Cloning Successive Generations of Industrial Hemp (*Cannabis sativa*) to Assess Cannabinoid Profiles

by
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Dedication

I dedicate this project to my family and friends who continued to support me and push me to reach for the stars−thank you Momma, Dad, Nicky, Marc, Nana, and Grandma for all the unconditional love. Another thanks to Dakota for helping me with my lack-of-technology skills and excessive HPLC dataset organization and input into excel. I will be forever filled with gratitude for each and every one of you.

Acknowledgements

I acknowledge Dr. John DuBois for his continuous guidance throughout my research project. His extraordinary support through the entire process of my research journey kept me motivated to succeed. Additionally, I give a shout out to Dr. Paul Kline. Thank you for running the thousands of samples via HPLC, allowing me to use your laboratory for my preparation of samples, and helping me interpret the HPLC datasets; without your help I would have not been able to complete my project. Another big thank you to Dr. Stephen Wright for reading my thesis from the very start and providing great feedback and other resources for me to use to ensure my writing is the best it can be. Lastly, thank you Dr. Ryan Otter for reading my thesis and asking me a variety of questions to get me thinking outside the box. All these professors are outstanding and are dedicated to help students thrive during their undergraduate research experience. I acknowledge the URECA (Undergraduate Research Experience and Creative Activity) program for my Silver Scholar Grant as well as GreenWay Herbal Products, LLC. for funding my research and allowing me to present my research at Scholars Week, TCHC (Tennessee Collegiate Honors Council), and NCUR (National Conference on Undergraduate Research).

Abstract

Industrial hemp (*Cannabis sativa*) has made a remarkable impact worldwide due to the plant's beneficial properties and versatile use. Vegetative cuttings (clones) of *C. sativa* are the preferred propagation technique to be the most effective to retain the same genetic information and to reduce hybridization and mutations. The objective of this project was to assess cannabinoid profile concentrations of successively cloned generations of 5 varieties: Cherry, Cherry Blossom, Cherry x Workhorse, Sour Space Candy, and The Wife. This research project focused on the idea that every cloned plant contains the exact same genetic information and, therefore, should have the same metabolic profile of cannabinoids through all the successive generations grown, which is shown to be true. The results of my study show that there is not a significant difference in cannabinoids over successive generations showing no major trends.

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Introduction

Industrial hemp *(Cannabis sativa)* has made a significant impact across the globe due to the plant's beneficial properties and versatile use. Over the past decade there has been an increase in research studies on *C. sativa* and how this single plant can revolutionize the pharmaceutical and agricultural industries [4]. *Cannabis sativa* contains many different chemical components, but two substances of interest are Δ9-tetrahydrocannabinol (THC) and Cannabidiol (CBD), which are both considered cannabinoids [16] (Figure 1 and Figure 2).

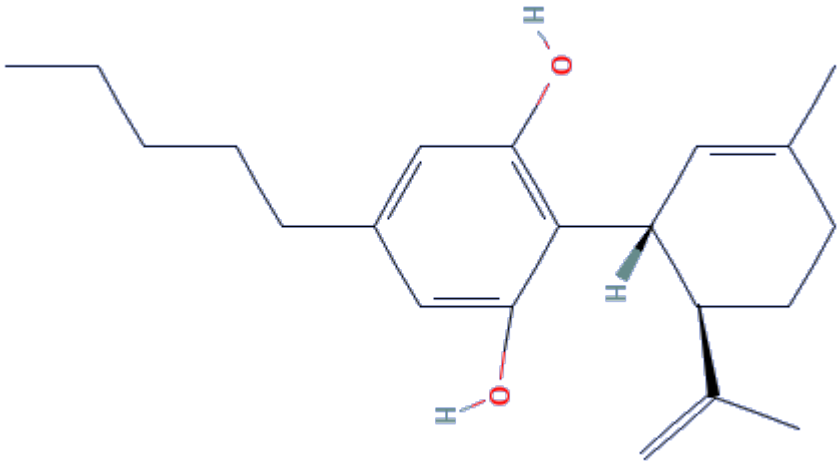


Figure 1. Chemical Structure of CBD (Cannabidiol) [11].

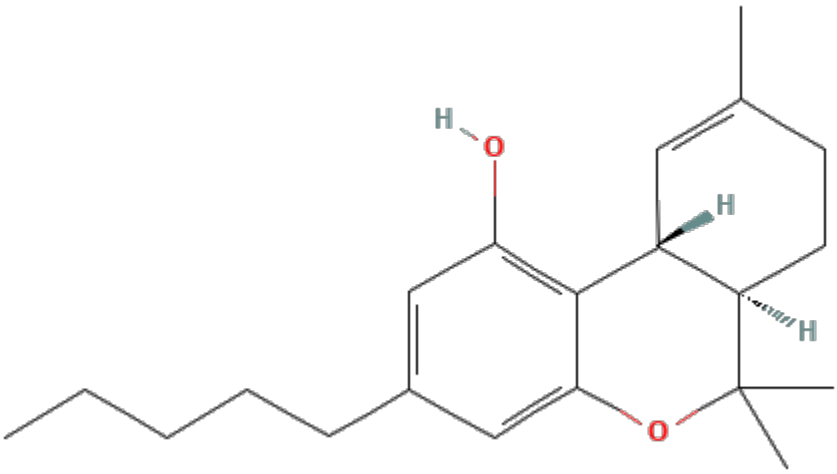


Figure 2. Chemical Structure of Δ 9-THC (Δ 9-tetrahydrocannabinol) [13].

These substances have different physical/mental effects on humans, as THC is known for its psychoactive effects, but CBD produces non-hallucinogenic therapeutic effects [10, 20]. Industrial hemp must contain an overall THC concentration under 0.3% for legality purposes [20]. The *C. sativa* plant naturally produces the acidic version of certain cannabinoids, for example, CBDA (Cannabidiolic acid) is the naturally occurring form of CBD [6]. As the plant undergoes harvesting and manipulation (storage, burning, etc.), a phenomenon called decarboxylation occurs; decarboxylation removes the carboxyl functional group (-COOH) from the chemical structure which ultimately turns CBDA (Figure 3) into CBD [6]. Each acidic version of a cannabinoid has different overall function, which goes back to the central dogma of structure determines function [6].

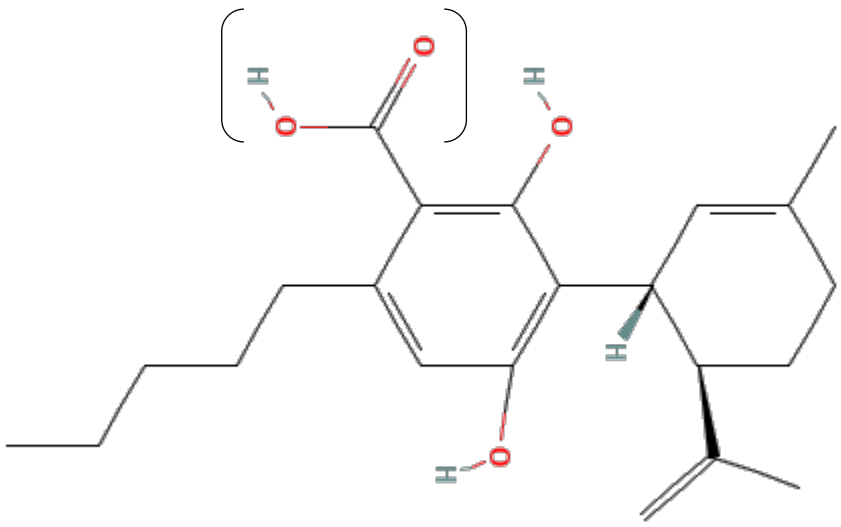


Figure 3. Chemical Structure of CBDA (Cannabidiolic acid). Bracketed functional group is the carboxyl group which gets removed during decarboxylation [12].

Cannabinoids are produced as secondary metabolites; compounds that are produced alongside metabolic pathways needed for basic plant life. Therefore, these compounds are produced to help the plant thrive in the environment but are not necessary to live [7]. Besides pharmaceutical usage of the plant, *C. sativa* has been known to be used for its natural hemp fibers to produce various goods such as carpets and ropes [3]. Hemp fibers attain an extremely high tensile strength (300-800 MPa)—high resistance to breaking under tension—which allows hemp fibers to be a substitution for synthetic fibers in polymer composite reinforcement [3]. Thus, the various properties of *C. sativa* have been accessed globally for new medical and materialistic applications.

Medical usage of *C. sativa* entails the consumption of the plant (flower) which then interacts with different receptor sites (Cannabinoid Receptors 1 and 2 respectively, CB1R and CB2R) in the cannabinoid and endocannabinoid systems that are a very large

subcategory in the central nervous system; endocannabinoids are fatty neurotransmitters that are human-produced agonists very similar to cannabinoids that are produced from *C. sativa* [2, 21]. Two endocannabinoids that are produced naturally in humans are *N*-arachidonoyl-ethanolamine and 2-arachidonoylglycerol that are derivatives from the eicosanoid, arachidonic acid, which has anti-inflammatory properties [21]. When these specific receptors are bound, there is a triggered cellular response that activates other receptors throughout the body that are responsible for other physiological functions—pain, hunger, and even memory [2, 21]. According to Zou and Kumar [21], the receptors are part of the G protein family where extracellular signals get transmitted to intracellular signals so therapeutic actions can occur.

Medical studies on *C. sativa* have concentrated on the therapeutic properties of the cannabinoid CBD and its ability to decrease symptoms associated with multiple diseases and chronic illnesses. Workman *et al.* [19] observed the differences between THC and CBD regarding the cannabinoid effects on multiple sclerosis patients; this was done by the observation of the patient's cerebral glucose metabolism and physical symptoms. The study's results showed that THC caused hypermetabolism as well as an intoxicating psychoactive feeling, but the CBD induced hypometabolism and overall reduced anxiety and agitation levels drastically [19]. Not only do the beneficial effects of CBD help mentally, but also physically; for instance, CBD demonstrates anti-inflammatory properties as well as properties to reduce symptoms of Alzheimer's disease, epilepsy, diabetes, and many more chronic illnesses [4, 18]. Cannabidiol (CBD) has multiple medically beneficial properties that will change the study of medicine

drastically and is the reason for additional research on *C. sativa* and its cannabinoid profiles.

Vegetative cloning is the process of taking a portion (cutting) of a plant to asexually grow an entirely new plant [14]. To retain cannabinoid production levels throughout multiple generations, vegetative cutting is the most effective propagation technique. Research has shown that cannabinoid profiles change due to different genetics and mutations as generations are grown via seeds [5]. In addition, the levels of the cannabinoids were over 4.1x greater in the plants grown vegetatively rather than those that grew from seeds [5]. Additionally, as plants are cloned, a mutation, commonly known as a loss-of-function gene, gets 'activated,' so in other words, the plants typically lose a function over multiple generations of vegetative cloning [1]. After observing multiple clonal generations the plants themselves become less robust as the generations continue [1]. Also, the plants that the cuttings are taken from are more prone to diseases as well as harmful insects [1].

Thesis Statement

This research project revolved around the idea that all cloned plants have the same genetic information—metabolic production of cannabinoids should remain consistent throughout successive generations. The goals for this study were (1) successfully grow successive clonal generations from vegetative cuttings and (2) harvest buds and analyze cannabinoid levels to see if the function of producing cannabinoids is lost.

Methodology

Five different varieties of *C. sativa* were used in this project, specifically: The Wife, Sour Space Candy, Cherry, Cherry Blossom, and Cherry x Workhorse. All the plants in this research were female. Cuttings were obtained for a new generation by the inspection of each plant (of the same variety and generation) for optimal stems that contained at least 3 nodes and were approximately 8 cm long to be placed in the cloner. In addition, a new razorblade had been used to ensure the cut was clean as well as at an angle to achieve the maximum surface area possible on the stem. The leaves were clipped using shears to reduce the loss of water while in the cloner. Each freshly cut stem had been coated with a rooting gel, Clonex, and then placed in a cloner (Botanicare, Model RESLPWHB-40) to obtain healthy root growth (Figure 4).

Figure 4. Fresh cuttings of C. sativa in water-based cloner.

The cloner was set to a 16h:8h ratio of light:dark schedule to guarantee that the cuttings remain in a vegetative state to prevent the process of flowering. This process normally took around 2-3 weeks for adequate root growth (Figure 5), and once it was achieved, the plants from which the cuttings were taken were moved into a growth chamber.

Figure 5. Healthy root growth of cutting that was placed in the cloner.

The new generation of plants (rooted cuttings) had been potted in 8 cm pots with MiracleGro potting soil to proceed growing to around 15 cm tall. At around 15 cm of growth, each plant was moved into a 15 cm pot (Figure 6). The plants were allowed to mature to around 1⁄2 to 1 m tall in the greenhouse. As these plants were maturing, cuttings for the next generation had been taken and placed in the cloner.

Figure 6. Small pots contain cuttings that had grown healthy roots. Large pots contain plants that have grown more than 15 cm and have had cuttings taken for the next successive generation. These plants are maturing in a vegetative state in the greenhouse until ~ 1 m tall.

After the plants reached the desired height and the next generation cuttings were rooting properly, they were placed in growth chambers set at 8h:16h light: dark to stimulate flowering (Figure 7). The growth chambers were 1.2 m x 2.4 m x 1.8 m tall with LED growth lights to help the plants induce flowering over a 3-4-week period.

Figure 7. *Cannabis sativa* in flowering chamber.

Once flowering had occurred, buds were harvested, and air dried (Figure 8) for two weeks before they were analyzed for cannabinoid content following the procedures developed in our laboratory [17]. High-Performance Liquid Chromatography (HPLC) was used to analyze the cannabinoid profiles of each sample.

