EFFECTS OF FREEZING AND ACCLIMATION ON COLD TOLERANCE AND CANNABINOID PROFILES OF *CANNABIS SATIVA* L. (HEMP)

A Project Report

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Master of Professional Studies

by

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ABSTRACT

Hemp (*Cannabis sativa*) is a high-value crop garnering newfound attention from researchers and consumers. While interest has emerged, a lack of substantiated research still exists regarding effects of adverse weather events on physiological health and secondary metabolite production of hemp. The aim of this experiment was to assess cold tolerance of hemp using the cultivars 'FINOLA' and 'AutoCBD'. Effects of cultivar, plant age, cold acclimation, frequency of cold treatments, and intensity of cold treatments were all considered in regard to their influence on physiological stress, biomass, and cannabinoid profile. In contrast to expectations, few effects of sequential cold treatments existed and were not moderated by cold acclimation, which tended to have negative effects across many responses. This detrimental effect of cold acclimation conditions was further observed in decreased total CBD% and total THC% compared to non-acclimated plants. These findings bear consideration when assessing the unpredictability of a changing climate on the heath and cannabinoid profile of hemp.

BIOGRAPHICAL SKETCH

Los Angeles native Andrei Galic's passion for plant life developed during his time studying Agricultural and Environmental Plant Sciences at California Polytechnic State University in San Luis Obsipo, CA. It was here, far from the urban jungles of Los Angeles, that his fascination with plants (particularly *Cannabis sativa*) transitioned into a yearning for practical learning in horticulture. A study abroad internship spent developing irrigation and greenhouse plans for farms in Kikuyu, Kenya was instrumental in opening his mind to the extent to which plant life can benefit humanity. This "learn by doing mentality" drove Andrei to pursue key horticultural experiences following his graduation from Cal Poly; experience was gained in plant protection when he worked IPM for MedMen, and an eye for detail in research and development was trained during his time at vertical farming startup Plenty. These foundational career experiences left him seeking further knowledge not always available in industry. As per the suggestion of his long time friend and current Cornell graduate student Conor Stephen, he pursued a Master of Professional Studies degree in Hemp Sciences at Cornell University. Research experience and industry skills learned in Ithaca further solidified his life mission in utilizing plants for the benefit of many.

ACKNOWLEDGMENTS

Firstly, I want to acknowledge and thank the Cornell faculty involved in advising me on this Capstone project. I am deeply grateful to Dr. Carlyn Buckler for leading the first iteration of the Hemp Science MPS program. This experience took place during a doubtful year of the pandemic, but Carlyn's poise as the program advisor helped our entire cohort push through to the end. I am also greatly appreciative to Dr. Larry Smart, who served as my primary advisor specific to this capstone project. In spite of the distance between Ithaca and Geneva, Larry made himself and his resources available to guide me throughout this experience, and showed trust in my abilities to independently problem solve. Next, I would like to thank Dr. Heather Grab, who also mentored me throughout this research. Heather was generous with her time dedicated to this project, as she taught me principles of data analysis and guided data collection of cannabinoids. Finally, I must express deep gratitude to Nicholas Kaczmar who also mentored me throughout this process. Nick made himself available to reflect back realistic expectations of logistical greenhouse processes and was always a friendly face to catch up with in Kenneth Post Laboratories.

Secondly, I want to acknowledge Cornell faculty and staff involved with the on campus greenhouses. Dr. Bill Miller was instrumental in accommodating my greenhouse, growth chamber, and bulb cooler needs and provided recommendations on experimental design of the project. In Kenneth Post Laboratories, Julie Blaha fertigated and applied pesticides to ensure my experimental units remained healthy throughout plant development. Nick Van Eck selflessly coordinated my use of a freezer unit located in the Guterman Bioclimatic Laboratory. I would also like to thank Nick Lepak for allowing me to use his lab's freezer unit.

Thirdly, I want to acknowledge my Hemp MPS cohort, and those that assisted me throughout this project. I'd like to thank Kady Maser for choosing to make substantial contributions to the HPLC portion of this research while she was still finishing her capstone project. I would like to thank Paul Reum and Anthony Barracco for helping process cannabinoid samples, and for Anthony's involvement in the first harvest.

Fourthly, I want to acknowledge my team at the Bridgen Lab, where I worked as a Micropropagation Lab Assistant. Conor Stephen, a long time friend and colleague, had the foresight to recommend me to apply to the Hemp Science program and was pivotal in sparking my interest in micropropagation. Victor Zayas's vast knowledge of the Plant Sciences inspired me to continue to learn more. Victoria Zheng's hard work ethic motivated me to work harder at my studies. Dr. Mark Bridgen's generous trust in my ability to learn micropropagation via tissue culture was greatly appreciated.

Fifthly, I want to thank Ethan Zohn and Dave Christian of MONTKUSH Farms for inspiring the concept of this research. Ethan is a motivational speaker and my role model from the television show 'Survivor' and was thoughtful in connecting me to his business partner Dave, who graciously gave me a tour of his Vermont Hemp Farm. Cultivation difficulties expressed by Dave in the form of early frost to his crop became the focus of this research.

Lastly, but certainly not least, I would like to acknowledge all those who have been adversely affected by the laws and stigmas surrounding the plant species *Cannabis sativa*. Legislation and public perception has evolved in recent times but must continue to progress to ensure equity and acceptance in this burgeoning industry.

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1. INTRODUCTION

Cannabis sativa L. (< 0.3% tetrahydrocannabinol hemp) is an annual, dioecious crop and agricultural commodity produced for its use in textiles, food, and cannabinoid medicine. With its industry projections nearing $6.3 billion by 2025, cannabidiol (CBD) is quickly being integrated into mainstream societal adoption (Yahn-Grode, 2021). Increasing commercial interest in hemp - *C. sativa* plants containing less than 0.3% Δ9-tetrahydrocannabinol (THC) on a dry weight basis as per United States' federal regulations - has rejuvenated involvement from researchers and cultivators alike.

A rapidly changing climate, however, poses several, unanswered questions pertaining to cultivating hemp in cold temperatures. Freezing damage on crops in the US causes more financial losses than any other weather-related abiotic stress (Snyder and Melo-Abreu, 2005). Toth et al. (2021) studied the influence of five abiotic or biotic stresses on cannabinoid accumulation and profile, ultimately concluding these stresses (with the exception of herbicide application) had no significant effect on ratios of cannabinoids. However, still very little is documented regarding the timing and intensity of cold temperature abiotic stress effects on the physiological health and total cannabinoid levels of hemp.

Hemp produces a variety of bioactive compounds that are most highly concentrated in the capitate stalked trichomes found on the apical inflorescences of female plants (Happyana et al., 2003; Livingston et al., 2020). This region of the plant is the most abundant producer of CBD, the primary legal cannabinoid gaining commercial interest within the United States and abundant in chemotype III hemp plants (de Meijer et al., 2003; Fernandez et al., 2020). Chemotype III plants (high in CBD and low in THC) are selected by hemp cultivators to

maintain compliance with federal regulations. While hemp differs from marijuana in its decreased concentration of the psychoactive cannabinoid THC, these plants are classified as the same species. Cannabigerol (CBG) is an additional cannabinoid garnering academic attention due to cannabigerolic acid's (CBGA) role as a precursor to cannabidiolic acid (CBDA) and tetrahydrocannabinolic (THCA) (Toth et al., 2020). Although day-neutral varieties - those that flower independent of day length - exist, hemp is primarily a photoperiodic crop whose flowering structures initiate with the introduction of short days (Salentijin et al., 2019). As winter approaches, the length of uninterrupted dark periods increases and transitions the plant from vegetative growth to flowering. Accumulation of cannabinoids and CBD:THC ratio significantly correlates with time elapsed after terminal flowering (Pacifico et al., 2008; Yang et al., 2020; Stack et al. 2021; Toth et al., 2021). The timing of harvest of mature hemp flowers, therefore, is largely dependent on the latitude of the cultivation site and distance from the equator.

The climate crisis of the 21st century has impacted life on Earth and poses several difficulties to cultivators of hemp. Increased variability in timing of precipitation and snowfall, duration of snow-cover, and frequency of freeze-thaw cycles threaten climate stability (Solomon et al., 2007). Early frosts can be particularly deleterious to cultivators at latitudes farther removed from the equator; cultivators at these latitudes must harvest later into the growing season due to hemp's photoperiodism. Stack et al. (2021) conducted a multi-site hemp flowering trial elucidating variation in cannabinoid accumulation, flowering time, and disease resistance among 30 high CBD cultivars. This trial provided cultivators with valuable information regarding timing of flowering. Understanding variation of flower bud initiation and development are crucial in guiding cultivar selection in regions prone to early frosts. Thus, breeding efforts for early flowering cultivars must continue alongside investigations of cold tolerance in hemp.

