The Impact of Insect Herbivory in the Level of Cannabinoids in CBD Hemp Varieties

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Abstract

Background

In the United States, industrial hemp is defined as a *Cannabis sativa* L. plant not containing more than 0.3% delta-9-tetrahydrocannabinol (D9THC) by dry weight. Plants respond to insect herbivore damage by changing their chemistry to counter the effects of herbivore attack. Here, we hypothesized that the corn earworm (*Helicoverpa zea*) infestation might impact the level of cannabinoids (Cannabidiol (CBD) and D9THC).

Results

In a laboratory trial, the CBD hemp, Cherry Blossom, and The Wife varieties were subjected to herbivore damage (HD), Mechanical damage (MD), and Control. After 24hrs of the treatments, we found a significant increase in CBD and D9THC in HD plants compared with MD and Control plants. Similar experiments were conducted in the field condition. A substantial increase in CBD and D9THC observed in herbivore damaged hemp plants compared to the control plants. However, in the field trial, the levels of cannabinoids were not significantly higher in The wife variety. Interestingly, the Corn earworm larvae fed with CBD and D9THC spiked diet showed a significant reduction in body mass, as compared to the larvae fed with the control diets.

Conclusions

The level of cannabinoids seems not genetically fixed somewhat; it is affected by insect herbivory. Our results suggest that CBD hemp plants are exposed to insect herbivory spikes in cannabinoid production and surpass the 0.3 % legal limit of D9THC. The growth and development of Corn earworm, the number one hemp pest in North America affected by cannabinoids. The increased concentration of CBD and D9THC observed in herbivore damaged hemp plants might be associated with the direct deterrence of the corn earworm larvae. Further research underway using different hemp varieties to assess if herbivory and other biotic stressors impact the level of cannabinoids.

Background

The 2014 Farm Bill passed, formally defines hemp as the crop as the plant *Cannabis sativa* L. and any part of such plant, whether growing or not, with a delta-9 tetrahydrocannabinol (D9THC) concentration of not more than 0.3 percent on a dry weight basis [1]. The recent Agriculture Improvement Act of 2018 removed hemp with less than 0.3% D9THC from controlled substances [2]. The 2018 bill made hemp a regular agricultural commodity. Moreover, the 2018 farm bill amends the 2014 farm bill definition of industrial hemp. The new definition of hemp by congress, quote "the plant *Cannabis sativa* L. and any part of that plant, including the seeds thereof and all derivatives, extracts, cannabinoids, isomers, acids, salts, and salts of isomers, whether growing or not, with a delta-9 tetrahydrocannabinol concentration of

not more than 0.3 percent on a dry weight basis" [3]. Following the Agriculture Improvement Act of 2018, many states launched Industrial hemp pilot programs.

"Hot hemp," a new term coined by hemp farmers to describe the spike of D9THC above 0.3%, must be confiscated by state agencies. To determine how hot the hemp is, growers must report to the state agencies at the initiation of flowering and before harvesting their hemp produce. In 2018 North Carolina Department of Agriculture and Consumer Services tested 400 samples, about 10% of the hemp samples going hot [4]. In Hawaii, more than half of the hemp crops grown for the 2019 pilot program are going hot [5] . In 2019 Wisconsin hemp growers lost about $40,000 on a crop of 70 hemp plants that tested at 0.5% D9THC content [6].

The differentiation between industrial hemp and marijuana is the 0.3% D9THC cap, this charming number based on the 1976 article published in the journal of Taxon by Canadian horticulturalists Ernest Small and Arthur Cronquist, entitled "A Practical and Natural Taxonomy in Cannabis," in which they provide the pivotal differentiation between "industrial hemp" vs "marijuana" that has been adopted by governments around the world. As directly quoted from the article, "It should be noted that we arbitrarily adopt a concentration of 0.3% D9THC (dry weight basis) in young, vigorous leaves of relatively mature plants as a guide to discriminating two classes of plants" [7].