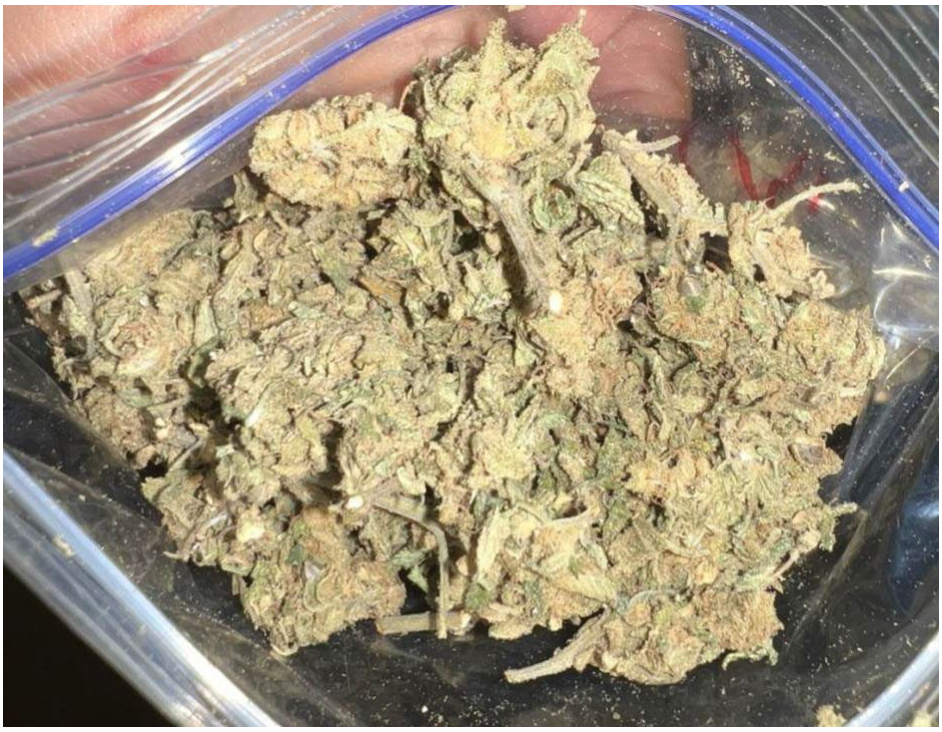


Figure 8. Air-dried bud sample collected before HPLC sample preparation.

Preparation for HPLC was initiated by taking 100 mg of air-dried bud sample (each generation for the varieties were completed individually) and placing it in a 50 mL centrifuge tube. Exactly 25 mL of 95% ethanol was placed in the 50 mL centrifuge tube (Figure 9). The goal was to have 10 plants per generation per variety (five samples made per plant).

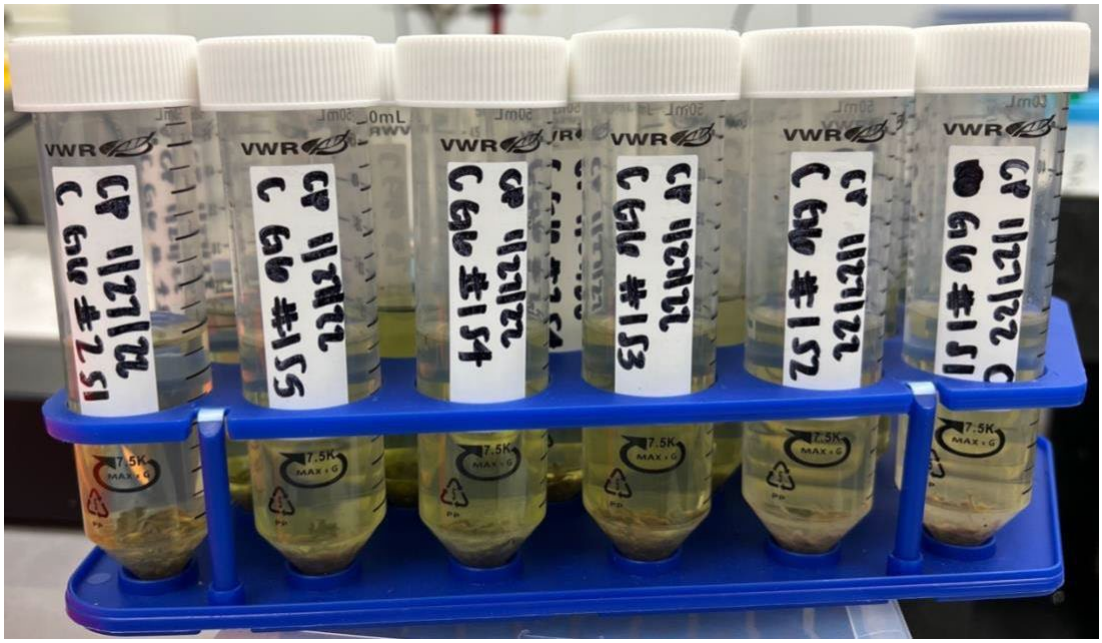


Figure 9. Labelled 50 mL centrifuge tubes containing 100 mg dried bud sample and 25 mL of 95% ethanol.

All samples were vortexed for 1 minute on speed level 10 and then placed in the centrifuge; the samples were centrifuged for 2 minutes, at 4° C, and at 2000 RPM. Syringes were prepared by the attachment of a Millex HV 0.45 µm Filter to the opening (each sample had its own syringe and filter to prevent contamination). Each sample had 1 mL of solution extracted which was placed in a 1.5 mL vial, labeled, capped, and then stored in a cold refrigerated room until they were analyzed (Figure 10).

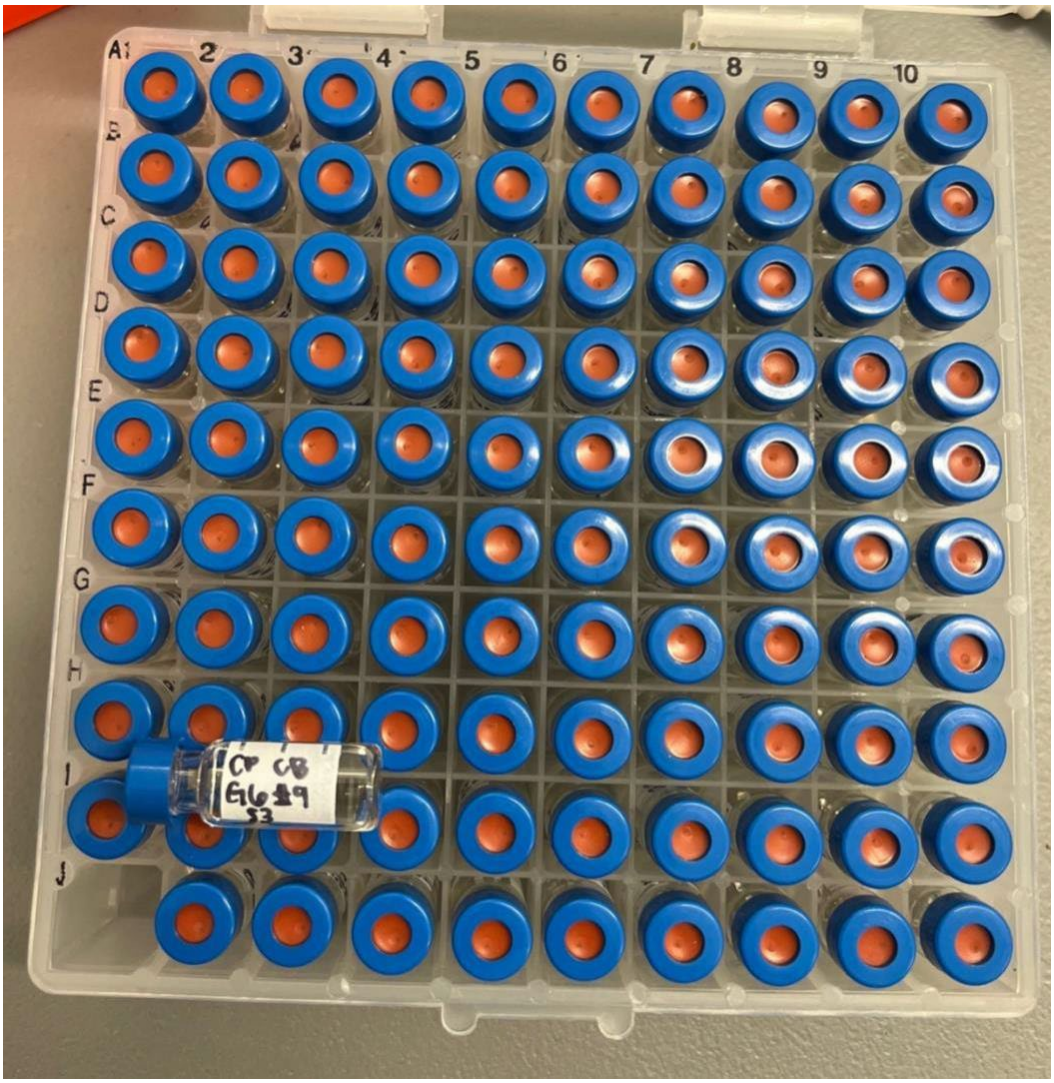
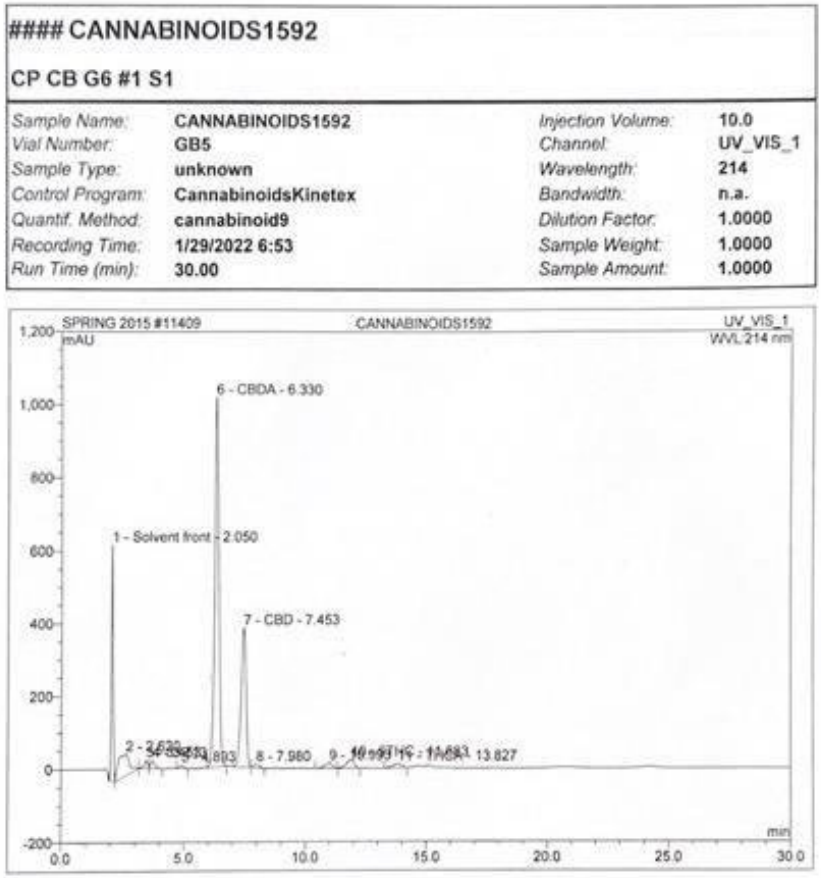


Figure 10. Prepared HPLC 1.5 mL glass vials with filtered *C. sativa* sample extract solution.

The HPLC system that had been used was the Dionex UltiMate 3000 and the specific column was the Phenomenex Kinetex EVO 5 µm C18 100 Å (150 x 4.6 mm) column. The mobile phases that eluted the cannabinoids consisted of methanol with 0.1% formic acid (B) and water with 0.1% formic acid (A). Additionally, the flow rate and temperature were 1.0 mL/minute and 50 degrees Celsius. The eluent method used for the

result is a linear gradient which after 45 minutes was 60% B/40% A to 95% B/5% A. Every hour the HPLC system graphed the cannabinoid level concentrations and took in a new sample to analyze (Figure 11).



CANNABINOIDS1592

CP CB G6 #1 S1

Sample Name:	CANNABINOIDS1592	Injection Volume:	10.0
Vial Number:	GB5	Channel:	UV_VIS_1
Sample Type:	unknown	Wavelength:	214
Control Program:	CannabinoidsKinetex	Bandwidth:	n.a.
Quantif. Method:	cannabinoid9	Dilution Factor:	1.0000
Recording Time:	1/29/2022 6:53	Sample Weight:	1.0000
Run Time (min):	30.00	Sample Amount:	1.0000

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.05	Solvent front	649.091	49.416	12.21	n.a.	BMB
2	2.62	n.a.	59.615	32.320	7.99	n.a.	BMB
3	3.45	n.a.	22.452	3.395	0.84	n.a.	bM
4	3.71	n.a.	22.126	4.200	1.04	n.a.	MB
5	4.89	n.a.	9.802	1.619	0.40	n.a.	BMB
6	6.33	CBDA	1015.140	202.204	49.97	n.a.	BMB
7	7.45	CBD	383.603	90.086	22.26	n.a.	BMB
8	7.98	n.a.	9.266	2.398	0.59	n.a.	BMB
9	10.99	n.a.	15.170	5.423	1.34	n.a.	BM
10	11.88	9THC	24.463	8.890	2.20	n.a.	MB
11	13.83	THCA	11.523	4.733	1.17	n.a.	BMB

Figure 11. Raw HPLC data of Cherry Blossom Generation 6, Plant 1, Sample 1 of 5.