Plant species develop varying mechanisms of tolerance to abiotic stresses. However, very little published research exists on the cold tolerance of hemp. A University of Vermont trial tested the effects of row cover on temperature near soil surface and the harvested concentrations of CBD in relation to cold temperatures. Results indicated that those plants containing row cover had higher average temperatures near the soil surface, but CBD concentrations were not significantly different (Darby et al., 2018). Another study sought to evaluate cold tolerance of seedlings of nine hemp varieties based on duration and intensity of cold acclimation periods. Findings indicated that while cold acclimation conferred cold tolerance differently in relation to cultivar, all cultivars experienced cell damage via electrolyte leakage during cold acclimation periods of 7 and 14 days and at 4°C ±1°C (Mayer et al., 2015). Research on other species has reported that plant age and photoperiodism are factors also affecting cold tolerance (Płażek et al. 2011; Lejeune-Hénaut et al. 2008). Understanding hemp's ability to tolerate cold temperatures was the purpose of the following experiment. Therefore, the objectives of this trial were to:

1. Understand the effect of plant age, cultivar, cold acclimation, frequency and intensity of cold, or their interaction on hemp's cold tolerance.
2. Evaluate the effects of cold temperatures on post-harvest biomass yield and cannabinoid content.

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN

Cold stress experiments consisting of four treatment groups were conducted to assess effects on plant health and cannabinoid levels. Day-neutral, chemotype III cultivars 'AutoCBD' and 'FINOLA' were selected for this trial. 'AutoCBD' (Phylos) is a feminized, high-cannabidiol cultivar and 'FINOLA' is a dioecious, grain cultivar. Male 'FINOLA' plants were culled immediately after staminate flowers were observed. Day-neutral cultivars were selected to accurately stagger planting date treatments. Seeds were planted at three times to form three age groups, with each planting occurring 14 days following the previous. In doing so, the stage of flower development was unique to each plant age treatment group on the day of cold stress, but all plant age treatment groups were harvested when plants were 75 days old. Half of the plants were exposed to a 10 day cold acclimation treatment to initiate a hardening response. One week prior to cold stress treatment, a stratified randomization based on height was used to group four biological replicates from each age group into four cold stress groups (n = 16 per group), each receiving a different cold stress treatment. Due to the dioecious nature and low germination of 'FINOLA', there were not enough female 'FINOLA' plants to form a treatment group of non acclimated older plants, and the acclimated and non acclimated groups of youngest plants contained 14 'FINOLA' plants each. As result, 96 'AutoCBD' and 76 'FINOLA' plants split between three age groups were divided into four cold stress treatment groups per acclimation treatment.

Frequency of cold exposure was tested via whole plant cold stress. Whole plant cold stress groups consisted of four plants receiving no cold exposure (control), four plants receiving

a single cold exposure, four plants receiving two consecutive cold exposures, and four plants receiving three consecutive cold exposure. Each consecutive whole plant cold stress occurred 24 hours following the previous exposure. Plants were exposed to -0.5° C for 3 hours in darkness within a bulb cooler (Geerlofs), and returned to cold acclimated conditions until harvest. Plants other than those receiving control treatment were exposed to initial whole plant cold stress on June 4th, 2021. A second exposure for two treatment groups continued June 5th, 2021. The remaining treatment group was subject to a final cold exposure that concluded on June 6th, 2021. Stress measurements were taken for each whole plant cold stress group four days after their respective final exposure. Although control treatments received no cold exposure, their second, comparative measurements occurred on the day following post stress measurements for plants receiving three cold exposures. Chlorophyll measurements were quantified as the change in Fv/Fm ($\Delta Fv/Fm_{WP}$) and in SPAD ($\Delta SPAD_{WP}$) as measured before and after whole plant cold stress (Table 1).

Detached leaf cold stress was the method selected to test intensity of cold temperatures in a modified freezer. Leaves from the upper half of the plant were detached with petiole intact and placed in ziplock bags. Only leaves from experimental units receiving the control whole plant cold stress were used for detached leaf cold stress. A total of 44 unique leaf samples were placed into the freezing unit for 3 hour periods for each temperature treatment. Samples were exposed to -2° C on June 4th, 2021, -4° C on June 5th, 2021, and -8° C on June 6th, 2021. Stress measurements were taken immediately after cold exposure. To quantify chlorophyll damage as a result of cold exposure intensity, initial Fv/Fm and SPAD values were identical to initial values used in quantifying damage resulting from frequency of cold exposure (initial Fv/Fm_{WP} and

$SPAD_{WP}$). However, in this case, $\Delta Fv/Fm_{DL}$ and $\Delta SPAD_{DL}$ values were generated using final Fv/Fm and SPAD values collected immediately after cold exposure (Table 1).

Table 1. Formulas used in analysis of chlorophyll fluorescence, SPAD, electrolyte leakage, and cannabinoids.

Value	Formula
ΔFv/Fm WP	post whole plant cold stress Fv/Fm - pre whole plant cold stress Fv/Fm
ΔFv/Fm DL	post detached leaf cold stress Fv/Fm - pre whole plant cold stress Fv/Fm
ΔSPAD WP	average post whole plant cold stress SPAD - average pre whole plant cold stress SPAD
ΔSPAD DL	average post detached leaf cold stress SPAD - average pre whole plant cold stress SPAD
EL WP	post whole plant cold stress EC / post autoclave EC
EL DL	post detached leaf cold stress EC / post autoclave EC
Total CBD %	CBD % + (CBDA %*0.877)
Total CBG %	CBG % + (CBGA %*0.878)
Total THC %	Δ9-THC % + (THCA %*0.877)

2.2 GROWING ENVIRONMENT

All plants were grown in Cornell University greenhouses and growth chambers in Ithaca, NY (Kenneth Post Laboratory: 42.449, -76.468). The oldest plants were seeded on March 28th, 2021, middle-aged plants on April 11th, 2021, and youngest plants on April 25th, 2021. Both cultivars were seeded 1 cm deep in 4.5-inch pots containing a commercial all purpose potting mix (Lambert LM-111). Direct seeding was done to avoid the potential of early flowering caused by transplant shock specific to day-neutral cultivars. Seeds were germinated in a propagation house and were fertigated everyday with a 21-5-20 150 ppm fertilizer solution (J. R. Peters, Inc.). After 14 days in the propagation environment, plants were moved to a higher light intensity greenhouse containing mature hemp plants. Plants were grown at ambient light conditions and exposed to daytime temperatures of 22.2° C and night temperatures of 19.4° C. All greenhouse plants received an additional daily fertigation. Plants received multiple pesticide applications on an as needed basis. Powdery mildew was controlled through applications of Cease (BioWorks),

Milstop (BioWorks), Ultra-Pure Oil (BASF), and JMS Stylet Oil (JMS Flower Farms). Thrips were targeted with applications of Acephate 97 UP (UPI), Talstar P (FMC), and Safari 20 SG (Valent). Akari 5 SC (SePRO) and Avid .15 EC (Syngenta) were applied for mite and aphid control. Cold acclimation period took place in a growth chamber kept at 10° C with a 14 h light: 10 h dark photoperiod starting May 24th for 10 days. The chamber was equipped with 24 dimmable LED boards (Horticultural Lighting Group) emitting 330 umols. Individual plants were fertigated on an as needed basis with fertilizer solution kept at growth chamber temperatures.

2.3 MEASURING COLD STRESS RESPONSES

Cold stress was quantified via chlorophyll fluorescence, SPAD measurements, and electrolyte leakage. Chlorophyll fluorescence was measured using a LI-6400XT portable photosynthesis system (LI-COR Biosciences). Middle leaflets of detached leaves were dark adapted for 20 minutes using dark adapting clips and subsequently inserted into the 6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences) to measure maximum quantum yield of PSII (Fv/Fm) (Maxwell and Johnson, 2000). Fv/Fm provides valuable insight into photosynthetic capacity of plants as a result of photoinhibition (He et al., 1996; Valladeres and Pearcy, 1997). Dark adapted chlorophyll fluorescence measurements are an especially useful diagnostic in assessing tolerance in relation to freezing damage (Groom and Baker, 1992). Chlorophyll content was measured using a SPAD 502 Plus Chlorophyll Meter (Konica Minolta). Photosynthesis can decrease as a result of freeze damage to chlorophyll (Wang et al., 2016). Chlorophyll content was measured as an indirect method to quantify abiotic stress to plants (Gitelson and Merzlyak et al., 1992; Takai et al., 2010; Park et al., 2013). Initial SPAD readings

were taken from the middle leaflets of three randomly selected leaves from the upper half of each plant. Initial Fv/Fm and SPAD measurements were taken prior to whole plant cold stress and also used as initial values for detached leaf cold stress. Final Fv/Fm and SPAD were taken again 4 days after final whole plant cold stress and immediately following detached leaf cold stress.

