THESIS

UNDERSTANDING THE PHYTOCHEMISTRY OF HIGH-CBD HEMP: EFFICACY OF COMMON ROW COVER MATERIALS FOR POLLEN EXCLUSION AND IMPACT ON FLOWER PHYTOCHEMISTRY

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2022

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ABSTRACT

UNDERSTANDING THE PHYTOCHEMISTRY OF HIGH-CBD HEMP: EFFICACY OF COMMON ROW COVER MATERIALS FOR POLLEN EXCLUSION AND IMPACT ON FLOWER PHYTOCHEMISTRY

Production of high-cannabidiol (high-CBD) hemp (*Cannabis sativa* L.) is steadily increasing in Colorado and across the United States. However, the impact of management practices for this crop remains relatively unexplored. For example, there is high potential for male hemp plants from fiber and grain cultivars to pollinate exclusively female high-CBD hemp plants grown in close proximity, but it is unknown how the phytocannabinoid content of high-CBD hemp flowers is affected by pollination. We hypothesized that high seed content resulting from pollination will negatively impact the phytochemical yield of high-CBD crops. In this study, three experimental pollen exclusion treatments were applied to two cultivars of high-CBD hemp, Cherry Uno and Wife. Treatments included non-woven thick row cover (largest pore size of approximately 50 microns), non-woven thin row cover (largest pore size approximately 200 microns), woven insect netting (average pore size 700x240 microns), and uncovered controls. A total of 60 high-CBD plants (clones) were planted in a randomized complete block design at the Colorado State University Agricultural Research, Development and Education Center South (ARDEC South) in Fort Collins, Colorado (lat. 40.611804 N; long. -104.997144 W; elevation 1525 meters). Total biomass and seed weights for 60 whole plants were evaluated. Additionally, 5 cm inflorescence samples were taken from each plant, in concordance with the 2019 Colorado Department of Agriculture (CDA) sampling protocol. Seeds and floral material were weighed

separately before samples were homogenized in preparation for phytocannabinoid analysis. Extracts were analyzed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) to determine the quantitative profiles of 20 phytocannabinoids. Results indicate that for the Cherry Uno cultivar, thick and thin row cover treatments effectively reduced pollination as compared to uncovered controls. The row cover treatments did result in a significant reduction of pollination for the Wife cultivar which may be due to later flowering in this cultivar. For Cherry Uno homogenized biomass, a significant reduction in CBD concentration of up to 2.7% (Control = 3.77% CBD, 0.13% Δ^9THC. Thin = 6.49% CBD, 0.21% Δ^9THC) Thin was also associated with increased seed percentage from pollination, likely a dilution effect. Taken together, our results suggest that implementation of strategies to minimize pollination and/or remove seeds from high-CBD hemp biomass could improve phytocannabinoid yield. More research is warranted to evaluate the economic viability of such strategies and the effectiveness across different cultivars and growing climates.

ACKNOWLEDGEMENTS

I am filled with gratitude for the support, encouragement, and understanding from the many people in my life that have shaped me. My advisor Dr. Jessica Prenni is an unwaveringly authentic person and dedicated advisor whose support and confidence has truly been transformative for my professional self. My committee members Dr. Mark Uchanski and Dr. Whitney Cranshaw offered invaluable insights that made the steep ascent from the Psychology Department to the Horticulture Department manageable. However, I wouldn't be where I am without psychology, and I feel forever indebted to Dr. Diann Gaalema, Dr. Alexandra Potter, and Sarahjane Dube who indoctrinated me into a world where I felt my voice was heard and my power was limitless. You saw a scientist in me when I couldn't see her myself. Dr. Jacqueline Chaparro offered consistently fantastic instruction into metabolomic sample processing and instrumentation. My lab group has been so supportive and I'm grateful for each one of you; Beth Mitchell, Katie Fromuth, Katie Nasiatka, Valerie Lindstrom, and Brandon Sandoval. Jane Davey and William Folsom at ARDEC South helped facilitate my projects in the field and their support was crucial to the success of these experiments. Brian Mitchell and Hannah Sutherland were also indispensable sources of insight and support with fieldwork and sample processing. Eddie Palumbo and Alex McCorkle of Charlotte's Web Hemp were wonderful collaborators and I hope we continue to collaborate in the future. Bill Althouse of the Fat Pig Society is a lovely resource and hemp pioneer and I'm lucky to have had access to his high-CBD hemp 'Unicorn 1' clones for a project on simulated hail damage. To my friends and family, I am so thankful, and I have much to be thankful for. My twin brother Chris Bowen, my sister Lindy Bowen, my sister-in-law

Corin Vallee, my sister-in-law Victoria Blakeborough, my dad and stepmom David and Alicia Bowen, and some of my closest friends, Dr. Laura Marengo, Eliana ‘Mummy’ Marengo, Sarah Buchter, Robbie O’Loughlin, Dr. Lauren Matheny, and Dr. Kevin Gittner. To my mother, thank you for teaching me to stand on my own two feet, for showing me that I’m self-reliant, resilient, and consistently able to scoff in the face of expectation in favor of forging a new path entirely. It is a truly invaluable lesson to face rejection and to beat on, without being borne back ceaselessly into the treacherous current of the past. To everyone, thank you so much. It has meant the world to me.

DEDICATION

I dedicate my thesis work to my dad, who taught me you can't please everyone, and why on earth would you want to?

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CHAPTER 1
Introduction

1.1 Overview

High-CBD hemp (*Cannabis sativa* L.) is a pharmacologically important annual angiosperm that produces bioactive phytocannabinoids and other secondary metabolites that demonstrate therapeutic potential for a wide variety of human health conditions [1-5]. The Agriculture Improvement Act of 2014 (2014 Farm Bill) marked the initiation of expanding *Cannabis sativa* L. research in the United States by allowing low-THC *Cannabis sativa* L. chemovars, including those colloquially known as high-CBD hemp, to be cultivated in small pilot programs. These pilot programs were implemented to study if there was a demand for hemp-derived products in the current market. The Agriculture Improvement Act of 2018 (2018 Farm Bill) is more expansive than its predecessor. The 2018 Farm Bill allows large-scale cultivation of hemp plants, as well as allowing hemp and hemp-derived products with >0.3% Δ^9THC to cross state lines. However, the transportation of hemp still results in issues at times, with individuals working with legal high-CBD hemp having their product mistaken for marijuana and be arrested[6]. Furthermore, despite hemp material with ≤ 0.3% Δ^9THC being federally legal in the United States, businesses carrying CBD products still face raids and product confiscation in states such as Texas where much tension surrounding the legality of CBD containing any amount of Δ^9THC at all, or even CBD products with 0% Δ^9THC[7].

Following a nationwide trend, Colorado saw a large increase in licensed hemp acreage between 2018 and 2019, but licensed almost 40,000 fewer acres in 2020 compared to 2019[8].

Despite this decrease in acreage, Colorado remains the largest hemp-producing state with 40,391 acres licensed in 2020[9]. Of the 40,391 acres licensed for hemp production only 6,166 acres of hemp were planted[10]. Considering that 730 acres of the 6,166 planted acres were disposed of by the state in 2020 for having a Δ^9THC content >0.3%[10], the marked decrease may come as no surprise to the producers feeling the punitive pinch of legislation that has remained stubbornly in place despite being an arbitrary concentration that was not intended to be used as a legal definition. Indeed, Small and Cronquist describe this designation in their 1976 article as follows:

"It will be noted that we arbitrarily adopt a concentration of 0.3% Δ^9THC (dry weight basis) in young, vigorous leaves of relatively mature plants as a guide to discriminating two classes of plants."

"…generally, approximately 2% of the dry weight of young leaves of mature plants, or of the average dry weight of the softer parts of the female flowering plant (leaves, small twigs, flowers) is comprised of cannabinoids. Since CBD (cannabidiol, the most common non-intoxicant cannabinoid) and THC collectively usually compose the bulk of the cannabinoids present, one can crudely adjust literature reports of cannabinoid concentration for comparison with our values on the basis that the concentration of CBD and THC should sum to roughly 2%."

Considering that the total amount of CBD and THC in the cannabis material tested for this study summed to 2%, whereas today a readily available 25:1 (CBD:THC) cultivar that reaches 0.3% Δ^9THC would be expected to accumulate 25x that concentration in CBD, which would equal 7.5% CBD. This means the CBD and Δ^9THC sum to roughly 7.8% in this modern example, almost 4x the concentration cited in the 1976 article. Furthermore, the theoretical maximum CBD concentration for high-CBD hemp is thought to be 20%. A 25:1 cultivar would therefore have the potential for summed CBD and Δ^9THC to equal almost 21%, and surely higher if minor cannabinoids such as CBC, CBG, THCV, etc., are also considered.

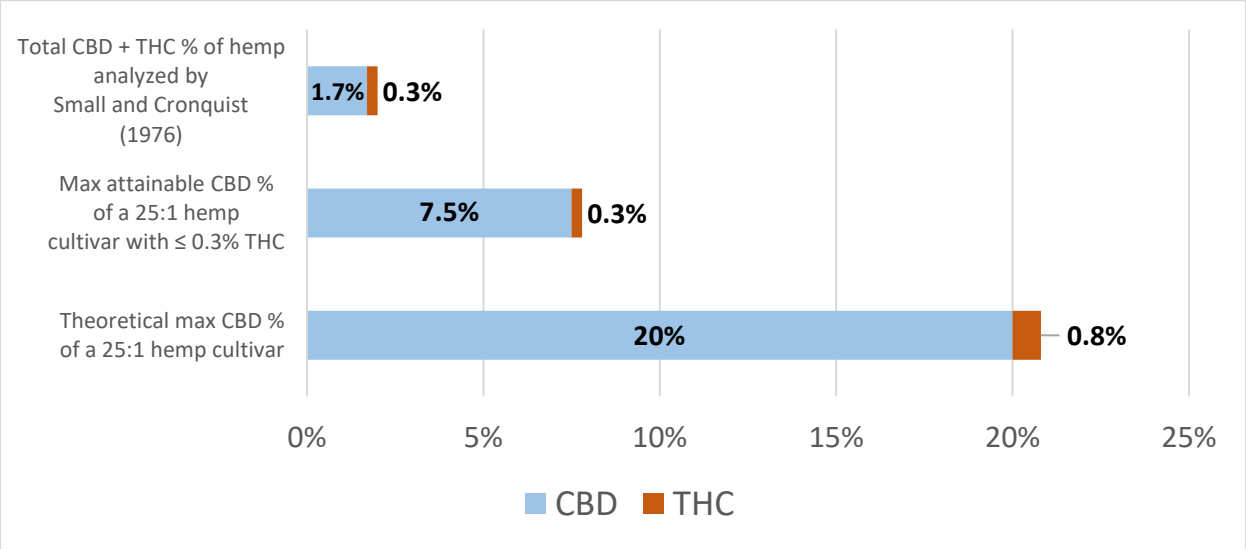

Figure 1: A comparison of the cannabis material analyzed by Small and Cronquist (1976), from which the arbitrary 0.3% Δ^9THC legal limit was adopted into law, which has a total cannabinoid content more than 4x lower than high-CBD hemp cultivated today and roughly 10x lower than the theoretical maximum CBD concentration attainable.

Table 1: U.S. national hemp acreage licensed, planted, and harvested 2018-2020[11].

Year	Licenses Issued	Acres Licensed	Acres Planted	Acres Harvested
2018	3,546	111,912	78,176	NA
2019	17,724	511,442	201,126	134,059
2020	13,475	336, 655	70,530	33,844

Cannabis sativa L. can be broadly divided into three categories based on distinct chemical profiles, known as the chemovar. Chemovars of cannabis with >0.3% Δ^9THC by dry weight are legally considered marijuana in the United States and many countries abroad, while chemovars high in CBD and containing <0.3% Δ^9THC are considered high-CBD hemp. Cultivars grown for production of fiber and/or grain typically have low CBD content as well as <0.3% Δ^9THC. However, at present, there is at least one commercial cultivar (ABOUND™, New West Genetics, Fort Collins, CO) has demonstrated CBD concentrations in the flower that are higher than levels typically found in fiber or grain cultivars.

