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

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Background

The business of industrial hemp (*Cannabis sativa*) has grown tremendously over the past decades both in agriculture and pharmaceuticals. Health benefits of cannabinoid products have been found for a variety of illnesses including multiple sclerosis (Collin *et al*. 2007) and acute paranoid schizophrenia and schizophreniform psychosis (Leweke *et al*. 2012). 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).

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). The process involves taking plant tissue to grow a theoretically genetically identical offspring. Recent studies have shown that plant propagation can lead to certain genetic changes known as somaclonal variations caused by gene mutations resulting in nongenetically identical offspring (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 research was designed to test the effects of cloning hemp varieties (Cherry, Cherry Blossom, and Cherry x Workhorse) through plant propagation on cannabinoid production.

Methodology

Stem Cuttings

At least 10 cuttings of about 6-10 cm were made from each of the following varieties: Cherry, Cherry Blossom, and Cherry x Workhorse. Cuttings were exposed to sixteen hours of artificial light and eight hours of darkness daily in a cloner to ensure plants stayed in a vegetative state and did not flower. Once cuttings developed root systems, they were potted. These plants began clonal generation 1. Cuttings were taken from clonal generation 1 plants of each variety to begin clonal generation 2 once generation 1 had grown to about 20-25 cm tall. Once clonal generation 2 cuttings were potted and growing, clonal generation 1 plants were placed in a growth chamber to initiate flowering. In the growth chamber, plants were exposed to 8 hours of light 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 high performance liquid chromatography (HPLC). It was a goal that at least ten samples of each clonal generation of each variety were analyzed.

HPLC Analysis

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. Next, 1 mL of the centrifuged solution was extracted and filtered into a small vial for HPLC analysis. HPLC analysis 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.

Cannabinoid concentrations of each variety were compared between generations. 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 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). The cannabinoid levels were calculated using the area under the standard curve and the samples' retention times. The averaged cannabinoid levels for each generation were compared for varieties Cherry x Workhorse, Cherry, and Cherry Blossom (Figures 1-3). 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 (Figure 1). Over the course from generation 1 to generation 3, production of CBDA, CBDVA, CBL, and CBDV all significantly decreased.

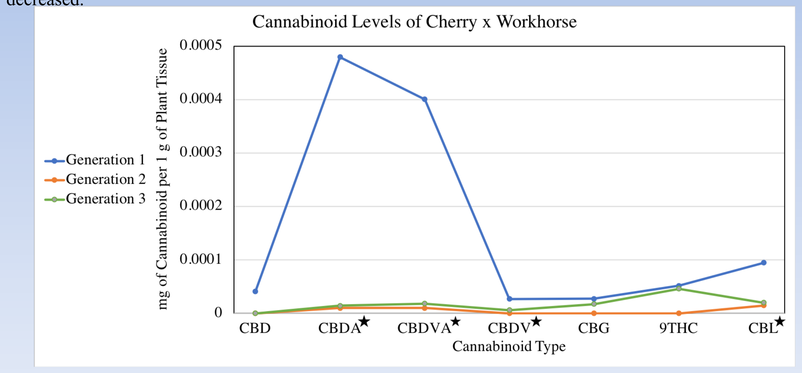

Figure 1. Averaged cannabinoid levels for plant variation Cherry x Workhorse. Some cannabinoids were not detected during HPLC analysis. Stars denote a significant difference in cannabinoid levels. (p = 0.05)

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 (Figure 2). Production of CBD, CBDA, CBDVA, CBDV, and CBL all significantly decreased.

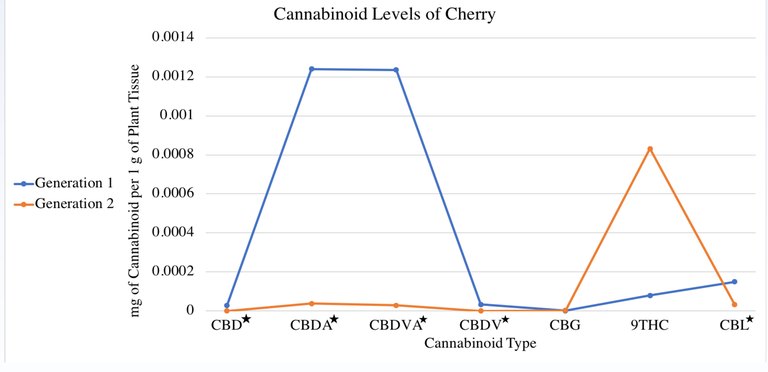

Figure 2. Averaged cannabinoid levels for plant variation Cherry. Some cannabinoids were not detected during HPLC analysis. Stars denote a significant difference in cannabinoid levels. (p = 0.05)

For Cherry Blossom, a significant difference was found between generation 1 and generation 2 for the cannabinoid CBDVA (Figure 3). 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.

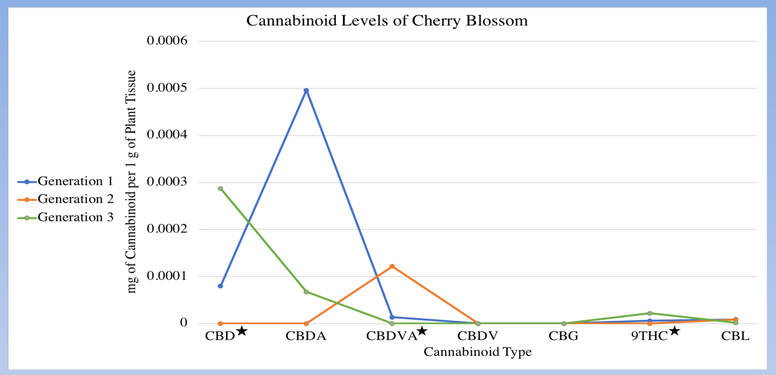

Figure 3. Graph of averaged cannabinoid levels for plant variation Cherry Blossom. Some cannabinoids were not detected during HPLC analysis. Stars denote a significant difference in cannabinoid levels. (pvalue = 0.05)

Conclusion

This study shows that there is a potential for somaclonal variation in the cloning of hemp that effect cannabinoid levels. 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.

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