Assessment of Cannabinoid Levels in Successively Cloned Generations of Industrial Hemp (*Cannabis sativa)*

by
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DEDICATION

I dedicate this project to my mom, my granny, and my late papa. Your love and support have helped me to push myself farther and achieve goals higher than I ever thought possible. I am forever grateful. This one is for you Papa and Uncle George.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. John DuBois for the opportunity to complete this project along with his extraordinary guidance and support. I would also like to acknowledge Dr. Paul Kline for his amazing help in running many analyses over the last year. I would never have made it to the end without these two amazing professors. Lastly, I acknowledge the URECA program and Greenway Herbal Products LLC. For funding my research and giving me opportunities to present my results at multiple research venues.

ABSTRACT

The business of industrial hemp (*Cannabis sativa*) has grown tremendously over the past decades, both in agriculture and pharmaceuticals because of its potential health benefits. Plant propagation using stem cuttings from stock plants has become the favorite method of growing hemp for farmers (Caplan *et al.* 2018). Recent studies have shown that plant propagation can lead to certain genetic changes known as somaclonal variations. This research was designed to test the effects of cloning hemp varieties (Cherry, Cherry Blossom, and Cherry x Workhorse) through plant propagation on cannabinoid production. Results showed significant differences in cannabinoid levels between clonal generations of each variety. The results of this study could be useful to farmers and hemp research centers such as the Tennessee Center for Botanical Medicine Research, and other hemp agricultural departments that must maintain cannabinoid consistency standards.

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1. **Cannabinoids**: (n) Any of a group of similarly related compounds that include the active constituents of cannabis.

2. **HPLC**: (n) High-performance liquid chromatography is a form of liquid chromatography that is designed to separate chemical compounds dissolved in solution.

3. **Tissue propagation**: (n) Asexual reproduction using plant tissue of a parent plant resulting in a genetically identical offspring.

4. **Somaclonal variation**: (n) Variation seen in plants that are produced through plant tissue propagation.

INTRODUCTION

Brief history of the *Cannabis* plant

Accounts of the cultivation of *Cannabis* date back at least 6,000 years to Neolithic times in China (Li 1973), but the beneficial pharmacological compounds we know today have only been discovered since the end of the nineteenth century. The first cannabinoid to be isolated was cannabinol (CBN; Figure 1) by a group in Cambridge that obtained a ruby red, viscous oil. In the 1930's, the research was expanded by Robert Cahn who obtained a pure sample of cannabinol and determined its full structure. It was initially assumed that cannabinol was the main active ingredient responsible for the psychoactive effects of *Cannabis* (Mechoulam and Hanus 2000). The second compound discovered was cannabidiol (CBD) by Mechoulam and Shvo in 1963, followed by the discovery of the main active compound, delta-9-tetrahydrocannabinol (d-9-THC) by Gaoni and Mechoulam in 1964. Although there are over 50 cannabinoid compounds that have been isolated, this project focused solely on cannabidiol levels, the cannabinoid that is most used from industrial hemp that can be measured using high-performance liquid chromatography (HPLC) (De Backer *et al.* 2009).

