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Research

Evaluation of the Effect of Harvesting Time on Three Varieties of Industrial Hemp (*Cannabis Sativa L*)

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ABSTRACT

Recently, the exponential growth of the legal cannabinoid market has revitalized the interest in this traditional in *Cannabis sativa L.* or industrial hemp in various industries around the world period. Today, strict regulatory mechanisms over this crop exist to control the expression of federally banned cannabinoids produced by the plant, hindering its industrial potential. As the interest in cannabis grows, and its production expands to new soils and climates, research is needed to assess how different cultivars will fare in these new regions. Here, were determining the changes in cannabinoid concentration for three CBD varieties of industrial hemp (*Cannabis sativa L*): Bubba Kush (BK), Emerald Flower (EF), and Golden Sunset (GS). Crops were cultivated under open-field conditions in a randomized block design. Plants were sampled weekly until senescence to assess the changes in Cannabidiol (CBD) and Tetrahydrocannabinol (THC) concentrations across time. A cannabinoid extraction and quantification was developed to quantify cannabinoids (post-anthesis) via HPLC-DAD. Total CBD and THC reached their peak concentration at 5-8 weeks within the study. After seven weeks, the decline of secondary metabolite concentration was observed, causing a decrease in cannabinoid concentration. Although the fluctuation of cannabinoids was dynamic within each variety, the study provides information and insights on the proper management and harvesting of *Cannabis sativa L* or industrial hemp in South Florida.

Keywords: Cannabidiol; Cannabinoids; *Cannabis sativa L*; Industrial Hemp; Post Anthesis; Secondary Metabolites; Tetrahydrocannabino

INTRODUCTION

Hemp (*Cannabis sativa L.*) has been cultivated throughout history to produce food, fiber, and medicinal products [1]. Despite the crop's versatility, it has been historically defined by the presence of a unique class of terpenophenolic metabolites commonly known as cannabinoids. These compounds are mostly found in the genus Cannabis and give the plant distinct phytochemical characteristics. Although scientists have identified more than a hundred different cannabinoids, many cultivars of C. sativa have been selectively bred to primarily produce tetrahydrocannabinol (THC), and more recently cannabidiol (CBD) and cannabigerol (CBG) [2]. These cannabinoids are of economic interest due to their putative therapeutic and medicinal properties [3]. Based on the chemical composition of the crop, cannabis varieties can be classified into two large categories: industrial hemp and marijuana. In the United States, cannabis plants containing a total dry-weight concentration of THC equal or higher than 0.3% are considered “marijuana”, a federally regulated substance [4]. Whereas cannabis plants with less than 0.3% total THC are classified as “industrial hemp”, making the crop legal for cultivation and commercialization [4].

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Throughout commercial cultivation of industrial hemp, the observation and management of total THC concentration is paramount [5]. Although the federally regulated substance Δ-9-THC is normally not abundant in plant varieties recommended for industrial hemp production, many biotic and abiotic factors can induce the production of cannabinoids period, In turn, this can make, some hemp plants of normally low THC cultivars reach THC concentrations that are above the legal limits. For example, one of the THC precursor molecules, tetrahydrocannabinolic acid (THCA), is often found in concentrations that vary from one variety to the other [6]. Notably, THCA can transform into Δ-9-THC via decarboxylation, a process that removes the carboxylic group from THCA [7]. Consequently, cannabis total THC is defined as the total amount of Δ-9-THC plus the "Δ-9-THC equivalent" after a hypothetical complete decarboxylation of THCA. Total THC is commonly calculated by implementing the formula: Total THC=ConcentrationΔ-9-THC (ConcentrationΔ-9-THCA × 0.877) [4]. Total THC then serves as a proxy for the accurate quantification of potential psychoactive activity of a cannabis plant.