Three alternative groups have been identified through genomic comparisons, which differ from the chemovar groups mentioned above. Leaf morphology of cannabis is genetically associated with the evolutionary origins of various cannabis chemovars, with narrow leaves (*sativa* colloquially) originating from a vast number of places geographically, and broad leaves (*indica* colloquially) showing less genetic diversity, possibly due to a smaller geographic range or more recent domestication[12]. Genetically, cannabis clusters by leaf morphology and chemovar into the following categories, as defined by Lynch et al., 2015: Broad-leaf drug type (BLDT), narrow-leaf drug type (NLDT), and hemp[12]. In this model, hemp encompasses fiber and grain cultivars (low Δ^9THC and low CBD). What is legally defined as marijuana and high-CBD hemp in the United States cluster together genetically and are therefore defined as drug-type in this model. More genetic variability is found between plants with narrow leaflets and broad leaflets than is found between cannabis with >0.3% Δ^9THC and <0.3% Δ^9THC. Additionally, the three genetic groups identified in this model also display unique cannabinoid and terpenoid profiles[12].

1.2 Pharmacology and Biology

Δ^9-tetrahydrocannabinol (Δ^9THC) and cannabidiol (CBD) are two of the most extensively studied cannabinoids, and the only two that are currently available by prescription in the United States. Δ^9-tetrahydrocannabinolic acid (Δ^9THCA) and cannabidiolic acid (CBDA) are acidic precursors to Δ^9THC and CBD. Δ^9THCA and CBDA are the cannabinoids produced by plants of the *Cannabis sativa* L. species, which are converted to Δ^9THC and CBD, respectively, when decarboxylation occurs in the presence of heat (e.g., smoking or vaporizing cannabis or through commercial processing and extraction). Synthetic Δ^9THC (dronabinol) and a synthetic substance similar to Δ^9THC (nabilone) are primarily prescribed for the treatment of nausea and vomiting in cancer patients undergoing chemotherapy, as well as symptoms related to HIV/AIDS[13]. Plant-derived CBD (Epidiolex™; the first plant-derived cannabinoid medication approved by the FDA), is available as a concentrated (100 mg CBD per milliliter of Epidiolex) clear, oral solution that is used to treat Lennox-Gastaut syndrome and Dravet syndrome; two rare forms of epilepsy[13].

In addition to these two major neutral cannabinoids, acidic (Δ^9THCA, CBDA, CBGA, CBCA), minor (CBG, CBN, CBC), and varinic (Δ^9THCV, CBDV, CBGV) cannabinoids have also exhibited promising *in vitro* and *in vivo* results for treatment of various human health treatments[4]. For example, these bioactive compounds have demonstrated preliminary anti-inflammatory, anti-microbial, anti-proliferative, anti-convulsive and neuroprotective properties. Furthermore, these minor cannabinoids serve as emerging treatment strategies for anxiety, nausea, diabetes, acne, metabolic syndrome, obesity, pain, colorectal cancer, breast cancer and more[4]. Many distillate and isolate products are readily available to consumers including those containing CBD, CBDV, CBC, CBG, CBGA, CBN, Δ^8THC, Δ^9THC, and Δ^9THCV. All but Δ^9THC products can be purchased online and shipped anywhere in the United States and many places abroad. Few studies have been

conducted that explore minor cannabinoids such as CBL, CBT, and Δ^8THC. One notable *in silico* study assessed bioavailability and drug likeness using Lipinski's rule of five for 16 cannabinoids (CBD, CBDA, CBN, CBC, CBG, CBL, CBV, CBDV, CBCV, CBGV, CBDL, CBE, CBT, Δ^9THC, Δ^9THCV, and Δ^8THC) and found that all 16 cannabinoids had potential as oral drugs[14]. Specifically, all 16 cannabinoids demonstrated good absorptivity, were either moderately active or active in terms of bioactivity and were either moderately active or active in terms of drug likeness. CBT was exceptional as it was the only cannabinoid that did not violate any of Lipinski's rule of five, also known as Pfizer's rule of five; all others violated cLogP. One *in vivo* study found Δ^9THCVA acts as an anandamide cellular uptake inhibitor, as did CBC, CBG, CBD, CBGV, CBDA, and Δ^9THCA[15], and a systematic review found that 14 cannabinoids (Δ^9THCA, Δ^9THCV, CBDA, CBDV, CBC, CBCA, CBCV, CBG, CBGA, CBGV, CBGVA, CBCVA, CBDVA, and CBN) had neuroprotective properties, in particular CBG and its derivatives, CBDV, Δ^9THCV, CBC, CBN, and Δ^9THCA[16]. Each cannabinoid acronym and the corresponding full form name are listed in Table 2 below. Taken together, these studies demonstrate that many cannabinoids have pharmacological potential, and suggests that consumers may benefit from products that retain or are enriched with specific minor cannabinoids. In addition, further research is warranted to elucidate therapeutic possibilities for the myriad of other phenolic compounds found in cannabis such as terpenes and terpenoids,[17-21] flavonoids,[22,23] bibenzyls,[24] stilbenoids[25,26], and hydroxycinnamic acids [27,28].

Table 2: A selection of cannabinoids and the respective acronyms for each.

Cannabinoid (full form)	Cannabinoid (acronym)
Cannabichromene	CBC
Cannabichromenic Acid	CBCA
Cannabichromenevarin	CBCV

Cannabichromanon	CBCN
Cannabichromevarinic Acid	CBCVA
Cannabidiol	CBD
Cannabidiolic Acid	CBDA
Cannabinodiol	CBDL
Cannabidivarin	CBDV
Cannabidivarinic Acid	CBDVA
Cannabielsoin	CBE
Cannabielsoic Acid	CBEA
Cannabifuran	CBF
Cannabigerol	CBG
Cannabigerolic Acid	CBGA
Cannabigerovarin	CBGV
Cannabigerovarinic Acid	CBGVA
Cannabicyclol	CBL
Cannabicyclolic Acid	CBLA
Cannabicyclovarin	CBLV
Cannabinol	CBN
Cannabinolic Acid	CBNA
Cannabiripsol	CBR
Cannabitriol	CBT
Cannabitriolvarin	CBTV
Cannabinodivarin	CBV
Delta-8-tetrahydrocannabinol	Δ8THC
Delta-9-tetrahydrocannabinol	Δ9THC
Delta-9-tetrahydrocannabinolic acid	Δ9THCA
Delta-9-tetrahydrocannabivarin	Δ9THCV
Delta-9-tetrahydrocannabivarinic acid	Δ9THCVA

There is also growing body of evidence supporting the idea that synergistic effects, colloquially known as the "entourage effect," may contribute to the therapeutic properties of cannabis extracts which include combinations of multiple cannabinoids[29,30] as well as other bioactive secondary metabolites such as terpenes and/or terpenoids[31,32]. It has been demonstrated that the endogenous endocannabinoid 2-arachidonoyl-glycerol shows enhanced activity in the presence of 2-acyl-glycerol esters, where alone the esters are inactive[33]. This effect has also been

noted for organisms other than cannabis. For example, combining multiple terpenes from the tropical Amazonian plant *Copaifera oleoresins* demonstrated synergistic effects that were more toxic than the terpenes alone to cells of *Trypanosoma cruzi*, a protozoan parasite responsible for the Chagas' disease endemic in Latin America[21]. Additionally, another study found that combining multiple terpenes was found to be more effective at inhibiting growth of a protozoa than the terpenes alone[34]. Since cannabinoids are also diterpenoids, findings such as these may translate to our understanding of potential mechanisms of action responsible for synergistic effects described for cannabis and help to explain how isolated compounds often do not exhibit the same bioactivity as the same compound within the background of a more complex matrix. Conversely, there is also some evidence suggesting that cannabis polypharmacy could results in negative interactions, and the potential for toxicity has also been reported and warrants further evaluation[35,36].

Cannabinoids demonstrate an atypical receptor binding profile and interact with a myriad of receptors, enzymes, and ion channels in humans. Class A G-protein-coupled receptors (GPCRs) involved with cannabinoid metabolism appear to include CB1, CB2, GPR55, 5-HT1A, and alpha-2 adrenoceptors[4]. Additional receptors and cation channels of interest include PPARγ, TRPA1, TRPV1, TRPV5, TRPV6, and more continue to be elucidated from ongoing research.

Cannabidiol (CBD) has demonstrated a variety of actions *in vitro* as well as *in vivo*. *In vitro,* CBD has been shown to act as a non-competitive negative allosteric modulator of CB1[37], and has also shown potent, possibly non-competitive antagonism of CB1 and CB2 receptor agonists via inverse agonism at low (1uM) concentrations[38]. Allosteric modulators of CB1 receptors show potential advantages for the treatment of central and peripheral nervous system disorders when compared to antagonists or orthosteric agonists[37], CB1 antagonists show potential as antipsychotic drugs[39], and inverse agonism of CB2 receptors accounts for at least some of the

anti-inflammatory properties that have been extensively documented in relation to CBD administration[40]. Δ^9THC has also been demonstrated to act as a partial agonist and a partial antagonist in mice [41].

Table 3: Cannabinoids interact with a variety of receptor families and have demonstrated various mechanisms of action in vitro *and* in vivo*, with some cannabinoids showing multiple mechanisms of action dependent on dose concentration[4]. Full agonist (+), partial agonist (pt+), antagonist (-), positive allosteric modulator (posAM), negative allosteric modulator (negAM).*

Receptor family	**Class or group**	**Receptor**	**Mechanism(s) of action and cannabinoids that interact**
G-protein-coupled receptors	Cannabinoid	CB1	(negAM) CBD (+; pt+) Δ^9THC
		CB2	(negAM) CBD (+; pt+) Δ^9THC
		GPR55	(+) Δ^9THC
	Serotonin	5-HT1A	(+) (posAM) CBD (+) CBDA (-) CBG (+) Δ^9THCA (+) Δ^9THCV
	Adrenoceptor	alpha 2	(+) CBG
Nuclear receptors	Nuclear hormone receptors	PPAR-γ	(+) CBG (+) Δ^9THCA
Ion channels	TRPA cation channels	TRPA1	(+) CBC
	TRPV cation channels	TRPV1	(+) CBDV
		TRPV5	(-) Δ^9THCV
		TRPV6	(-) Δ^9THCV

Cannabinoids appear to interact directly or indirectly with a multitude of pathways and receptors involved in numerous health conditions and symptomology. Nausea is one example of a symptom that can be brought about in numerous, complex ways. Multiple cannabinoids may act

as possible therapeutics for nausea treatment through a variety of pathways. Nallathambi et al., 2018 describes how the activation of four different neural pathways can all result in a sensation of nausea when the appropriate stimuli are relayed to a sensory nucleus located in a portion of the medulla oblongata and lower pons, the nucleus tractus solitarius. The central nervous system (CNS) contains many receptor types including CB1, CB2, and GPR55 receptors. Multiple cannabinoids have shown promising effects in terms of regulation of nausea due to their ability to modulate not only cannabinoid receptors but also some serotonin, adrenoceptor, and nuclear hormone receptors, as well as several TRP cation channels. Cannabinoids interact with a myriad of receptor types through different mechanisms of action (Table 3). For example, CBDA and Δ^9THCA have been shown to prevent nausea and vomiting in rats by enhancing CB1 and 5-HT1A receptor activation[42]. Furthermore, GPR55, the orphan receptor, is blocked by CBD but activated by Δ^9THC[43], which may also have implications in the pathogenesis of nausea.

