of incidence of BCTV in dandelion, prickly lettuce and chicory in relation to other weed species that tested positive (Table 3.2).

Life history of beet leafhoppers in sugar beet and hemp. There was a significant difference in mean of nymphs produced between viruliferous and non-viruliferous BLH on sugar beet Viruliferous BLH produced 19.4 ± 1.8 (n=27) and non-viruliferous BLH produced 13.9 ± 1.1 (n=39) nymphs (u = 338.5, *p-value = 0.0143*) (Fig. 3.5A), demonstrating a fitness advantage of more offspring produced by viruliferous leafhopper. There was no statistical difference of mean observed in development time (u = 291.5*, p-value = 0.225*), number of adults produced (u = 161, *p-value = 0.059*) or nymph survival (u = 358, *p-value = 0.358*) (Fig. 3.5 B-D).

There were no statistical differences of means observed between nymphs produced (u = 52.5, *p-value = 0.107*), development time (u = 291.5, *p-value = 0.646*), adults produced (u = 67, *p-value = 0.507*) or nymphal survival (u = 79, *p-value = 0.979*) between viruliferous and non-viruliferous BLH treatments on hemp (Fig. 3.6 A-D).

Discussion

Beet curly top virus has challenged agriculture production for over 100 years and remains a challenge because of the relationship between the virus and BLH vector. The mobility of BLH, the persistence of BCTV in viruliferous BLH, the polyphagous feeding, and wide range of BCTV susceptible plants are all characteristics that make the BCTV-BLH complex difficult to manage. In this study, evaluation of the seasonal abundance of BLH in hemp fields and the timing and level of incidence in BCTV associated with BLH activity was performed. To further understand the relationship between the BLH vector and BCTV, life history assays were performed to evaluate if the virus has any influence on BLH fitness and fecundity.

In Colorado, hemp grown in the Western Slope had higher incidence of BCTV than northern fields. Higher rates of BCTV infection correlate to the level of exposure crops have to viruliferous BLH. There was higher level of detection of BCTV 6% (2/36 pooled insect samples) in BLH samples in the western survey region than in the northern survey region 0% (0/27 pooled insect samples), which may in part explain the higher incidence in western region compared to northern region. . Although there was overall low BCTV observed in surveyed BLH, these finding align with observations of level of BCTV viruliferous BLH and BCTV incidence in hemp fields, which corresponds to findings from previous studies in other agricultural crops (Bennett and Wallace, 1938, Bennett, 1971, Chen et al., 2010).

Beet leafhoppers and BCTV infection impacts different crops by regions, such as chili pepper in New Mexico and tomato in California, demonstrating regional crop preferences by BLH populations (Creamer et al., 2003, Chen at el., 2010). Crop preferences could be correlated to migration of BLH from different biotype populations by region, and this should be further investigated. Hence, it is possible that BCTV infection and transmission capacities may differ by

hemp cultivars. We hypothesize that hemp varieties in the northern region may have some level of natural resistance to BLH and BCTV. Hemp resistance breeding should be further studied and developed as a pest management strategy in hemp cropping systems.

Beet curly top virus was detected most frequently from weed reservoirs dandelion, prickly lettuce, and chicory. In total, BCTV was detected from nine different weed species from this study, some of which have not previously been detected in the BCTV-BLH transmission complex. Weed reservoirs contributing to BCTV transmission cycles in New Mexico chili cropping systems include kochia, London rocket, Russian thistle and pig weed (Cook, 1967, Creamer et al., 2003, Lam et al., 2009). These are weed species that have not been previously recognized to facilitate BCTV transmission cycles. These BCTV weed reservoirs differ from previous findings of globe mallow, ground cherry, flixweed, common mallow, nightshades (*Solanum elaeagnifolium* and *Datura* sp), spurred anoda, amaranth, and *Chenopodium* sp. Weed species occur at different times and in different regions of the western United States, resulting in different weeds will have a different role in transmission between geographic regions. Dandelion, prickly lettuce and chicory are winter bridge annual weed species, that facilitate virus transmission between seasons. Northern Colorado experiences higher precipitation in comparison to western Colorado, which experiences higher drought. Moister conditions are favored by weed species that can thrive in non-irrigated regions of surrounding hemp fields (National Drought Mitigation Center, 2020). Increased moisture conditions contribute to higher BCTV incidence in the northern surveyed weeds in comparison to weeds surveyed from around western hemp field. This study observed higher BCTV incidence in weeds than in hemp crop, which is different from the findings from surveyed chili pepper crop in New Mexico which found greater BCTV incidence in chili crop than weed reservoirs (Creamer, et al., 2003).