A modified electrolyte leakage assay was conducted referencing previous protocols (Sukumaran et al. 1972; Ristic and Ashworth et al. 1993). Petioles were detached from leaf samples and were placed in 50 mL glass tubes containing 45 mL of deionized water. Tubes were placed on a shaker and agitated at 100 rpm for 16 hours. Electrical conductivity (EC) of solution was taken using a HI8733 Multi-range EC Meter (Hanna Instruments) after agitation. Vessels were autoclaved for 20 minutes to effectively lyse all cells. After cooling to room temperature, EC readings were taken again. Electrolyte leakage was calculated as the ratio of initial EC measurements divided by the final EC measurements. Plant cells leak electrolytes after damage to membranes (Murray et al., 1989; Campos et al., 2003). Measuring EC before and after cell lysis results in a percentage of leaked electrolytes indicating damage due to cold stress. Electrolyte leakage assay was conducted only after the whole plant and detached leaf cold stress treatments were completed.

2.4 POST HARVEST MEASUREMENTS

Weight and cannabinoid data were collected on all 'AutoCBD' plants. Due to the staggered planting schedule, harvest occurred 75 days after seeding on 6/10, 6/24, and 7/8 for the oldest, middle, and youngest age groups respectively. Samples collected for high pressure liquid chromatography (HPLC) were collected from the top 10cm of apical inflorescence and freeze dried using a Pharma Freeze Dryer (Harvest Right). Remaining plant biomass was cut where the

stem meets the soil surface and placed in brown paper bags to be dried at ambient greenhouse temperatures. After 10 days of drying in the greenhouse the dried biomass was weighed. Total dry weight was calculated by adding dry weight of plant biomass with the dry weight of the HPLC sample.

After being stored at -2° C, freeze-dried HPLC samples were granulated by hand to a uniform sample consistency. Individual samples were weighed to 100 mg and mixed with 10 ml of methanol using a VWR Vortexer 2 at room temperature. Samples were diluted 20 fold with methanol and filtered using a Captiva 0.45 µm regenerated cellulose. Samples were then analyzed using an Agilent 1220 Infinity II LC system using a Poroshell 120 2.7 µm column (3x50 mm). Run conditions included a column temperature of 50° C beginning with an isocratic 1 ml/min ratio of 60:40 methanol + 0.05% formic acid to ultrapure water + 0.1% formic acid for the first minute. This was followed by a 6 minute gradient to 77% methanol followed by an additional 90 second gradient to 95% methanol. UV absorbance was measured at 230 nm. Calibration standards were used for quantification in the range of 1-250 µg/ml and included THCA, THC, CBDA, CBD, CBGA and CBG (Agilent). Total percentage of cannabinoids was calculated using formulas in Table 1. Total cannabinoids were measured on a dry weight basis and analyzed as a percentage. A single chemotype II 'AutoCBD' plant was not included in cannabinoid analysis to avoid misinterpretation of data.

2.5 STATISTICAL ANALYSIS

All statistical analysis was executed using R Studio version 1.4.1717 (R Core Team). A four-way analysis of variance (ANOVA) test was performed to determine if there was an effect of cultivar, acclimation period, plant age, cold frequency or intensity, or an interaction between

these factors on the cold tolerance and cannabinoid profile of a plant. Separate models were fit for each response variable and distributional assumptions were confirmed by evaluating model diagnostic plots. Each full model was simplified by backwards stepwise simplification via the `step` function in base R. Post hoc Tukey tests were used to evaluate differences among treatment levels via the `emmeans` function in the package emmeans (Lenth, 2021).

3. RESULTS

3.1 FREQUENCY OF COLD EXPOSURE IN WHOLE PLANTS

Cold tolerance, as measured by $\Delta Fv/Fm_{WP}$, varied with cold acclimation ($F = 101.466$, $p < 0.0001$) and plant age ($F = 53.490$, $p < 0.0001$), as well as cultivar ($F = 6.365$, $p < 0.05$). The oldest plants experienced greater average $\Delta Fv/Fm_{WP}$ than did middle age and youngest plants for both cold acclimated and non acclimated plants (Figure 1). Significant two-way interactions existed but none involved cold treatment (Table 1). Although whole plant cold stress ($F = 2.409$, $p = 0.0695$) was the only treatment group not statistically significant, its interaction with both plant age and exposure to cold acclimation was significant ($t = 4.228$, $p < 0.001$). Control plants for the oldest non-acclimated 'AutoCBD' plants had greater $\Delta Fv/Fm_{WP}$ than one cold exposure ($t = 4.254$, $p < 0.001$), two cold exposures ($t = 3.338$, $p < 0.05$), or three cold exposures ($t = 4.658$, $p < 0.0001$). However, these effects were not observed in other cultivar and cold acclimation treatment interactions. $\Delta SPAD_{WP}$ was influenced by cultivar ($F = 13.052$, $p < 0.001$) and plant age ($F = 9.102$, $p < 0.001$) (Table 1). The largest range of values within this interaction of treatments occurred within non-acclimated 'AutoCBD' plants. No significant effects of any cold treatment were present.

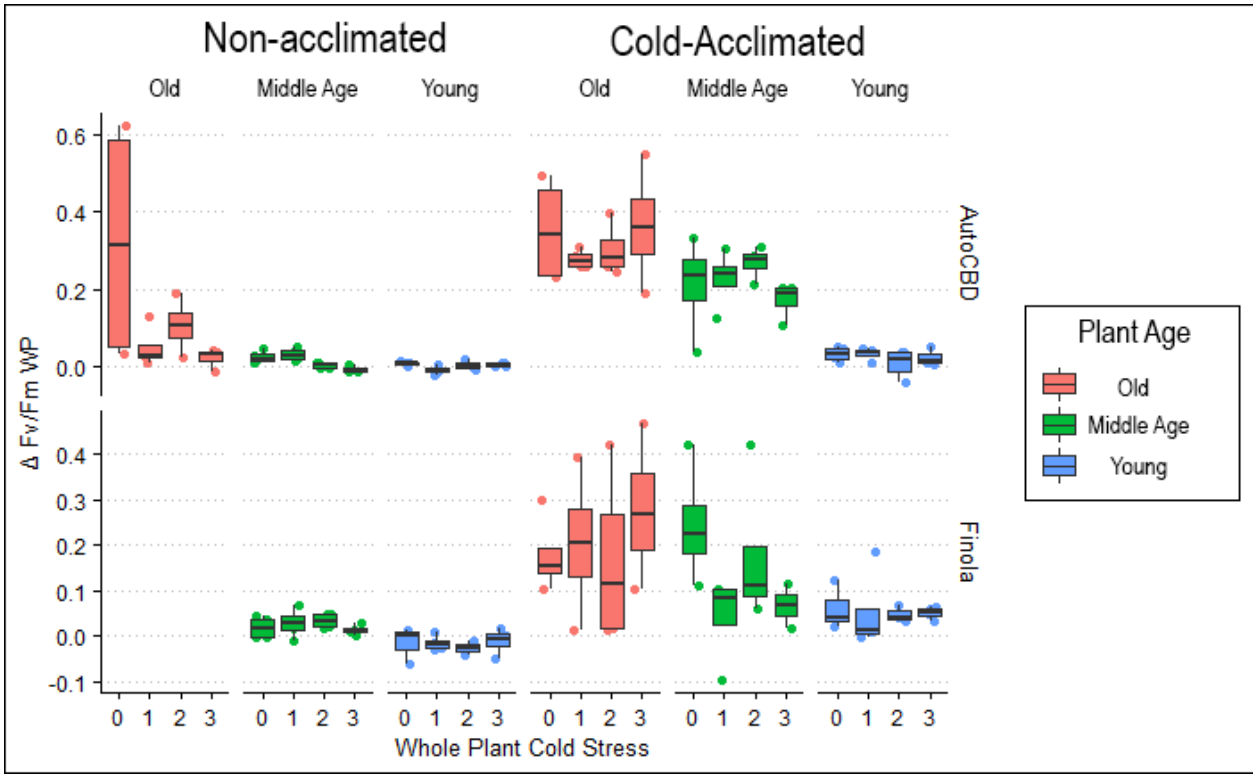

Figure 1. $\Delta Fv/Fm_{WP}$ box plots comparing whole plant cold stress and plant age for cold acclimated and non-acclimated plants 'AutoCBD' and 'FINOLA' plants. Data represents the difference between Fv/Fm taken before and after whole plant cold stress. Non-acclimated plants received no cold acclimation period and cold acclimated plants were subject to 10 days at 10° C. Cold stress consisted of no exposure (control), a single 3 hour -0.5° C exposure, two consecutive 3 hour - 0.5° C exposures separated by 24 hours, and three 3 hour -0.5° C exposures separated by 24 hours. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively). Y axis scale differs between acclimation treatments to accurately reflect data.