While cannabis is predominantly known for its psychoactive compound THC, there are other non-psychoactive phytochemicals that accumulate in the plant [8]. These phytochemicals have generated commercial interest, leading to further research of cannabis for its secondary metabolite production. Recently, the cannabinoid cannabidiol (CBD) has gained popularity because of its application in pharmaceutical and medical industries [9,10]. When cultivating industrial hemp for CBD production, the value of the crop is mainly determined by the total concentration of CBD within the plant material [11]. Commonly, CBD and THC can reach peak concentrations simultaneously, between 5-8 weeks post anthesis [12,13] Therefore, observing cannabinoid levels throughout the growth cycle of the plant is crucial to maximize cannabinoid harvest while remaining federally compliant [14] Hence, the goal of this research is to determine how harvesting period in different cannabis plant varieties affect the cannabinoid compound production within the crop. The objectives of the study are to

1) Determine the relationship between plant growth and accumulation of cannabinoids, CBD, and THC

2) Identify the best harvesting period to optimize the cannabinoid production in each variety.

MATERILAS AND METHODS

General experimental procedures

The study was conducted at a Florida International University/Green Point Research Project Partner Farm, located in Homestead, FL. The study evaluated three CBD-dominant day length sensitive industrial hemp varieties. Seedlings of Bubba Kush (BK), Emerald Flower (EF) and Golden Sunset (GS) were obtained from Green Point Research (Jasper, FL) and were chosen for this study, due to their potential approval for sale in the state of Florida. 540 plants total were configured in a randomized block design with three replications per variety under open field conditions.

Varieties were seeded in 72-cell liners containing potting mix for germination. Seedlings were grown under high pressure sodium halide bulbs for a minimum of 14 hours daily to maintain vegetative growth. Overhead irrigation was applied to the seedlings. After 2 weeks of seedling root establishment, liners were removed from the greenhouse to allow gradual light penetration from 35% to 55%. This aided in the hardening process for future outdoor transition of the plants. Three weeks after germination, the seedlings were transplanted to the open field site. The field site consisted of a plasticulture raised beds system 30 inches wide and 8 inches high. Spacing between plants was approximately 1 foot, with a row spacing of 40 inches wide. Irrigation in the field was applied by a piping system within the raised beds. Soil at the field site was made up of a sandy loam, which is representative of farming operations in South Florida. The average temperature of the field during the study was approximately 29°C. Prior to planting, the Helena Professional Slow-release fertilizer NPK (6-12-12) was applied approximately at a rate of 195 kg per hectare to the field.

In order to monitor the growth and development of the varieties cultivated, leaf chlorophyll concentrations were measured bi-weekly utilizing a Spectrum Technologies Inc Soil Plant Analyses Development (hereafter SPAD) 502 Plus Chlorophyll Meter (Konica Minolta, Japan). 36 plants were randomly sampled per variety biweekly utilizing Freidenreich, et al. SPAD sampling method [15]. The SPAD meter was utilized to determine chlorophyll content as a proxy for the health of the plant [16]. Plant height was also measured weekly across all varieties. During the last week of the experiment, samples were collected to record final dry biomass weight (oven dried at 65°C for 72 hours).

While monitoring cannabinoid accumulation over time, plant samples were collected weekly. Collection started once 50% of a variety's population displayed their first pistillate flowers (post anthesis) and ended with plant senescence (Table 1). Samples were collected utilizing a modified version of Florida Department of Agriculture and Consumer Services (FDACS) sampling protocols [17]. 10 random samples from each variety were collected weekly. Each sample was collected from the primary stem, measuring 8 to 10 inches from the tip of the stem (including flowers, leaves, and stems). Size and weight of the sample varied depending on the variety and harvest date, but this is expected under current FDACS guidelines. Each plant was sampled only once to avoid confounding variables such as stress induced secondary metabolite production. Biomass was dried in a forced air dehydrator at 35°C for 24 hours to avoid decarboxylation of cannabinoids. Plant samples were then randomly combined to create "composite samples" comprised of equal amounts of 5 different plants.

Table 1: Timeline of the field work and harvesting/sampling period in Days after sowing (D.A.S.)