Beet leafhopper abundance was greater in hemp from the northern survey region than in the western Colorado hemp fields. Abundance of BLH peaked in the northern hemp fields in June and a month later in the western hemp fields observed, despite BLH activity occurring earlier in the western survey region than the northern survey region. The higher moisture of northern Colorado supports weed species, which in-turn host BLH populations in the north resulting in higher BLH abundance (National Drought Mitigation Center, 2020).

Beet leafhopper migrate north to Colorado beginning as early as March through May from warmer mild wintered states Texas, Arizona and southern New Mexico (Romney, 1939, Creamer et al., 2003). Beet leafhopper flights can disperse anywhere from 100 to 300 miles, invading agricultural districts and feeding on wild plants throughout May and June (Annand, 1931, Annand and Davis, 1932, Wintermantel et al., 2003).

There was an overall greater abundance of BLH in 2020 than in 2021, these finding maybe associated with previous season precipitation. In 2019, there was relatively higher rainfall for Colorado with an average precipitation of 15.09 inches, whereas average rainfall for 2020 was 12.96 inches (Climate.colostate.edu). Wet winter and spring seasons with higher precipitation result in the prolonged presence of weed species and the early germination in the following season (Hills, 1937, Murphy et al., 2012).

Beet curly top virus was detected before beet leafhopper activity was detected in the western survey region supporting evidence that BCTV overwinters in these annuals and is picked up by BLH as they migrate into Colorado from eastern Utah from southern states Arizona and New Mexico. Beet curly top virus was detected in hemp from western fields at the end of June, which was a month after hemp was planted. Beet leafhopper activity peaked three weeks later. This provides evidence that BCTV was sourced from weed reservoirs prior to BLH populations

reaching peak abundance. There was no BCTV detected in hemp in northern Colorado hemp fields, however BCTV was detected in weed reservoirs in early June, two weeks before BLH abundance reached its initial peak. Both regions had a similar trend of BCTV detection prior to BLH peaks by 2-3 weeks. This is different from what was observed in hemp in Arizona, where BLH abundance peaked before BCTV infected hemp was collected in July from south-eastern Arizona fields (Hu et al., 2021). However, many studies evaluating BCTV incidence do not measure corresponding BLH abundance in association with BCTV throughout the season. They typically measure BCTV incidence at the end of the season, close to harvest. Understanding when BCTV infection in crops begins and corresponding leafhopper seasonal abundance is insightful for the development of new pest management strategies and when to apply them to the pest system.

This study found positive impacts of BCTV on BLH, by increasing vector fecundity. There was no difference in BLH life history traits between viruliferous and non-viruliferous BLH reared on hemp. Beet curly top virus infected BLH produced more offspring than non-viruliferous BLH, which is a beneficial characteristic to both BLH and BCTV. An increase in offspring is an evolutionary achievement by BLH enhanced by BCTV, but also, more nymphs increase transmission and disease incidence benefiting the virus (Sisterson, 2009). There have been limited investigations devoted to understanding the relationship between BVTC and BLH vector. There is one study that demonstrated BLH nymphs reared on BCTV infected sugar beet require up to 10 days longer to develop into adults. This is seemingly a negative impact BCTV has on BLH, as it delays the amount of time it takes to reach sexual maturity for reproduction. Slower development time by BLH is favorable to BCTV, as nymphs are more efficient at transmission (Bennett, 1962). The development stage of adolescent insects is focused on feeding

for successful development into adults. Delayed development ensures more time dedicated to feeding and in turn virus transmission. Unlike the previous study, I did not find statistical difference in development time between BLH reared in infected and non-infected hemp and sugar beet hosts. The difference in findings could be due to differences in experimental design. Bennett observed the development of individual viruliferous and non-viruliferous BLH nymph development, whereas my study was initiated using viruliferous and non-viruliferous adult pairs and measure developing offspring on sugar beet infected by parents.