In cold frequency exposures to whole plants, cold tolerance measured by electrolyte leakage (EL_{WP}) was significantly affected by both plant age ($F = 39.305$, $p < 0.0001$) and whole plant cold stress treatment ($F = 17.072$, $p < 0.0001$) (Table 1). Cold tolerance was also affected through interactions between whole plant cold stress and cultivar ($F = 3.093$, $p < 0.05$), cold acclimation ($F = 2.875$, $p < 0.05$), and plant age ($F = 2.353$, $p < 0.05$). Plants of cultivar 'AutoCBD' consistently displayed significant variation between control whole plant cold stress and one cold exposure in the oldest ($t = 4.292$, $p < 0.001$) and middle age group ($t = 2.723$, $p < 0.05$), but not in the youngest. Additionally, non-acclimated and cold acclimated plants both had lower EL_{WP} compared to the one and two cold exposure groups. Control whole plant cold stress

and plants exposed to two cold exposures showed similar variation ($t = 4.761$, $p < 0.0001$, *t.ratio* = 2.697, $p < 0.05$, $t = 4.174$, $p < 0.001$, and $t = 3.594$, $p < 0.05$) respectively. In both treatment groups of cold acclimation, the oldest plants of both cultivars experienced a higher percentage of EL_{WP} than did middle age or younger plants (Figure 2). Furthermore, whole plant cold stress control treatment consistently leaked a greater percentage of electrolytes than plants receiving one and two exposure(s) of whole plant cold stress, and often the same was true in plants receiving three exposures of whole plant cold stress.

Table 2. Summary of significant effects of cultivar, cold acclimation, plant age, whole plant cold stress, their interactions and mean values for 'AutoCBD' and 'FINOLA'. Cold tolerance was quantified with $\Delta Fv/Fm_{WP}$ (difference between Fv/Fm taken prior to and after whole plant cold stress), $\Delta SPAD_{WP}$ (difference in mean SPAD values taken prior to whole plant cold stress and after whole plant cold stress), and EL (ratio between EC of leaf sample after whole plant cold stress and EC of leaf sample after cell lysis).

	Δ Fv/Fm WP	Δ SPAD WP	EL WP
AutoCBD	0.12	34.43	0.17
FINOLA	0.08	32.75	0.17
Cultivar	*	***	n.s.
Cold Acclimation	***	n.s.	n.s.
Plant Age	***	***	***
Whole Plant Cold Stress	n.s.	n.s.	***
Cultivar x Cold Acclimation	*	n.s.	n.s.
Cultivar x Plant Age	n.s.	***	n.s.
Cold Acclimation x Plant Age	***	n.s.	***
Cultivar x Whole Plant Cold Stress	n.s.	n.s.	*
Cold Acclimation x Whole Plant Cold Stress	n.s.	n.s.	*
Plant Age x Whole Plant Cold Stress	n.s.	n.s.	*
Cultivar x Cold Acclimation x Plant Age	*	n.s.	n.s.
Cold Acclimation x Plant Age x Whole Plant Cold Stress	***	n.s.	n.s.

n.s. = not significant;
* $p < 0.05$;
** $p < 0.01$;
*** $p < 0.001$.

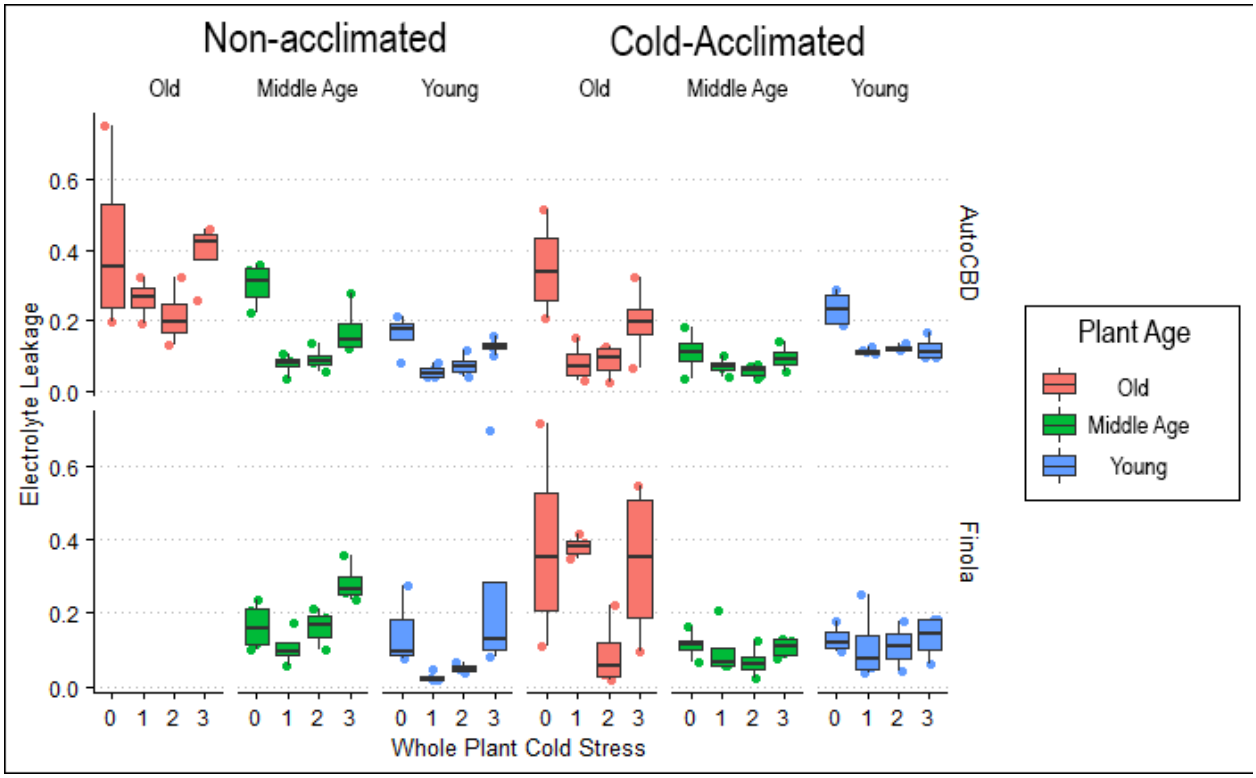

Figure 2. EL_{WP} box plots comparing whole plant cold stress and plant age for cold acclimated and non-acclimated 'AutoCBD' and 'FINOLA' plants. Data represents the ratio between EC of leaf sample after whole plant cold stress and EC of leaf sample after cell lysis. Non-acclimated plants received no cold acclimation period and acclimated plants were subject to 10 days at 10° C. Cold stress consisted of no exposure (control), a single 3 hour -0.5° C exposure, two consecutive 3 hour - 0.5° C exposures separated by 24 hours, and three 3 hour -0.5° C exposures separated by 24 hours. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively).

3.2 INTENSITY OF COLD EXPOSURE IN DETACHED LEAVES

Cultivar ($F = 8.542$, $p < 0.05$), cold acclimation ($F = 14.921$, $p < 0.001$), and detached leaf cold stress ($F = 31.424$, $p < 0.0001$) showed significant effects in relation to cold tolerance (Table 2). $\Delta Fv/Fm_{DL}$ was the only stress measurement that experienced a statistically significant four-way interaction, which occurred between cultivar × cold acclimation × plant age × detached leaf cold stress ($F = 6.583$, $p < 0.05$). With the exception of non-acclimated 'FINOLA' plants, -8° C showed significant variation to -2° C and -4° C in plants of non-acclimated 'AutoCBD' ($t = -4.156$, $p < 0.001$, $t = -4.087$, $p < 0.001$), cold acclimated 'AutoCBD' ($t = -5.022$, $p < 0.001$, $t =$

-4.618, $p < 0.0001$), and cold acclimated 'FINOLA' ($t = -3.633$, $p < 0.001$, $t = -3.451$, $p < 0.05$).