Plants heavily rely on 'chemical weapons' to defend against insect attack. The plant defense against herbivores could be direct and indirect. The direct defense is when plants repel or deter herbivores using plant secondary metabolite compounds. Moreover, plants use their physical structures such as spines, trichomes, and leaf epidermis toughness that increase plant fitness in herbivores' presence are also direct defenses. The indirect defense is when plants release herbivore-induced plant volatiles (HIPVs) that attract natural enemies of the herbivore ([8, 9]. The chemicals involved in direct and indirect plant defence could be induced and/or constitutively produced in plants. The induced defence is triggered by insect injury, whereas the constitutive defence always presents whether there is an insect injury or not in the plant system. The induced and constitutive accumulation of secondary metabolites in plants was found to be strongly dependent on a variety of environmental factors such as light, temperature, soil, water, soil fertility and salinity, and biotic factors such as plant disease and insect herbivory [10]. This study will focus on the impact of insect herbivory on the level of cannabinoids with an emphasis on D9THC.

Recent studies show insect herbivory significantly changes plants' chemistry [9, 11, 12] . For instance, the mint plant, *Mentha aquatic*, synthesizes and emits increased levels of secondary metabolites (terpenoid) upon damage by mint beetle, *Chrysolina herbacea*, and herbivory was found to up-regulate the expression of genes involved in terpenoid biosynthesis. The level of terpenoids on the surface of the inflorescence of female *C. sativa* plants, glandular-trichomes produce and accumulate a terpene-rich resin, and cannabinoids [13]. Terpenes are important components of cannabis resin as they define some of the unique organoleptic properties and may influence the medicinal qualities of different cannabis strains and varieties [13].

Hemp production has been illegal for so long, and with the complicated legality surrounding hemp, there are limited studies on hemp plant-insect interactions. Virtually no pest control products have been registered. Even if products were to be registered, labelling products for a new crop takes years. Consequently, hemp growers have very few options for controlling the pests that are damaging their crops. The damage is associated with yield loss and the hotness of the hemp, farmers might completely lose their produce. Apparently, corn earworm (*Helicoverpa zea*) emerged as one of the key pests of hemp in many states. This study tests whether the heavy infestation of corn earworm affects the level of essential cannabinoids (Cannabidiol (CBD) and delta-9- tetrahydrocannabinol (D9THC).

Results

Herbivory increase the level of cannabinoids

The level of cannabinoids was higher in plants damaged by insect herbivores when compared with control plants. In a laboratory trial, the CBD hemp, Cherry Blossom varieties showed a significant increase in the level of CBD in herbivores damaged (HD) plants when compared with mechanical damaged (MD) and Control plants ($p=0.002$). There was no significant difference between the MD and Control plants ($p=0.152$) (Figure 1 and S1).

Similarly, the level of D9THC was significantly higher in HD Cherry Blossom plants when compared with MD and Control plants ($p=0.000$). There was no significant difference between the MD and Control plants ($p=0.084$) (Figure 2). Interestingly the trend in the levels of cannabinoids in the wife variety was similar to that of Cherry blossom varieties. The levels of CBD and D9THC were significantly higher in HD plants when compared with MD and Control plants at $p= 0.022$ and $p = 0.010$ level of significance, respectively (Figure 1 & 2).

In the Cherry Blossom variety, the percentage of CBD to the dry weight of HD, MD, and Control plants was 17%, 6%, and 8% in a laboratory trial, respectively (Figure 1). In the wife variety, the percentage of CBD to the dry weight of HD, MD, and Control plants were 12%, 7%, and 6%, respectively (Figure 1). The levels of D9THC in HD, MD, and Control Cherry Blossom were 1.2%, 0.33%, and 0.36% of the dry weight, respectively (Figure 2). Similar trends recorded in the wife variety that is 0.93%, 0.26%, and 0.37% of the dry weight of HD, MD, and Control plants, respectively (Figure 1).

In a field trial, the CBD hemp, Cherry Blossom varieties showed a significant increase in the level of CBD in herbivores damage with cage (HDC) plants when compared with control cage (CC) and Control without a cage (CWC) plants ($p=0.044$). There was no significant difference between the CC and CWC plants ($p=0.561$) (Figure 3). In Cherry Blossoms, the level of D9THC was significantly higher in HDC plants when compared with CC and CWC plants ($p=0.029$) (Figure 4). However, the levels of CBD and D9THC were not significantly different among the HDC, CC, and CWC the wife plants (Figure 3&4).