The cannabinoid level concentrations were compared to standards through their individual Peak Area Retention Time, and clonal generation cannabinoid levels were compared through ANOVA (Analysis of Variance). This process was used on each successive generation for all five varieties of *C. sativa* to compare the cannabinoid profiles. The equations used for calculation of cannabinoid % mass are as follows:

Step 1: Find X, the concentration of particular cannabinoid for particular sample

(Peak Area Standard) / (0.1 mg/mL concentration standard) = (Peak Area Sample) / (X mg/mL)

Step 2: Fill in X from Step 1 and calculate % mass

(X) (50 mL total extracted volume) / (100 mg hemp extracted total) * 100

Results

Cherry

Ten successive generations of Cherry were cloned, flowered, harvested, and analyzed by HPLC. All cannabinoids had been analyzed via ANOVA with alpha = 0.05. Every generation that had detectable results had an F calculated value less than the F critical value; this means that we fail to reject the null hypothesis that there is no difference between successively cloned generations of cannabinoid % mass values. Thus, there is no difference between successive generations of Cherry. The cannabinoid CBDVA remained consistent throughout successive generations (Figure 12). Also following this trend had been the cannabinoid CBDA, the data showed consistency across successive generations (Figure 14). The cannabinoid Δ9THC showed a rapid decrease across successive cloned generations, but the decrease had not been significant enough to make this cannabinoid have an F calculated value larger than the F critical value (Figure 16). The cannabinoids CBDV, CBD, and CBG analyzed in Cherry showed no trend due to experiment error because the data did not stay consistent, increase, or decrease (Figure 13, 15, 18). There had been a slight trend for the cannabinoid CBL. For CBL, the production had stopped after generation 2; there was not considered a difference in this cannabinoid because there was only a small concentration before the production stopped (Figure 17).

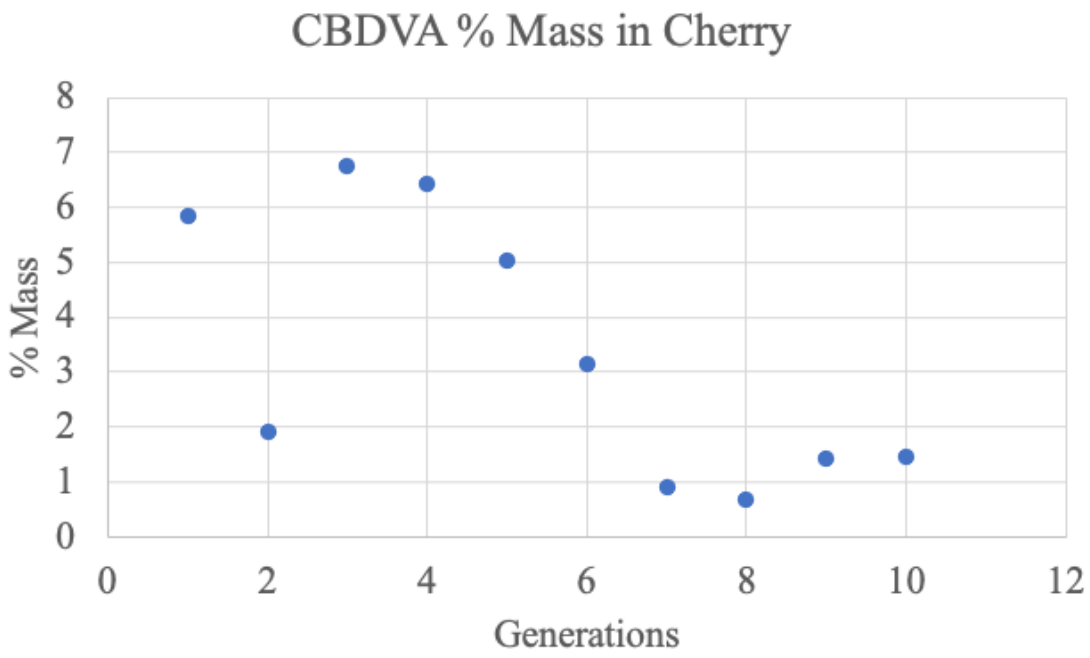


Figure 12. CBDVA % mass in Cherry variety. The p value was 0.992301. The F calculated value was 0.1762394 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDVA.

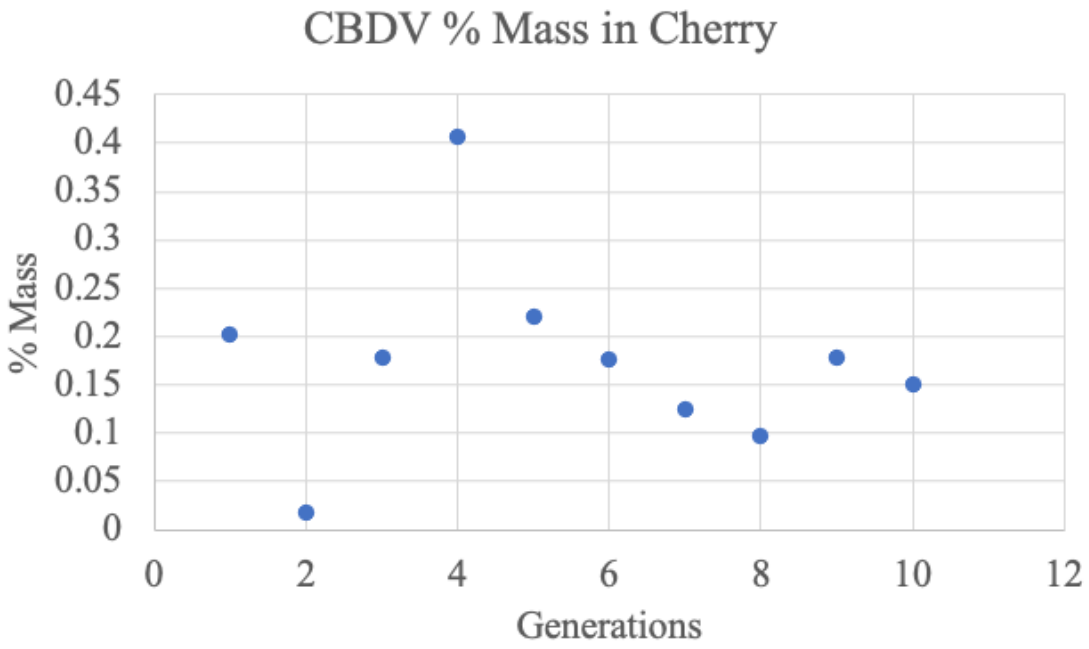


Figure 13. CBDV % mass in Cherry variety. The p value was 0.976219. The F calculated value was 0.2482533 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDV.

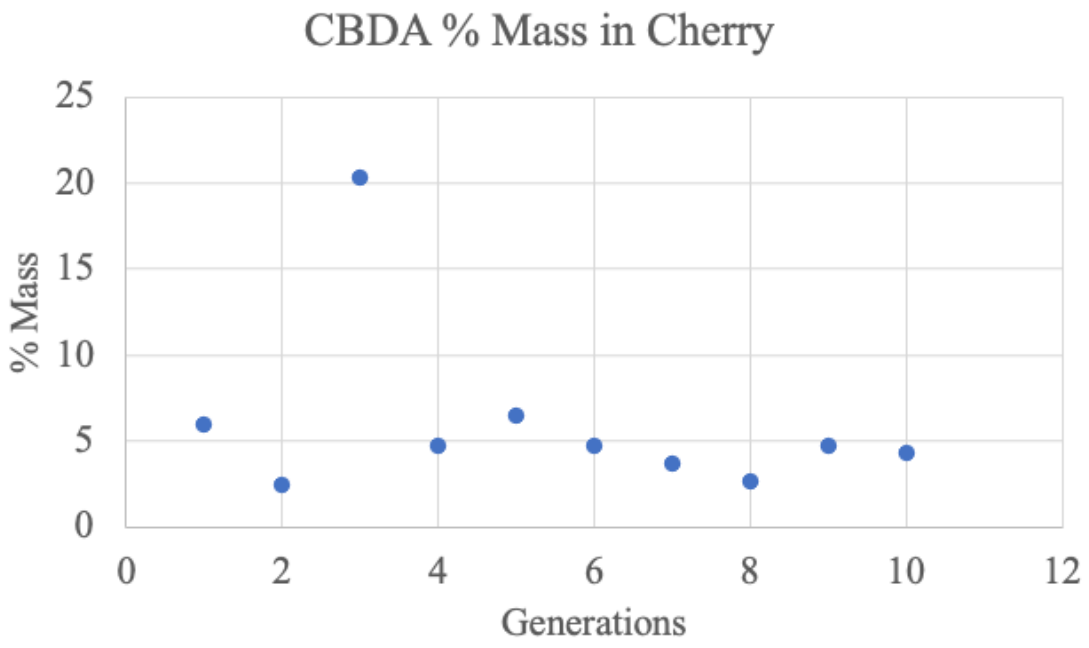


Figure 14. CBDA % mass in Cherry variety. The p value was 0.76171. The F calculated value was 0.614548 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDA.

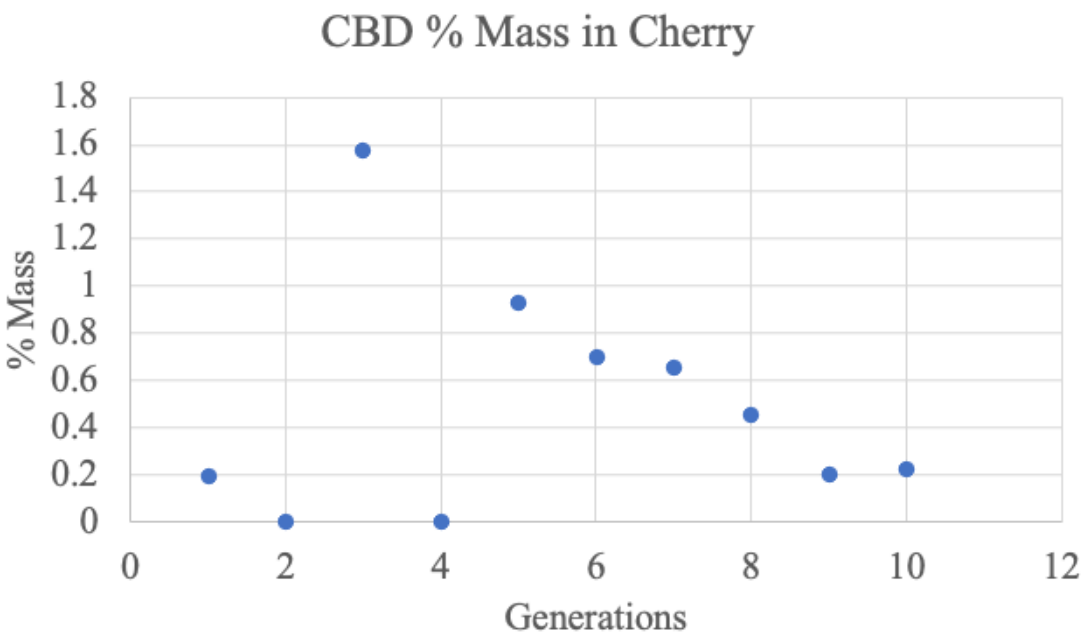


Figure 15. CBD % mass in Cherry variety. The p value was 0.96868. The F calculated value was 0.27158 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBD.

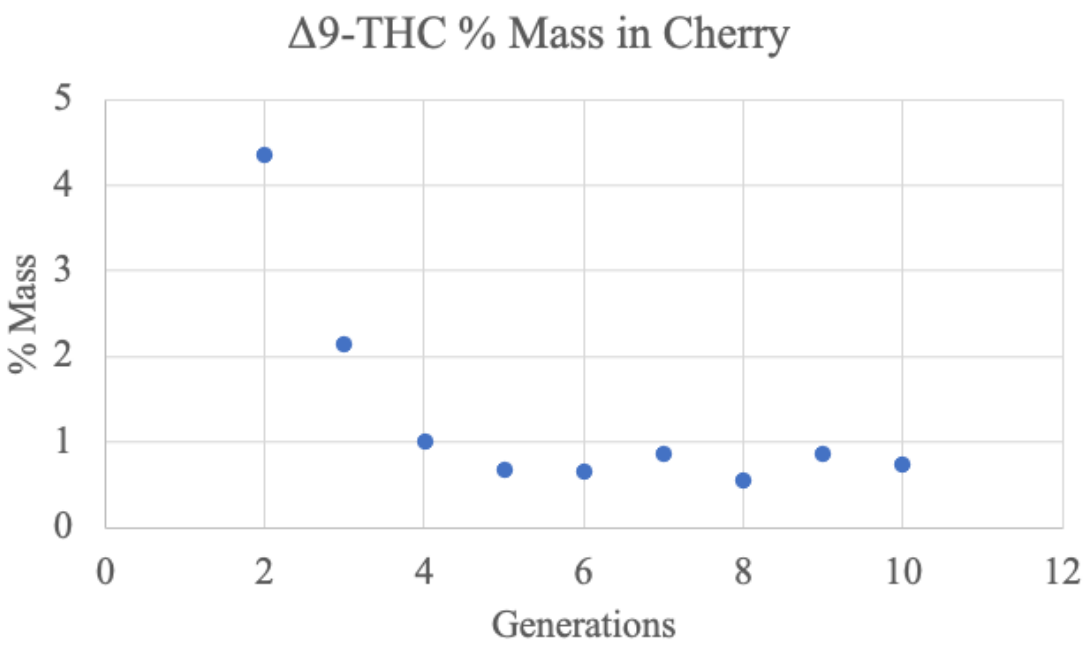


Figure 16. Δ9-THC % mass in Cherry variety. The p value was 0.994754. The F calculated value was 0.154977 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid Δ9-THC.