Cannabinoids also act in the body by indirectly elevating endogenous human endocannabinoid levels through competition with fatty acid amide hydrolase (FAAH), which may result in elevated anandamide levels. It has been suggestion that inhibition of FAAH could have therapeutic potential by limiting hydrolysis of endocannabinoids, therefore raising endogenous cannabinoid levels[15]. Elevated levels of the endocannabinoid anandamide has been correlated with nausea and vomiting from motion sickness. Specifically, participants in a study on motion sickness induced nausea and vomiting were found to have a reduction in blood concentration of anandamide and had consistently lower 2-AG levels before and after developing acute motion sickness. Participants who did not develop motion sickness during the task were found to have increased anandamide and 2-AG levels after the task was complete, suggesting that motion sickness may be related to impairment of the endocannabinoid system[16]. Furthermore, participants who developed

motion sickness demonstrated a significant decline in CB1 receptor expression, but CB2 receptor expression did not change for either group[29]. CBD and Δ^9THC consumption appears to also elevate anandamide levels, but seeing as neither CBD or Δ^9THC inhibit FAAH activity a possible explanation is that the competition between FAAH and other substrates that bind to fatty acid-binding proteins (FABPs) elevate endocannabinoid levels[29]. CBD and Δ^9THC bind to at least three human FABPs, limiting the FABPs that may bind with and metabolize anandamide, 2-AG, and other fatty acid amides[29]. Inactivation of FAAH and the subsequent elevation in fatty acid amides may help treat pain[15,30], anxiety, depression, insomnia, and inflammation[30].

1.3 Processing Methods for Cannabis Biomass

Recently, there has been a surge in marketing of so called *full-spectrum* products. This marketing strategy capitalizes on debated principles associated with the idea that the entourage effect provides additional benefits to consumers who choose full-spectrum hemp products as compared to CBD distillate or isolate products. However, because these products are not regulated by the FDA as dietary supplements and there remains a paucity of research on entourage effects, there is no present consensus on what denotes a "high-quality" cannabis product. Phrases such as "full-spectrum extract", "whole plant extract", and "broad-spectrum extract" further muddy the waters for consumers and healthcare providers.

The composition of a commercial cannabis extract will in large part be determined by the genetics of the starting plant material[44]. However, the impact of processing methodology on the resulting product is not well understood. Commonly utilized commercial extraction approaches include the use of solvents (e.g., ethanol and isopropanol) to more advanced technologies using supercritical CO_2. Solvent extraction represents the lowest cost option; however, this method runs the risk of leaving behind trace organic solvent contamination. This is more of a concern with

hydrocarbon solvents such as methanol and butane which are toxic for human consumption. Extraction with supercritical CO_2 requires investment in specialized equipment but has multiple advantages including "tunability" by modifying temperature and pressure conditions for more precise and consistent extraction. Furthermore, supercritical CO2 systems have the potential to reuse CO_2 and this technology also eliminates the possibility of trace hydrocarbon solvents being present in the final product.

1.4 Analytical Methods for Detection of Cannabinoids

There are many analytical options for cannabinoid analysis, though some are ill-suited for some cannabinoids, for reasons that will be discussed below. The choice of instrumentation platform depends on the compounds of interest, the matrix of the sample, and practical considerations such as cost and time. Common to all analytical options are two components: a separation component which disentangles the individual compounds from one another within the matrix, and a detector which detects the molecule and can inform on the molecule's abundance. The detector will either be a mass spectrometer (MS), or a nonspecific detector such as flame ionization (FID) for gas chromatography, and photodiode array (PDA) for liquid chromatography. Liquid and gas chromatography (LC and GC, respectively) are the most common approaches for compound separation.

GC and LC separate compounds based on chemical properties (e.g., polarity, hydrophobicity, etc.) and utilize partition equilibrium chromatography which involves the equilibrium formed between compounds in either the gas or liquid phase and a solid phase within the chromatographic column. Mass spectrometry enables the detection of both mass and relative abundance which makes it a highly sensitive and specific detector often hyphenated to a GC or LC system. Mass spectrometers can also isolate and fragment molecules to generate fragmentation

spectra (MS/MS) which provide additional structure specificity. LC-MS/MS has been reviewed to be the ideal quantification method for cannabinoids as it can be used to accurately quantify both the acidic and neutral versions of cannabinoids[45]. However, this system is also the most expensive and requires technical expertise to use.

Compared to liquid chromatography systems coupled with an MS detector, systems that utilize a photodiode-array (PDA) detectors have less specificity but are much more affordable. If the cannabinoids of interest can be chromatographically separated in time, the use of appropriate analytical standards can enable accurate quantitation using a PDA detector. However, the primary disadvantage of this approach is the lack of specificity, meaning a PDA detector is not able to resolve co-eluting compounds. In particular, given the high number of potential cannabinoids that have been described[46] and the chemical similarity of these compounds[14], it is likely that there will be co-eluting compounds that will result in non-specific absorbance, inflating the signal in the PDA detector if this method of analysis is used in cannabinoid analysis.

Gas chromatography systems can also be coupled to multiple types of detectors, such as MS or flame ionization detection (FID). Cannabinoids are nonpolar molecules that are synthesized in an acidic form in the cannabis plant (e.g., cannabidiolic acid) which decarboxylate when heated to form a neutral cannabinoid (e.g., cannabidiol). The heat produced by the GC source to volatize compounds within a sample will also decarboxylate acidic cannabinoids, making quantification of acidic cannabinoids impossible on this instrument without a time-consuming derivatization step[47]. Common applications of the various analytical instruments, and the corresponding drawbacks and advantages of each system are below (Table 4).

Table 4: Applications, limitations, and advantages of common analytical systems used to identify and quantify cannabinoids.

Instrument	Limitations	Advantages
GC-FID flame ionization detector	• Cannabinoids decarboxylate in the source [47] • FID oxidizes analytes. • No MS, less specificity and sensitivity.	• Good for routine analysis of organic compounds. • Simple • Versatile • Ease of operation
GC-MS	• Cannabinoids decarboxylate in the source [47] • Labor-intensive sample prep, derivatization. • Limited utility • More expensive than GC-FID	• Good for volatile analytes. • Robust • High specificity • MS separates co-eluting analytes with differing m/z.
LC-PDA	• No MS, less specificity. Requires standards to identify compounds based on chromatographic separation.	• Fast • Much less expensive than MS systems. • Ease of operation
LC-MS	• Expensive • Require stable isotope labeled internal standards for absolute quantitation.	• MS separates co-eluting analytes with differing m/z.

GC, with either an MS or FID detector, is well suited to measure volatile compounds, which are compounds of low molecular weight that become volatile when heated. Alternatively, non-volatile compounds such as sugars can be made volatile through a chemical derivatization process and are then suitable to undergo GC-MS analysis. GC approaches are ideal for the analysis of small polar or volatile compounds, such as organic acids, sugars, terpenes, and terpenoids. Conversely, LC is most suited for nonpolar compounds such as cannabinoids, most carotenoids, most flavonoids, etc.

Hemp potency testing for regulatory compliance often reports results in terms of "total THC." Total THC is calculated by either (1) decarboxylating the sample using heat and then quantifying only Δ^9THC, or (2) by quantifying Δ^9THC and Δ^9THCA separately and then calculating the total Δ^9THC assuming 100% potential conversion of THCA to Δ^9THC. However, both approaches can be inaccurate because they assume 100% decarboxylation efficiency. While >99% decarboxylation of acidic cannabinoids such as CBDA and THCA may be possible in commercial extraction facilities with specialized equipment, this is not likely to occur in various real-world conditions where cannabis flower or extractions are being decarboxylated by consumers through smoking, vaporizing, etc.

Furthermore, Δ^9THCA itself holds significant therapeutic potential, and there will likely be increased demand for Δ^9THCA in this acidic, non-intoxicating form[4]. Currently, Δ^9THC is the only cannabinoid known to be both intoxicating and psychoactive[31], with all other cannabinoids (including Δ^9THCA) appearing thus far to be non-intoxicating and psychoactive[48]. It should be noted that the terms psychoactive and psychotropic are synonyms which are often confused with the term intoxicating when cannabinoids are described, adding confusion for consumers trying to understand the terminology. Psychoactive or psychotropic substances affect how one thinks or feels without causing impairment, such as caffeine or aspirin, while psychoactive *and* intoxicating substances affect how one thinks or feels and also causes inebriation, such as alcohol or Δ^9THC[49].

1.5 Field Management for Hemp Production

Field crops such as corn and tomatoes have been extensively studied in the United States to determine optimum conditions and management practices for various cultivars and varieties. Extensive research is needed to determine optimum conditions and practices for high-CBD hemp, which is typically grown with wide spacing like a specialty vegetable crop. Genetic lineage,

morphology of leaflets, number of flowering weeks required for optimum potency, and ideal cultivar climate (e.g., continental, tropical, etc.) are all thought to be important variables to consider when reviewing the myriad of high-THC cannabis information generated through lay sources.

Until recently, diploid cultivars of CBD hemp which contain two complete sets of chromosomes have been the only commercially available option for producers. However, the development of triploid cultivars (containing three homologous sets of chromosomes instead of two; plants are sterile and incapable of producing seeds) has provided an alternative option.

It has been speculated that the width of cannabis leaflets may be a meaningful metric in terms of determining suitability for various environments. For example, when purchasing high-CBD hemp and marijuana clone plugs alike the following words are seen as descriptors: *sativa*, *indica*, and *hybrid*. *Sativa dominant* and *indica dominant* are also commonly seen, often with a percentage applied to either (e.g., 60% sativa, 40% indica) with plants deemed *sativa* or *sativa dominant* generally coming with recommendations to allow them to develop flowers indoors for 9 to 10 weeks to achieve maximum potency, whereas plants deemed *indica* or *indica dominant* may have a recommended flowering period of just 8 weeks when grown indoors using supplemental lighting. *Sativa* plants with narrow leaflets are thought to originate in climates with longer growing seasons that accommodate a longer flowering period, whereas *indica* plants with broad leaflets are thought to have originated in harsher, colder climates with shorter growing seasons.

Anecdotal observations on certified high-CBD hemp clones "Unicorn 1" grown at Colorado State University in Fort Collins, Colorado in 2019 and 2020 demonstrated that despite Unicorn 1 having a narrow-leaflet phenotype and being described by the breeder as sativa dominant, Unicorn 1 plants begin to flower earlier (mid-July onset of flowering) than some of their

so-called *indica* and *hybrid* counterparts; The high-CBD hemp cultivars Cherry Uno and Wife are frequently described by plant nurseries in Colorado as being *indica dominant* (Cherry Uno) and a *50/50 sativa/indica hybrid* (Wife). Which cultivars are considered sativa or indica seem to be largely a game of telephone played across the decades. It is not well understood how the appearance of the leaflets is regulated within the genome, and while they cluster genetically by leaflet width[12], the development of "hybrid" cultivars is continuously expanding as desirable traits are identified, cross bred, and propagated. Leaflet width seems to land somewhere in between skinny and broad when plants with either leaflet type are cross bred together. In other words, it does not appear to be so straightforward such that plants with broader leaflets reliably flower earlier than plants with more narrow leaflets. These results suggest that there may be additional genetic and/or environmental factors contributing to flower development. This is critically important in climates such as the Front Range of Colorado, where the average first frost date is October 1st.

Some traits of *Cannabis sativa* L. are strongly influenced by genetic factors, including days to grain maturity, Δ^9THC concentration, and CBD concentration, while others are strongly influenced by the environment and the genotype X environment interactions, such as plant height and water use[44]. Cultivars of cannabis can have differing efficiency pattens of water use under the same conditions, with more efficient cultivars likely being better suited for semi-arid climates with 10-20 inches of precipitation annually. Chandra et al., 2011 demonstrated how several high-THC chemovars and several low-THC, fiber-type chemovars of cannabis have differing temperatures at which optimum photosynthetic response occurs as well as differing carotenoid content, with the high-THC chemovars ranging from 0.22-0.40 mg/g of total carotenoids and fiber chemovars ranging from 0.13-0.28 mg/g of total carotenoids[50].