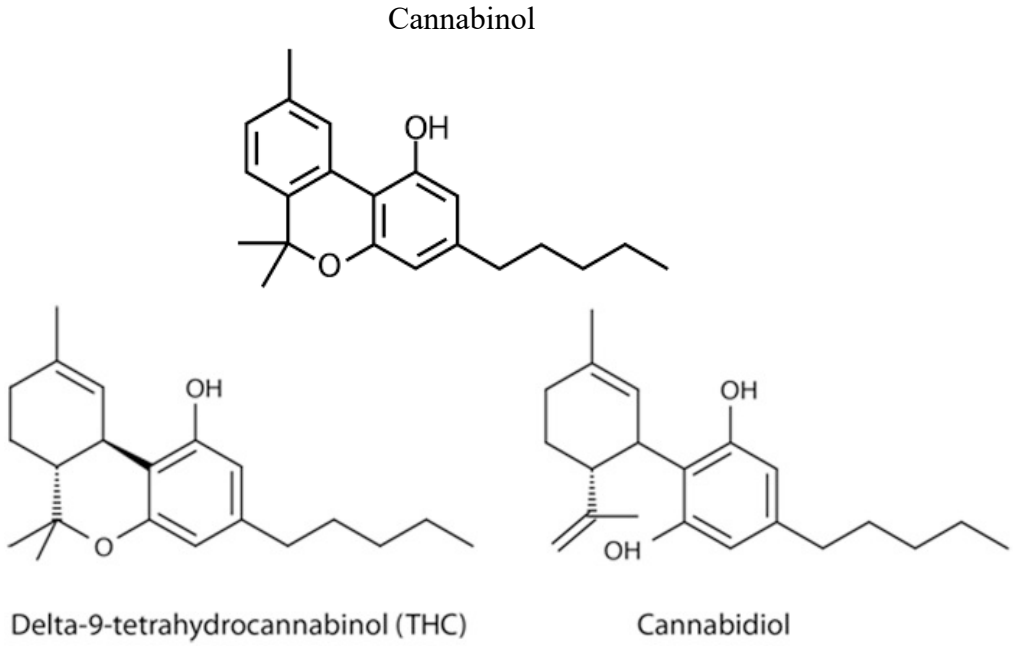

Figure 1. Chemical structures of cannabinol, delta-9-tetrahydrocannabinol, and cannabidiol.

Uses for *Cannabis*

Cannabinoid products have been tested against a variety of illnesses, with many having positive outcomes. A study completed by Collin *et. al.* (2007) involving 189 multiple sclerosis patients with spasticity showed a significant reduction in spasticity. *Cannabis* derived "Sativex," a drug with a 1:1 ratio of THC:CBD, was delivered oromucosally. Another study by Leweke *et al.* (2012) involved 42 patients suffering from acute paranoid schizophrenia and schizophreniform psychosis. Patients demonstrated significantly-reduced psychopathological symptoms of acute psychosis. Natural CBD was as effective as a common antipsychotic drug "Amisulpride."

Cannabis has also demonstrated efficacy as a source of all-natural fibers for use as insulation material and for bio-composites in automotive applications (Carus *et al.* 2013).

As *Cannabis* has demonstrated its usefulness in a variety of socioeconomic sectors, it has been deemed a multi-faceted crop. The economic value of hemp is maximized by harvesting at the end of maturity, so all plant biomass can be used.

Current industrial hemp farming techniques and somaclonal variation

Most of the research into *Cannabis* and its 144 naturally-occurring compounds, otherwise known as cannabinoids, revolves around THC and CBD. Although both compounds originate from the *Cannabis* plant, CBD does not cause an intoxicating "high" sensation like THC (Freeman *et al.* 2019). The use of products derived from the plant genus *Cannabis* for recreational, pharmaceutical, and industrial purposes has greatly increased over the past decade as a new perspective on *Cannabis*-derived products has emerged. Legislation involving *Cannabis* has laxed, allowing for medicinal use in 33 U.S. states and for non-medicinal use in 10 U.S. states (Freeman *et al.* 2019). Because of the future pharmacological potentials of CBD and the quality demands of the pharmaceutical industry that require prescription medicines to have consistent levels of active ingredients (Potter 2013), this study addressed the effects cloning has on CBD consistency through asexual stem propagation by which this study can be applied to mass industrial hemp farming.

Most modern-day *Cannabis* production involves the use of greenhouses, artificial light, and soilless growing methods. Plant propagation using stem cuttings from stock plants has become the favorite method for farmers as it has proven to be the most cost-efficient method to growing genetically uniform plants with consistent rates of growth and cannabinoid production compared with propagation from seed (Caplan *et al.* 2018). Also,

field cultivated *C. sativa* plants are limited by their heterozygosity and their susceptibility to diseases and pests when compared to plants cultivated through tissue propagation (Boonsnongcheep and Pongkitwitoon 2020). It is expected that since the clonal generations grown from cuttings will have the same genetic makeup of the stock plant, CBD levels will be consistent, excluding differences caused by environmental conditions. However, there has been very little concrete evidence to prove that the CBD level of plants produced though propagation using stem cuttings remains consistent over many clonal generations. Coffman and Genter (1979) compared CBD levels of plants grown from propagated seeds versus propagated stem cuttings. Cannabidiol concentrations were nearly 4x greater in plants propagated from cuttings versus seeds. Potter (2009) examined the effects of the irradiance level, day length, and duration of flowering period on cannabinoid levels. Results showed that irradiance levels, day length, and flowering period all had a positive correlation with cannabinoid potency and yield. No previous literature has examined the effects on CBD consistency of cloning generation after generation through stem propagation of *Cannabis sativa*. According to Bilodeau *et al.* (2019), commercial growers in the cannabis industry are still referring to unreliable information, given the lack of peer-reviewed reports on cannabis production. This research is necessary as evidence has suggested that clonal generations are not always genetically identical and can demonstrate somaclonal variation caused by gene mutations due to multiple possible stress factors such as wounding, lighting conditions, and imbalances of media components (Jiang *et al.* 2011, Krishna *et al.* 2016). This can be a serious problem for farmers who seek to preserve elite genotypes (Krishna *et al.* 2016).