-8° C had the highest $\Delta Fv/Fm_{DL}$ values on average (Fig 3).

Table 3. Summary of significant effects of cultivar, cold acclimation, plant age, whole plant cold stress, their interactions and mean values for 'AutoCBD' and 'FINOLA'. Cold tolerance was quantified with $\Delta Fv/Fm_{DL}$ (difference between Fv/Fm taken prior to whole plant cold stress and after detached leaf cold stress), $\Delta SPAD_{DL}$ (difference in mean SPAD values taken prior to whole plant cold stress and after detached leaf cold stress), and EL (ratio between EC of leaf sample after detached leaf cold stress and EC of leaf sample after cell lysis).

	Δ Fv/Fm DL	Δ SPAD DL	EL DL
AutoCBD	0.16	0.88	0.23
FINOLA	0.07	-3.96	0.13
Cultivar	**	***	***
Cold Acclimation	***	***	n.s.
Plant Age	n.s.	n.s.	*
Detached Leaf Cold Stress	***	***	***
Cultivar x Plant Age	**	***	n.s.
Cold Acclimation x Plant Age	*	**	**
Cultivar x Detached Leaf Cold Stress	*	*	*
Cold Acclimation x Detached Leaf Cold Stress	n.s.	n.s.	**
Cultivar x Cold Acclimation: Plant Age	n.s.	*	n.s.
Cultivar x Plant Age x Detached Leaf Cold Stress	n.s.	***	n.s.
Cultivar x Cold Acclimation x Plant Age x Detached Leaf Cold Stress	*	n.s.	n.s.

n.s. = not significant;
* $p < 0.05$;
** $p < 0.01$;
*** $p < 0.001$.

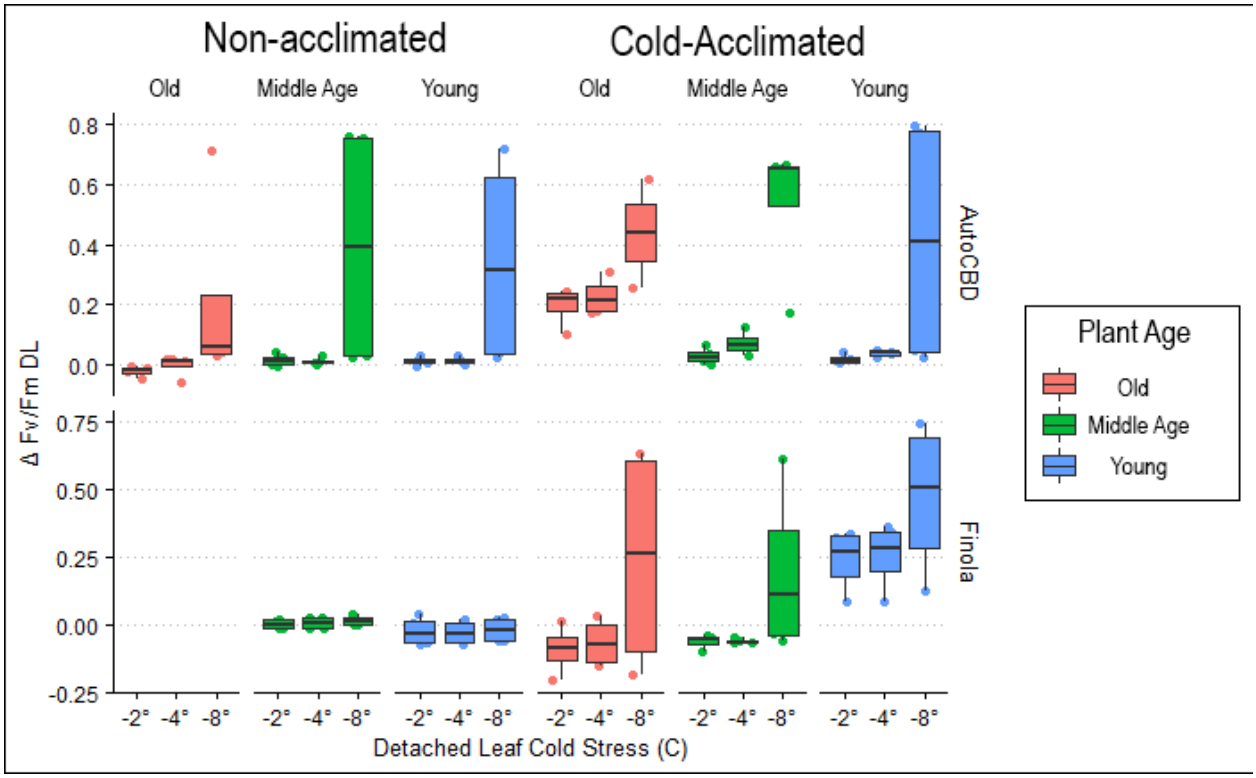

Figure 3. $\Delta Fv/Fm_{DL}$ box plots comparing detached leaf cold stress and plant age in cold acclimated and non-acclimated 'AutoCBD' and 'FINOLA' plants . Data represents the difference between Fv/Fm taken before whole plant cold stress and after detached leaf cold stress. Non-acclimated plants received no cold acclimation period and cold acclimated plants were subject to 10 days at 10° C. Detached leaf cold stress consisted of a single 3 hour -2° C exposure, a single 3 hour -4° C exposure, and a single 3 hour -8° C exposure. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively). Y axis scale differs between acclimation treatments to accurately reflect data.

Chlorophyll data measured through $\Delta SPAD_{DL}$ was significantly affected by cultivar (F = 34.10, $p < 0.0001$), cold acclimation (F = 16.411, $p < 0.001$), and detached leaf cold stress (F = 10.640, $p < 0.0001$). Two significant interactions, cultivar × plant age × detached leaf cold stress (F = 5.45, $p < 0.001$) and cultivar × detached leaf cold stress (F = 4.090, $p < 0.05$), occurred involving the effect of cold temperature intensity on cold tolerance. It is important to note that $\Delta SPAD_{DL}$ was the response variable that most commonly displayed an approximately equal distribution of positive and negative change after induction of cold treatment (Fig 4). This may be due in part to lower degree of accuracy in SPAD readings than in Fv/Fm or EC measurements.