Beet curly top virus is a persistent non-propagative virus that is maintained in the insect's body through molts and for extended periods ranging from weeks to months post acquisition without replicating within the vector. Persistent viruses can have a range of effects on associated vectors, from positive, neutral to negative (Lowe and Strong, 1963, Miller and Coon, 1964, Keough et al., 2016). Persistent non-propagative viruses can have direct impacts on associated insect vectors, such as increased mobility, phloem ingestion and higher fecundity in aphid species *Myzus persicae,*Sulzer in association with *Turnip yellows virus* (Chesnais et al., 2020). Indirect impacts made by the viruses target the plant host and can inhibit plant defenses executed by the jasmonic pathway to upregulate nutrients for the insect vector to thrive and improve conditions to increase survival and fecundity as well as virus transmission (Rajarapu et al., 2021). Regardless of these impacts being direct or indirect, these life history modifications influenced by the virus have all evolved in favor of virus transmission. The impact of BCTV on BLH is likely associated with increased nutrient availability to BLH by BCTV infected sugar beet, resulting in an indirect effect on BLH. A strategy than can explore whether or not these benefits are direct or indirect would be to rear viruliferous and non-viruliferous BLH on a non-susceptible host such as corn, to determine if the source of beneficial induced by BCTV. Because

BCTV does not replicate within BLH, it is likely an indirect benefit induced by the plant. The difference observed was in the number of offspring produced by BLH adults that were viruliferous or non-viruliferous. Virus infected sugar beet hosts help increase BLH fecundity. There were no differences observed in BLH offspring development on infected or non-infected sugar beet. If there was, that would demonstrate an indirect effect of the virus on BLH.

This study reveals the ability of BLH to survive and reproduce on hemp by evaluating the performance of 150 adult viruliferous and non-viruliferous BLH, in life history assays on hemp. Of these adults, 57 ± 4% survived a seven-day period. We were aware of BLH ability to feed on hemp at a minimum of the similar capacity they do on chili pepper and tomato because of their vectoring capacity and the observance of BCTV infected hemp. However, this provides insight on the ability for BLH to use hemp as a host. BLH have limited ability to replace themselves on hemp, with a mean of each female being able to replace herself with 1.375 ± 0.337 (n=24) adults. In turn, mated pairs are unable to replace themselves with two leafhoppers, indicating they are unable to generate a sustainable population on this host. There are positive impacts by BCTV on BLH when utilizing sugar beet host, but not experienced on non-host hemp.

Understanding that there is a fitness advantage of viruliferous BLH in producing more offspring, it would be of great interest to evaluate the differential gene expression between viruliferous and non-viruliferous BLH. Identification of differentially expressed genes would provide target genes that can be utilized for RNAi technology development. This could be used in the development of a biopesticide that can inhibit virus transmission at a genomic level, and therefore would also be specific enough to only impact BLH species and not threated incidental species that may occupy similar or overlapping niches. There is also room for investigation of whether virus infected sugar beet and or hemp have push-pull responses to BLH. If differences in

preference between viruliferous and non-viruliferous insects for virus infected and non-infected hemp and sugar beet exist, this could serve as the precursor for volatile organic compound (VOC) analysis and lure development as a trapping strategy. The emission of unique VOCs by BCTV infected plants is another strategy that the virus could be manipulating the vector or infected plant to increasing virus transmission and disease.

With the current regulations limiting pesticide use in hemp production, breeding programs focusing on pest and pathogen defenses is critical in pest management of this crop. Susceptible crops that become infected serve as reservoirs that contribute to transmission cycles. The development and incorporation of resistant hemp cultivars will help lower BCTV incidence and yield loss. Weed control through herbicide application and periodic mowing will reduce weeds that host BLH populations and serve as BCTV reservoirs. Continued communication between Colorado State University Research and Extension with growers will guide research focus to address grower concerns. These relationships will increase communication in agricultural research and production and facilitate the implementation of problem-solving strategies to promote more sustainable agricultural practices.