This study tests the assumption that clonal generations of plants have a consistent CBD level when compared with parent stock plants.

THESIS STATEMENT

The objective of this research was to assess cannabinoid levels in successively cloned generations of *Cannabis sativa*. It was expected that cloned generations of hemp would have no variations. Therefore, each generation should have the same level of cannabinoids.

METHODOLOGY

Stock plants already in the Department of Biology served as the source for clonal generations. These varieties were*:* Cherry, Cherry Blossom, and Cherry x Workhorse. All plants were female. At least ten cuttings of 6-10 cm from each variety of stock plant were placed into a cloner (Botanicare, Model RESLPWHB-40). Cuttings were collected using new, sharp razor blades to prevent vascular tissue collapse. The exposed tips of cuttings were dipped in rooting gel (CLONEX, Growth Technology) and sat for 15 minutes for gel to absorb. Clones were exposed to sixteen hours of light and eight hours of darkness daily. Artificial light was used to supplement natural light to ensure plants stayed in a vegetative state and did not flower. Once root systems of clonal generation 1 had developed enough, plants were potted in small pots using MiracleGro potting soil. Plants were watered three times weekly and fertilized biweekly. Once clonal generation 1 plants had grown to 15 cm tall, the plants were transferred into larger pots to continue maturing. This process was repeated for each subsequent clonal generation. Cuttings were taken from clonal generation 1 plants of each variety to begin clonal generation 2 once generation 1 had grown to 20-25 cm tall. The maturation of a plant from a cutting (6-10cm) to a potted plant (20-25cm) took about 45 days. Once clonal generation 2 cuttings were potted and growing, clonal generation 1 plants were placed in a 1.2- meter x 2.4-meter x 1.8-meter tall growth chamber to initiate flowering. In the growth chamber, plants were exposed to 8 hours of light using LED growth lights and 16 hours of darkness daily. Once plants produced flowers, the buds were removed from plants and air dried. Once dried, the buds' cannabinoid levels were analyzed using HPLC. It was a goal that at least ten samples of each clonal generation of each variety were analyzed. Due to the

unpredictability of plants, some variations in some generations had less samples. At least five bud samples were taken from each of the ten plants of each clonal generation of each variety. To prepare samples for HPLC analysis, 100 mg of dried buds were placed into 50 mL centrifuge tubes along with 25 mL of 95% Ethanol. Tubes were vortexed for one minute at speed level 10. The tubes were centrifuged for 2 minutes, at 4°C, at 2000 RPM. A syringe was used to extract 1 mL of liquid from the centrifuge tube. A Millex HV 0.45 μm Filter Unit was attached to the syringe to filter the liquid into a 1.5 mL vial. Vials were capped and stored in a refrigerated room to await analysis.

HPLC was completed using a Dionex UltiMate 3000 liquid chromatography system. Separation of solvents was completed under standard conditions on a Phenomenex Kinetex EVO 5 μm C18 100 Å (150 x 4.6 mm) column where the flow rate was 1.0 mL/minute. The column temperature was 50° C. The column's mobile phase was methanol with 0.1% formic acid (B) and water with 0.1% formic acid (A). The linear gradient was from 60% B / 40% A to 95% B / 5% A in 45 minutes. Every hour, the HPLC system automatically took one sample and recorded its cannabinoid concentration. Cannabinoids retention times were compared to retention times of known cannabinoid samples for identification. The following cannabinoid levels were analyzed for each generation of each variation: cannabidiol (CBD), cannabidiolic acid (CBDA), cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), and cannabigerol (CBG), 9-tetrahydrocannabinol (9THC), cannabicyclol (CBL). These cannabinoid levels were used to compare generations as other cannabinoids could not be accurately determined using retention times. Retention times of other cannabinoids were too similar to differentiate. Statistical analyses were performed using Microsoft Excel. Analyses One-Way Analysis

of Variance (ANOVA) was used to detect significant differences. When a significant difference was detected with the One-Way Analysis of Variance, the Tukey test was used to determine differences between generations.