1.6 CBD:THC Ratios of CBD Hemp

The value of a CBD hemp crop is directly related to the CBD yield that is achievable within the current legal framework. The legal definition of hemp varies by country and ranges from 0.2% to 1% Δ^9THC by mass. The metabolic processes for synthesis of CBD and Δ^9THC in the plant are genetically linked in such a way that as CBD content increases over the course of the growing season, Δ^9THC also steadily increases. For producers in the United States and Canada, the legal restriction of 0.3% Δ^9THC constrains the upper limit of CBD concentration that hemp farmers can achieve. This constraint is more pronounced producers in the European Union where the legal restriction is 0.2% Δ^9THC. Cultivars with a theoretical yield ratio from 20:1 to 30:1 CBD:THC are the most efficient cultivars currently available in the United States and thus far, cultivars with a yield above 30:1 have been illusive to breeders. It is important to note that to stay within the legal limit of Δ^9THC using the available cultivars, producers will often have to harvest their crop before maximum CBD content is achieved.

If a crop exceeds 0.3% Δ^9THC , producers are required to dispose of the acreage associated with sample in question, potentially resulting in a significant profit loss. For example, of the 6,166 acres of hemp planted in Colorado in 2020, 730 acres were disposed of for exceeding the 0.3% Δ^9THC limit. Raising the Δ^9THC concentration limit from 0.3% to 1%[10] has been proposed as a viable way to increase security for farmers. For example, if the legal restriction were to be raised to 1% Δ^9THC, the 20:1 cultivars that are commonly available today could be allowed to reach their maximum CBD yield potential, while all but removing the concern of their crop becoming hot. This would significantly increase profitability for growers and decrease risks associated with high CBD hemp production. . This would also enable increased production of minor cannabinoids, or other secondary metabolites, representing another potential value of this crop for farmers.

Certain management practices may raise or lower cannabinoid content, which could have both positive and negative implications depending on the cannabinoid in question and the context of how the product will be consumed. Management practices include (for example) how and when the plants are transplanted to the field, plant spacing, row spacing, pruning strategies, irrigation techniques, use of organic or plastic mulch, type and application method/timing of fertilizer, protection from hail and frost, as well as harvesting and processing protocols. How plants are spaced within and between rows affects how large the plants can grow, though there is ongoing speculation regarding the benefits of growing larger plants spaced further apart with 3- or 4-foot centers and 6-foot rows at 1,500-2,000 plants per acre compared to planting an acre of an autoflowering cultivar using 15 cm centers, with 10,000 or more plants per acre. Drip irrigation is common, but flood irrigation could be a viable option for hemp as well. Research results (unpublished) obtained by Colorado State University Extension research scientist K. Russell in Yellow Jacket, Colorado (high desert arid environment, elevation 2103 meters, 8" rain annually) on the effects of black, white, and striped black and white plastic mulches on water use efficiency showed that plastic color did not affect water use efficiency for either a low water (12") or a high water (22") treatment. Furthermore, no differences were found between yield, stalk diameter, or number of branches between plastic treatments (K. Russell, personal communication, June 23, 2020). Finally, there is a distinct lack of basic fertility studies to determine the optimal level of fertilizer to apply in conventional and organic systems between various regions.

It has been speculated that abiotic stress such as defoliating injuries from hail damage or insects may also impact the concentration of cannabinoids found in the inflorescence. Preliminary, unpublished studies suggest that simulated hail damage defoliation at various developmental stages does not appear to alter CBDA or Δ^9THCA levels. Physical damage may be more important

to consider during processing once the biomass has been dried, as a portion of the cannabinoid-rich trichomes that coat the flowers and small leaves of female cannabis plants can be lost during handling.

Cannabis is an angiosperm that is typically dioecious, meaning male flowers (that produce cannabis pollen) form on distinctly male plants and that female flowers (which are fertilized by cannabis pollen) form on distinctly female plants. For dioecious species, both male and female plants are needed for seeds development to occur. Most flowering plants are hermaphroditic, which implies that the female and male reproductive structures are found within the same floral structure. Indeed, 95% of angiosperms are considered hermaphroditic plants[51]. Cannabis is sometimes monoecious, but not hermaphroditic, because the female and male reproductive structures form distinctly from one another when both are present on a cannabis plant. In contrast, hermaphroditic plants have female and male reproductive organs within the same floral structure[51]. Female pistillate cannabis inflorescences are dense, indeterminate, compound racemes while male staminate cannabis inflorescences are determinate panicles (branched racemes) which bear distinctive banana-shaped anthers at the end of each stamen when the flowers mature[52]. Cannabaceae is a small family that consists of Cannabis (typically dioecious), Humulus (dioecious), and Celtis (monoecious). Most flowering plant species are monoecious, meaning male and female reproductive organs are both found within each individual plant when flower development begins. Some hardy dioecious genera examples include kiwi (Actinidia spp.), mulberries (Morus spp.), hollies (Ilex spp.), and junipers (Juniperus spp.). Pollination of hollies is necessary to produce the ornamental (and poisonous) red berries. Likewise, juniper and mulberry trees (also kiwifruit vines) require pollination to produce the flavorful, edible berries that certain species within these respective genera are cultivated for. Genus Humulus (hops) is an example of

a flowering dioecious plant in which the pollination of the female flowers by the male pollen is unwanted and avoided outside of a plant breeding context.

Pollination in hops is intentionally avoided by growing only female hop plants because seeded hops have lower levels of essential oils and resins that are desirable for the flavor profile of beer[53]. Hop seeds are a one-seeded fruit known as an achene. This is also the case with hempseeds, and another common example is strawberries seeds. Terpenoid metabolism in glandular hop trichomes corresponds mainly (~90%) to the seven enzymes of the *non-mevalonate*, or methyl-D-erythritol phosphate, (MEP) pathway produced in the plasmid, and to a much smaller extent (~10%) terpenoid metabolism corresponds to the six enzymes of the cytosolic mevalonate (MVA) pathway[54]. In contrast, cannabinoid biosynthesis necessitates fatty acid biosynthesis, acetyl-CoA synthesis through the cytosolic MVA pathway to form olivetolic acid via the polyketide pathway, as well as geranyl diphosphate (GPP) which is synthesized in the MEP pathway from pyruvate[48]. Finally, GPP and olivetolic acid combine to form cannabigerolic acid (CBGA), the precursor to all other phytocannabinoids[48]. While a similar analysis of terpenoid metabolism has yet to be completed using the glandular trichomes of cannabis, is it interesting to note that while both of the primary volatiles found in hops (monoterpene and sesquiterpene compounds) are synthesized overwhelmingly though the MEP pathway in the plasmid, monoterpenes found in cannabis are synthesized through the MEP pathway but sesquiterpenes are synthesized through the MVA pathway. This discrepancy in metabolite allocation across the various pathways leaves open the possibility of hop and cannabis flowers responding differently in terms of secondary metabolite production in relation to seeded vs seedless flowers. Hop growers do not appear to have a difficult time avoiding pollination of hop cones in the current market although, like cannabis, hops are also wind pollinated. In this regard, the pollination of Cannabis

is a problem unique to this industry that demands Cannabis-specific research in terms of the impact of pollination and seed formation in high-CBD hemp flower.

Thus, further research is needed to elucidate the implications of pollination in cannabis. Cannabis is wind-pollinated, with pollen traveling up to 5 km upwind[55]. Once pollinated, female CBD hemp flowers will develop seeds. The value of high-CBD hemp biomass is based on the phytochemical content derived primarily from the flowers. Accidental pollination of cultivars grown for flower is likely a common occurrence for hemp cultivated outdoors, as evidenced in anecdotal reports from hemp farmers and/or cannabinoid extraction facilities that consistently observe at least some seeds in much of the CBD hemp biomass grown outdoors. Farmers and extraction facilities alike are concerned about the potential implications of producing a crop that yields highly seeded CBD hemp biomass, both in terms of market value for farmers and for product quality and consistency for cannabinoid extractions. Pollination and the resulting process of seed maturation has the potential to affect cannabinoid content through either metabolic reallocation of key molecules such as glucose, or simply through dilution by seed content in the biomass to be extracted.

Seed removal from hemp biomass is labor-intensive and often does not occur prior to homogenization and extraction. The implication of high seed content in hemp biomass has not been thoroughly studied. However, in addition to a potential reduction in cannabinoid yield, the primarily lipid-containing endosperm of the seeds could also cause problems for the various instrumentation used in processing and extracting. The study presented in this thesis explored (1) the potential for using common row cover material to reduce pollination and (2) the impact of increased seed content on the cannabinoid profile of high CBD hemp.

CHAPTER 2

Understanding the Phytochemistry of High-CBD Hemp: Efficacy of Common Row Cover Materials for Pollen Exclusion and Impact on Flower Phytochemistry

2.1 Introduction

There is a notable lack of research on how management practices impact hemp flower quality. Fertilizer macronutrients and macronutrient concentrations, timing and number of fertilizer applications, plant spacing within row, row spacing, use of mulch, pruning practices, and inches of water per week all have the potential to cause significant variance in crop yield and quality. Likewise, the impact of cannabis genetics remains understudied, but available data suggest that variables such as pigment content, optimum temperature, and temperature range for optimal photosynthetic response varies significantly between drug-type and fiber-type cannabis chemovars grown under controlled environmental conditions[50]. Cannabis is typically considered a photoperiodic plant that requires a short-day length to begin flowering, however during the 2019, 2020, and 2021 field seasons at Colorado State University it was observed that different cultivars grown under identical field treatments began flowering as early as mid-July or as late as the first week of September (data unpublished). The choice of propagation material (seed or clones) may also impact the final crop yield and/or quality due to anecdotal evidence that the rooting structure differs between the two.

Pollination of hemp flower is a unique concern specific to production of high-CBD cultivars where the value of the crop is directly tied not only to dry matter yield, but also to the yield of extractable phytochemicals. When plants are grown in the vicinity of pollen sources, pollination occurs resulting in seed development. Male cannabis plants produce pollen which fertilize the female plants, leading to grain (hemp seed) formation. Male cannabis plants are

important for the cultivation of hemp grain, whereas high-CBD hemp and high-THC marijuana will ideally contain high levels of cannabinoids and no seeds. Male cannabis plants typically release pollen from numerous pollen sacs by mid-July, with some male flowers persisting into September. Pollen appears to peak in late July and early August before the male plants senesce in the field, leaving only the grain-producing female plants to be harvested. In climates with short growing seasons (e.g., Colorado) it is necessary for high-CBD hemp plants to begin flowering in July so that 8-10 weeks of flower development may occur for optimal phytochemical yield. However, with the onset of flowering also comes with the potential for seed generation if cannabis pollen is present in the area. Importantly, cannabis is wind-pollinated, and pollen has been shown to travel up to 5 km downwind and 1 km upwind[55].

Pollination can be reduced in several ways. Female cannabis flowers can be grown at a distance that would preclude pollen from reaching the female flowers, the female flowers can be covered with material or grown inside a structure that physically blocks any wind-borne pollen, or newly developed triploid cultivars may be utilized (commercially available starting in 2021). Maintaining a proper distance between a pollen source and female cannabis flowers with legislation (e.g., dictating that certain counties not grow any pollen-producing male cannabis plants) might be effective in theory, but could prove challenging in practice considering the tendency of rouge male cannabis plants to crop up in unlikely places due to distribution of hemp grain by birds or other means, as well as the ability of female cannabis plants to spontaneously develop male flowers, i.e. spontaneously becoming a monoecious plant with the ability to self-pollinates. This phenomenon is not well understood and to our knowledge has not been studied in an evidence-based fashion.