Tables and Figures

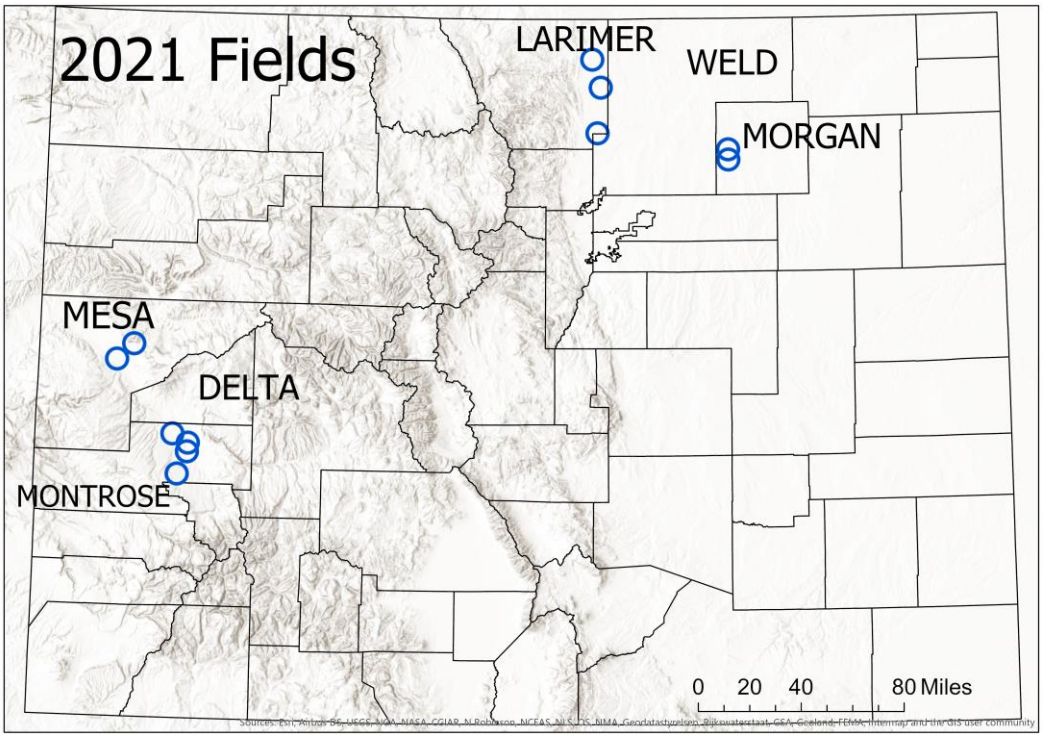

Figure 3.1 Maps of hemp fields in northern and western Colorado surveyed. Field locations of hemp fields are indicated by blue circles located in Larimer and Morgan county in the north and Mesa and Montrose county in the western survey region.