RESULTS

The cannabinoid levels were calculated using the area under the standard curve and the samples' retention times. The averaged cannabinoid levels for each generation are compared for varieties Cherry x Workhorse, Cherry, and Cherry Blossom in Figures 2-4. Stars denote a significant difference in cannabinoid levels.

For Cherry x Workhorse, a significant difference was found between generation 1 and generation 2 and between generation 1 and generation 3 for the following cannabinoids: CBDA, CBDVA, CBL, and CBDV. Over the course from generation 1 to generation 3, production of CBDA, CBDVA, CBL, and CBDV all significantly decreased.

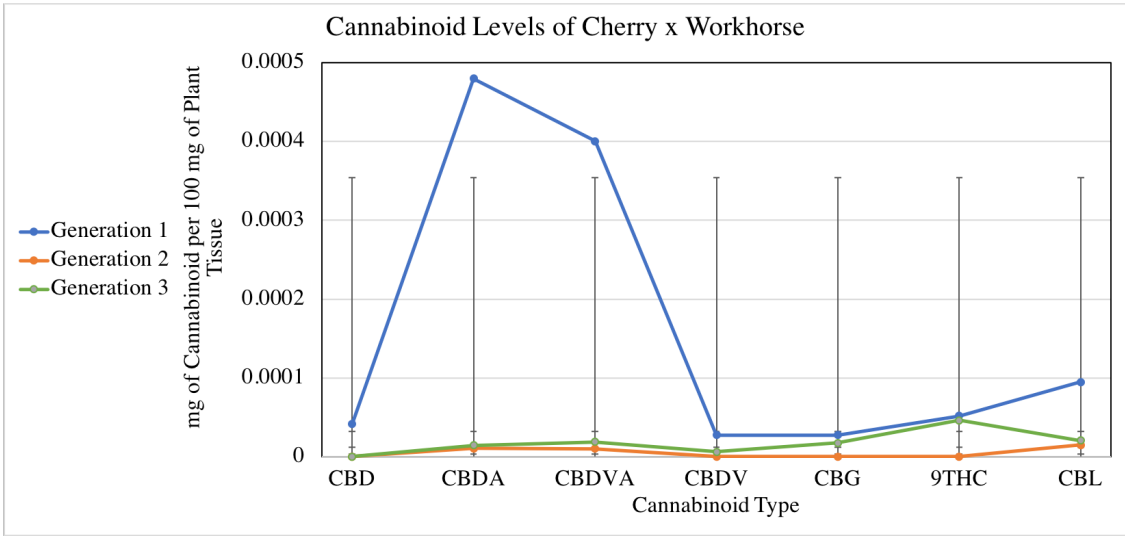

Figure 2. Graph of averaged cannabinoid levels for plant variation Cherry x Workhorse. Stars denote a significant difference in cannabinoid levels. ($\alpha = 0.05$)

For Cherry, only two generations were compared using an ANOVA. A significant difference was found between generations 1 and 2 for the following cannabinoids: CBD, CBDA, CBDVA, CBDV, and CBL. Production of CBD, CBDA, CBDVA, CBDV, and CBL all significantly decreased.