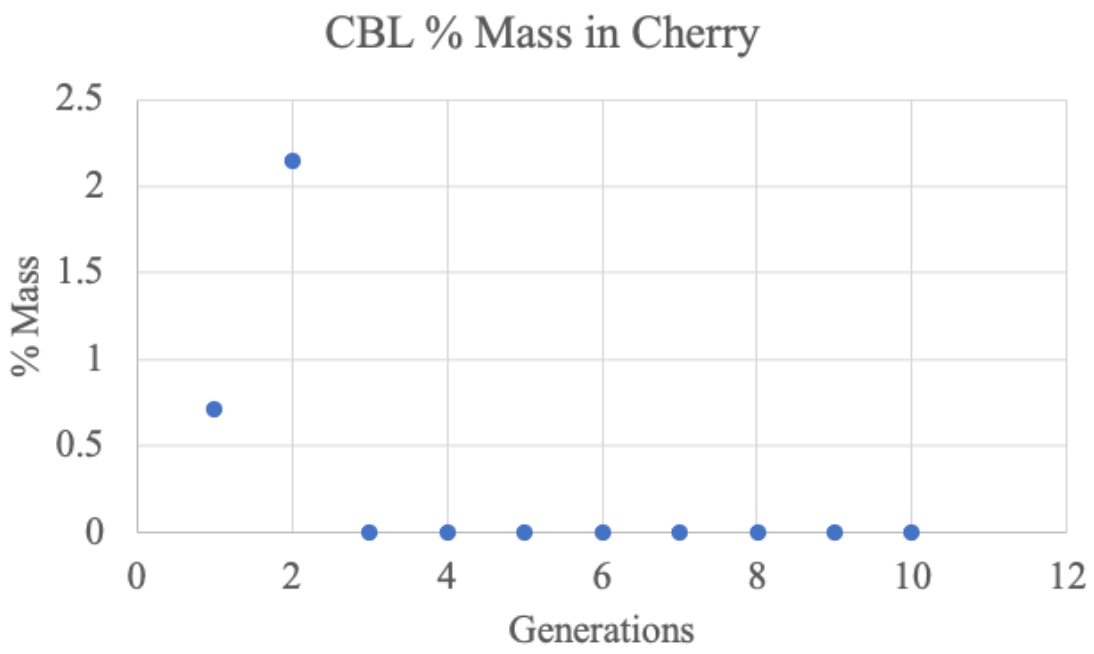


Figure 17. CBL % mass in Cherry variety. The p value was 0.989861. The F calculated value was 0.191017 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBL.

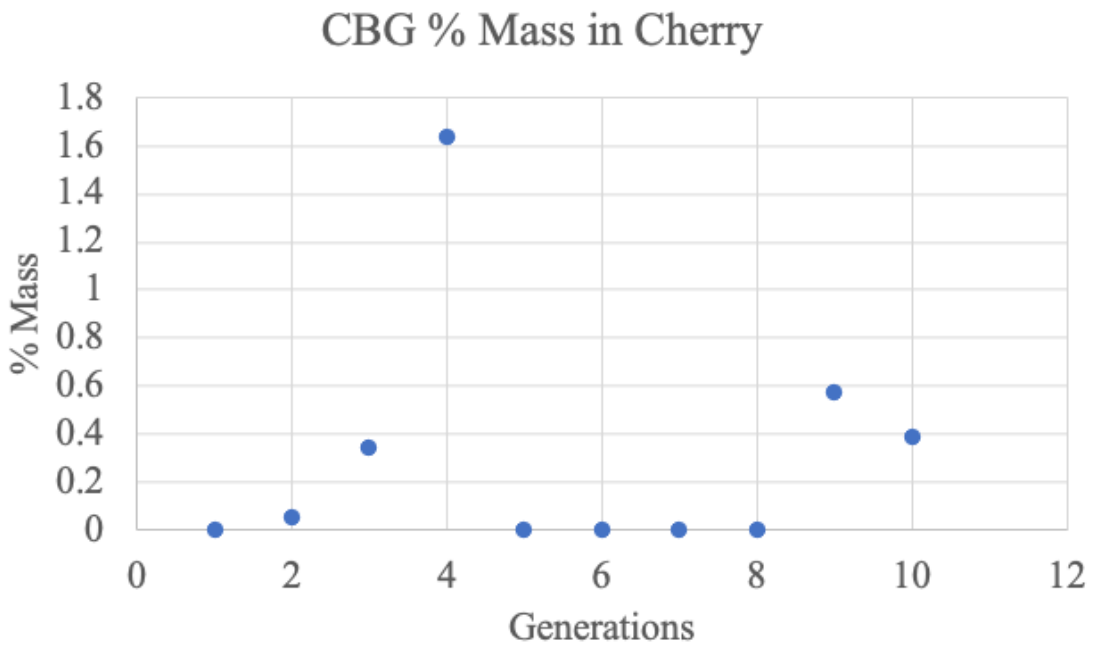


Figure 18. CBG % mass in Cherry variety. The p value was 0.970114. The F calculated value was 0.267404 and the F critical value was 3.020383; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBG.

Cherry Blossom

Ten successive generations of Cherry Blossom were cloned, flowered, harvested, and analyzed (Generations 8 and 9 not analyzed) by HPLC. Two cannabinoids that were not present were CBDV and CBG. All cannabinoids had been analyzed via ANOVA with alpha = 0.05. Every generation that had detectable results had an F calculated value less than the F critical value; this means that we fail to reject the null hypothesis that there is no difference between successively cloned generations of cannabinoid % mass values. Thus, there is no difference between successive generations of Cherry Blossom. The cannabinoid CBDVA had remained consistent throughout successive generations (Figure 19). All the other cannabinoids analyzed for Cherry Blossom (besides CBL) had showed no trend due to experimental error; this is because the cannabinoids did not remain consistent, increase, or decrease over time (Figures 20, 21, 22). The cannabinoid CBL had stopped being produced after generation 3; there was not considered a difference in this cannabinoid because there was only a small concentration before the production stopped (Figure 23).

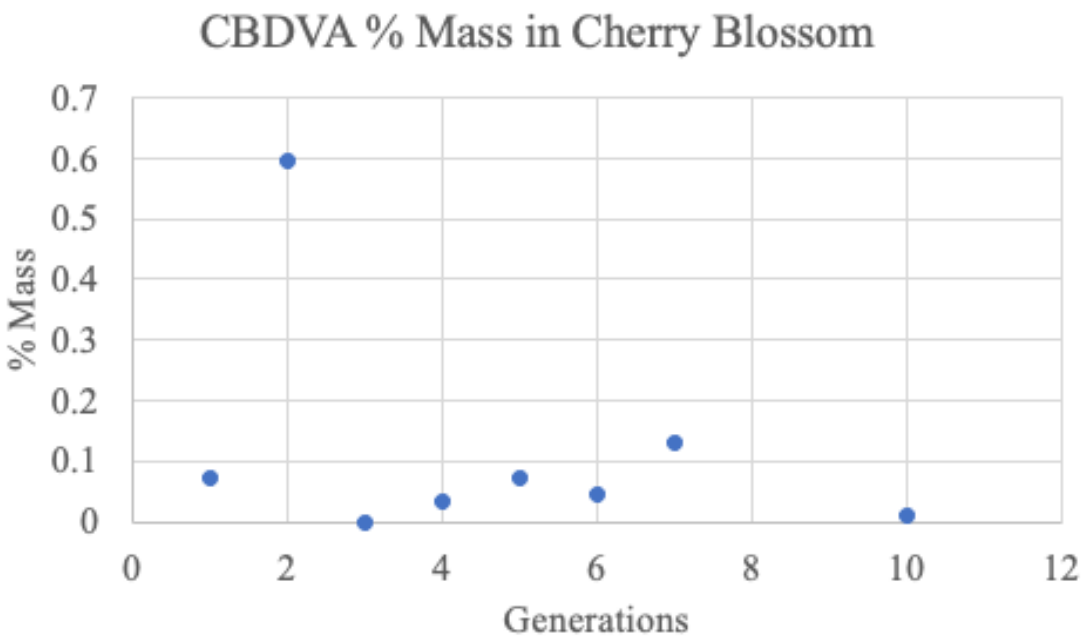


Figure 19. CBDVA % mass in Cherry Blossom variety. The p value was 0.0.687111. The F calculated value was 0.715257 and the F critical value was 3.38813; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDVA.

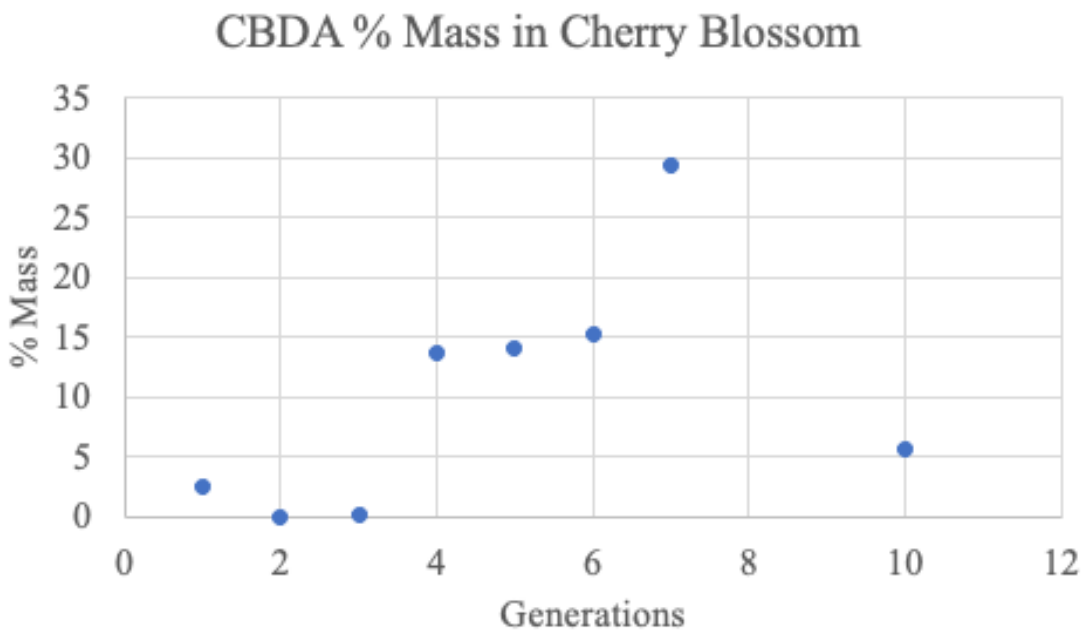


Figure 20. CBDA % mass in Cherry Blossom variety. The p value was 0.771503. The F calculated value was 0.59637 and the F critical value was 3.38813; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDA.

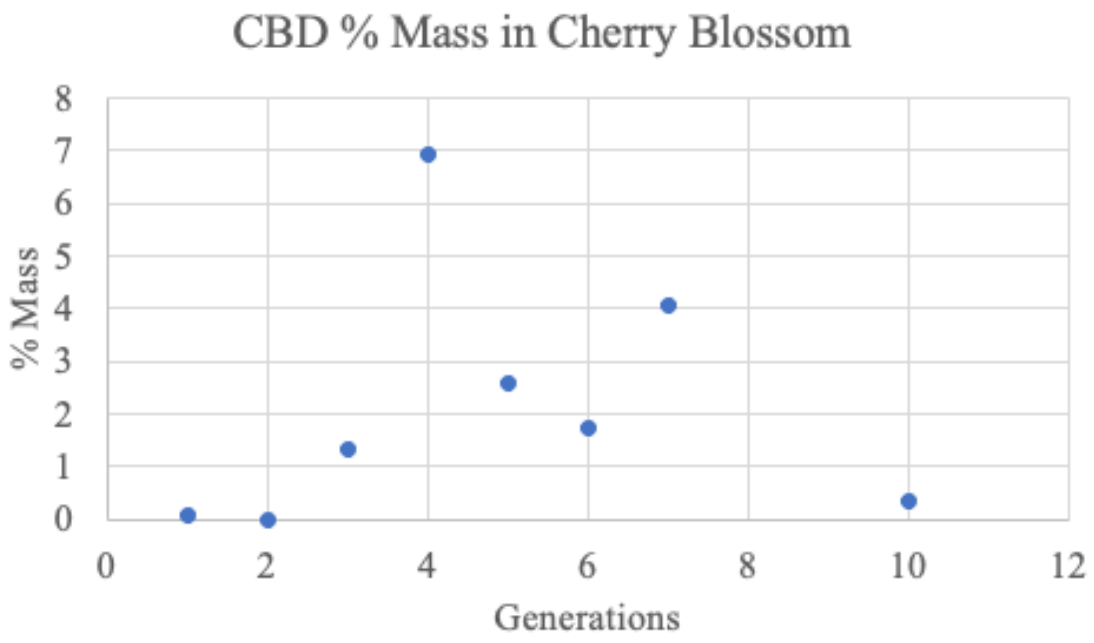


Figure 21. CBD % mass in Cherry Blossom variety. The p value was 0.374408. The F calculated value was 1.267546 and the F critical value was 3.38813; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBD.

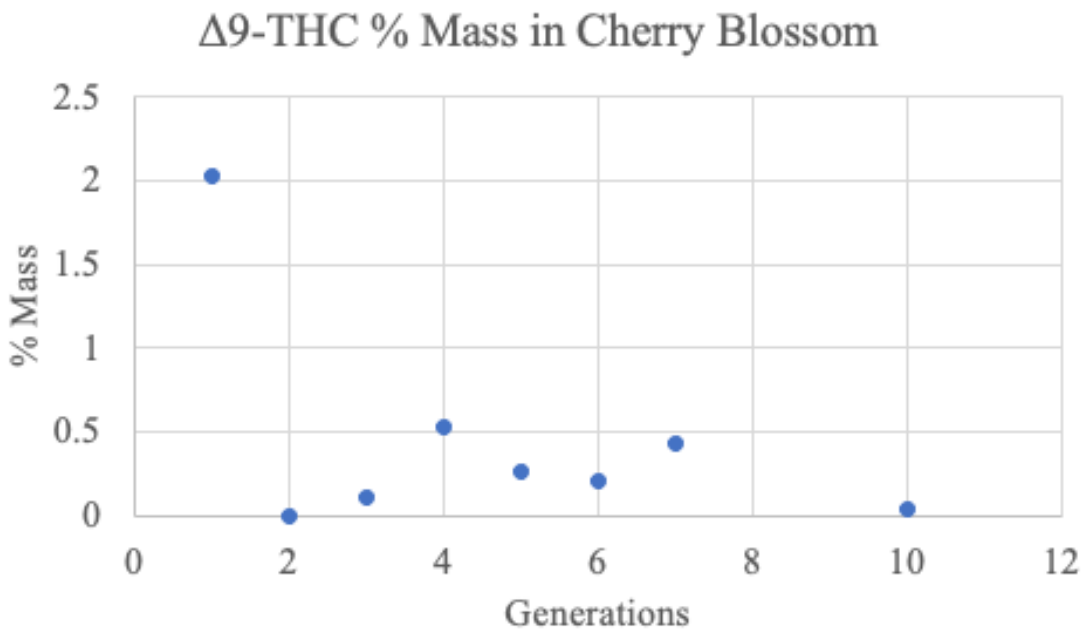


Figure 22. Δ9-THC % mass in Cherry Blossom variety. The p value was 0.74228. The F calculated value was 0.690901 and the F critical value was 3.38813; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid Δ9-THC.