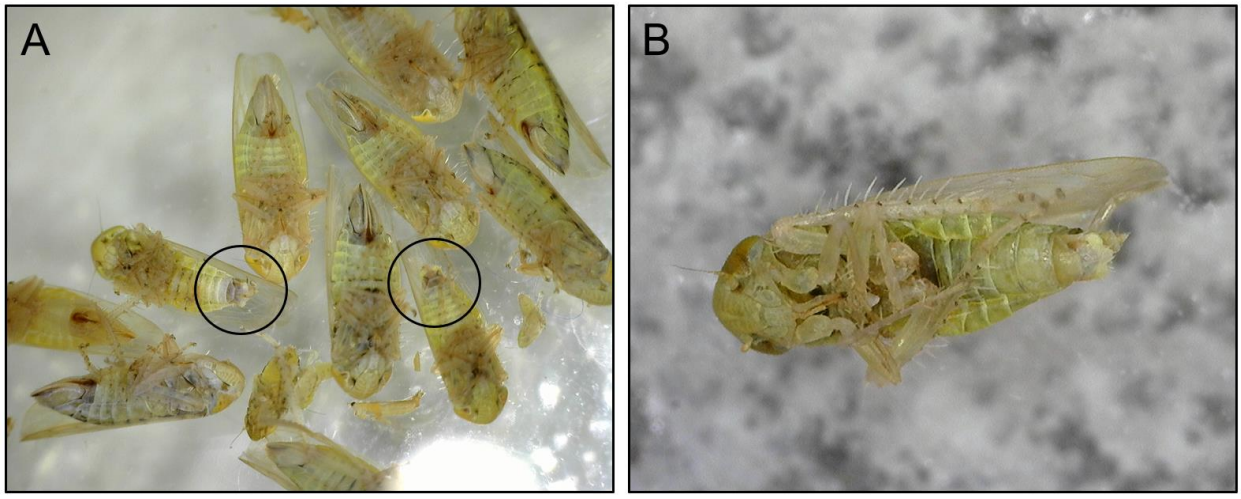

Figure 3.2 Micrographs of field captured beet leafhopper under a dissecting scope, exposing ventral characteristics of BLH. The key characteristic for identification of beet leafhopper morphologically is the shape of the male subgenital plate. A) includes both male and female beet leafhopper with circles emphasizing the subgenital plates of males. B) is a higher magnification image of a male beet leafhopper. Male characteristics are required for external morphological species level identification of BLH.