Growth Timeline/Date	Harvest (Sample Collection)	D.A.S (Days After Sowing)
Germination/Sowing August 17th-September 10th 2020	Pre-Sample Collection	1-25
Field Transition September 11th 2020	Pre-Sample Collection	26
Vegetative Growth September 12th-September 20th 2020	Pre-Sample Collection (Pre-flowering)	27-35
September 21st 2020	Harvest 1	36
September 28th 2020	Harvest 2	43
October 5th 2020	Harvest 3	50
October 12th 2020	Harvest 4	57
October 19th 2020	Harvest 5	64
October 26th 2020	Harvest 6	71
November 2nd 2020	Harvest 7	78
November 9th 2020	Harvest 8	85
Termination of Experiment November 13th 2020	Senescence	89

Material was grounded to a fine powder and passed through a 1.0 mm sieve. Finally, samples were stored in a -80°C freezer until chemical analysis.

HPLC-DAD analysis of samples

A protocol for cannabinoid extraction was developed and optimized by the Florida International University Industrial Hemp Pilot Project research team. During the extraction process, 30 mg of each sample was extracted with 1.2 ml of 100% methanol (MeOH) in a Fisher Scientific Bead Mill 24 for five minutes at 6 m/s. Lastly, extractions were transferred to 0.2-micron Nylon filters to prepare for HPLC analysis.

Standards for cannabidiolic acid (CBDA), cannabidiol (CBD), tetrahydrocannabinolic acid (THCA), Delta-9-tetrahydrocannabinol (Δ-9-THC) were obtained from Cayman Chemical (Ann Arbor, MI) and Restek Pure Chromatography (Bellefonte, PA). Analysis was carried out with an 1100 HPLC-DAD system by Agilent Technologies (Waldbronn, Germany). The mobile system consisted of (A) H2O/Acetonitrile 5%/0.1% Formic Acid/0.1 M Ammonium Formate and (B) Acetonitrile/0.1% Formic Acid. The final gradient elution was: 0.00-6.00 min isocratic at 25% B: 75% A, then 6.00-8.00 min ramp to 33% B: 67% A; and 8.00-13.00 min ramp to 100% B. The flow rate was a constant 1.7 mL/min with an injection volume of 5 µl. Post-run equilibration time was 7 min in between samples. The chromatograms were acquired at 210 nm and 280 nm. Column used was a Restek Raptor C18 Column 2.7 µl, 1.50 × 4.6 mm (Belforte, PA). Concentrations of all cannabinoids evaluated were calculated utilizing integrated peak area in combination with 7 points standard calibration curves for all compounds (from 0.5 mg/ml to 8 ug/ml). Total THC and total CBD were calculated using the equations below.

- Total THC=ConcentrationΔ-9-THC (ConcentrationΔ-9-THCA × 0.877)

Equation 1: Total THC Equation for accumulation [4].

- Total CBD=Concentration CBD (Concentration CBDA × 0.877)

Equation 2: Total CBD Equation for accumulation [4].

Statistical analysis

Data analysis was performed in JMP15. One-way and two-way ANOVAs were done to detect any differences or changes in total THC and total CBD accumulation across sample periods and hemp varieties. Additionally, a multiple linear regression analysis was conducted to determine the effects that interactions between time and variety had on total THC and total CBD concentrations. . Finally, p-value was considered statistically significant at p=0.05.

RESULTS

Physical analysis

During this study, relative chlorophyll concentrations varied significantly over time for all varieties (DF=2, F=0.7077, P<0.05); (Figure 1). Generally, varieties experienced the highest relative chlorophyll levels during the month of October in Harvest 2 (47.39 ± 7.8) and Harvest 4 (45.23 ± 4.8); (Figure 1) and the lowest values during the first two weeks of the study (Pre-flowering; 33.86 ± 16.2) and Harvest 6 (38.65 ± 9.6). The tallest stem heights recorded were observed in the Golden Sunset variety ($\bar{x}$=55 cm), followed by Bubba Kush ($\bar{x}$=53.12 cm). The Emerald Flower variety had significantly lower stem heights ($\bar{x}$=43.17 cm; DF=2, F=0.0402, P<0.05) (Table 1).