Pollen exclusion materials are traditionally used in horticultural plant breeding to ensure that a particular cultivar is pollinated with specific pollen from a known source and no cross-contamination occurs due to pollinators or wind moving the pollen to flowers where it is not desired. Materials used for excluding pollen vary depending on the pollen vector in question. Most biotic pollinators will be excluded with woven mesh insect netting, however species that are wind pollinated require materials with smaller pore sizes to effectively exclude pollen grains. Materials are rarely assessed to quantitatively determine efficacy of a material at blocking wind-borne pollen. In one study of the few studies on this topic, Neal et al. (2004) compared similar pollen exclusion coverings including large polyester mesh (pore size 2387 X 3623 µm), small polyester mesh (pore size 185-269 X 839 µm), cotton muslin (pore size 0-223 µm), and nylon filter fabric (pore size 6 µm)[56]. The results found that the cotton muslin with a pore size of approximately 200 µm was nearly as effective as nylon filter fabric with 6 µm pore size[56]. The selection of a natural cotton vs synthetic material will also be an important factor depending on the crop in question[56]. Additionally, the choice of material may influence the microclimate experienced by the plant, with synthetic fabrics drying more quickly that natural fibers, and materials with larger pore sizes facilitating evaporation. Durability is also a factor as cotton fibers tend to unravel over time becoming more effective as the microscopic pore openings become clogged with the unraveling fibers, whereas nylon based thin row covering tend to develop snags and tears over time making them less effective.

Here, we evaluate the efficacy of three commonly used horticultural materials (insect netting, thin row covering, thick row covering) for pollen exclusion during field production of high-CBD hemp cultivars. We additionally determine the impact of increasing seed concentration on the phytochemical profile of hemp biomass. The results of this study represent

an important first step towards the development of informed recommendations regarding the management practices aimed at reducing pollination in field grown high-CBD hemp.

2.2 Materials

A total of 60 high-CBD hemp rooted clone plugs (30 each of two cultivars; Cherry Uno and Wife) were planted in a certified organic field in 2019. The experiment was repeated with the same number and cultivars in 2020. Plants were cultivated at the Agricultural Research, Development and Education Center South (ARDEC South) at Colorado State University in Fort Collins, Colorado (lat. 40.611804 N; long. -104.997144 W; clay loam soil).

Three common horticultural materials were utilized as row cover (Figure 3). The materials included a woven insect netting with pores of approximately 700 µm in diameter (similar to Agtec insect mesh HDPE 50), Agribon+ AG-19 thin row covering with pores of approximately 200 µm in diameter, and a thick row covering (similar to Agribon+ AG-50; also known as frost blanket) with pores of approximately 50 µm in diameter. The materials were clipped to electrical conduit that was bent uniformly across the plant, to act as support for the low tunnels. Materials were affixed to the soil along all four edges with sod staples (Figure 2).

Figure 2: An image of the low tunnels that were constructed to exclude pollen from high-CBD hemp plants in the 2019 and 2020 pollen exclusion experiments.

Table 5: Specifications of the three common horticultural materials that were used to construct the low tunnels used in the pollen exclusion experiments in 2019 and 2020.

Treatment	**Pore size**	**Hemp pollen grain diameter**	**Weave**	**Manufacturer**
Insect netting	250 X 700 µm	~20 µm	Woven	Similar to Agtec HDPE 50
Thin row cover	50-200 µm		Non-woven	Agribon+ AG-19
Thick row cover (frost blanket)	0-50 µm		Non-woven	Similar to Agribon+ AG-50

2.3 Methods

Plants were cultivated organically outdoors in a semi-arid climate at the Agricultural Research, Development and Education Center South (ARDEC South) at Colorado State University in Fort Collins, Colorado (lat. 40.611804 N; long. -104.997144 W; elevation 1525 meters). The CBD hemp clone plugs were purchased from the same local nursery for both the 2019 and 2020 field seasons. Clones were transplanted in the field 6/11/2019 and 6/10/2020. Planted were spaced on 3-foot centers, with rows spaced 1.8 meters apart. A randomized complete block design with 3 blocks was planted per season with individual plants as the experimental unit. Drip tape irrigation with 20 cm emitter spacing and a flow rate of 0.33 gallons per hour (GPH) was utilized for both seasons (Irritec 5/8" P1 drip tape). Approximately 2.5 cm of water was supplied per week with the irrigation schedule dependent on precipitation. Granular 13-0-0 fertilizer (Nature Safe, allowed in organic systems) was applied at a rate of 150 lbs. per acre for both seasons, split over three side dress events that were applied 2 weeks apart between the end of June and the end of July for both seasons. All cultivation practices were in accordance with regulations affecting Certified Organic production. There was a known source of hemp pollen managed at ARDEC South that grew within 50 meters of the female high-CBD hemp plants in this experiment, for both years of data.

A randomized complete block design with elements of a split plot was utilized. Specifically, three blocks were utilized, with cultivar randomized into groups of 5 within each block. Each group of 5 was then randomized to receive the various treatments, with each treatment occurring within each block exactly once.

Materials were assessed September 19th, 2020, at midday in full sun conditions. Photosynthetically active radiation (PAR) readings were obtained using an Apogee MQ-500

Full-spectrum Quantum Sensor. PAR readings were taken in direct sunlight, and five readings were taken for each of the three pollen exclusion materials. Materials were spread evenly over the meter, 15 cm above the sensor.

Hemp pollen grains were examined by light microscopy and were observed to have had an average diameter of approximately 20 µm. As illustrated in Figure 3B, all materials had pore sizes larger than the average diameter of the hemp pollen observed.

Temperatures were recorded using a HOBO 4-Channel External Data Logger U12-008. Each probe was covered with a solar radiation shield and mounted to wooden stakes. One probe was placed inside one low tunnel from each treatment, with the control probe being placed near uncovered control plants.

Whole plants were harvested at soil level and dried for at least 2 weeks before weights were recorded. Total dry biomass measurements included all above-ground plant parts. Individual plants were kept separate in open 30-gallon paper lawn/leaf refuse bags while drying to ensure all seeds remained with the appropriate plant. Once dry, plant biomass (flower, leaf, very small stems, and seed) was removed from the large stalks and weights were recorded for both the stalk and the combined plant biomass. Seeds were cleaned using an Agriculex Inc. CB-2A large column blower after the plant biomass was passed through a large screen by hand to uncouple seeds from the floral structures. Total marketable flower/leaf biomass was determined by subtracting seed weight and stalk weight from the total dry biomass measurement for each plant. Seed % was calculated using total seed weight and total dry biomass for each plant in 2019 and 2020. Seed % was calculated using total seed weight and total flower weight for each plant in 2020, but this data was not available for 2019 plants. Data was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons to evaluate

each treatment relative to the uncovered control plants. Statistical analysis was performed in GraphPad Prism (Version 8.2.1).

For metabolomic sample collection, three 5 cm inflorescence samples were taken from each plant for phytocannabinoid analysis. Metabolism was quenched by placing the freshly harvested plant material on dry ice during transportation and storing samples at -80°C with subsequent lyophilization. Lyophilized plant material was homogenized using a bead beater to grind each sample into a fine, homogeneous powder. Seeds and flower material were first separated and weighed individually before being homogenized together for extraction. Stems > 1 cm in length were discarded while stems <1 cm in length homogenized well using the bead beater and were included in the sample.

Homogenized inflorescence material (20mg +/- 0.5mg, including seeds and stems < 1 cm) was weighed into 2 mL glass vials. Samples were extracted using an 80% MeOH extraction consisting of LC-MS grade methanol and LC-MS grade water. Deuterated internal standards Δ^9THC-d3 and CBD-d3 were spiked into the 80% MeOH extraction at a concentration of 25 ng/mL before the spiked mixture was added to the samples. A 12-point standard curve was prepared with the following ng/mL concentrations: 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, 2000, and 4000 ng/mL.

Table 6: Selected Reaction Monitoring (SRM) transitions for targeted phytocannabinoid analysis.

Phytocannabinoid	RT (min)	Primary Transition	Confirmatory Transition	Polarity	CE (eV)	Dwell Time (ms)
CBT	14.65	315.1 -> 193.1	315.1 -> 122.9	+	-34	138
CBDA	3.70	357 -> 107	357 -> 245.1	-	56	150
CBDV	2.42	287.1 -> 164.9	287.1 -> 122.8	+	-46	150

CBDVA	2.11	329 -> 107	329 -> 217	-	37	150
CBD	4.66	315.1 -> 193	315.1 -> 122.9	+	-31	115
CBL	11.16	315.1 -> 81.1	315.1 -> 165.1	+	-37	250
CBLA	13.67	357 -> 191	357 -> 216.9	-	56	138
CBCO	2.98	257 -> 135	257 -> 146.9	-	34	150
CBC	12.55	315.1 - >193.1	315.1 ->122.8	+	-33	250
CBCA	13.87	357 -> 136	357 -> 147.9	-	42	138
CBG	4.45	317.1 -> 122.9	317.1 -> 193	+	-51	115
CBCV	5.72	287 -> 122.9	287 -> 164.9	+	-52	250
CBN	7.09	309 -> 279	309 -> 107.9	-	46	300
Δ^9THC	9.16	315.1 -> 192.9	315.1 -> 122.9	+	-36	342
Δ^9THCA	12.45	357 -> 245	357 -> 191	-	38	250
Δ^8THC	9.76	315.1 -> 193	315.1 -> 122.8	+	-62	360
CBGA	4.18	361.1 -> 343.1	343.1 -> 134.9	+	-20	115
CBNA	9.11	353 -> 279	353 -> 221.9	-	49	342
THCVA	5.69	329 -> 217.1	329 -> 163	-	41	250
THCV	4.23	287 -> 165.1	387 -> 122.9	+	-33	115
Internal Standard	**RT (min)**	**Primary Transition**	**Confirmatory Transition**	**Polarity**	**CE (eV)**	**Dwell Time (ms)**
CBD-d3	4.66	315.1 -> 193	315.1 -> 122.9	+	-31	115
Δ^9THC-d3	9.16	315.1 -> 192.9	315.1 -> 122.9	+	-36	342

UPLC-MS/MS Analysis. Five microliters of extract were injected onto an LX50 UPLC system equipped with a LX-50 solvent delivery pump (20-µL sample loop, partial loop injection mode; PerkinElmer, Shelton, CT, USA). An ACQUITY UPLC HSS T3 column (1 x 100 mm, 1.8 µM; Waters Corporation, Milford, MA, USA) was used for chromatographic separation. The

column was maintained at 50°C, Mobile phase A consisted of LC-MS grade water with 0.1% formic acid and mobile phase B was 100% acetonitrile. Elution gradient was initially at 59% B for 11.5 min, which was increased to 99% B at 16.50 min, then decreased to 59% B at 21.5 min. The column was re-equilibrated for 4 mins for a total run time of 25.50 min. The flow rate was set to 200 µL/min. Detection was performed on a PerkinElmer QSight 220 triple quadrupole mass spectrometer (MS) with an electrospray ionization source operated in selected reaction monitoring (SRM) switching from negative and positive mode ionization. SRM transitions for each compound were optimized through analysis of authentic standards (Table 6). The MS had a drying gas 120 (arbitrary units), a hot-surface induced desolvation (HSID) temperature of 250°C, electrospray voltage was kept at -4500 eV or 4500 eV, and a nebulizer gas flow at 350 (arbitrary units). The MS acquisition was scheduled by retention time with 1.5 min windows.

UPLC-MS/MS Data Analysis. Data processing was performed using Simplicity 3Q™ software (Version 1.5, PerkinElmer). Peak retention times corresponding to SRM transitions for each cannabinoid were validated against authentic standards. Quantitation was performed by generating a standard curve from 20 authentic cannabinoid standards from 1.95-4000 ng/mL concentrations. All signals were normalized to internal standard and concentration of unknowns were determined based on linear regression of the calibration curve. Peak areas were integrated and exported for statistical analysis performed in GraphPad Prism (Version 8.2.1). Data was analyzed using a 2way ANOVA with a Dunnett's multiple comparisons test to evaluate each treatment versus the control plants.