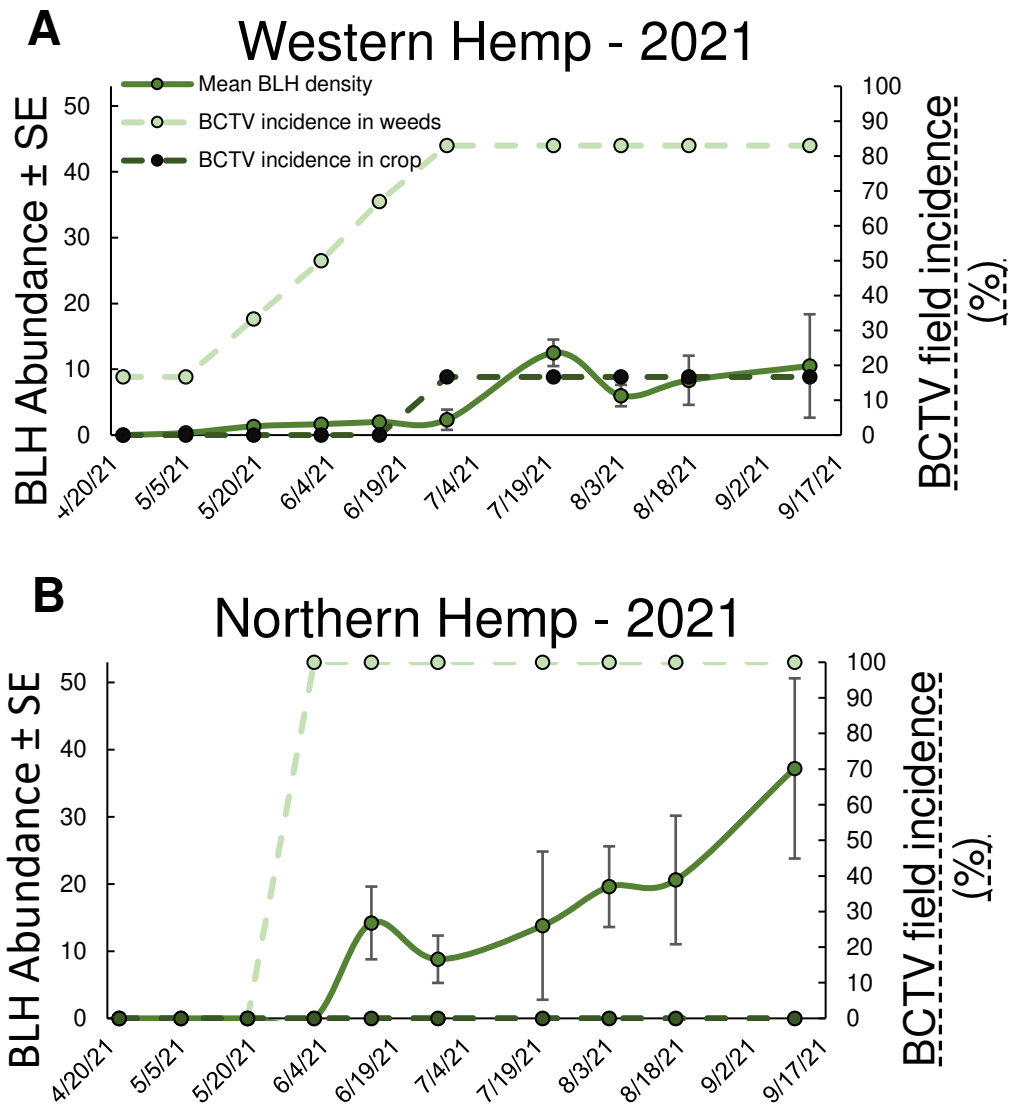

Figure 3.3 Beet curly top virus (BCTV) field incidence in hemp and weeds and corresponding abundance of beet leafhoppers in western slope of Colorado. A) western region in 2020. B) western hemp region in 2021, and C) northern region in 2021. Field incidence was calculated as the number field where BCTV was detected from either hemp crop from the individual field by date or by pooled weeds by species from designated field by date over total number of fields within the specific region. Beet curly top virus was detected from hemp and weed samples using BCTV ELISA assay. Beet leafhopper abundance was calculated as total number of leafhoppers per 400 sweeps in 0.5-acre area by sample date. Beet leafhopper abundance includes associated standard error to BLH collected by date.

Table 3.1 BCTV incidence in pooled weed samples from northern and western hemp fields in 2021. Single asterisks (*) indicate statistical significance in the percentage of BCTV occurrence in dandelion, prickly lettuce, and horseweed in relation to other weed species **$\alpha < 0.05$, all values are included in Table 3.2**. Double asterisks (**) indicate statistical significance that there was a higher percentage of BCTV detected in pooled weeds from northern sites than in the western survey region (u = 10.396, *p-value = 0.001*).

BCTV incidence in pooled weed samples – 2021			
Weed species	**Overall**	**North**	**West**
Dandelion *	44% (16/36)	52% (14/27)	22% (2/9)
Prickly lettuce *	32% (18/56)	35% (12/34)	27% (6/22)
Field bindweed	4% (3/73)	5% (1/22)	4% (2/51)
Chicory *	27% (3/11)	--	27% (3/11)
Russian thistle	4% (2/47)	0% (0/16)	3% (1/31)
Kochia	2% (2/85)	3% (1/32)	6% (2/31)
Red root pigweed	2% (1/53)	0% (0/22)	2% (1/53)
Horseweed	50% (1/2)	100% (1/1)	0% (0/1)
Alfalfa	2% (1/54)	5% (1/21)	0% (0/33)
% BCTV+ pooled weeds out of BCTV associated pooled weed samples	11% (47/417)	17% (30/175) **	7% (17/242)
% BCTV+ pooled weeds out of total weeds surveyed this season	**4% (47/1090)**		

Table 3.2 Evaluation of incidence between BCTV positive weed samples. The proportions of BCTV incidence were evaluated between each weed species to determine if there was a statistically supported association to BCTV incidence by weed species. Statistical analysis was performed using a Fisher's Exact Test. Odds ratios and p-values associated with each comparison of weed species is found by following the column and row associated with both weed species.

Proportion of BCTV + incidence between weed species								
	Prickly lettuce 44%	Bindweed 4%	Chicory 27%	Russian thistle 4%	Kochia 2%	Red root pig weed 2%	Horse weed 50%	Alfalfa 2%
Dandelion 44% odds ratio p-value	1.679 0.272	**18.037** **P<0.0001**	2.101 0.485	**17.346** **P<0.0001**	**31.940** **P<0.0001**	**39.873** **P<0.0001**	0.805 1	**40.639** **P<0.0001**
Prickly lettuce 32% odds ratio p-value		**10.850** **P<0.0001**	1.259 1	**10.449** **0.0003**	**19.256** **P<0.0001**	**24.078** **P<0.0001**	0.480 1	**24.541** **P<0.0001**
Bindweed 4% X-squared p-value			**0.120** **0.027**	0.965 1	1.772 0.663	2.216 0.638	0.049 0.104	2.258 0.636
Chicory 27% odds ratio p-value				**7.997** **0.042**	**14.654** **0.010**	**18.015** **0.014**	0.408 1	**18.360** **0.014**
Russian thistle 4% odds ratio p-value					1.835 0.616	2.292 0.560	0.054 0.120	2.336 0.600
Kochia 2% odds ratio p-value						1.251 1	0.029 0.068	1.275 1
Red root pig weed 2% odds ratio p-value							0.027 0.072	1.019 1
Horseweed 50% odds ratio p-value								37.817 0.071