The Golden Sunset variety had significantly heavier plants ($\bar{x}$=30.2 g) than Bubba Kush ($\bar{x}$=10 g) and Emerald Flower

varieties (x̄=8 g; DF=2, F=0.0001, P<0.05) (Figure 2). The highest mortality rate was observed in Emerald Flower populations (51.7%), followed by Bubba Kush (45.6%) and Golden Sunset (29.4%).

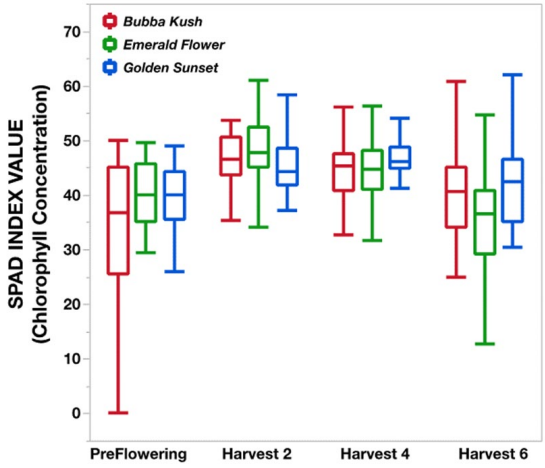

Figure 1: Mean Leaf Chlorophyll concentrations across time and variety. Boxplots show all data points between the 25th and 75th percentile, central boxplot line represents the 50th percentile. Whiskers show maximum and minimum values. Varieties are distinguished by colors: a) Bubba Kush=Red, b) Emerald Flower=Green and c) Golden Sunset=Blue. In this graph, harvesting periods (x-axis) represent a two-week time interval. One-way ANOVAs were conducted to determine the effects of time and variety on chlorophyll concentraions Chemical analysis

Figure 2: Above: Typical differences among the three CBD-varieties tested. Below: Images showing the average differences between "Type A" and "Type B" branching structures

Chemical Analysis

Total THC % and total CBD % across sampling periods and varieties: Cannabinoid accumulation was monitored for all varieties across harvesting periods through HPLC-DAD. To meet USDA requirements, total THC% and total CBD % values are expressed as "milligrams of compound per gram of dried plant material" [4]. All three varieties of industrial hemp tested in the field study reached reproductive growth within the first week of transplanting. This is the result of a 12-hour photoperiod in south Florida on average. During the study, cannabinoid concentrations fluctuated across time and hemp variety. In general, varieties experienced their highest total THC and total CBD accumulation in between Harvest 5 and Harvest 7, some surpassing the current 0.3% THC federal limit in some samples (Figures 3 and 4). The varieties evaluated had different mean total THC and total CBD levels. One of the tested varieties (Bubba Kush) showed total THC concentrations twice as high as the other varieties (Figure 4).

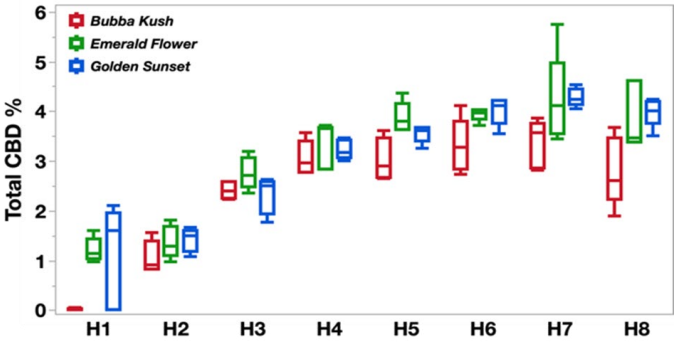

Figure 3: Mean Total CBD concentrations across all harvest periods. Boxplots represent the variation in total CBD concentration for each variety at each harvesting period. Varieties are distinguished by color.