2.4 Results

Days to flower, days to metabolomic sample collection, and days to harvest for both cultivars are listed in Table 7 with Cherry Uno beginning to form flowers approximately 9 weeks (58-65 d; early- to mid-August) after transplanting and Wife beginning to flower approximately 11 weeks (80-81 d; late August to early September) after transplanting. Cherry Uno flowered for approximately 6 weeks for both seasons, and Wife flowered for approximately 4 weeks for both seasons (Table 7). Cherry Uno plants were smaller in terms of total biomass (2019 + 2020 mean biomass = 203 grams) when compared to the total biomass of Wife (2019 + 2020 mean biomass = 593 grams). Cherry Uno had smaller plants that flowered 2 weeks earlier than Wife, so while the biomass measurements for Wife are greater than for Cherry Uno, Cherry Uno had more substantial flower development when the crop was sampled for metabolomic analysis. Harvest index for high-CBD hemp cultivated indoors has been described in a study published in 2021 as the ratio of total inflorescence dry weight to the total dry weight of all aboveground biomass[57], but for the 2019 and 2020 field seasons a harvest index measurement for high-CBD hemp had yet to be published. Furthermore, harvest index for high-CBD hemp grown outdoors will likely have to account for seed weight in this equation as highly seeded hemp flower appears to dilute the cannabinoid concentration of high-CBD hemp biomass (Figure 11).

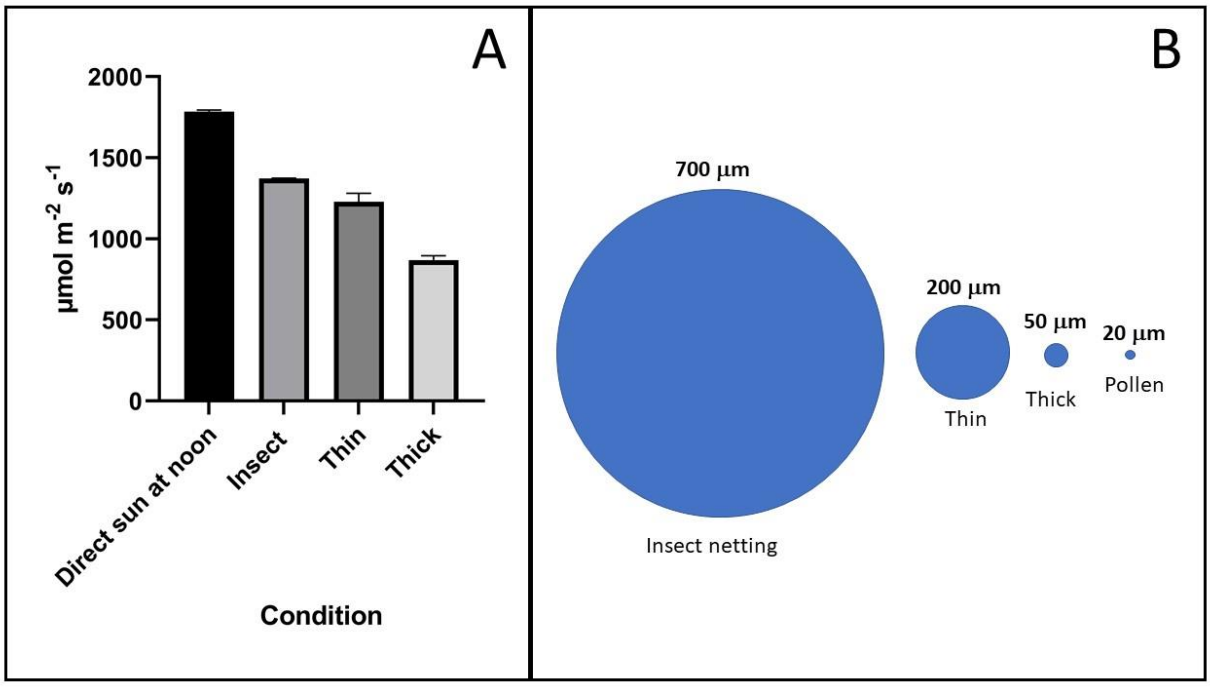

Figure 3A: Average photosynthetically active radiation (PAR) transmission (µmol $m^{-2}s^{-1}$) for direct sunlight at noon in mid-August 2020 versus PAR transmission through the selected pollen exclusion materials. Figure 3B: Pore sizes of pollen exclusion materials versus the average diameter of a hemp pollen grain.

Table 7: Cultivar differences between Cherry Uno and Wife in number of days between transplant to the field and (1) onset of flower development, (2) sample collection, and (3) the plants were harvested from the field.

Cultivar	Field season	Days to flower	Sample collection	Days to harvest	Approximate number of weeks flowering
Cherry Uno	2019	58 d	99 d	104 d	2019: 6 weeks
	2020	65 d	95 d	111 d	2020: 6 weeks
Wife	2019	81 d	99 d	104 d	2019: 4 weeks
	2020	80 d	95 d	111 d	2020: 4 weeks

The amount of photosynthetically active radiation (PAR) under each covering treatment inside the low tunnels is detailed in Figure 3A. Direct sunlight at 12:00 pm in mid-August

averaged 1784 µmol m^{-2} s^{-1} (± 4.38), insect netting transmitted ~77% of radiation at an average of 1372 µmol m^{-2} s^{-1} (±1.36), thin row covering transmitted ~69% of radiation at an average of 1229 µmol m^{-2} s^{-1} (±23.36), and thick row covering transmitted ~49% of radiation at an average of 869 µmol m^{-2} s^{-1} (±12.16). The pore sizes varied considerably across the three treatments. The insect netting had the largest pore sizes at 700 µm (0.7 mm), thin row covering pores measured as wide as 200 µm (0.2 mm) across, and thick row covering pores measured as wide as 50 µm (0.05 mm) across.

Table 8: Results from one-way ANOVA of average 2020 temperatures under each of the low tunnels compared to the temperatures recorded near the uncovered control plants. One-way ANOVA was followed by Dunnett's test for multiple comparisons to evaluate each treatment relative to the uncovered control plants.

	Temperature		
Year	Control vs. Insect	Control vs. Thick	Control vs. Thin
2020	0.3074	<0.0001	0.9958

Thick row covering resulted in significantly cooler temperatures as compared to the ambient temperature experienced by the uncovered control plants (Figure 4). This result was unexpected based on previously reported data that demonstrated that covering with synthetic material did not create a microclimate for the plant[56]. No significant difference in temperature

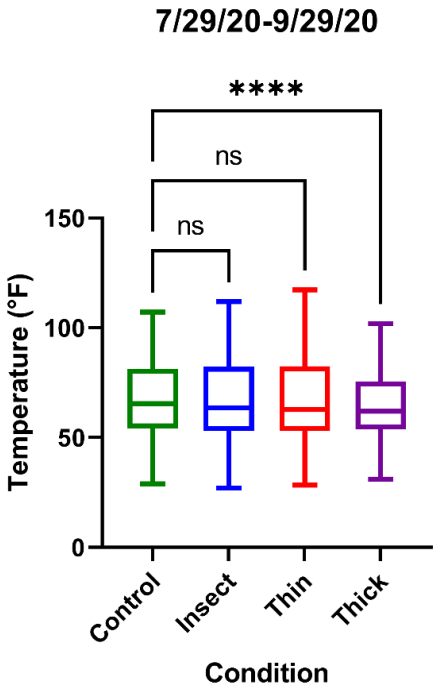

Figure 4: Temperature differences between treatments from 7/29/2020-9/29/2020.

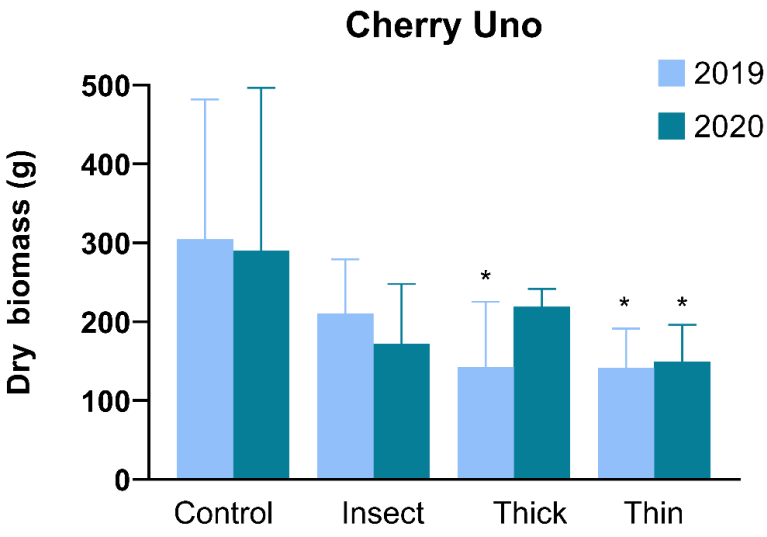

Figure 5: Cherry Uno total dry biomass by treatment in grams for 2019 and 2020.

was observed inside the insect netting or thin row covering compared to the temperature recorded near the uncovered control plants. A significant difference in total plant biomass was

observed for both thick and thin row covers compared to the control in 2019 and for thin row cover in 2020 (Figure 6A). No significant differences in plant biomass were observed for Wife between treatments and control in either year (Figure 7).

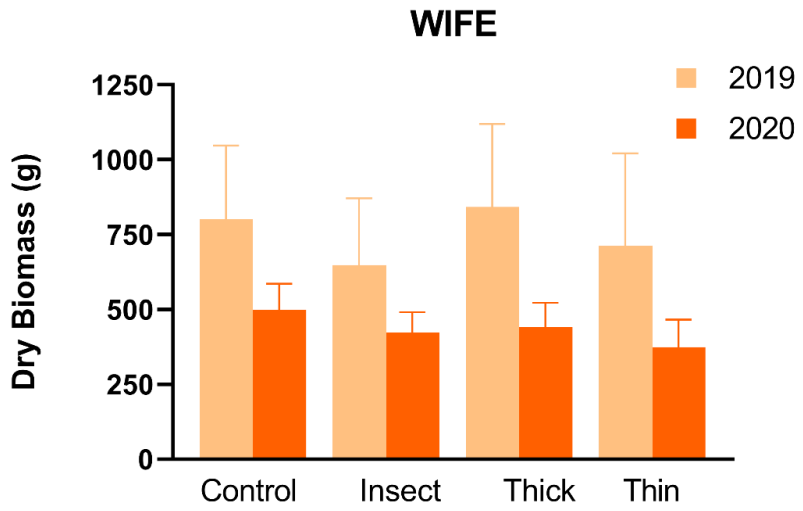

Figure 6: Wife total dry biomass by treatment in grams for 2019 and 2020.

Table 9: Results from one-way ANOVA of 2019 and 2020 biomass from the 4 treatments for Cherry Uno and Wife. One-way ANOVA was followed by Dunnett's test for multiple comparisons to evaluate each treatment relative to the uncovered control plants.

	2019 Biomass			2020 Biomass		
Cultivar	Control vs. Insect	Control vs. Thick	Control vs. Thin	Control vs. Insect	Control vs. Thick	Control vs. Thin
Cherry Uno	0.32	0.0417	0.0407	0.0764	0.1955	0.0215
Wife	0.636	0.8871	0.9871	0.8615	0.8615	0.1574

In 2019, the seed percentage of the total biomass for cultivar Cherry Uno plants under the thick and thin row cover treatments was significantly less (thick p=0.0096, thin p=0.0119, Table 12) than in the Cherry Uno uncovered control plants (Figure 8A). This result was replicated in

2020 for the thick row cover treatment (p=0.0182, Table 12). Although not significant the trend was the same for the thick row cover treatment in 2020. Conversely, none of the treatments resulted in a significant decrease in seed % for the wife cultivar in either crop year. Wife did not have significant differences for any treatment. Data for seed % using only total flower weight instead of total plant biomass was only available for 2020, and only the thick row covering

	2019 seed % of total biomass			2020 seed % of total biomass		
Cultivar	Control vs. Insect	Control vs. Thick	Control vs. Thin	Control vs. Insect	Control vs. Thick	Control vs. Thin
Cherry Uno	0.0506	0.0096	0.0119	0.973	0.0182	0.2978
Wife	0.4125	0.9226	0.0672	0.342	0.3823	0.7408

Table 10: Results from one-way ANOVA of seed % of total biomass for Cherry Uno and Wife. One-way ANOVA was followed by Dunnett's test for multiple comparisons to evaluate each treatment relative to the uncovered control plants.