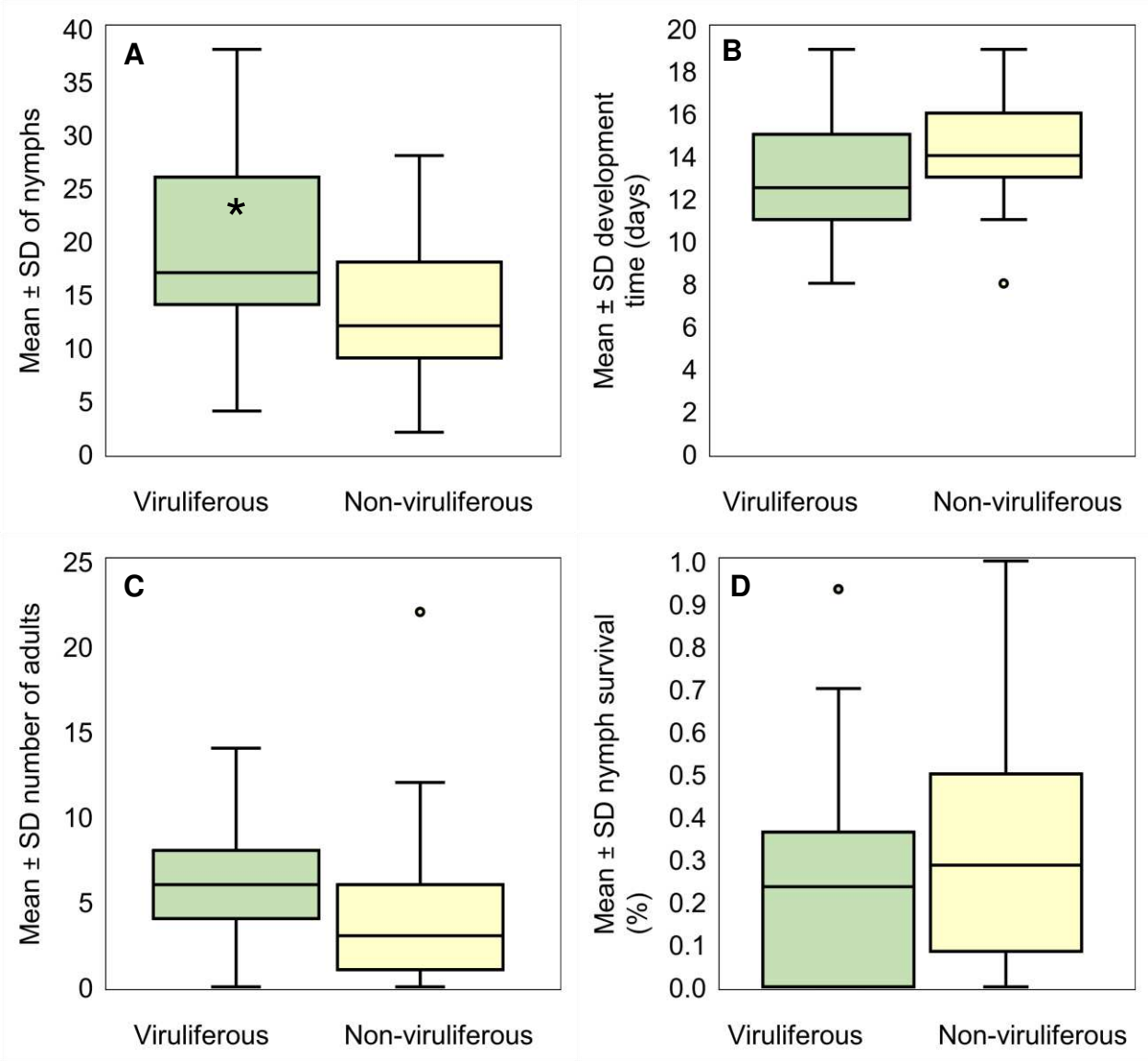

Figure 3.5 Life history of viruliferous and non-viruliferous BLH on sugar beet host. The box depicts the mean ± standard deviation. The whiskers represent the interquartile range. A) number of nymphs produced between treatments, marked by an asterisk (*) with a corresponding *p-value = 0.014*, B) development time, C) number of adults produced, D) nymph survival. There was no statistically significant difference of mean in development time, number of adults produced and nymph survival between treatments on sugar beet host.