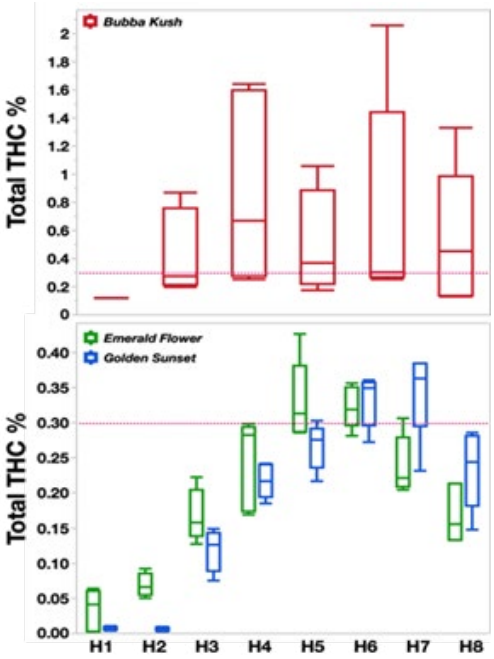

Figure 4: Mean Total THC concentrations across all varieties, at all harvest periods. Due to the high THC content found in Bubba Kush, this variety is plotted separately in the upper figure pane. Varieties are distinguished by color. Each harvesting period represents one week transpired. Red- dotted line denotes

0.3% total THC federal limit at the time of the study. One-way ANOVAs were conducted to determine the effects of time and variety in total THC concentration, see main text.

Golden Sunset plants surpassed the federal THC limit at Harvest 6 (0.330 ± 0.04% total THC) and peaked during Harvest 7 (0.344 ± 0.06% total THC; (Figure 4). Here, peak CBD concentrations coincided with peak THC concentrations (4.279 ± 0.18% total CBD; (Figure 3). In the following week (Harvest 8), total THC concentrations fell below the federal limit (0.234 ± 0.06% total THC) but CBD levels remained high (3.973 ± 0.28%). This meant that legal harvesting was possible near maximum CBD accumulation in the plant (Figure 3).

Like Golden Sunset, the Emerald flower variety total THC was surpassed before reaching peak total CBD concentration at Harvest 5 (0.393 ± 0.06% total THC). Total, THC fell below the legal threshold at Harvest 7 (Figure 4). Coincidently, total CBD also remained high by Harvest 8, allowing for legal harvest during peak CBD production (Figure 3).

The last variety Bubba Kush had a high accumulation of CBD and THC throughout reproductive growth. Peak concentration of total CBD and total THC was reached at Harvest 4 (0.439 ± 0.30%THC) (Figure 4) and Harvest 7 (3.355 ± 0.47% CBD; (Figure 3), respectively. Total THC concentrations did not decrease below the federal limit after Harvest 7. To reach compliance Bubba Kush could have only been harvested during Harvest 3 (2.410 ± 0.16 total CBD), resulting in significantly lower total CBD concentrations in comparison to Harvest 7 (DF=7, $F<0.0001$, $p<0.0001$) (Figure 3).

A multiple linear regression analysis was performed to test the effects of variety and harvesting period on total THC and total CBD concentrations. Here, only 38% of the variation in THC% across the experimental samples was explained by the combined effect of variety and harvesting period ($R2=0.39$, $F=7.64$, $P>0.0001$). Contrastingly, 89% of the variation in total CBD concentrations was attributed to the combined effect of variety and harvesting period ($R2=0.89$, $F=84$, $P<0.0001$). Both results confirming that variety and harvesting are the two most significant factors determining total THC and CBD in an industrial hemp crop (Figure 3 and 4). Notably, interactions between variety and harvesting time were not significant. For this reason, the model with the variety/harvesting period interaction was excluded from the analysis.

DISCUSSION

Effects of harvesting time on growth parameters

Relative chlorophyll concentrations were monitored for all industrial hemp varieties throughout the experiment. Chlorophyll levels are a useful indicator for plant health [18]. Stressors such as nutrient deficiencies, dehydration and diseases produce changes in chlorophyll concentrations and appearance of the leaf [18]. Biweekly chlorophyll measures did not significantly differ ($p>0.05$) among varieties tested. However, chlorophyll concentrations significantly changed ($p<0.05$) over time. The lowest SPAD values were generally observed during the first two weeks of the study (Pre-flowering; 33.86 ± 16.2) and Harvest 6 (38.65 ± 9.6), which could have corresponded with transplanting and the start of senescing, respectively (Figure 1), [19]. Tressors such as high temperatures, nutrient depletion and transplanting of the crop may have negatively influenced chlorophyll production during the growth cycle [19].