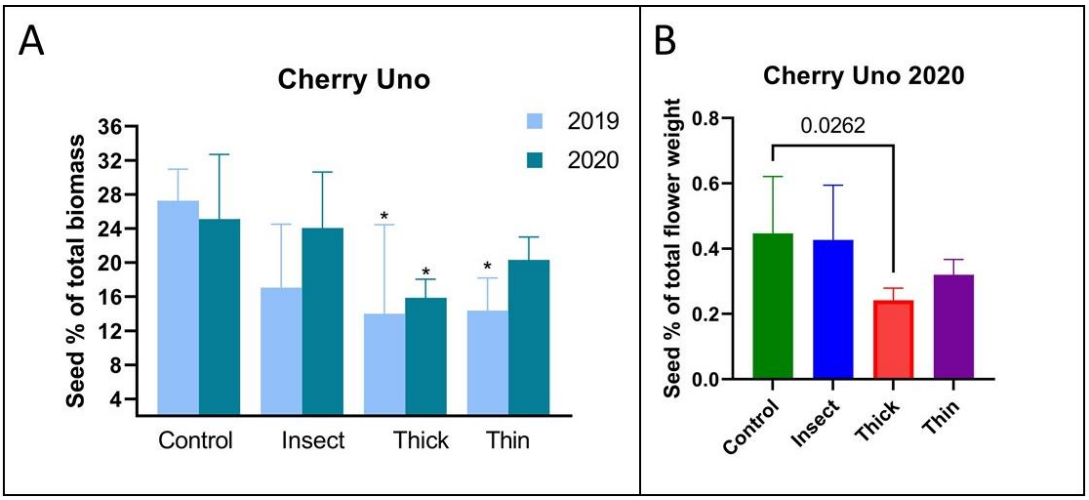

Figure 7A: Total seed weight as a percentage of total dry biomass for Cherry Uno 2019 and 2020. Light blue bars represent Cherry Uno plants grown in 2019, and darker blue bars represent Cherry Uno plants grown in 2020. Figure 7B: Total seed weight as a percentage of total dry flower weight for Cherry Uno in 2020.

treatment showed significantly less seeds compared to the uncovered controls (Figure 7B). Additionally, Figure 7B highlights that when seed percentage is compared to total dry flower weight instead of total dry biomass, the ratio is even more stark with marketable biomass (flower and leaf) of uncovered control plants having up to 40% seeds by weight. Lastly, the data trends suggest that plants under the insect netting treatment also had reduced seed content when compared to controls, but this was not statistically significant for either cultivar or year.

Most significant cannabinoid differences were found when comparing treatments in Cherry Uno, but not for Wife. Wife varied significantly in 2019 and 2020 for CBGA content, but otherwise all other significant differences were seen only in Cherry Uno. Of the twenty cannabinoids evaluated, three cannabinoids were significantly higher between some of the treatments versus the control plants for Cherry Uno in 2019 (CBCO, CBGA, and THCVA [Table 12]) and eleven cannabinoids were significantly higher between some treatments and control

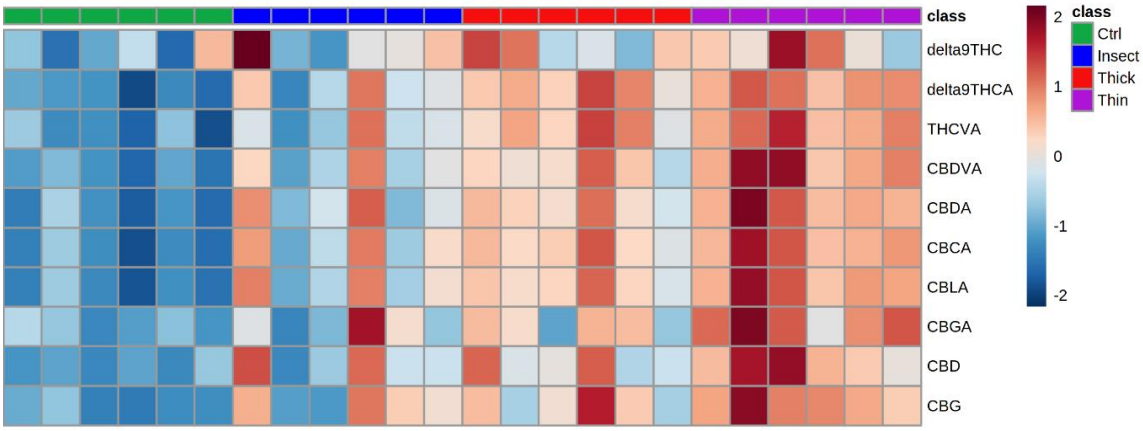

Figure 8: A heatmap showing relative abundance (as a z-score) of all cannabinoids that were found to differ significantly between treatments for Cherry Uno in 2020. The treatments are along the top of the heatmap: green = uncovered control, blue = Insect netting, red = thick row covering, purple = thin row covering. 6 biological replicates per condition.

plants for Cherry Uno in 2020 (CBCA, CBD, CBDA, CBDVA, CBG, CBGA, CBLA, Δ^9THC,

Δ^9THCA, THCV, and THCVA [Table 12]). No cannabinoids were significantly different between treatments and control plants for Wife in 2019. CBGA was significantly higher in 2020 for Wife plants (Table 12), but this was the only cannabinoid result which indicated the control plants had significantly more CBGA than the plants under the thick row covering. Figure 9 is a heatmap showing significant cannabinoid changes in 2020 for Cherry Uno. On the right half of the heatmap an increase in cannabinoids is generally seen, which correlates with the thick and thin row coverings. Control and insect netting treatments showed relatively less of these cannabinoids, indicated in shades of blue. The heatmap shows relative abundance between each of the six replicates that were analyzed for each treatment. Each sample's abundance is shown in one column of the heatmap, and the rows of the heatmap each indicate a different cannabinoid that was evaluated across the sample set.

Figure 10A and 10B show cannabinoids with significant differences between treatments in 2019 and 2020. In 2019, CBGA and CBCO were significantly higher under the thick row cover treatment when compared to the controls. Figure 10C -F show cannabinoids with significant differences between treatments in 2020. In 2020, CBCA, CBDA, and Δ^9THCA were significantly higher in all three treatments when compared to controls, but CBGA was only significantly higher for plants under the thin row covering treatment. Limit of detection (LOD) and limit of quantitation (LOQ) for each phytocannabinoid (Table 8) and p-values for all one-way ANOVA analyses are shown below (Table 9).

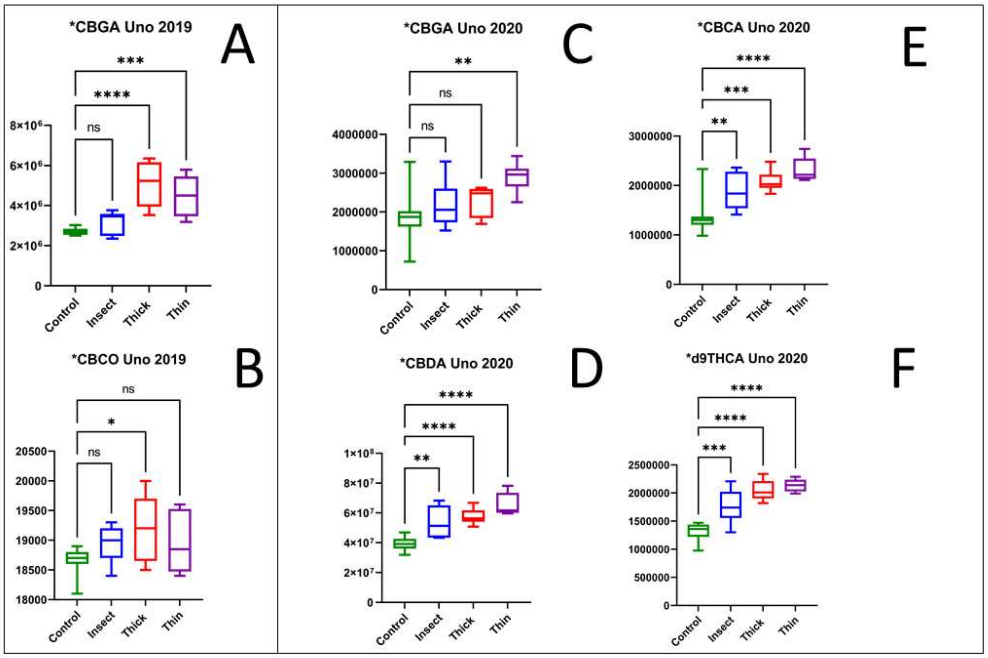

Figure 9A+B: Significantly different cannabinoids between treatments for 2019 Cherry Uno included CBGA and CBCO. Figure 9C-F: Significantly different cannabinoids between treatments for 2020 Cherry Uno included CBGA, CBDA, CBCA, and Δ^9THCA.

The CBD:THC ratio of each treatment for Cherry Uno 2020 was evaluated by comparing the amount of CBDA and Δ^9THCA quantified in the metabolomic samples. Including the miniscule concentration of CBD and Δ^9THC found in the samples did not affect the CBD:THC ratio trends. No significant differences between treatments were observed in the CBD:THC ratio achieved in inflorescences for Cherry Uno in 2020. Ratios varied from 25:1 to 30:1 for control

plants, 25:1 to 33:1 for insect netting, 25:1 to 31:1 for thick row covering, and 28:1 to 34:1 for thin row covering (Figure 11).

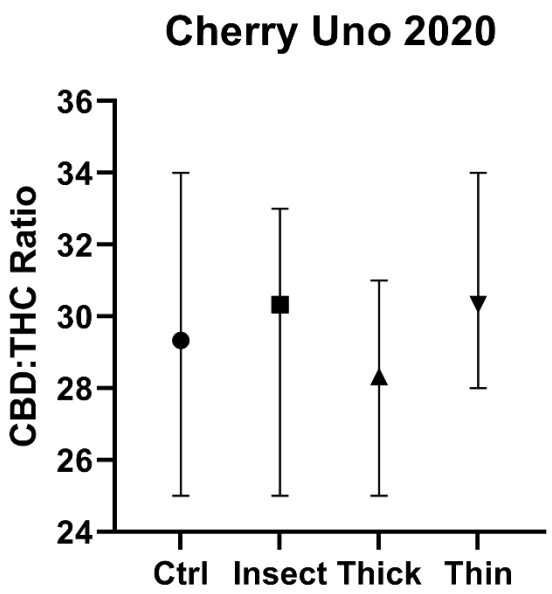

Figure 10: No significant difference in CBD:THC ratio was seen between treatments for Cherry Uno in 2020, which had the most significant cannabinoid differences between treatments.

Table 11: Limit of detection (LOD) and limit of quantitation (LOQ) for 20 phytocannabinoid analyses.

Phytocannabinoid	LOD (ng/g)	LOQ (ng/g)
CBT	538.5	1795
CBDA	1144.5	3820
CBDV	82.85	276
CBDVA	772	2570
CBD	1963	6545
CBL	17750	59150
CBLA	334.5	1115
CBCO	640.5	2131
CBC	350	1165.5
CBCA	1535.5	5115
CBG	304.5	1016
CBCV	489.5	1633.5
CBN	29.31	97.55
Δ^9THC	123.3	411.3
Δ^9THCA	154.55	515

Δ^8THC	529	1765
CBGA	291.5	973
CBNA	427.05	1425
THCVA	103.5	344
THCV	101.65	339

Table 12: Results from one-way ANOVA on 20 cannabinoid concentrations from 4 treatments for Cherry Uno in 2019 and 2020 field seasons. Treatments included: Uncovered controls, insect netting, thick row cover, and thin row cover. One-way ANOVA was followed by Dunnett's test for multiple comparisons to evaluate each treatment relative to the uncovered control plants.

