The Effect of Elicitor Stimulation on Cannabinoid Production by Industrial Hemp (*Cannabis sativa*) Varieties in a Hydroponic System

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Dedication

I dedicate this thesis to my parents, Ray and Kay Bailey. Without their love and support I would not have been able to graduate college or complete this thesis. Their help along the way has enabled me to be successful, and for that, I am forever grateful.

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Abstract

Industrial hemp (*Cannabis sativa*) has caught the attention of both the agricultural and pharmaceutical realms around the world. The interest in the plant stems from the unique cannabinoid properties it possesses. This thesis was designed to test if different hemp varieties (Canda, Cherry, and Cherry Blossom), grown in hydroponic systems, would produce more or less levels of cannabinoids when treated with one of two elicitors (salicylic acid or methyl jasmonate). For each variety, the elicitor was either sprayed on the leaves or administered into the hydroponic system, allowing for root uptake. For each variety, the control group was also grown, elicitor-free. The results of this study showed that the methyl jasmonate elicitor, the leaf treatment, and the variety Cherry produced the greatest level of cannabinoid production. Methyl jasmonate was able to increase cannabinoid production of cannnabidioloic acid (CBDA) and cannabichromene (CBC) in all three varieties, while not increasing tetrahydrocannabinol (THC). The results of this study suggest that methyl jasmonate could be of use to hemp research centers, such as the Tennessee Center for Botanical Medicine Research, and other hemp agricultural departments.

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1. **Methyl Jasmonate**: (n) an organic compound used in plant defense mechanisms and many developmental pathways.
2. **Salicylic Acid**: (n) a bitter plant chemical compound that can be used as a fungicide and is a component of aspirin and dyestuffs.
3. **Aeroponics**: (n) a plant-growth technique, where the roots hang suspended in the air while nutrient solution is taken up in mist form.
4. **Cannabinoids:** (n) any of a group of similarly related compounds that include the active constituents of cannabis.
5. **Elicitor:** (n) something that evokes or draws out a response**.**
6. **Germination:** (n) the growth of a plant from a seed or spore after a period of dormancy.
7. **Hydroponics:** (n) the cultivation of plants in sand, gravel, or liquid, with added nutrients but without soil.
8. **HPLC**: (n) High-performance liquid chromatography is a form of liquid chromatography that is designed to separate chemical compounds dissolved in solution.

Introduction

Industrial hemp (*Cannabis sativa*) is an important component in the agricultural and pharmaceutical industries due to its production of cannabinoids, phytochemicals, terpenes, phenolic compounds, cellulose fibers, and other biosynthetic compounds (Christelle *et al.* 2016). The biological makeup of the hemp plant is composed of woody fibers along the outside of the stem, while the core is lignified with a cortex coating of blast fibers surrounding it (Guerriro *et al*. 2013). Research in the industrial hemp field has shown growing interested in the use of cannabinoids, which are found in highest abundance in the buds and are commonly used in oil form due to their calming and healing properties. Precursors of the hemp cannabinoids come from the polyketide and plastidal-2-C-methyl-D-erythritol-4-phosphate biosynthetic pathways (Sirikantaramas *et al.* 2007). The polyketide pathway produces olivetolic acid (OLA), while the plastidal pathway produces geranyl diphosphate (GPP). The alkylation of OLA and GPP produces CBGA, which is the most important precursor to cannabinoids present in industrial hemp (Fellermeier and Zenk, 1998).

Although in nature there are ninety total cannabinoids present across multiple plant species, the goal of this project was to test thirteen target cannabinoid compounds that the High-performance liquid chromatography (HPLC) can detect (De Backer *et al.* 2009). Cannabinoids come in three major types: D-tetrahydrocannabinol (D -THC), cannabidiol (CBD) and cannabinol (CBN) (Stefanidou *et al.* 1998).

In order to synthesize these compounds, industrial hemp needs to be cultivated first through cuttings and/or seed germination (Arnsika *et al.* 2013). Hemp can grow from cuttings of mature plants. Cutting about 10 centimeters from the top of a mature

hemp plant and placing it in an aeroponic system allows the plant to grow sustainable roots (Knight *et al.* 2009*)*. Once the young plants obtain roots, hydroponic systems can be used to grow nutrient rich industrial hemp in a controlled environment. This process is accomplished by allowing the plants to have maximum root uptake potential by growing them in a nutrient rich solution (Knight *et al.* 2009).