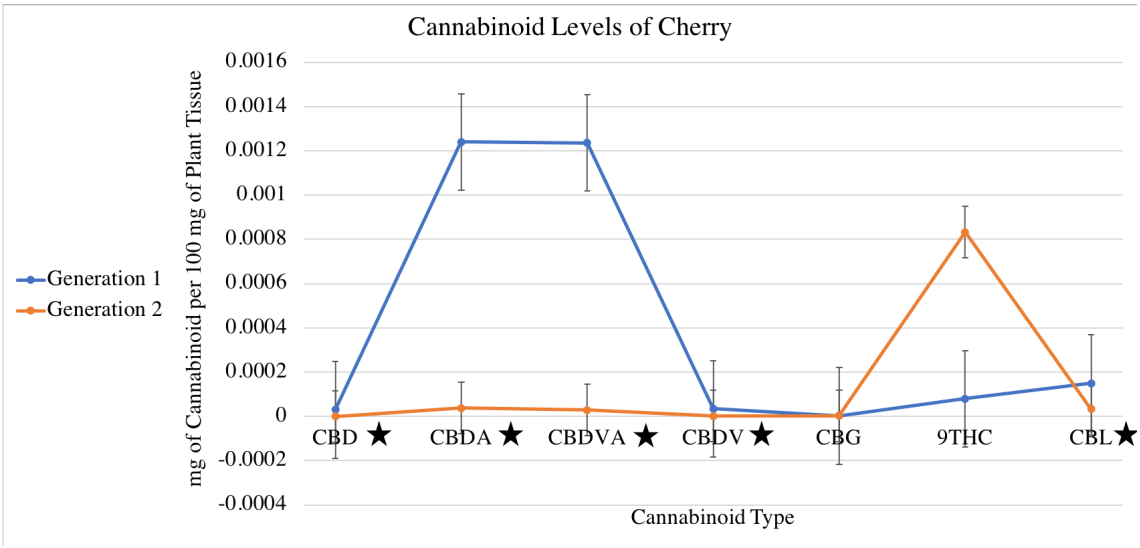

Figure 3. Graph of averaged cannabinoid levels for plant variation Cherry. Stars denote a significant difference in cannabinoid levels. ($\alpha = 0.05$)

For Cherry Blossom, a significant difference was found between generation 1 and generation 2 for the cannabinoid CBDVA. A significant difference was found between generation 1 and generation 3 for the following cannabinoids: CBD and 9THC. A significant difference was found between generation 2 and generation 3 for the following cannabinoids: CBD, CBDVA and 9THC. The levels of CBD and 9THC significantly decreased then increased from generation 1 to generation 3. However, the level of

CBDVA significantly increased then decreased over the course from generation 1 to generation 3.

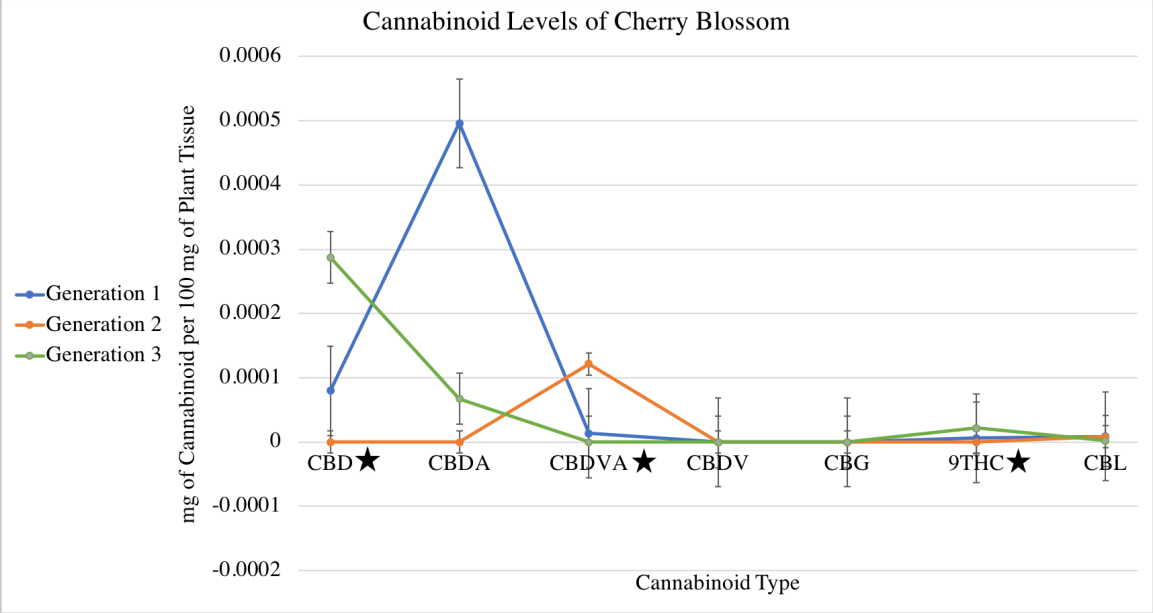

Figure 4. Graph of averaged cannabinoid levels for plant variation Cherry Blossom. Stars denote a significant difference in cannabinoid levels. ($\alpha = 0.05$)