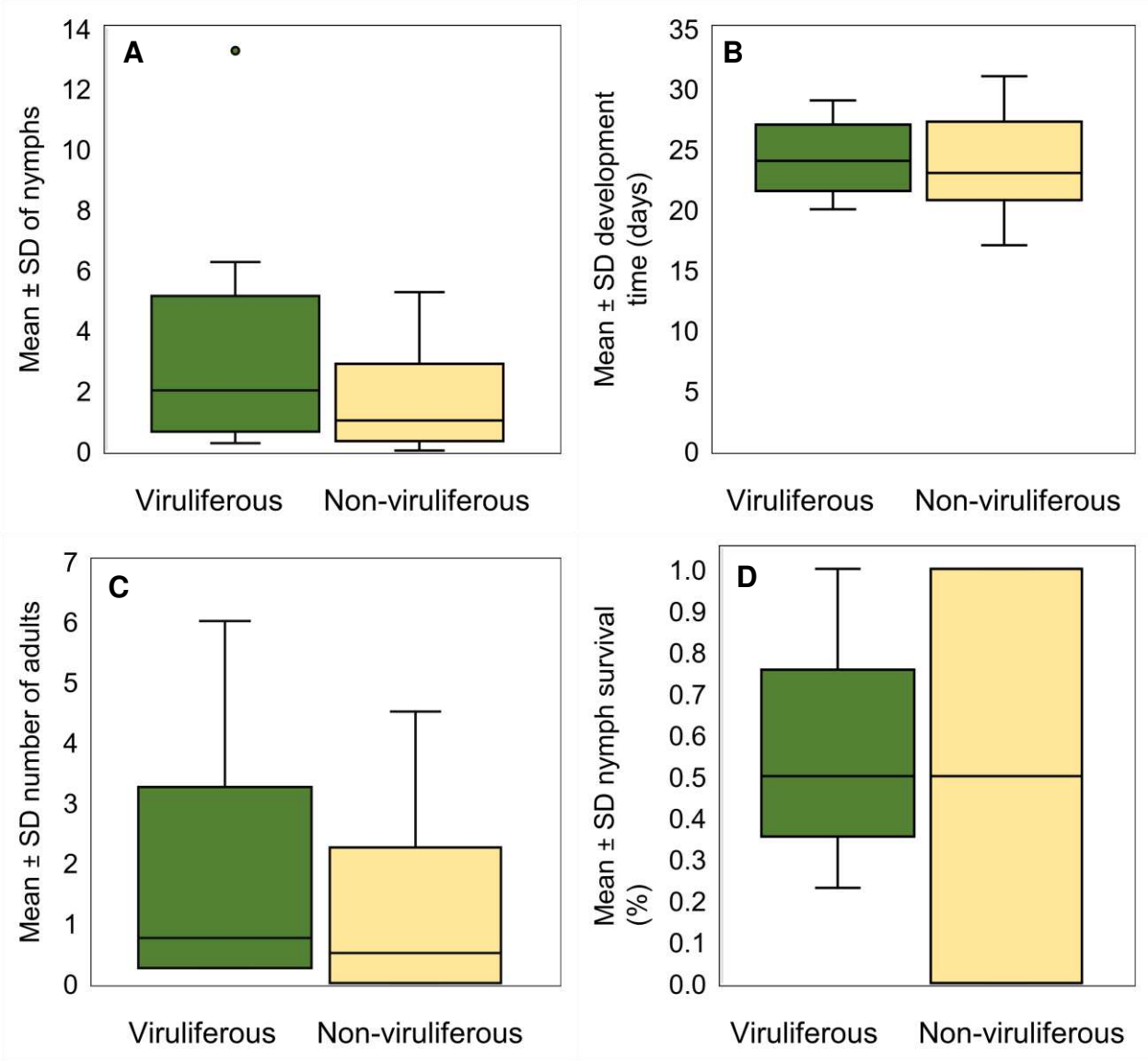

Figure 3.6 Life history of viruliferous and non-viruliferous BLH on hemp host. The box depicts the mean ± standard deviation. The whiskers represent the interquartile range. A) number of nymphs produced, B) development time, C) number of adults produced, and D) nymph survival. There was no statistically significant difference of mean in nymphs produced, development time, number of adults produced or nymph survival between treatments on hemp host.

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