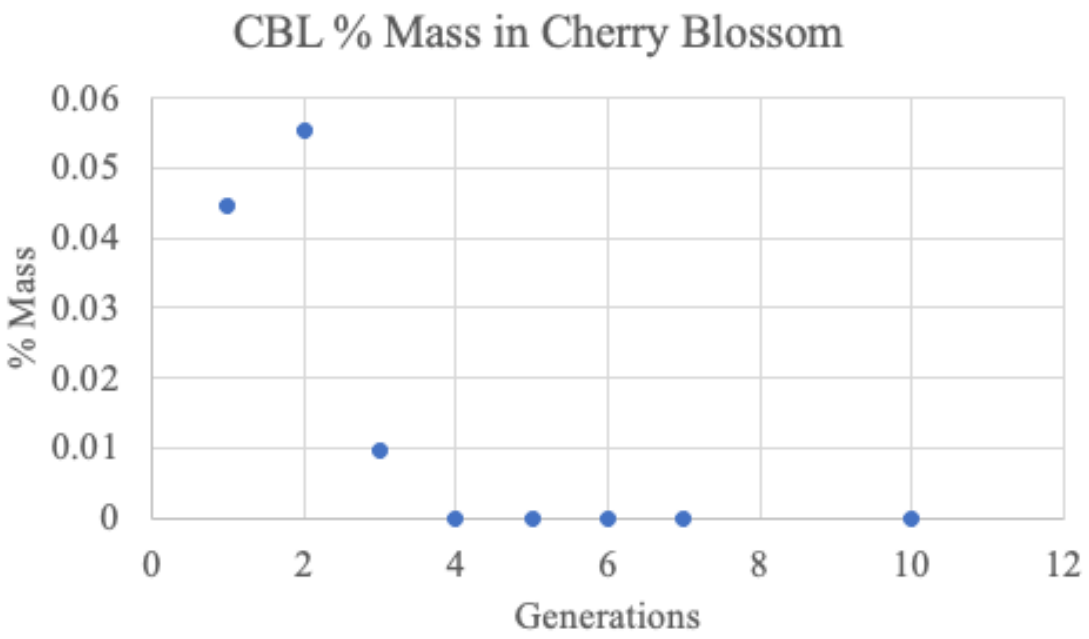


Figure 23. CBL % mass in Cherry Blossom variety. The p value was 0.686863. The F calculated value was 0.715611 and the F critical value was 3.38813; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBL.

Cherry x Workhorse

Ten successive generations of Cherry x Workhorse were cloned, flowered, harvested, and analyzed (generation 7 not analyzed) by HPLC. All cannabinoids had been analyzed via ANOVA with alpha = 0.05. Every generation that had detectable results had an F calculated value less than the F critical value; this means that we fail to reject the null hypothesis that there is no difference between successively cloned generations of cannabinoid % mass values. Thus, there is no difference between successive generations of Cherry x Workhorse. The cannabinoid CBDVA had remained consistent throughout all analyzed generations (Figure 24). Likewise, the cannabinoid CBDV had remained consistent throughout all analyzed generations (Figure 25). The randomness for the following cannabinoids can be explained by experimental error: CBDA, CBD, Δ9-THC, and CBG (Figures 26, 27, 28, and 30). These cannabinoids showed no trend over being cloned successively. Lastly, the cannabinoid CBL shows a decrease in % mass levels over time and had stopped being produced after generation 3; there was not considered a difference in this cannabinoid because there was only a small concentration before the production stopped (Figure 29).

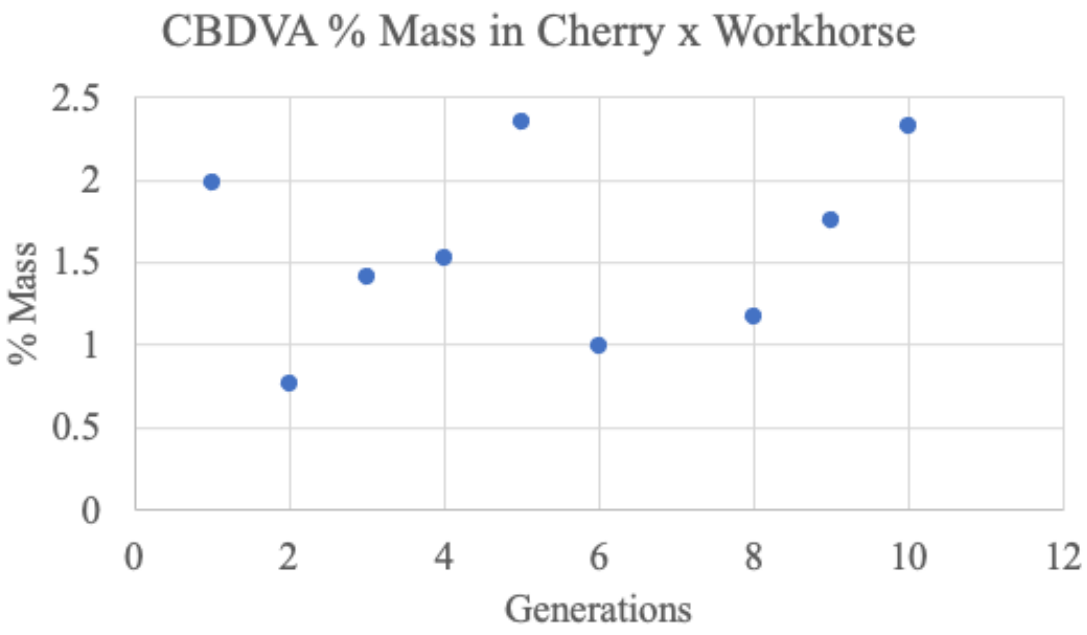


Figure 24. CBDVA % mass in Cherry x Workhorse variety. The p value was 0.79526. The F calculated value was 0.566098 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDVA.

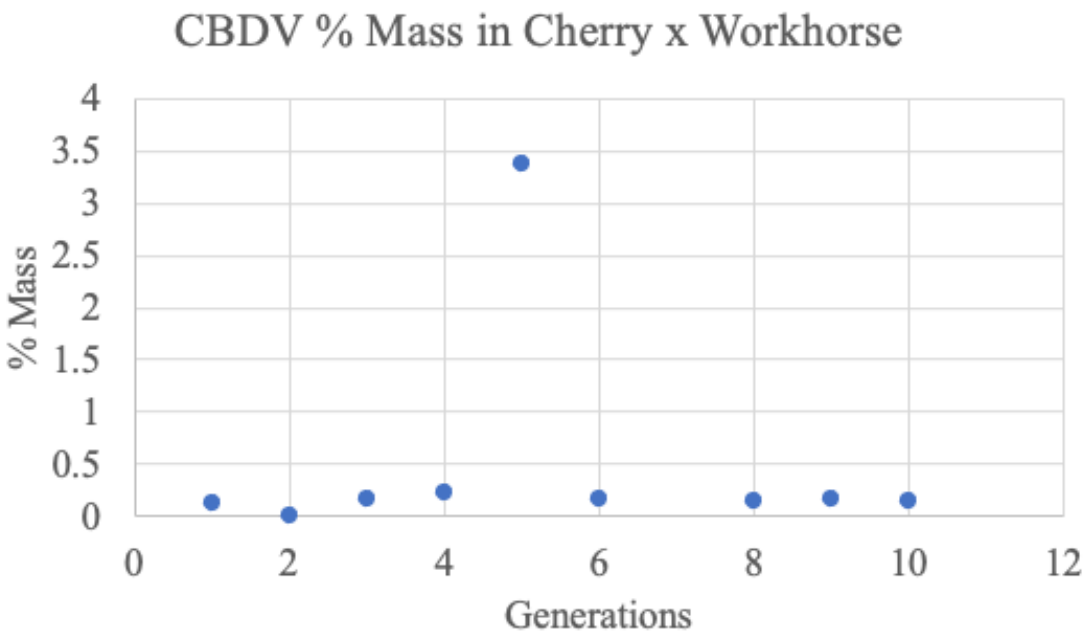


Figure 25. CBDV % mass in Cherry x Workhorse variety. The p value was 0.90633. The F calculated value was 0.399044 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDV.

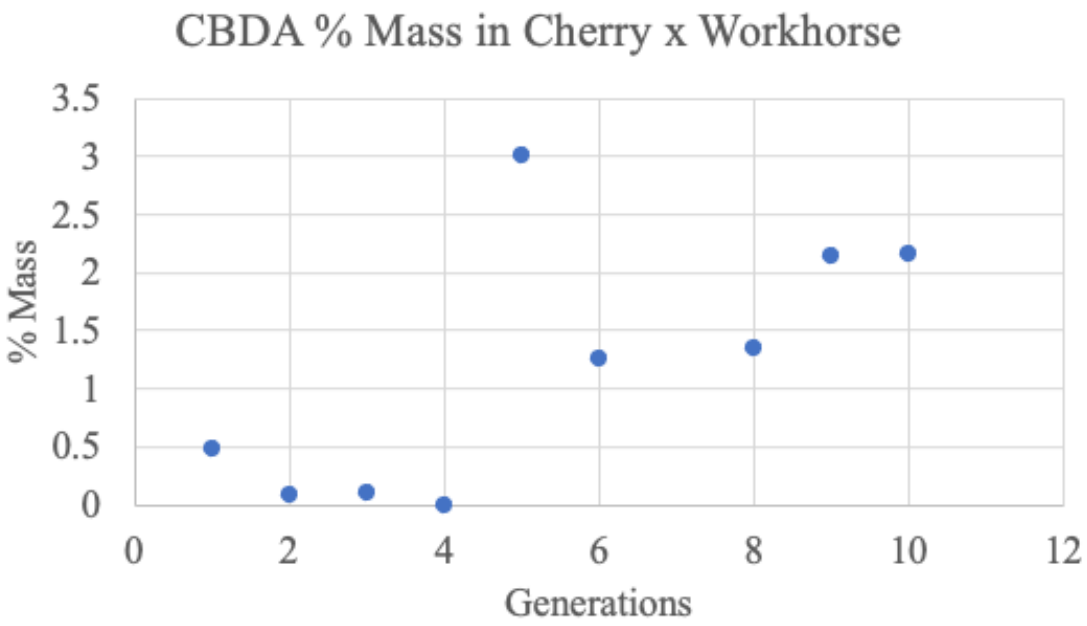


Figure 26. CBDA % mass in Cherry x Workhorse variety. The p value was 0.669192. The F calculated value was 0.74042 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDA.

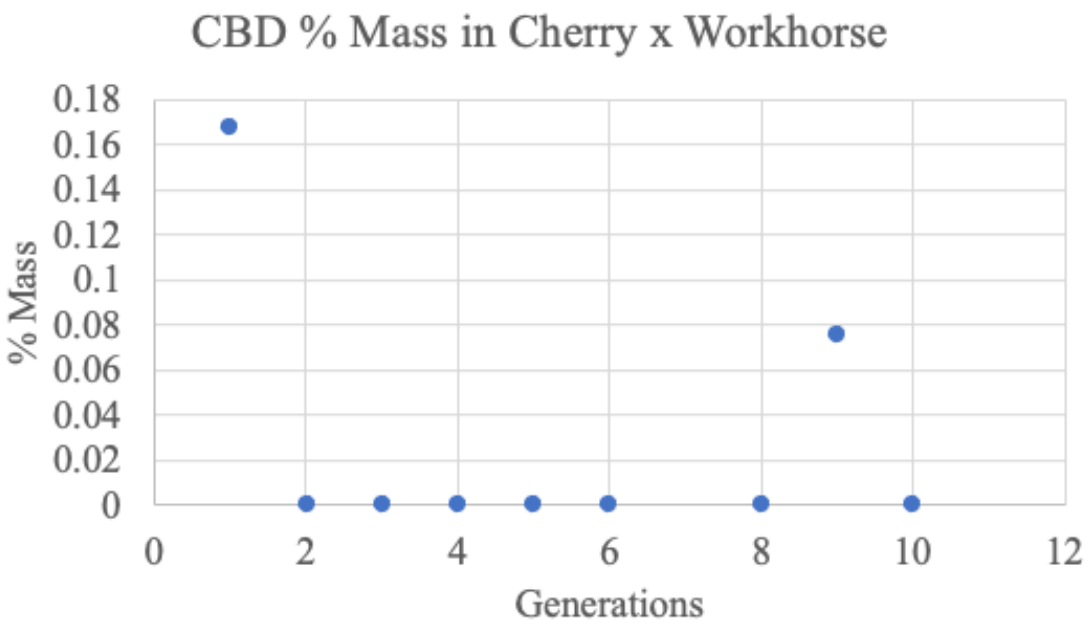


Figure 27. CBD % mass in Cherry x Workhorse variety. The p value was 0.93692. The F calculated value was 0.342431 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBD.