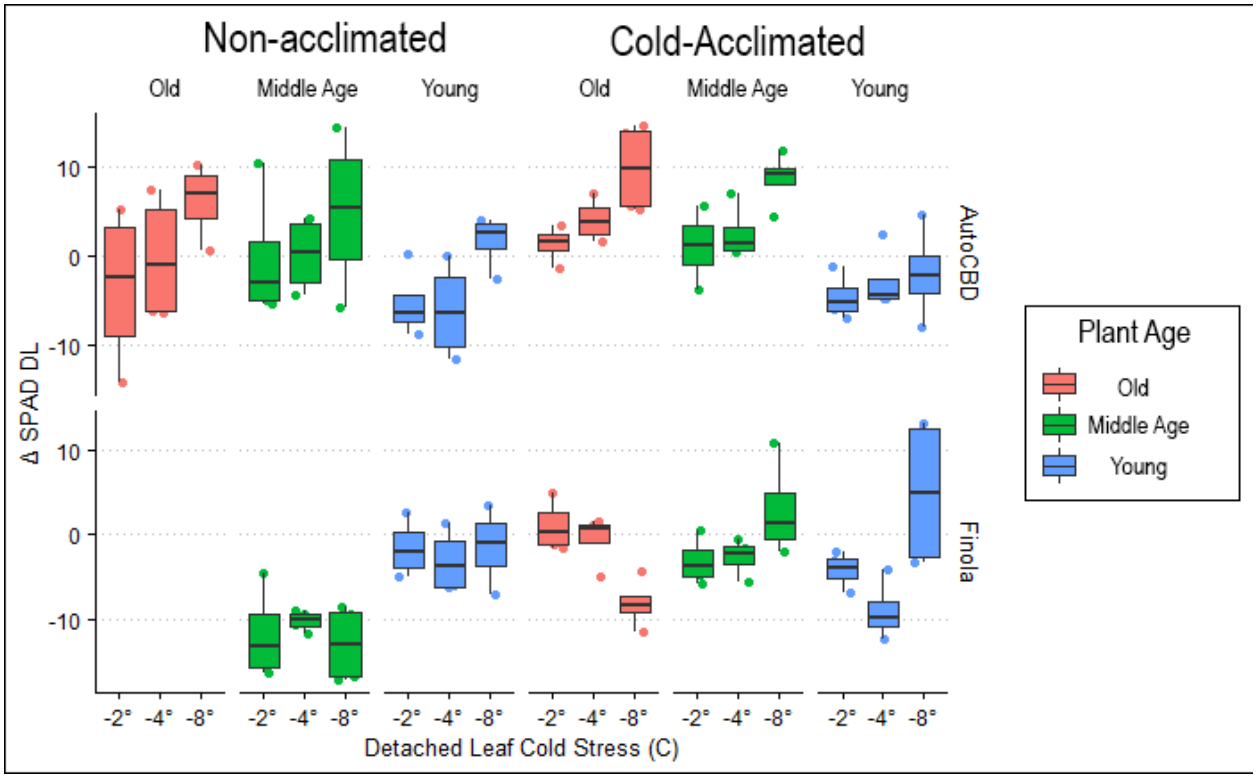

Figure 4. $\Delta SPAD_{DL}$ box plots comparing detached leaf cold stress and plant age. Data represents the difference in mean SPAD taken before whole plant cold stress and after detached leaf cold stress. Detached leaf cold stress consisted of a single 3 hour -2° C exposure, a single 3 hour -4° C exposure, and a single 3 hour -8° C exposure. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively).

Electrolyte leakage as a result of cold intensity treatments (EL_{DL}) showed significant effects of cultivar ($F = 17.108$, $p < 0.0001$), plant age ($F = 3.870$, $p < .05$), and detached leaf cold stress ($F = 12.316$, $p < 0.0001$) on cold tolerance (Table 2). Significant interactions among treatment groups was also observed in cultivar × detached leaf cold stress ($F = 4.086$, $p < 0.05$), and cold acclimation × detached leaf cold stress ($F = 6.962$, $p < 0.01$). Significant differences existed between -2° C and -8° C in cold acclimated 'AutoCBD' ($t = -4.921$, $p < 0.0001$) and 'FINOLA' ($t = -2.657$, $p < 0.05$) plants. Plants receiving -8° C treatment most commonly had highest average EL values across cultivars, plant age, and acclimation treatment.

3.3 CANNABINOIDS AND WEIGHT

Postharvest data was collected from only ‘AutoCBD’ plants. Total CBD % decreased with cold acclimation ($F = 176.311$, $p < 0.0001$). Although no significant effect of cold treatment was observed, there was a significant interaction between cold acclimation and cold treatment ($F = 3.024$, $p < 0.05$); however, pairwise contrasts among cold stress treatment levels were not different. Comparing the total CBD % showed that plants receiving no acclimation treatment had approximately twice the mean total CBD than did the acclimation treatment group (Table 4, Figure 5). Similar effects of cold acclimation ($F = 16.417$, $p < 0.01$) and plant age ($F = 3.611$, $p < 0.05$) were observed on total CBG after cold exposure, again with no effect of cold stress treatment. CBG was the cannabinoid that showed the least variation of effects and interactions of treatments (Figure 5).

Table 4. Summary of means and significant effects of cold acclimation, plant age, whole plant cold stress, and their interactions on cannabinoids and weight for 'AutoCBD'. Total percentage of CBD, CBG, and THC was quantified by adding the percent of each cannabinoid's neutral form to the product of the percentage of each cannabinoid's acid form multiplied by the cannabinoid's molecular weight (0.877, 0.878, and 0.877) respectively. Ratio of CBD:THC was calculated by dividing Total CBD (%) by total THC (%). Weight was measured as the total weight of whole plant dry biomass (grams).

Mean	**Total CBD (%)**	**Total CBG (%)**	**Total THC (%)**	**Total CBD : Total THC**	**Weight (g)**
No acclimation	6.93	0.19	0.31	22.79	9.60
Cold acclimation	3.38	0.13	0.12	34.02	6.54
Old	5.53	0.18	0.22	26.19	7.98
Middle age	5.04	0.15	0.22	28.58	7.96
Young	4.60	0.13	0.19	31.48	8.06
Control	5.51	0.19	0.23	27.07	7.69
1 cold exposure	4.99	0.15	0.20	28.27	8.12
2 cold exposures	4.75	0.14	0.19	27.23	7.88
3 cold exposures	5.04	0.14	0.22	31.91	8.30
Cold Acclimation	***	***	***	***	***
Plant Age	n.s.	*	n.s.	n.s.	n.s.
Cold Stress	n.s.	n.s.	n.s.	n.s.	n.s.
Cold Acclimation x Plant Age	***	***	***	*	*
Cold Acclimation x Cold Stress	*	n.s.	**	*	n.s.

n.s. = not significant;
* $p < 0.05$;
** $p < 0.01$;
*** $p < 0.001$.

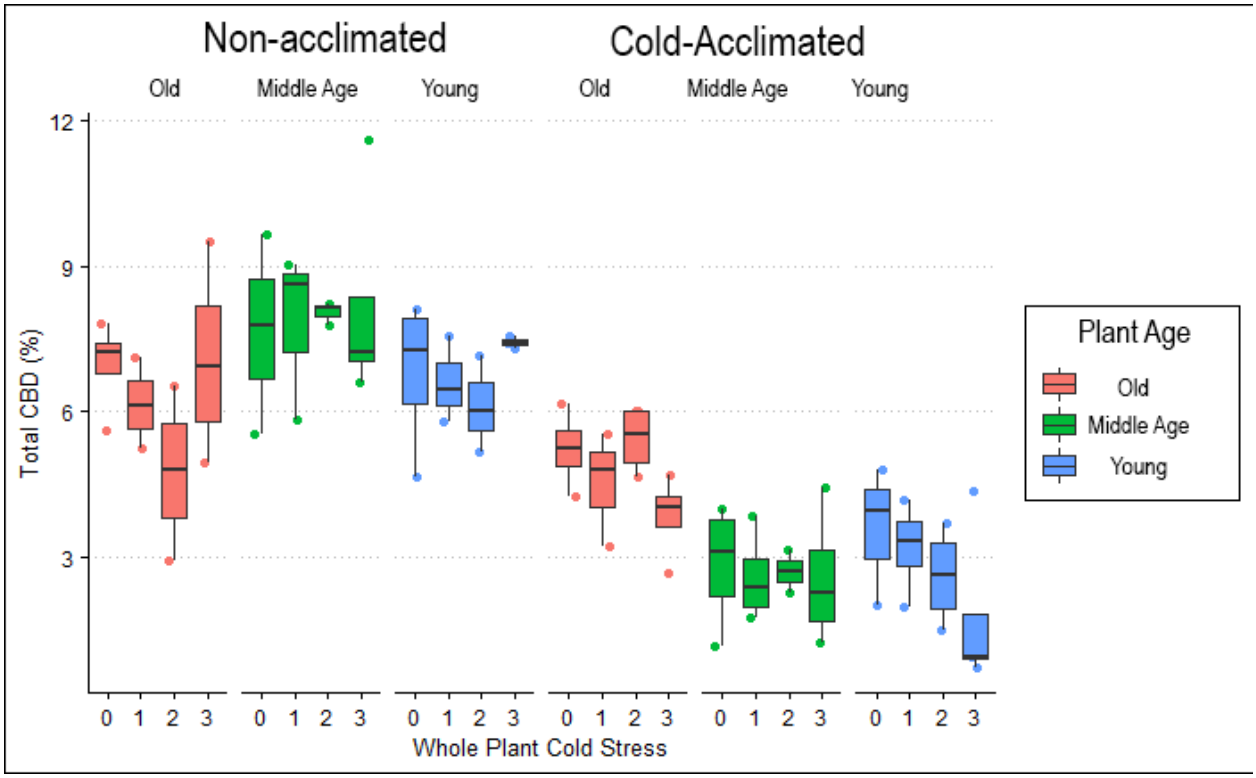

Figure 5. Total CBD (%) box plot comparing whole plant cold stress to acclimation treatment and plant age in 'AutoCBD'. Data represents the percent of CBD's neutral form added to the product of the percentage of CBD acid form multiplied by the cannabinoid's molecular weight (0.877). Plants receiving cold acclimation treatment were subject to 10 days at 10° C. Whole plant cold stress consisted of a control treatment receiving no cold exposure, a single 3 hour -0.5° C exposure, two consecutive 3 hour - 0.5° C exposures separated by 24 hours, and three 3 hour -0.5° C exposures separated by 24 hours. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively).