Plant height and dry weight biomass was recorded to compare growth and development throughout variety and time. Golden Sunset plants had the largest stems and heaviest biomass recorded. Differences among dry biomass weight and size are likely linked to Golden Sunset's height, fan leaf production, internodal branching and abundance of flowers (Figure 3); {20]. These plants had lateral branching throughout the main stem, providing better coverage, structure, and space for flower development [21,22]. In comparison, Bubba Kush and Emerald Flower did not perform as well in the field trial. The majority of Bubba Kush and Emerald Flower plants showed the "type A" morphotype (Figure 2). Type A plants produced a large singular cluster of flowers on top of the main stem with little to no branching (Figure 2). The "type B" individuals displayed a more branched architecture with the presence of multiple flower clusters (Figure 2). Due to the novelty of cannabis on the U.S agricultural market, regulation of this crop is still in its initial stages [4]. As a result, cannabis crop farmers face problems when trying to pass federal total THC regulation because of lack of uniformity in seed genetics [23].

During our study, mortality rates were high among all varieties tested. Substantial loss of individuals in Bubba Kush and Emerald Flower varieties may be attributed to heavy rainfall, high temperatures poor genetics, disease, and sampling protocols. The FDACS sampling protocol requires the first 8 to 10 inches from the top portion of the main stem to be harvested for cannabinoid testing. This kind of substantial sampling is likely to translate in an increase mortality rate across all sampled individuals [21]. Among our varieties, Golden Sunset had the lowest mortality rate. This cultivar's branching structure and abundant fan leaves may have protected the crop from some environmental stressors such as pests and excess moisture [24] Bubba Kush and Emerald Flower's structure could have left them increasingly susceptible to abiotic stressors and disease-causing pathogens [22]. Finally, On November 8th, 2020, hurricane Etna produced strong winds and heavy rains in our field site (Homestead, Fl). The inclement weather toppled and stripped several plants, likely increasing the mortality. As a result, the experiment was concluded at Harvest 8 to avoid any confounding variables within our data.

Effects of harvesting time on cannabinoid production

Among the varieties tested, total THC and total CBD fluctuated across time and variety. Nevertheless, the pattern of accumulation for total THC and CBD was similar throughout time. Both compounds experienced their maximum accumulation rates 5 to 7 weeks post-anthesis. Emerald Flower and Golden Sunset

varieties had high total CBD accumulation without surpassing the total THC limit. This would allow for the legal harvest of both crops near maximum CBD concentration, increasing overall profitability of the crop. Bubba Kush total THC concentrations showed very high variation. However, total CBD concentrations in Bubba Kush plants followed the same pattern of accumulation as the other varieties. Several similar studies including Pacifico et al. (2007), and Baldini et al. (2018), evaluated the accumulation of cannabinoids over time. Despite varying environmental conditions and genetic differences on each study, they concur that maximum cannabinoid accumulation occurs 5 to 7 weeks post-reproductive growth. These results are consistent with our findings.

Several abiotic and biotic stressors can influence the accumulation of THC and CBD in industrial hemp plants [25]. A multiple linear regression analysis was conducted to test the effects of variety and harvesting period on total THC and total CBD concentrations. The analysis demonstrated that variety and harvesting interval were significant predictors of total CBD and total THC in the crops tested. Notably, we found a large difference in the amount of variation of total CBD and THC explain by the combined effect of variety and harvesting time. Total CBD production was more stable and predictable than total THC. This result was heavily affected by the large concentrations of THC in Bubba Kush. Indeed, after assessing the statistical association between the total THC and CBD for each of our varieties independently, both Emerald Flower and Golden Sunset showed a tight relationship between the concentrations of these two cannabinoids (Figure 5). This is a very desirable trait for any industrial help cultivar, as it allows predicting the potential yield of CBD that is possible before the levels of THC become illegally high. Contrastingly, we did not find any significant relationship between the total CBD and THC in the individuals of Bubba Kush (Figure 5). Thus, suggest that, for some industrial hemp cultivars, the expression of CBD metabolic pathways can be less linked to the THC metabolic pathways. As a result, legal cultivation of industrial hemp is difficult to achieve without the acquisition of proper genetic varieties for cultivation, and the consistent observation of