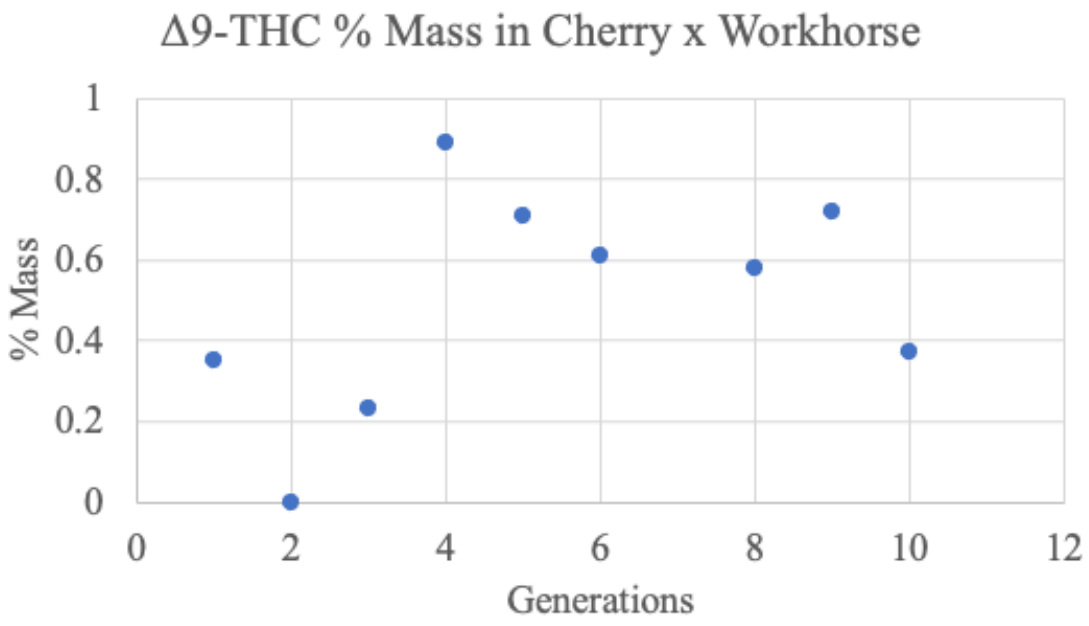


Figure 28. Δ9-THC % mass in Cherry x Workhorse variety. The p value was 0.898164. The F calculated value was 0.412846 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid Δ9-THC.

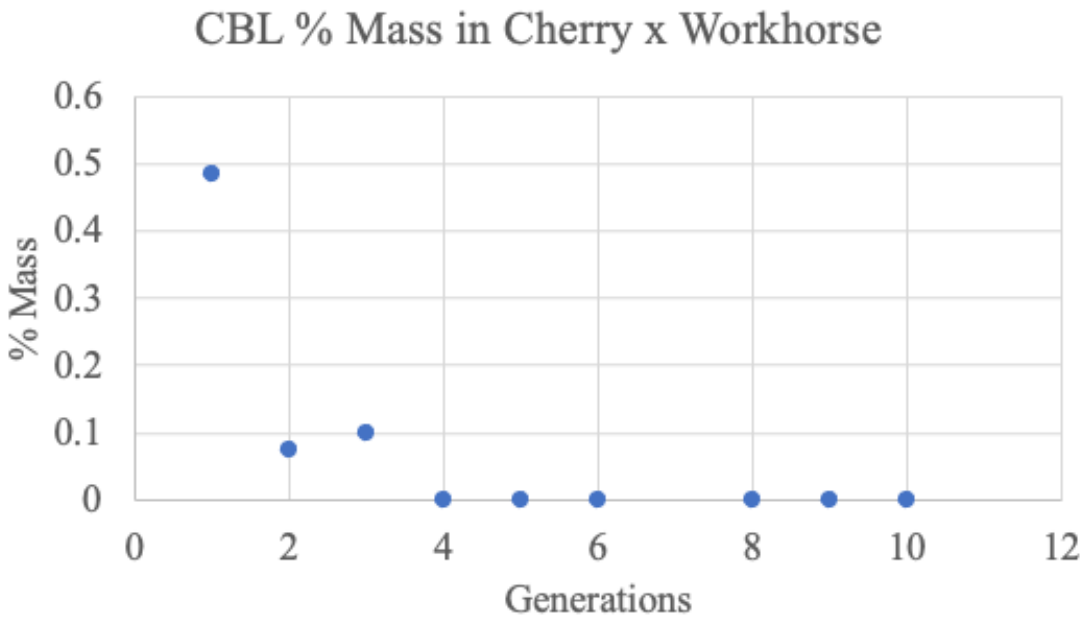


Figure 29. CBL % mass in Cherry x Workhorse variety. The p value was 0.943388. The F calculated value was 0.329032 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBL.

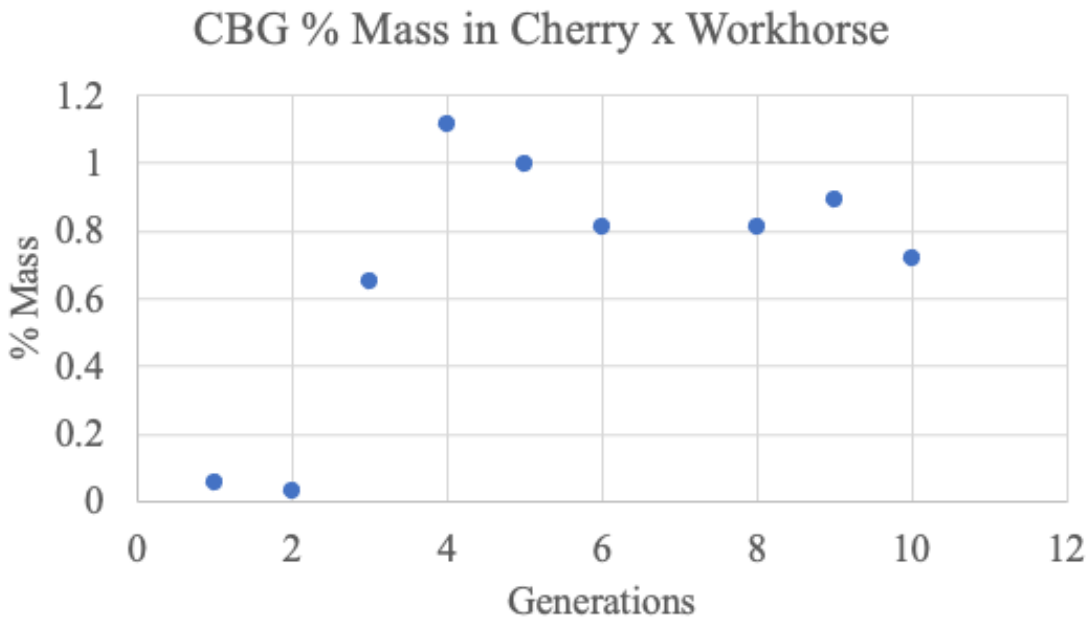


Figure 30. CBG % mass in Cherry x Workhorse variety. The p value was 0.86668. The F calculated value was 0.462864 and the F critical value was 3.178893; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBG.

Sour Space Candy

Seven successive generations of Sour Space Candy were cloned, flowered, harvested, and analyzed (generation 5 not analyzed) by HPLC. There was one cannabinoid that was not detected in every successive generation: CBG. All cannabinoids had been analyzed via ANOVA with alpha = 0.05. Every generation that had detectable results had an F calculated value less than the F critical value; this means that we fail to reject the null hypothesis that there is no difference between successively cloned generations of cannabinoid % mass values. Thus, there is no difference between successive generations of the same cannabinoid in Sour Space Candy. There is no trend for CBDVA, CBDV, CBDA, CBD, and Δ9-THC analyzed for Sour Space Candy as the % mass levels do not remain consistent, increase, or decrease (Figures 31, 32, 33, 34, 35). The cannabinoid CBL stopped being produced after generation 3; there was not considered a difference in this cannabinoid because there was only a small concentration before the production stopped (Figure 36).

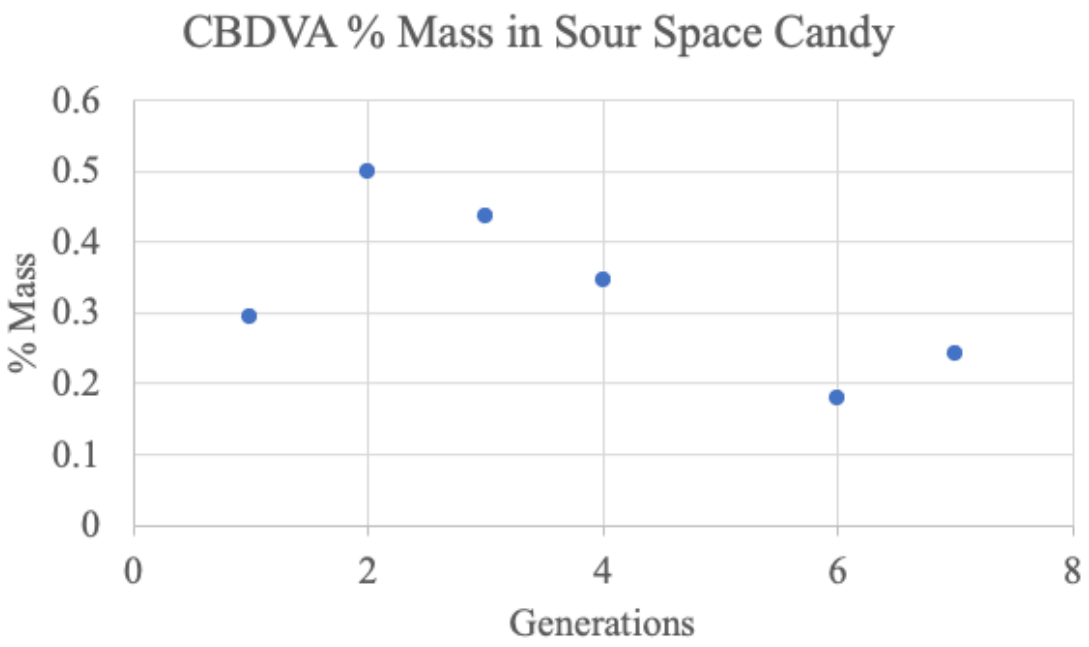


Figure 31. CBDVA % mass in Sour Space Candy variety. The p value was 0.8556766. The F calculated value was 0.3994066 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDVA.

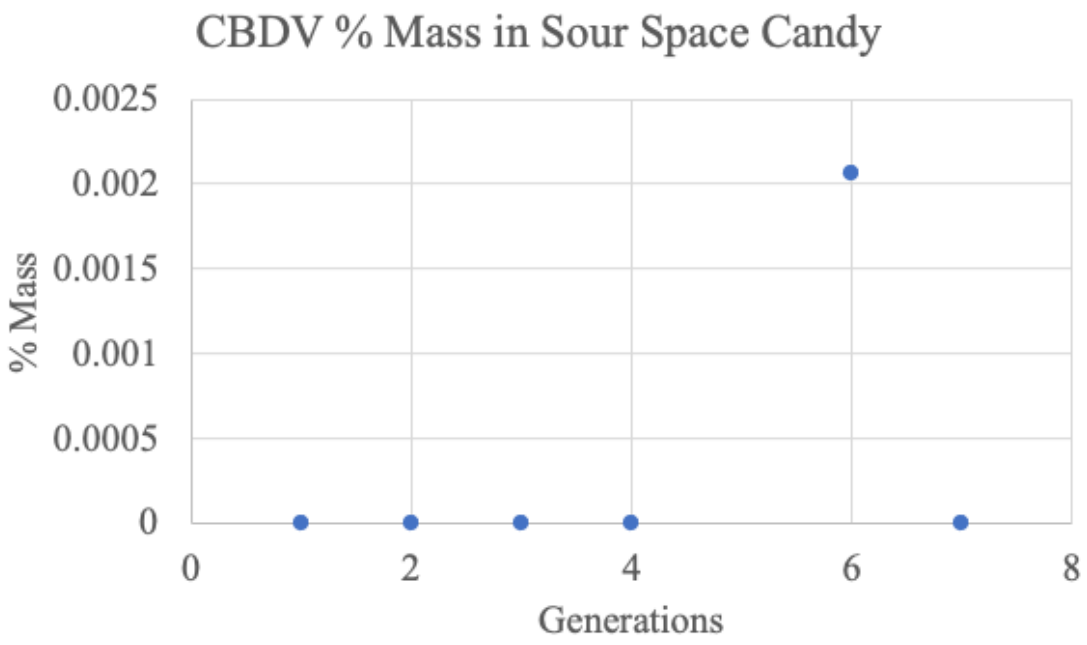


Figure 32. CBDV % mass in Sour Space Candy variety. The p value was 0.8641148. The F calculated value was 0.3860969 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDV.

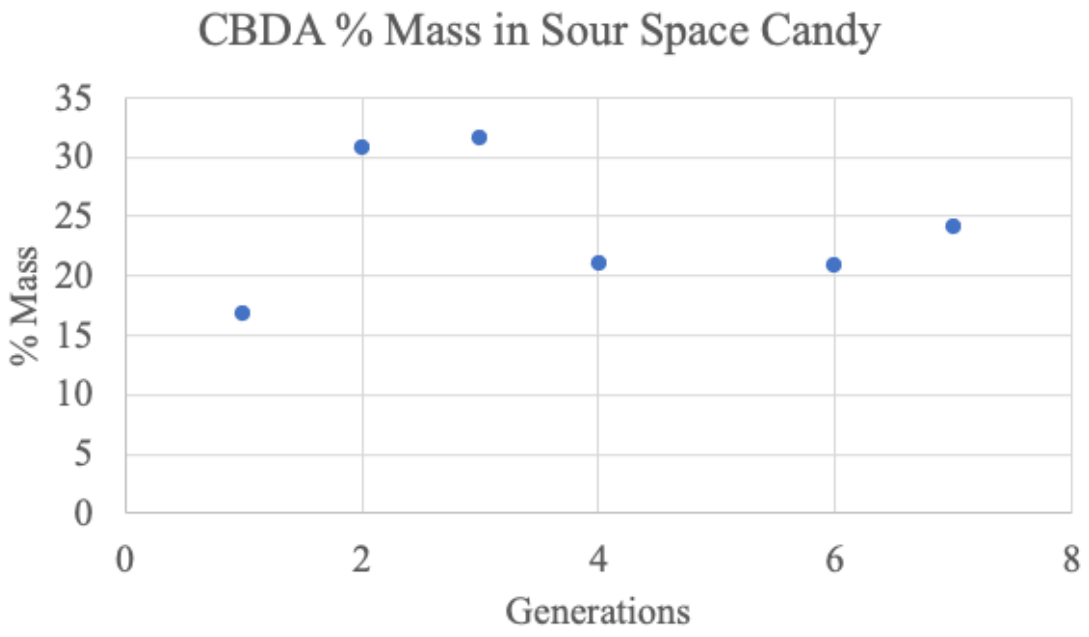


Figure 33. CBDA % mass in Sour Space Candy variety. The p value was 0.9881642. The F calculated value was 0.1263014 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDA.