DISCUSSION

This study will be beneficial to hemp growers that use tissue propagation by showing that cannabinoid levels can vary significantly depending on the clonal generation. In addition, this is the beginning of a study that may lead to the discovery that cloning multiple generations of hemp leads to significantly decreased or increased production of certain cannabinoids. Studies such as those done by Krishna et al. (2016) have already proven that genetic mutations are possible and frequent when cloning plants, and the exact basis for somaclonal variation is still far from being completely understood. This study shows that there may be mutations affecting the production of cannabinoid levels in hemp. This is important for hemp growers that must adhere to certain consistency standards for licensure accordance. According to the results of this study, there is a potential for somaclonal variation in the cloning of hemp that effect cannabinoid levels, including THC. Many states require hemp farmers to maintain a THC level lower than a certain percentage. According to the results of this study, THC levels were higher in cloned generations of Cherry Blossom and Cherry. According to Krishna et al. (2016), it is still not possible to predict the outcome of a somaclonal program (Karp 1992) as it is random and lacks reproducibility. This is a great risk to licenses of hemp farmers. THC levels could rise unexpectedly as plants are cloned over and over. There are no other studies published to compare cannabinoid levels in successfully cloned generations of hemp. Overall, the results of this study are useful to industrial hemp research centers such as the Tennessee Center for Botanical Medicine Research, cannabinoid producers, and the pharmaceutical field to test for the effects of cloning on cannabinoid production.

In order to further this study, varieties should be cloned for more consecutive generations to have a more accurate understanding of the effects of cloning on cannabinoid levels.

WORKS CITED

Bilodeau, S., Wu, B., Rufyikiri, A., MacPherson, S., Lefsrud, M. 2019. An update on plant photobiology and implications for cannabis production. Frontiers in Plant Science, 10: 296.

Boonsnongcheep, P., Pongkitwitoon, B. 2020. Factors affecting micropropagation of *Cannabis sativa* L.: A Review. Pharm Sci Asia, 47 (1): 21-29.

Caplan, D., Stemeroff, J., Dixon, M., Zheng, Y. 2018. Vegetative propagation of cannabis by stem cuttings: effects of leaf number, cutting position, rooting hormone, and leaf tip removal. Canadian Journal of Plant Science, 98: 1126-1132.

Carus, M., Karst, S., Kauffmann, A., Hobson, J., Bertucelli, S. 2013. The European Hemp Industry: Cultivation, processing and applications for fibres, shivs and seeds. European Industrial Hemp Association, 17: 1-9.

Coffman, C., Genter, W. 1979. Greenhouse propagation of *Cannabis sativa* L. by vegetative cuttings. Economic Botany, 33: 124-127.

Collin, C., Davies, P., Mutiboko, I., Ratcliffe, S. 2007. Randomized controlled trial of cannabis-based medicine in spasticity caused by multiple sclerosis. European Journal Neurology, 14: 290–296.

De Backer, B., Debrus, B., Lebrun, P., Theunis, L., Dubois, N., Decock, L., Verstraete, A., Hubert, P., Charlier, C. 2009. Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. Journal of Chromotography B, 877: 4115-4124.

Freeman, T., Hindocha, C., Green, S., Bloomfield, M. 2019. Medicinal use of cannabis-based products and cannabinoids. British Medical Journal, 365: 1141.

Gaoni, Y., Mechoulam, R. 1964. Isolation, structure and partial synthesis of an active constituent of hashish. J Am Chem Soc, 86: 1646–1647.

Jiang, C., Mithani, A., Belfield, E., Klingler, J., Zhu, J., Ragoussis, J., Mott, R., Harberd, N. 2011. Regenerant Arabidopsis lineages display a distinct genome-wide spectrum of mutations conferring variant phenotypes. Current Biology, 21: 1385-1390.

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., Sadh, R. 2016. Somaclonal variations and their applications in horticultural crops improvement. Biotech, 6(1): 54.

Leweke, F., Piomelli, D., Pahlisch, F., Muhl, D., Gerth, C., Hoyer, C., Klosterkötter, J., Hellmich, M., Koethe, D. 2012. Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. Translational Psychiatry, 2: 94.

Li, H. 1973. An archaeological and historical account of cannabis in China. Economic Botany, 28: 437–448.

Mechoulam, R., Hanus, L. 2000. A historical overview of chemical research on cannabinoids. Chemistry and Physics of Lipids, 108: 1-13.

Mechoulam, R., Shvo, Y., Hashish, I. 1963. The structure of cannabidiol. Tetrahedron, 19: 2073–2078.

Potter, D. 2009. The propagation, characterization, and optimization of *Cannabis sativa* L as a phytopharmaceutical. PhD Thesis, Department of Pharmaceutical Science Research King's College London.

Potter, D. 2013. A review of the cultivation and processing of cannabis (Cannabis sativa L.) for production of prescription medicines in the UK. Drug Testing and Analysis, 6: 31-38.