Elicitors have been used to stimulate additional chemical production. When added, elicitors stimulate a chemical defense response in the hemp and allow a greater amount of cannabinoid production to form within the plant. One elicitor, methyl jasmonate, is known for stimulating this chemical defense response in plants (Zhang *et al.* 2015). Methyl jasmonate is a methyl ester form of its precursor jasmonic acid (Staswick 1992). Studies show that methyl jasmonate can stimulate the phytoalexin plant defense pathway, leading to the production of stress-signaling molecules by the plant (Staswick 1992). Another elicitor, salicylic acid, is also known for stimulating an induced chemical response in plants (Zwart *et al.* 2017). Salicylic acid is also chemically known as 2-hydroxy-benzoic acid or orthohydrobenzoic acid (Arif 2015). Salicylic acid comes in natural forms, such as willow bark, wintergreen leaves, and sweet birth, but the salicylic acid used for this project is artificially produced.

The effects of elicitors on the industrial hemp can be accessed by HPLC chemical analysis of cannabinoid production (De Backer *et al.* 2009). The HPLC is a form of column chromatography that takes an analyte, or solute, dissolved in a solvent and pumps it at high pressure through a column (Petrova and Sauer 2017). The chemical makeup of the solution determines its retention time during the stationary phase of the process. The compounds with the strongest attraction to the immobilized material in the column elute

the slowest, while the least attracted compounds elute quicker. Separation of the compounds in the sample can thus be performed through this isocratic elution of retention times (Petrova Sauer 2017). The HPLC detects the different types of CBD, which are the less intoxicating form of phytocannabinoids, and THC, which is known for its potent psychotic effects (Citti *et al.* 2019). The HPLC allows for a quantitative analysis and comparison of cannabinoid production for the three varieties of hemp based on their method of treatment (root uptake, leaf spraying, control) and their applied elicitor (salicylic acid or methyl jasmonate).

Rationale

This project with Dr. DuBois is part of a continuing study at Middle Tennessee State University involving the industrial hemp cannabinoid production, chemical defense response, and elicitor stimulation mechanisms. This study analyzed 13 target cannabinoid compounds from three different varieties (Cherry, Cherry Blossom, and Canda) of industrial hemp (*Cannabis sativa*) through hydroponic growth and elicitor stimulation. Plants of the three varieties were grown for 7-8 weeks in a nutrient-enhanced hydroponic system, and were stimulated using two different elicitors, and chemically analyzed using HPLC analysis to determine the cannabinoid production. The 13 cannabinoids that were assessed were: cannabidivarin, cannabidarinic acid, tetrahydrocannabinvarin, cannabidiol, cannabigerol, cannabidiolic acid, cannabinol, cannabigerolic acid, delta-9 tetrahydrocannabinol, delta-8 tetrahydrocannabinol, cannabichromene, delta-9 tetrahydrocannabinolic acid, and cannadichromenic acid.

Analysis compared elicitor uptake by the roots verses the leaves in stimulating chemical production.

Thesis Statement

Industrial hemp is a plant with a host of different capabilities and important chemical compounds. One objective of this study was to determine if the elicitors methyl jasmonate and salicylic acid will produce greater amounts of cannabinoids in the industrial hemp. A second objective was to determine if elicitor uptake by the roots or spraying on the leaves increased cannabinoid production.

Methods and Materials

Hydroponics: Three different varieties of industrial hemp (Cherry, Cherry Blossom, and Canda) were grown in hydroponic systems. Plant preparation for hydroponics was accomplished through seed germination or cuttings. Seeds were placed on a wet paper towel in a 10 cm diameter petri dish and allowed to germinate. The seedlings were put into a 4 cm black foam plug, set into a 27.5 cm by 14 cm Styrofoam float, and floated in a 28.5 cm by 15 cm, 3-liter tub filled with nutrient solution (Figure 1). The tubs were covered with aluminum foil in order to help decrease algae production. For cuttings, several stems were cut off a mature plant and put into an aeroponic system. The aeroponic system allows plant roots to grow via non-mediated, oxygenated mist, and the cuttings remained in this system until the roots were at least 10 centimeters in length before transfer to the hydroponic system. The hydroponic system was aerated using tubing and an air stone attached to an air pump and held 8 plants per system. To make the nutrient solution, four mL of Canna-Vega A and four mL of Canna-Vega B were mixed with two liters of water, and the pH adjusted to 5.9-6.0 by using the base KOH.

Figure 1. Three hydroponic systems used during this study.

Elicitor Stimulation: The two elicitors tested on each industrial hemp variety were methyl jasmonate and salicylic acid. Each of the three varieties (eight individual plants) were tested with one of the elicitors in three different ways; root uptake, leaf spraying, or no elicitor (control). For plant root uptake, the elicitors were chemically integrated into the nutrient solution. For salicylic acid, 27.6 mg/L of solution was used (Rahimi *et al*. 2014). For methyl jasmonate, 44.9 mg/L of solution was used (Ali *et al*. 2007). For root uptake, at week 7 of growth, each variety was transferred from Canna-Vega solution to elicited solution. Each variety sat on the elicited solution for a 24-hour period, then taken off the media and harvested. For leaf spraying, at week 7 of growth, each of eight plants was sprayed with an elicitor, while continuing to grow on normal nutrient solution. The plants were sprayed for 5 full seconds with a misting bottle, harvested after a 24 period

had been completed, and left to dry on a rack for 7 days. For the control group for each variety, no elicitor was used. At growth week 7 the plants were removed from their hydroponic system and left to dry on a rack for 7 days.