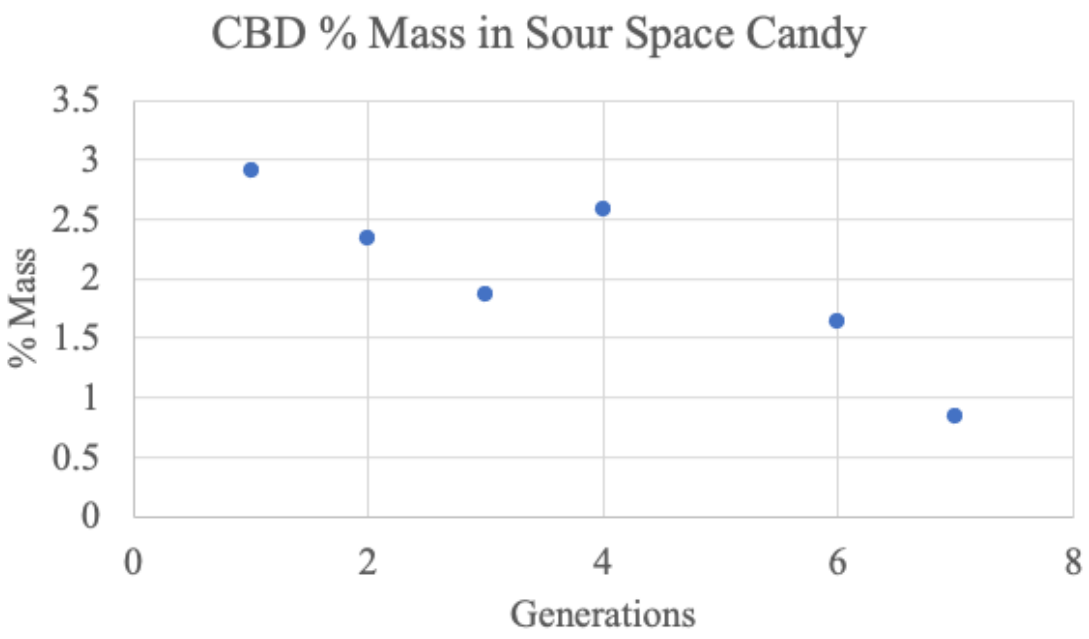


Figure 34. CBD % mass in Sour Space Candy variety. The p value was 0.884422. The F calculated value was 0.3533805 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBD.

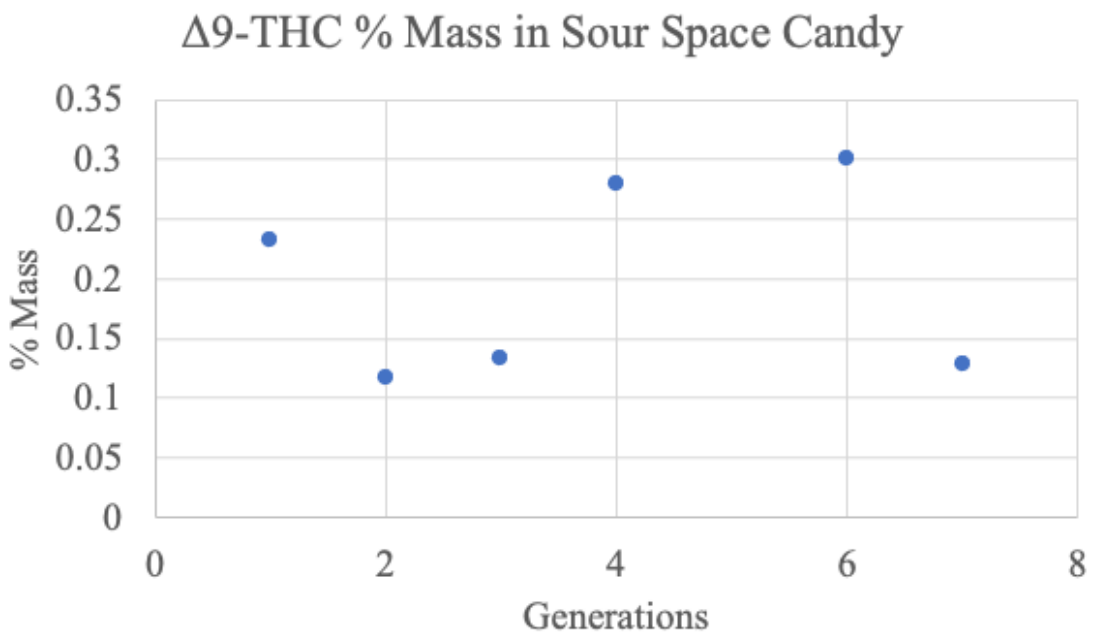


Figure 35.Δ9-THC % mass in Sour Space Candy variety. The p value was 0.8480999. The F calculated value was 0.4112489 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid Δ9-THC.

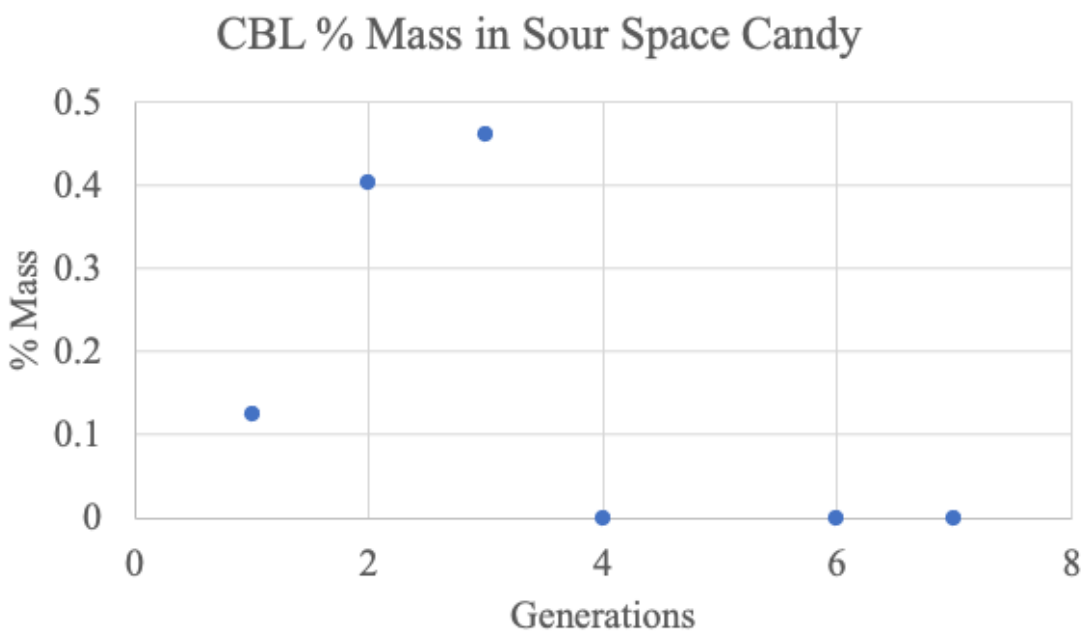


Figure 36. CBL % mass in Sour Space Candy variety. The p value was 0.8756019. The F calculated value was 0.367727 and the F critical value was 4.2838657; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBL.

The Wife

Six successive generations of The Wife were cloned, flowered, harvested, and analyzed by HPLC. Two cannabinoids that were not detected were CBDV and CBG. All cannabinoids had been analyzed via ANOVA with alpha = 0.05. Every generation that had detectable results had an F calculated value less than the F critical value; this means that we fail to reject the null hypothesis that there is no difference between successively cloned generations of cannabinoid % mass values. Thus, there is not a difference between successive generations of The Wife. All the cannabinoids except CBL showed no trend as the % mass levels did not remain consistent, increase, or decrease (Figures 37, 38, 39, and 40). The cannabinoid CBL had stopped being produced after generation 2; there was not considered a difference in this cannabinoid because there was only a small concentration before the production stopped (Figure 41).

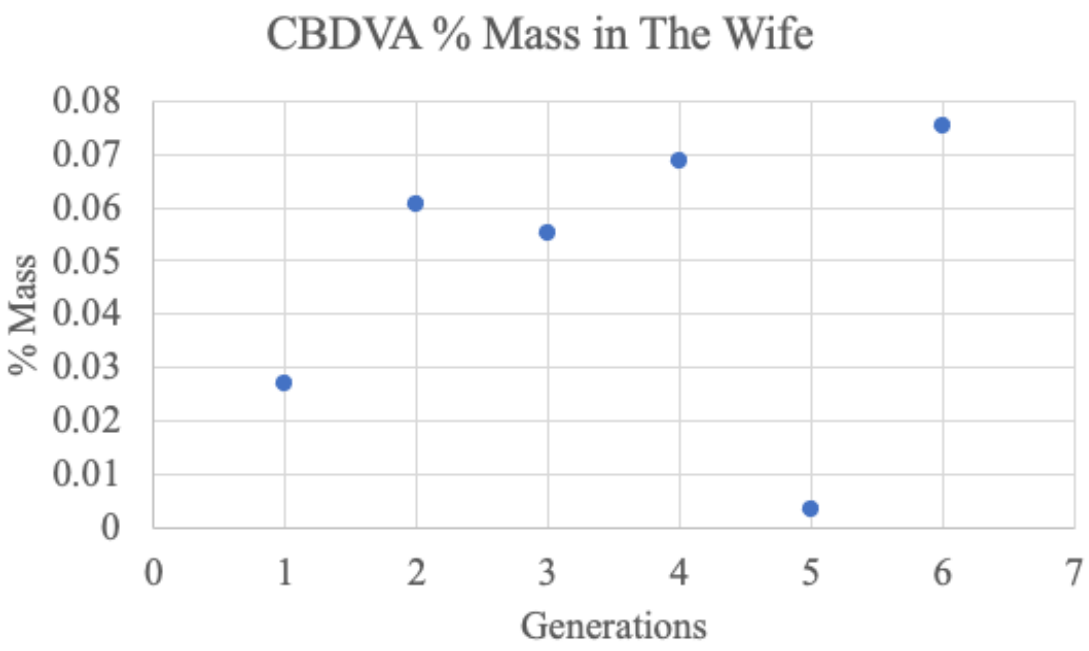


Figure 37. CBDVA % mass in The Wife variety. The p value was 0.931962. The F calculated value was 0.237425 and the F critical value was 4.387374; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDVA.

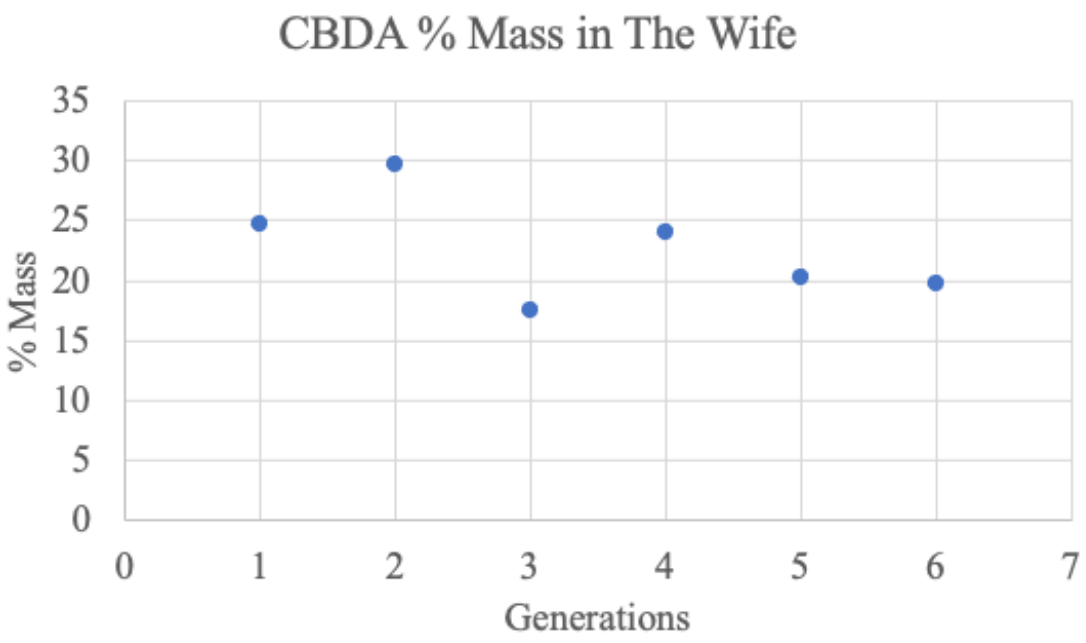


Figure 38. CBDA % mass in The Wife variety. The p value was 0.998982. The F calculated value was 0.034936 and the F critical value was 4.387374; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBDA.