In contrast, the mean total percentage of CBD and THC were more than twice as high in non acclimated plants compared to those receiving cold acclimation (Table 4). Out of the 88 plants sampled, 23 plants (26%) exceeded regulatory limits for THC (total THC > 0.3%). Effects of cold acclimation were deemed statistically significant ($F = 221.977$, $p < 0.0001$). Furthermore, the mean ratio between total CBD and total THC was distinctly different when comparing cold acclimated (34.02) and non-acclimated (22.79) plants. Weight was significantly affected by cold acclimation ($F = 122.661$, $p < .0001$) as exemplified by comparison of mean weights in non acclimated (9.60 grams) and cold acclimated (6.54 grams) plants.

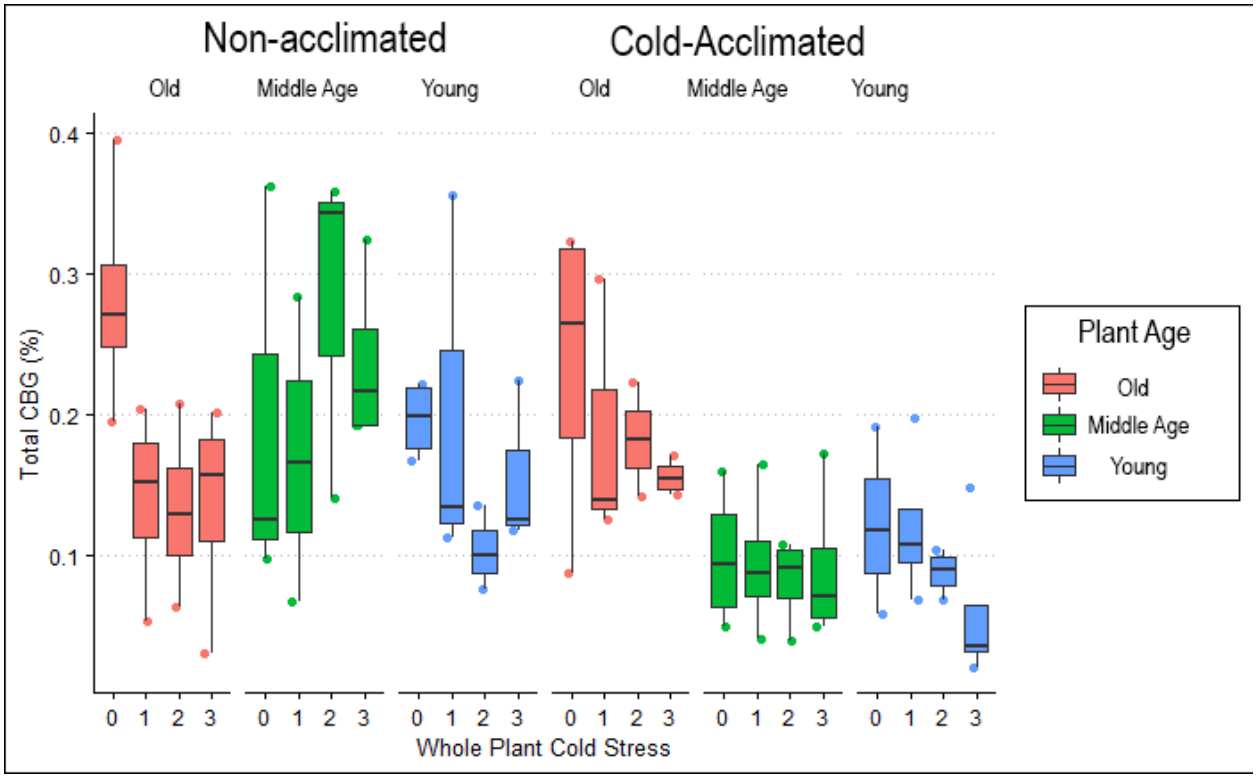

Figure 6. Total CBG (%) box plots comparing whole plant cold stress to acclimation treatment and and plant age in 'AutoCBD'. Data represents the percent of CBG added to the product of the percentage of CBGA multiplied by the cannabinoid's molecular weight (0.878 and 0.877) respectively. Plants receiving cold acclimation treatment were subject to 10 days at 10° C. Whole plant cold stress consisted of a control treatment receiving no cold exposure, a single 3 hour -0.5° C exposure, two consecutive 3 hour - 0.5° C exposures separated by 24 hours, and three 3 hour -0.5° C exposures separated by 24 hours. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively).

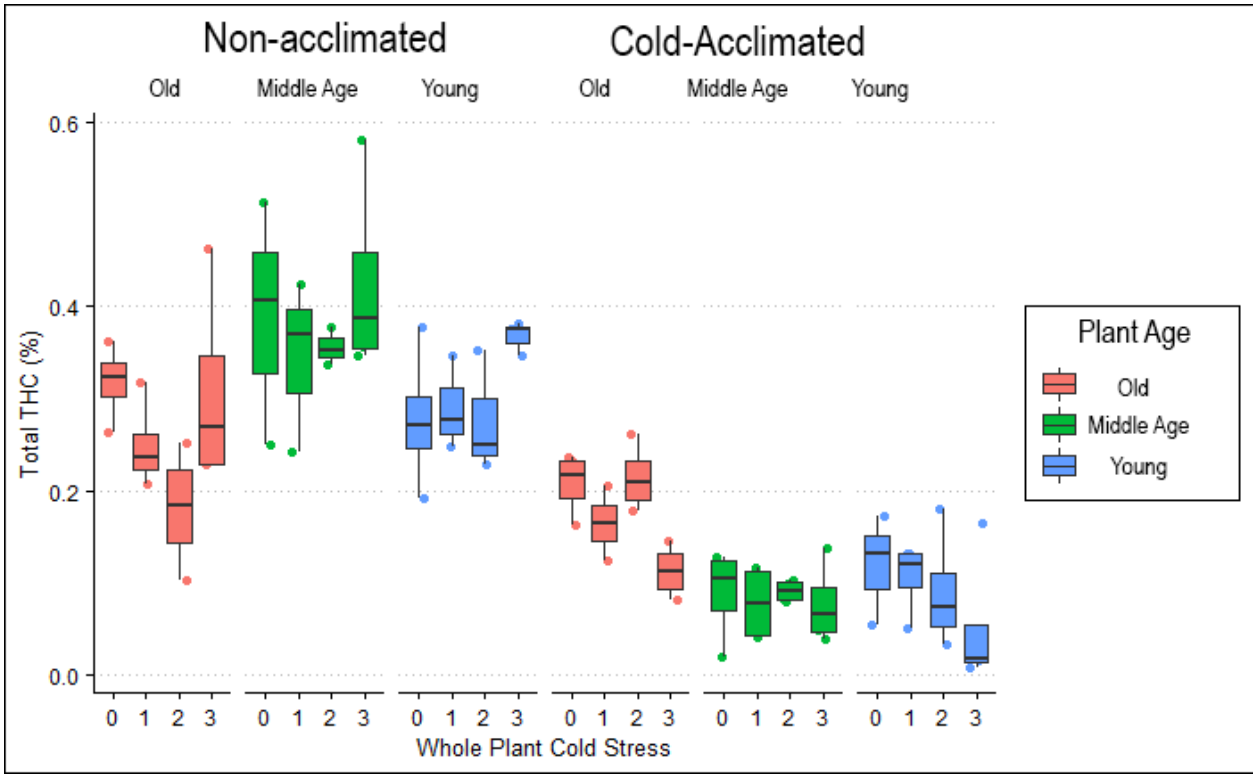

Figure 7. Total THC (%) box plots comparing whole plant cold stress and to acclimation treatment and and plant age in 'AutoCBD'. Data represents the percent of THC added to the product of the percentage of THCA multiplied by the cannabinoid's molecular weight (0.878) respectively. Plants receiving cold acclimation treatment were subject to 10 days at 10° C. Whole plant cold stress consisted of a control treatment receiving no cold exposure, a single 3 hour -0.5° C exposure, two consecutive 3 hour - 0.5° C exposures separated by 24 hours, and three 3 hour -0.5° C exposures separated by 24 hours. Plant age treatments include oldest plants, middle age plants, and youngest plants (68, 54, and 40 days old on the day of whole plant cold stress respectively).