Chemical Analysis: One hundred grams of the dry flowering bud material was collected and put into a 50 mL centrifuge tube. This centrifuge tube was filled with 45 mL of 95% ethanol, mixed on a vortex spinner for 60 seconds, and centrifuged at 3,000 rpm at 21 degrees Celsius for 2 minutes. The ethanol and suspended molecules were pumped through a 0.2 micron syringe filter, and placed into a HPLC vial. The final volume in the vial was 0.5 mL. The amounts of cannabinoids were determined by high performance liquid chromatography (HPLC) on a Dionex UltiMate 3000 liquid chromatography system consisting of a quaternary solvent pump/mixer, temperature-controlled autosampler, temperature-controlled column compartment, and multiwavelength UV-Vis detector. Separation was carried out, through standard conditions, on a Phenomenex Kinetex EVO 5 μm C18 100 Å (150 x 4.6 mm) column with a flow rate of 1.0 mL/min. The mobile phase consisted of methanol with 0.1% formic acid (B) and water with 0.1% formic acid (A) with a linear of gradient from 60% B/40% A to 95% B/5% A in 45 min. The column temperature was 50 ºC, while the autosampler temperature was 32 ºC and the UV-Vis detector at 214 nm. The HPLC system extracted one sample each hour and graphed its cannabinoid chemical content. Chemical compound identification was accomplished by comparing retention times to standard matrix samples. A quantitative analysis of the sample peak areas was performed to determine which cannabinoids were produced and the amounts of each one. Cannabinoid production was assessed between

plants stimulated by salicylic acid, methyl jasmonate, and the control group and was compared with chemical production based on elicitor uptake by roots versus leaves.

Statistical Analyses: Statistical analyses were performed using Sigma Stat (v.3.1) software. Analyses included t-tests (when only comparing two groups) and One-Way Analysis of Variance (when comparing root uptake, leaf uptake and control). When a significant difference was detected with the One-Way Analysis of Variance, the Tukey test was used to determine differences between groups.

Results

The results of testing the hemp varieties Cherry, Cherry Blossom, and Canda when elicited with salicylic acid and their controls are shown in Tables 1-3. Each variety produced different types of cannabinoids in differing levels when analyzed with the HPLC. The cannabinoid levels were measured qualitatively, using the area under the standard curve and the retention time.

The results show that salicylic acid decreased cannabinoid production of the Cherry variety (Table 1). In the two treatment methods, Cherry cannabinoid (THC, CBDA, and CBC) production decreased compared to the untreated control group. This shows that salicylic acid was not a useful elicitor for this study when tested on the Cherry variety.

Table 1. Data showing the average +/- SD production of various cannabinoids taken from the Cherry hemp after absorption by salicylic acid (SA). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties of treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoid (mAU*min)
Root Uptake SA	ND	0.144+/-0.001(7)	0.243+/-0.0006(7)	0.129
Leaf Uptake SA	0.781[1]+/-1.116(7)	0.391+/-0.098(7)	0.257+/-0.020(7)	0.476
Control	3.724+/-1.899(7)	0.437+/-0.143(7)	0.168+/-0(7)	1.443

[1]Significant difference from control (p=0.018)

For Cherry Blossom, salicylic acid decreased the production of THC (Table 2). However, there appears to be an increase in the production of CBDA in both treatments compared to the control group, but the increase was not significantly different from the control. CBC increased in the leaf uptake method, but did not change compared to the control in the root uptake method. The total cannabinoid production for the two treatments elicited with salicylic acid is still lower compared to the total cannabinoid production for the control group. These results show that Cherry Blossom, when elicited with salicylic acid, does not produce a significance increase of cannabinoid production.

Table 2. Data showing the average+/- SD production of various cannabinoids taken from the Cherry Blossom hemp after absorption by salicylic acid (SA). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties or treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoids (mAU*min)
Root Uptake SA	0.669[1]+/-1.273(8)	0.751+/-1.673(8)	ND	0.710
Leaf Uptake SA	1.180+/-2.875(8)	0.883+/-0.041(8)	0.883+/-0.007(8)	0.695
Control	5.094+/-1.883(6)	0.467+/-0.0407(6)	ND	2.781

[1]Significant difference from the control (p=0.037)

In Canda, salicylic acid does not significantly affect production of THC, CBDA or CBC compared to the control group (Table 3). These results show that Canda, when elicited with salicylic acid, does not significantly increase production of CBDA.