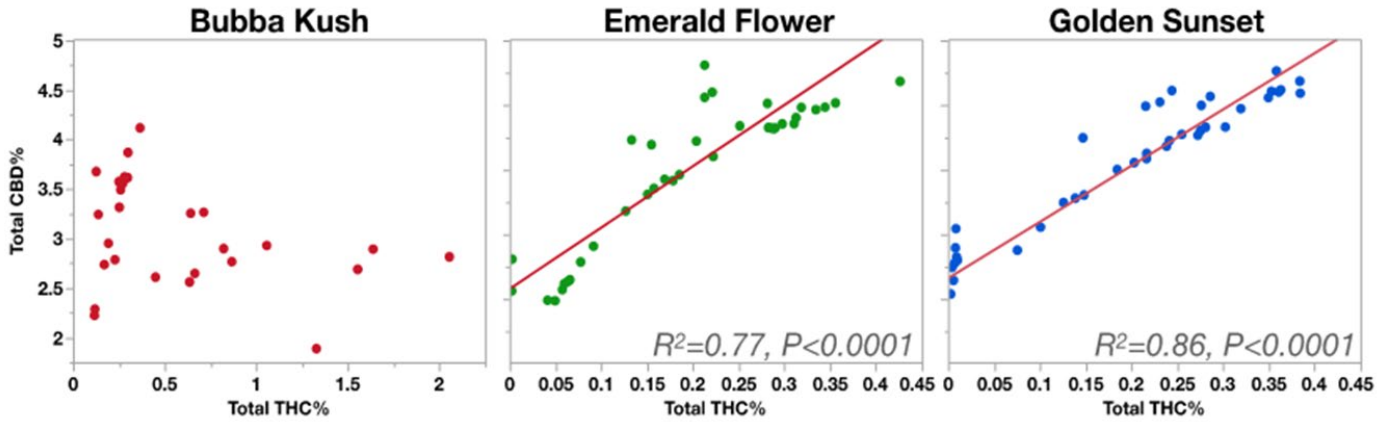

Figure 5: Simple linear regressions between total THC and total CBD for each of our experimental industrial hemp varieties. Regression lines (red) and statistical results are presented only where the regression was statistically significant.

the total THC levels throughout growth [26,27]. Although these factors are significant, more research is needed to understand the other abiotic and biotic stressors that drive cannabinoid accumulation.

CONCLUSION

In summary, more research is needed to understand how abiotic and biotic stressors drive cannabinoid accumulation in *Cannabis sativa L.* The research showed that variety and harvesting time were significant predictors of total THC and total CBD accumulation. Moreover, this study helps provide insights on how legal limitations affect production of Total THC and total CBD. In several of the aforementioned studies, they revealed that cannabinoid concentration can fluctuate even within the same genetic variety depending on abiotic and biotic factors. This meaning that the exact same cannabis crop can be grown in two different areas under the same conditions but have varying cannabinoid concentrations. This presents many problems for farmers investing in industrial hemp cultivation, due to the risk of financial loss if not managed correctly. To combat this, farmer's must first take into consideration the genetic varieties they are selecting, and then monitor their crops' cannabinoid content consistently post anthesis. Such practices can help to mitigate the risk of surpassing the total THC federal limit and maximize the success and profitability of the crop. Although variety and harvesting period were significant predictors for the accumulation of cannabinoids in this study, more research is needed to understand the mechanisms that drive cannabinoid accumulation. Farmers, policy makers, and researchers must work together to regulate this new emerging crop market safely.

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