4. DISCUSSION

Understanding the effects of cold temperatures on plant health and production of secondary metabolites is essential in combating uncertainties posed by a changing climate; however, few studies have addressed this topic. Here we find that, non acclimated plants and young plants across both cultivars exhibited the least amount of physiological stress in response to consecutive cold stress treatments though consecutive exposures to cold temperatures did not consistently result in greater amounts of photoinhibition in any treatment group. In combination with these findings, we also observed that AutoCBD plants subject to the cold acclimation period produced significantly less CBD, CBG, and THC and yielded less biomass, suggesting that cold acclimation acted as a plant stress as opposed to a protective, hardening mechanism. Furthermore, detached leaves exposed to -8° C received a magnitude of damage not observed in either -4° C or -2° C across any method of plant stress quantification. Together these findings suggest that hemp may be quite tolerant to short periods of frost prior to harvest though prolonged cold weather may reduce overall yields.

4.1 COLD TEMPERATURE EFFECTS ON PLANT HEALTH

Based on whole plant values of $\Delta Fv/Fm_{WP}$ and EL_{WP}, this experiment suggests that consecutive cold exposures did not elicit increased plant stress in a manner that was consistent in oldest and cold acclimated plants. Previous research has indicated that cold tolerance increases as plants age (Warnock et al., 1993; Płażek et al. 2011; Lim et al., 2014). However, the data observed in this trial indicated an opposite effect: mean $\Delta Fv/Fm_{WP}$, $\Delta SPAD_{WP}$, and EL_{WP} data was highest for oldest plants of both cultivars. The conflicting results may be partly attributed to

a higher incidence of pest pressure present during the early stages of growth when the oldest planting group was the only age group present in the growing environment. If this is the case, damage from cold exposures on hemp may exacerbate stressors such as from pests or phytotoxicity of pesticide application. Future research should explore the potential for multiple stressors to interact in driving plant health. Furthermore, control plants receiving no cold exposures often displayed values of plant stress greater than plants receiving one, two, and even three cold exposures. This discrepancy in results may be attributed to the timing of the second comparative measurements taken in control plants and used in calculating $\Delta Fv/Fm_{WP}$, $\Delta SPAD_{WP}$, and EL_{WP}. These measurements were taken three days after post-stress measurements in plants receiving one cold exposure, two days after plants receiving two cold exposures, and one day after plants receiving three cold exposures. Future iterations of such research should aim to record control comparative measurements one day prior to post stress measurements of plants receiving one cold exposure.

Effects of cultivar were apparent, with 'FINOLA' expressing greater cold hardiness than 'AutoCBD'. Furthermore, 'FINOLA' also expressed greater ranges of purple pigments in plant tissue than did 'AutoCBD' (Figure S1), but only in cold acclimated plants. The higher incidence of purple pigment may be attributed to a relationship between anthocyanin production and cold acclimation, leading to increased cold tolerance (Christie et al., 1994; Schulz et al., 2015). This relationship may help in explaining why 'FINOLA' experienced lower ΔFv/Fm and EL values than 'AutoCBD'. The increased cold tolerance of 'FINOLA' in this trial is partially supported by the findings of Mayer et al. (2015), which established that 'FINOLA' was one of nine tested cultivars whose electrolytes leaked the least when exposed to a 7 day acclimation period at 4° C The results of this experiment differed from Mayer's, however, in that plants of both cultivars in

this experiment showed greater susceptibility to cold damage in individuals that received cold acclimation treatments, as opposed to an increased tolerance to cold. The inclusion of cold acclimation as a treatment group was intended to increase a plant's potential to tolerate cold exposures as reported in previous research (Gilmour et al., 1988; Thomashow, 1999). On the contrary, our data suggests that a 10 day acclimation period at 10° C did not protect plants from future cold exposures but instead caused greater damage to leaf tissue than did a series of 3 hour exposures at -0.5° C.

These results are not surprising, however, when compared with data gleaned from the freezing intensity experiment. Plants did not experience a significant effect of cold intensity as measured by any quantification method when exposed to temperatures as low as -4° C. When exposed to -8° C, cold tolerance of plants in all treatment groups was significantly affected for every stress quantification method. Similar correlation between increased cold damage in cold acclimated plants observed in whole plant cold stress was further observed in this detached leaf trial, with the exception of electrolyte leakage measurements. Although mean electrolyte leakage differed greatly between cold acclimated and non acclimated plants at -8° C, it was approximately equal between acclimation treatments across all temperature treatments. This observed difference in quantified tissue damage between $\Delta Fv/Fm_{DL}$ and EL_{DL} may be in part attributed to secondary damage experienced by leaves during incubation in distilled water for EC measurements (Ehlert and Hinch, 2008).

4.2 COLD TEMPERATURE EFFECTS ON BIOMASS AND CANNABINOID PROFILE

Overall, cold treatment had very few effects on plant biomass, with the exception of reduced weight in plants that were cold acclimated. In contrast, cannabinoid profiles were

influenced by cold stress. The greatest impact of cold temperatures on cannabinoid content was the decrease in total CBD and THC % when exposed to cold acclimated conditions. Total THC concentrations declined more significantly than did total CBD concentrations when exposed to 10° C for 10 days. This pattern of cannabinoid expression was further evidenced when comparing populations of chemotype III plants that exceeded 0.3% total THC in cold acclimated (0%, n=0) versus non-acclimated (55%, n = 24) plants.

The mean CBD:THC across all samples was 28.7, in agreement with previously documented mean CBD:THC ranges (Stack et al., 2021; Toth et al., 2021). This trend is supported by extensive literature linking expression of chemotype primarily to genotype (Campbell et al. 2019; de Meijer et al., 2003; Mandolino et al., 2003). CBD:THC ratios were also affected by cold stress driven primarily by changes in THC concentrations. CBD:THC ratio was lowest in non-acclimated young plants following three cold stress exposures, but was highest following three cold exposures in acclimated plants. Variation in concentrations of CBD and THC are not uncommon in scientific literature (Yang et al., 2020; Stack et al., 2021; Toth et. al., 2021); however, in many instances, environmental drivers of variation are often not substantiated. Cold acclimation had a strong interaction with plant age. Plants exposed to whole plant cold stresses earliest in their flower development (youngest plant age treatment) expressed the lower mean concentrations of all total cannabinoids when compared to older plants, but only in cold acclimated groups. Although literature exists elucidating trends of cannabinoid accumulation, further studies may find value investigating how the variable timing of plant stress during a plant's flower development ultimately affects its accumulation of cannabinoids .

In conclusion, these results have allowed us to understand how cold temperatures impair physiological development of hemp and alter intra-chemotype cannabinoid ratios and

concentrations found in its flowering structures. Future trials assessing hemp's cold tolerance should evaluate consecutive exposures at lower temperatures and/or greater durations. Furthermore, trials in field settings may advance initial conclusions of this controlled environment experiment. Work conducted here suggests additional research may be necessary in understanding how timing of other environmental plant stressors - or combinations of stressors - during a plant's flower development ultimately affects its accumulation of cannabinoids. In light of recent advancements in crop insurance technologies, developing an extensive understanding for hemp's capacity to tolerate cold temperatures - particularly early frosts prior to harvest - may help cultivators mitigate the adverse weather effects influencing plant health and cannabinoid profiles (FAO and WUR, 2021). Understanding the impact of a fluctuating global climate on the health and secondary metabolite synthesis of hemp must continue to be prioritized by breeders and cultivators who face the immediate realities of unpredictable climate patterns of the 21st century.

APPENDIX

Figure S1: Comparison of pronounced, dark purple hues in cold acclimated ‘FINOLA’ compared to non-acclimated and cold acclimated ‘AutoCBD’.

Table S1. Summary of means and significant effects of cold acclimation, plant age, whole plant cold stress, and their interactions on neutral:acidic ratio of cannabinoids.

Mean	**Total Neutral : Total Acid**	**Total CBD : Total CBDA**	**Total CBG : Total CBGA**	**Total THC : Total THCA**
No acclimation	0.02	0.02	0.02	0.04
Cold acclimation	0.00	0.00	0.00	0.00
Old	0.01	0.01	0.01	0.01
Middle age	0.01	0.01	0.01	0.03
Young	0.01	0.01	0.00	0.03
Control	0.01	0.01	0.00	0.03
1 cold exposure	0.01	0.01	0.01	0.01
2 cold exposures	0.01	0.01	0.01	0.01
3 cold exposures	0.01	0.01	0.01	0.03
Cold Acclimation	***	***	*	***
Plant Age	n.s.	**	n.s.	*
Cold Stress	n.s.	n.s.	n.s.	n.s.
Cold Acclimation x Plant Age	***	***	n.s.	*
Cold Acclimation x Cold Stress	n.s.	n.s.	n.s.	n.s.

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