Table 3. Data showing the average +/- SD production of various cannabinoids taken from the Canda hemp after absorption by salicylic acid (SA). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties or treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoids (mAU*min)
Root Uptake SA	ND	3.718+/-2.132(8)	ND	3.718
Leaf Uptake SA	ND	4.269+/-2.726(8)	ND	4.269
Control	ND	2.946+/-2.860(7)	ND	2.9466

The results of testing the hemp varieties Cherry, Cherry Blossom, and Canda when elicited with methyl jasmonate and their controls are shown in Tables 4-6. Each variety produced different types of cannabinoids in differing levels when analyzed with the HPLC. The cannabinoid levels were measured qualitatively, using the area under the standard curve and the retention time.

The results show that methyl jasmonate decreased the production of THC for the Cherry variety (Table 4). However, methyl jasmonate increased the production of CBDA and CBC for both treatments compared to the control group. The increase in CBDA by leaf uptake was statistically significant. The total cannabinoid production for both treatments greatly increased compared to the control group. These results show that Cherry, when elicited with methyl jasmonate, produces an increase in cannabinoid production.

Table 4. Table of raw data showing the average +/- SD production of various cannabinoids taken from the Cherry hemp after absorption by methyl jasmonate (MJ). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties of treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoids (mAU*min)
Root Uptake MJ	1.828+/-0.034(8)	66.738+/-70.532(8)	4.511+/-2.876(8)	24.359
Leaf Uptake MJ	1.498[1]+/-2.876(8)	98.462[2]+/-160.02(8)	3.539+/-0.0001(8)	34.499
Control	3.724+/-1.899(7)	0.437+/-0.143(7)	0.168+/-0(7)	1.443

[1]Significant difference from the control (p=0.034)
[2]Significant difference from the control (p=0.015)

The methyl jasmonate treatment decreased the production of THC for the Cherry Blossom variety (Table 5). Methyl jasmonate significantly increased the production of CBDA for both treatment methods compared to the control group. The total cannabinoid production for Cherry Blossom, when elicited with methyl jasmonate, greatly increased in both treatments compared to the control group. These results show that in this system Cherry Blossom, when elicited with methyl jasmonate, will have an increased cannabinoid production.

Table 5. Data showing the average +/- SD production of various cannabinoids taken from the Cherry Blossom hemp after absorption by methyl jasmonate (MJ). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties of treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoids (mAU*min)
Root Uptake MJ	3.606+/-2.1473(8)	37.943[1]+/-180.00(8)	1.722+/-0.475(8)	14.424
Leaf Uptake MJ	2.618+/-0(8)	46.211[1]+/-147.07(8)	1.897+/-0.671(8)	16.909
Control	5.094+/-1.883(6)	0.467+/-0.047(6)	ND	2.781

[1]Significant difference from the control ($p<0.001$)

Methyl jasmonate had no effect on THC production for the Canda variety (Table 6). However, methyl jasmonate significantly increased the production of CBDA in both treatments compared to the control group. These results show that Canda, when elicited with methyl jasmonate, in this system increases cannabinoid production.

Table 6. Data showing the average +/- SD production of various cannabinoids taken from Canda hemp after absorption by methyl jasmonate (MJ). Parenthesis (n) includes the number of samples. ND stands for None Detected. Some types of cannabinoids were not detected in the different varieties of treatments.

	THC (mAU*min)	CBDA (mAU*min)	CBC (mAU*min)	Total Cannabinoids (mAU*min)
Root Uptake MJ	ND	29.441[1]+/-17.016(7)	5.138+/-0.004(7)	17.289
Leaf Uptake MJ	ND	26.663[2]+/-60.453(7)	5.415+/-0.173(7)	16.039
Control	ND	2.946+/-2.861(7)	ND	2.9466

[1]Significant difference from the control (p=0.026)
[2]Significant difference from the control (p=0.034)

Discussion

This study can help the broader industrial hemp field by showing several factors. Hemp plants, in this study, can successfully be grown in a stable, hydroponic environment. In addition, this study revealed that methyl jasmonate aids in producing significantly greater CBDA in all three industrial hemp varieties. Lastly, the best variety for cannabinoid production under these conditions was Cherry, and the most successful treatment method for cannabinoid production was leaf spraying. This study also shows that methyl jasmonate, for each variety and treatment method, did not increase the production of THC, but only CBDA and CBC. It is possible that these hemp varieties may not have the proper biological mechanisms to use methyl jasmonate to increase THC production. There are no other studies published to compare methyl jasmonate use on cannabinoid production on 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 greater cannabinoid production.

In order to further this study, other varieties of high CBD hemp should be tested using methyl jasmonate. More than 8 plants should be tested for each variety and treatment method to improve statistical analysis. If leaf application is the best application method, the plants could also be tested grown in soil rather than in a hydroponic system using the same elicitors.

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