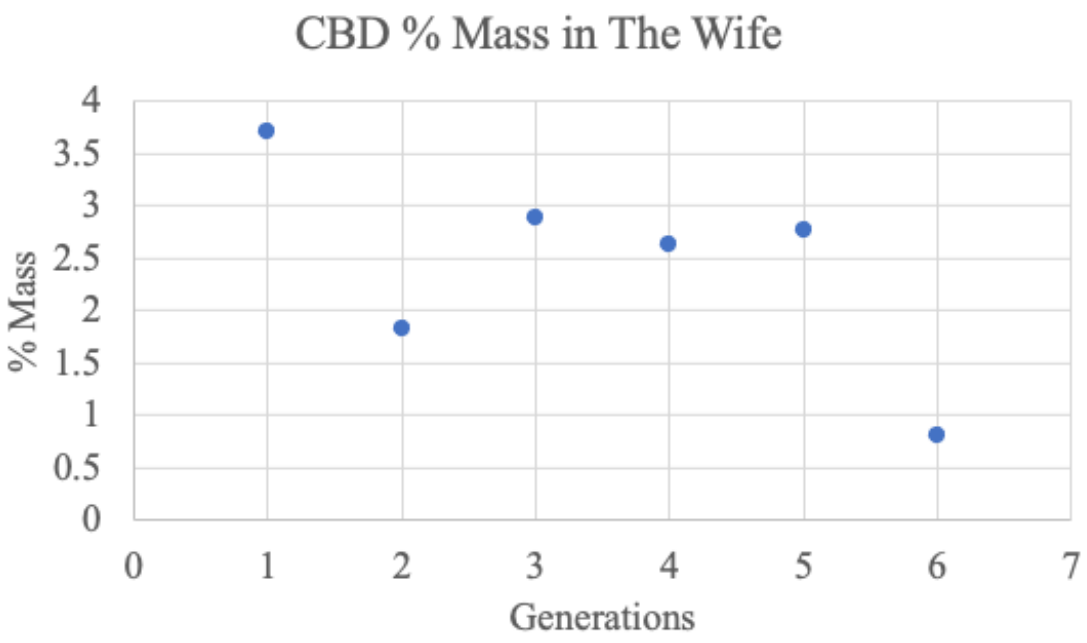


Figure 39. CBD % mass in The Wife variety. The p value was 0.892414. The F calculated value was 0.30635 and the F critical value was 4.387374; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBD.

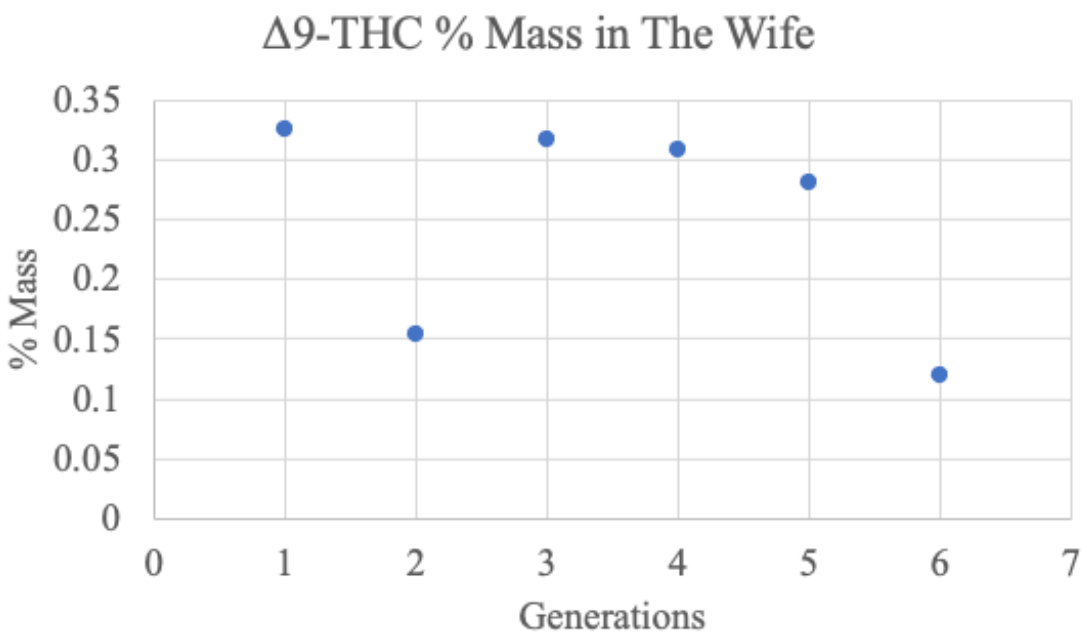


Figure 40. Δ9-THC % mass in The Wife variety. The p value was 0.653994. The F calculated value was 0.6873563 and the F critical value was 4.387374; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid Δ9-THC.

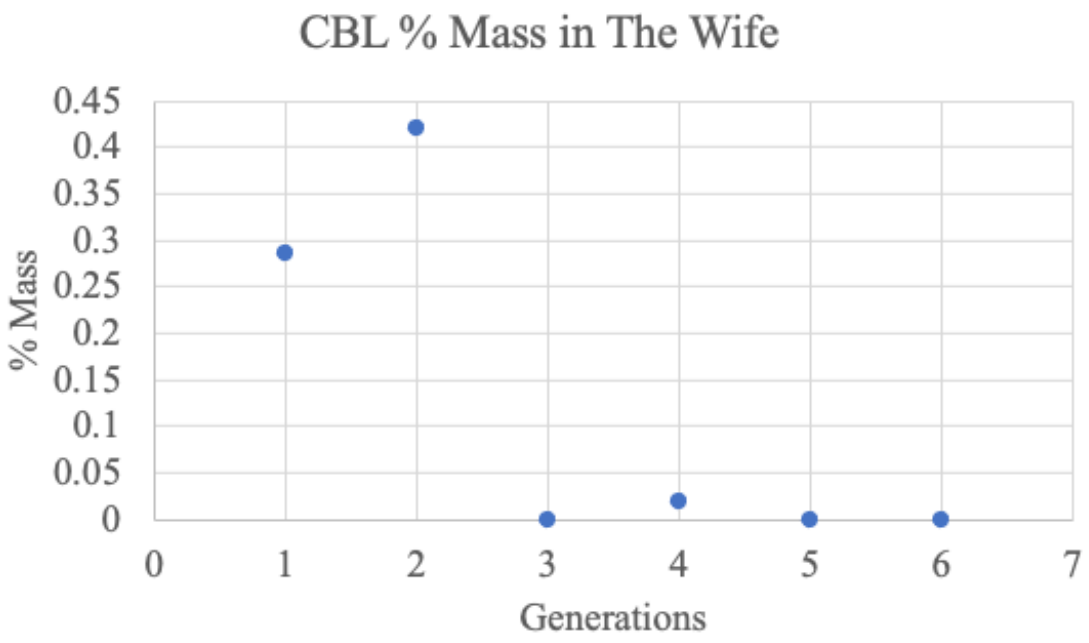


Figure 41. CBL % mass in The Wife variety. The p value was 0.949703. The F calculated value was 0.202624 and the F critical value was 4.387374; there is no statistical difference between % mass in successive generations of *C. sativa* for the cannabinoid CBL.

Discussion

A horticulture research study on cloning [9], stated that morphological changes, such as the growth of male organs, had occurred after the seventh successive generation. This observation indicated that the mutations occurred due to the nucleotide sequences in the genome of the cuttings taken for the new plant [9]. Therefore, those mutations will continue in the next successive generations, that alter the metabolic profile of *C. sativa* which effects the overall % mass of cannabinoids in the plant.

The results of my study show that there is not a significant difference in cannabinoids over successive generations although the % mass levels were variable but with no trends. The only cannabinoid to show slight decrease over successive generations had been Δ9-THC in the variety Cherry (Figure 16). All the generations of Sour Space Candy have high CBDA % mass levels around 30%; Sour Space Candy is known to have high CBDA/CBD levels (CBDA decarboxylates into CBD), so each successive generation grown exhibited this trend (Figure 32). An objective of this research had been to observe if the plants lose the function of producing cannabinoids overtime which had been shown in some cannabinoids: this occurred for CBL in every variety grown; there was not considered a difference in this cannabinoid in any variety because there was only a small concentration produced before the production stopped completely (Figures 17, 23, 29, 36, and 41). CBDV and CBG had not been produced in any successive generation in the following varieties: The Wife and Cherry Blossom. The idea that all the cloned plants should have the same exact metabolism throughout multiple successive generations has been shown with these data for every variety.

According to a research study conducted by Punja *et al.* [15], *C. sativa* is naturally a dioecious (female and male flowers are on separate plants) species but can turn to a monoecious (female and male flowers on the same plant) species spontaneously or turn under certain physical/chemical conditions. Female plants that undergo environmental stressors such as late harvest, changes in photoperiod, non-ideal temperatures, or hormone additives can cause male organs to grow; if the plants had been placed in the flowering chamber too early in development to flower, the extended darkness period specifically triggers this formation [15]. From generation 5 onward for the variety Cherry x Workhorse, the plants had all reverted to hermaphroditism (Figure 42). Generation 5 and 6 had been in the chamber together; when the plants had been harvested and dried seeds had been found from the generation 6 samples. These seeds had then been germinated and planted to show whether the seeds were viable which they were. All the successfully germinated seeds had produced healthy plants which happened to be all female. The amount of pollen produced by hermaphroditic plants is known to be significantly lesser in quantity than pollen produced by male plants [8]. Therefore, this allows for the assumption that the hermaphrodites still carried a XX genotype regardless of the flowers present in the monoecious plant and had viable pollen [8].

This research is beneficial for future vegetative growing projects because the study shows that there is no significant trend with cannabinoid levels over successive generations. For legal purposes, *C. sativa* plants must maintain a THC concentration below 0.3% for the plant to be considered industrial hemp [20]. Thus, *C. sativa* growers need to be extremely aware of the THC concentrations in the plants because of the lack of stable cannabinoid levels. This research study is useful to the Tennessee Center for

Botanical Medicine Research as well as other industrial hemp research facilities that need to understand different ways to propagate *C. sativa* in an efficient manner without affecting cannabinoid levels.

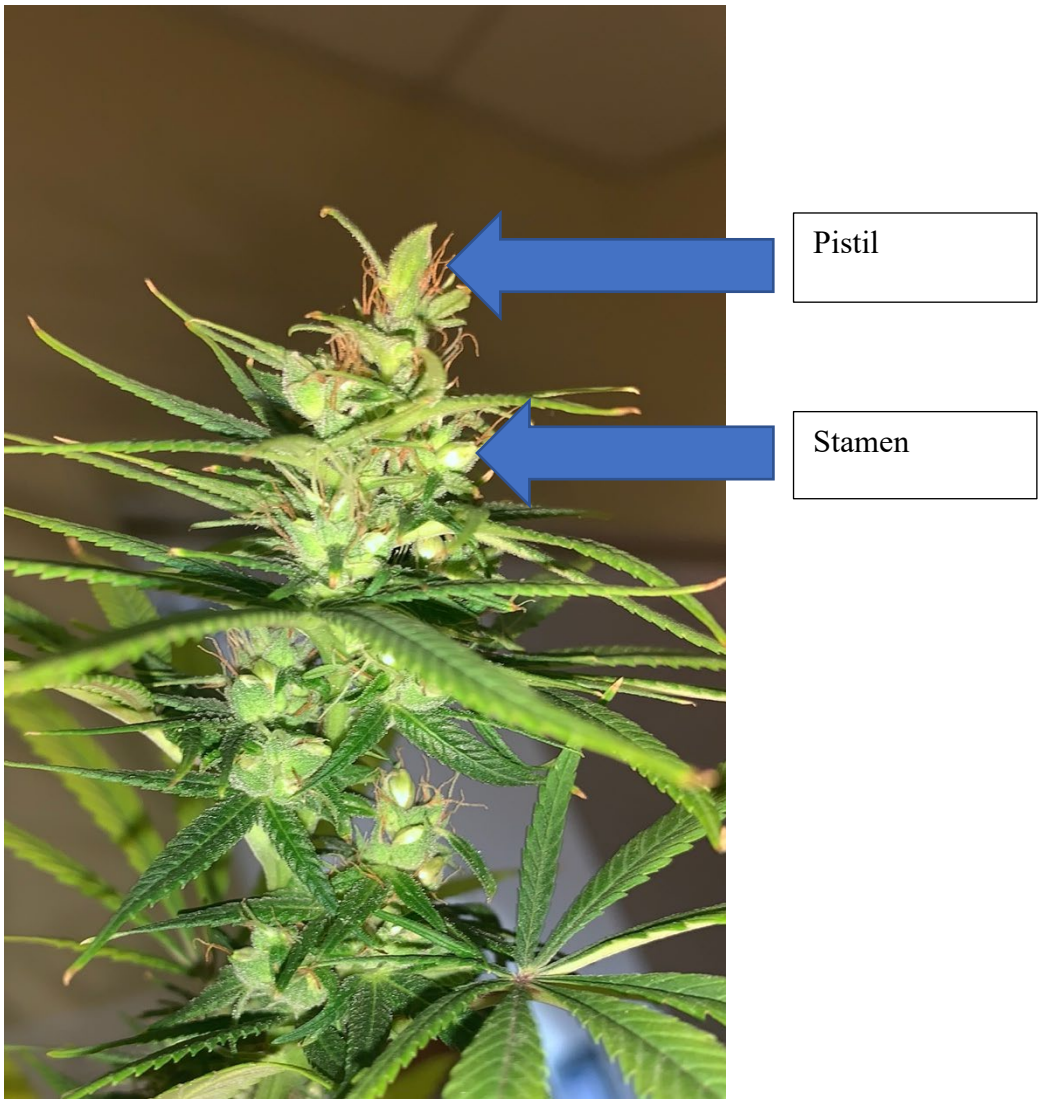


Figure 42. Generation 5 of Cherry x Workhorse reverted to hermaphroditism. Pistil (female) and Stamen (male) organs are labeled.

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