Compound	Uno 2019 cannabinoid p-values			Uno 2020 cannabinoid p-values		
	Control vs. Insect	Control vs. Thick	Control vs. Thin	Control vs. Insect	Control vs. Thick	Control vs. Thin
CBC	>0.9999	0.0838	0.9031	0.9339	0.3565	0.1636
CBCA	0.2786	0.1348	0.7713	0.008	0.0003	<0.0001
CBCO	0.3301	0.0409	0.3734	0.5782	0.9535	0.1856
CBCV	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CBD	>0.9999	0.5105	0.9944	0.0122	0.002	<0.0001
CBDA	0.4112	0.4885	0.8741	0.0009	<0.0001	<0.0001
CBDV	<LOD	<LOD	<LOD	0.0012	<0.0001	<0.0001
CBDVA	0.1812	0.1035	0.2176	<LOD	<LOD	<LOD
CBG	0.6923	0.2602	0.2521	0.0019	0.0002	<0.0001
CBGA	0.427	<0.0001	0.0004	0.5599	0.3216	0.0021
CBL	0.443	0.6694	0.992	0.6101	0.9742	0.214
CBLA	0.4736	0.4447	0.9864	0.0086	0.0006	<0.0001
CBN	>0.9999	0.9985	0.6987	0.4011	0.669	0.204
CBNA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CBT	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Δ^8THC	<LOD	<LOD	<LOD	0.5574	0.876	0.1319
Δ^9THC	0.9543	0.6705	0.9687	0.1346	0.0695	0.0267
Δ^9THCA	0.633	0.2055	0.8991	0.0003	<0.0001	<0.0001
THCV	<LOD	<LOD	<LOD	<0.0001	<0.0001	<0.0001
THCVA	0.795	0.0196	0.1623	0.0032	<0.0001	<0.0001

In 2020, Cherry Uno demonstrated significant higher concentrations in CBDA concentration when comparing thick row covering or thin row covering to the uncovered control plants. Control plants had a mean CBDA concentration of 3.77% compared to 5.75% and 6.49% mean CBDA % for thick and thin row cover, respectively (Figure 11B). Control plants also had lower levels of Δ^9THCA with a mean value of 0.13% compared to 0.21% Δ^9THCA for both thick and

thin row coverings. CBDA and Δ^9THCA concentrations alone were used for this comparison, as adding in the miniscule concentrations of CBD and Δ^9THC did not change the descriptive statistics in Figure 11B. Regulatory compliance testing often reports the major cannabinoids in terms of total CBD and total Δ^9THC. This calculation is addressed in the discussion below.

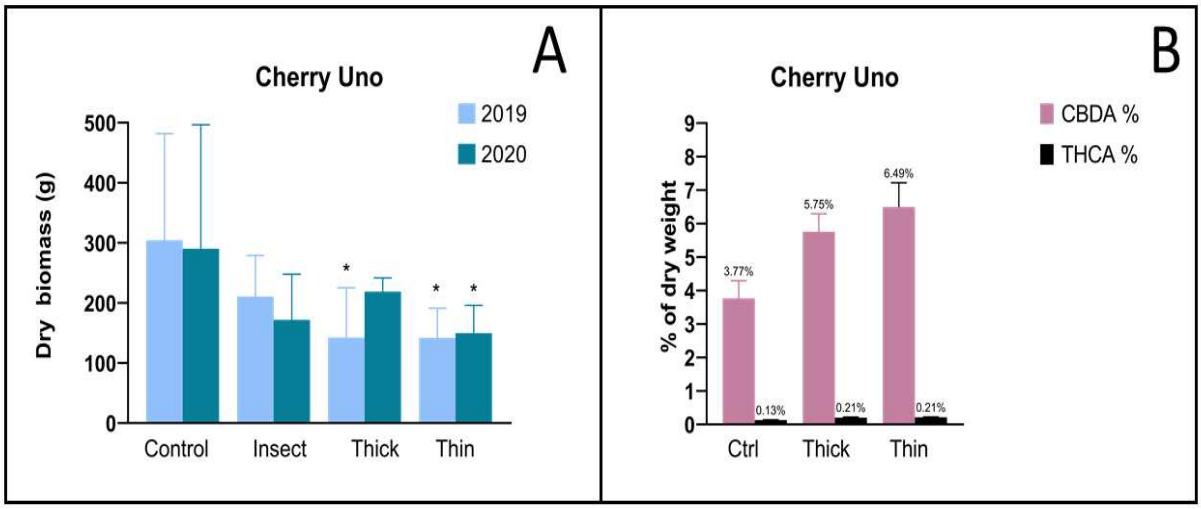

Figure 11A: Thick and thin row covering treatments had significantly less biomass compared to control plants. Figure 11B: Homogenized biomass from thick and thin row covering treatments had significantly more CBDA than the biomass from uncovered control plants.

2.5 Discussion and Future Directions

The results of this study demonstrate that both thick and thin row cover were successful at reducing pollination and seed content in the marketable biomass of high-CBD hemp plants. This suggests that row cover could be used as a mechanism to reduce pollination in high-CBD hemp cultivated outdoors. Optimization of this strategy as a management practice would necessitate additional evaluations of material type and timing of treatment application, as well as exploring different regions and climates. Research assessing how the timing of covers relates to pest infestations would be beneficial as well.

Though CBGA was statistically significant between Wife control in 2020 and Wife thick row cover in 2020, the magnitude of the difference was not large, with the mean difference between Wife 2020 control plants and Wife 2020 thick row cover plants being just over 1,000,000 ng/g, or 0.1% CBGA. Similarly, CBGA concentrations for thick and thin row covers for Cherry Uno in 2019 demonstrated roughly 0.2% more CBGA when compared to control plants. Of the treatments for Cherry Uno 2020 that had higher concentrations of cannabinoids compared to controls, THCVA was a miniscule difference (+0.0002-0.0004% THCVA), and CBDA demonstrated the largest difference when treatments were compared to 2020 controls (Insect vs Control +1.4% CBDA; Thick vs Control +1.8% CBDA; Thin vs Control +2.5% CBDA). The nine additional cannabinoids that were significantly higher in treatments for Cherry Uno 2020 (CBCA, CBD, CBDVA, CBG, CBGA, CBLA, THC, THCA, and THCV) showed concentrations +0.01-0.21% greater when compared to Cherry Uno 2020 controls.

It is notable that significant reductions in pollination were only observed in Cherry Uno, which suggests a potential cultivar difference on the row covering treatment. Alternatively, because Cherry Uno plants flowered for 2 weeks longer than Wife plants the flowers were visually much larger and its possible Wife flowers did not develop enough for trends to become apparent, which the data trend supports. For example, though there was no statistical significance found for seed percentages when comparing Wife treatments to control plants using one-way ANOVA for 2019 or 2020, when averaging the years together the same trend is seen in Wife: Control 7.36%, Insect 7.11%, Thick 5.46%, and Thin 3.94% of the total dry biomass of Wife plants was comprised of seeds on average. Studies to assess how various cultivars perform under the various coverings would be an important step to take as well, as only 2 cultivars were

evaluated in this experiment and cultivar-specific effects were noted in terms of Cherry Uno consistently having significant differences in the various measures.

Pollen exclusion with coverings in high-CBD cannabis could prove to be the most efficient way to ensure that the biomass produced is primarily flower material with few seeds, which appear to dilute the concentration of CBDA in the flower material significantly. Triploid cultivars that are sterile and do not produce seeds could be an attractive alternative to the current diploid varieties, but research into triploid cultivars is needed to determine how these genotypes perform. Studies including triploid cultivars are currently underway at Colorado State University for the 2021 growing season. Alternatively, diploid cultivars could be grown in a greenhouse or high tunnel environment to physically block the pollen from fertilizing the hemp flowers. High tunnels would be a cheaper alternative and possibly a good option for producers just beginning in the hemp industry, whereas greenhouses represent a larger investment and have larger maintenance costs associated with them. Furthermore, if harvest index and/or the quality of the biomass in terms of extractable compounds is significantly increased when high-CBD hemp is grown indoors, the cost of this infrastructure could prove to be a worthwhile investment. Growing indoors would also avoid hail damage and the threat of early frost (multiple harvests per year are possible indoors) though pest management can quickly become a significant challenge for an indoor crop. Compared to an indoor crop, plants cultivated outdoors will typically avoid pest issues due to natural predators being present alongside the pests. Lastly, if 5 km of distance can be maintained downwind between a known source of cannabis pollen and a high-CBD hemp farm, it is likely that a farmer will not have a seeded product. However, ensuring that this distance is maintained and that no rogue male cannabis plants inadvertently take root within a 5 km area could prove challenging.

Total CBD and total Δ^9THC are the values often reported in hemp potency testing for regulatory compliance. Total CBD and Δ^9THC values are often calculated by analyzing samples with an instrument such as GC-FID that decarboxylates all of the acidic cannabinoids, leaving only the neutral forms of CBD and Δ^9THC which are then quantified. Alternatively, total CBD and total Δ^9THC can be calculated with a formula based on the concentration of CBDA and Δ^9THCA. This formula assumes that decarboxylation is 100% efficient, which is unlikely in real-world settings. In theory, when a molecule of CO_2 is released from an acidic cannabinoid during decarboxylation, a specific amount of weight is lost. For Δ^9THCA ($C_{22}H_{30}O_4$), the weight of 22 carbon atoms (12.01 amu), 30 hydrogen atoms (1.008 u), and 4 oxygen atoms (16.000 amu) are added together, which equals 358.46 amu. For Δ^9THC ($C_{21}H_{30}O_2$), the molecule weight equals 314.45 amu. For total Δ^9THC calculations, that is translated to Δ^9THC weighing 87.72% of what a molecule of Δ^9THCA weighs. Thus, the 0.13% Δ^9THCA in the uncovered controls would be calculated as having 0.11% Δ^9THC, and the thick and thin row covering treatments with 0.21% Δ^9THCA would be calculated to be 0.18% Δ^9THC.

Another approach the pollination problem is to add an additional step to the processing of hemp biomass. Industrial seed cleaners can be utilized to remove the seeds from a farmer's biomass prior to the material being sent for extraction. This represents an extra expense by requiring another instrument, as well as the additional time needed to remove the seeds. At least one hemp processing and extracting company in Northern Colorado has this equipment that can remove hempseed from hemp biomass, but it is likely that this type of service would not be widely available or feasible for many farmers to utilize nationwide. A final consideration is that hempseed itself is a commodity and could be separated and potentially sold for a profit. This would be dependent on when the plants were harvested, as grain has typically not fully matured

until the end of September in Fort Collins, Colorado. If high-CBD hemps plants were harvested early due to exceeding the legal limit of Δ^9THC or because of the threat of an early cold snap, grain may still be immature and would not represent a marketable commodity.

Like specialty vegetable crops, high-CBD hemp plants with the highest possible harvest index will likely be the most profitable for producers. How harvest index is assessed for high-CBD hemp will have to factor in how much of the biomass is comprised of seeds, as this material contains no cannabinoids inside of the seed hull, but may comprise upwards of 25% of total biomass weight (stalk, flower, and seed). Furthermore, when only marketable biomass (flower weight) is considered, freely pollinated high-CBD hemp flower may contain more than 40% seeds by weight. Thick and thin row covering treatments resulted in plants that weighed significantly less than the uncovered control plants, but homogenized biomass from the thick and thin row coverings had approximately 2% and 3% more CBDA, respectively, when compared to the concentration of CBDA in the uncovered control plant biomass.

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