Field trial results revealed that for the Cherry Blossom variety, the percentage of CBD to the dry weight of HDC, CC, and CWC plants was 14%, 6.5%, and 8.5%, respectively (Figure 3). In the wife variety,

the CBD percentage to the dry weight of HDC, CC, and CWC plants were 9%, 5%, and 5%, respectively (Figure 3). The levels of D9THC in HDC, CC, and CWC Cherry Blossom were 0.86%, 0.28%, and 0.42% of the dry weight, respectively (Figure 4). In the wife variety, 0.18%, 0.10%, and 0.10% D9THC of the dry weight recorded in HDC, CC, and CWC plants, respectively (Figure 4).

Cannabinoids affect larval growth

The third instar larvae fed in cannabinoid spiked artificial diet showed a significant bodyweight loss when compared with the larvae fed in control diets. Insects feed-in D9THC and CBD treated diet significantly reduced their weights than the larvae fed on an untreated and methanol treated diet ($p < 0.05$). The average larval weight of the larvae fed in D9THC and CBD spiked diet was 0.28 g and 0.31 g, respectively. The larvae's weight fed with an untreated and methanol treated diet was 0.50 g and 0.41 g, respectively (Figure 5).

Discussion

Hemp plants exposed to insect herbivory showed increase in cannabinoid production. The production of secondary metabolites in plants recognized as a key plant defences mechanism against abiotic and biotic stressors [9]. Resistance against pests relies on chemical cocktails that might directly repel or deter the herbivore, and/or indirectly eavesdrops the natural enemy of the herbivore. Biotic stress caused by insect herbivory significantly influences the biochemical plasticity [14].

The level of CBD and D9THC in herbivore damaged hemp plants increases almost at a similar rate. The biosynthesis of cannabinoids starts with geranyl diphosphate (GPP), and the olivetolate geranyltransferase (GOT) catalyzes the synthesis of olivitolic acid (OA). Cannabigerolic acid (CBGA) synthase regulates the biosynthesis of cannabidiolic acid (CBDA) and tetrahydrocannabinol acid (THCA) from OA [15]. Insect herbivory seems to affect the regulation of CBGA synthase or/and the enzyme (GOT) involved the synthesis of OA from GPP.

Phenotypically and genotypically segregated CBD hemp cultivars accumulated more than 0.3% D9THC by dry weight [16]. Moreover, this study suggests that massive insect pest infestation might increases the level of D9THC above the legal limit. Similar trends observed in the Cherry blossom and The wife varieties of hemp in the percent concentration of cannabinoids by dry weight. Even some control plants showed more than 0.3% D9THC, but when compared with HD plants, the percent concentration of D9THC in the control plants was lower.

The oral secretions of tobacco hornworm induces enzymes that mediate the plant defense signaling pathways [17]. In fact, many compounds found in oral secretions from herbivores like inceptins and cheliferous are perceived by plants and stimulate defense against plants [14]. In this study, there was no significant difference between the MD and control plants in the level of cannabinoids. However, the level

of CBD and D9THC were higher in herbivore-damaged plants, which might indicate that the hemp plants recognize the elicitors in the oral secretions of the corn earworm.

Corn earworm feed with artificial diet spiked with cannabinoids showed reduced growth compared with the corn earworm larvae fed on control diets. The increased concentration of CBD and D9THC observed in herbivore damaged hemp plants might be associated with the deterrence of the corn earworm larvae. Several reports demonstrate that plants damaged by insect herbivory increase the level of secondary metabolites to deter insect herbivores directly or indirectly release some herbivore-induced plant volatiles to attract the natural enemies of the insect herbivore [9, 14, 18].

Conclusions

Corn earworm emerged as one of the key insect pests of hemp in the US [19]. Massive infestation might turn the hemp plants "hot" and pass the legal limit in the US. Our results demonstrate that corn earworm infestation on CBD plants boosts the level of CBD and D9THC above the legal limit. Further research needs to be done using multiple varieties to substantiate the observed increase in cannabinoids levels in herbivore damaged plants. Moreover, studies on the impact of other biotic stressors such as plant pathogens, sucking insects, and abiotic stressors such as drought, nutrient deficiencies, temperature, soil salinity in the level of essential cannabinoids need to be done to substantiate the results of this study further.

Methods

Plant Growth

Growth Chamber: Feminized Hemp varieties, Cherry Blossom, and The Wife seeds were purchased from FortunaHemp. The seeds were sown in a sterilized potting soil (ProMIX BXM) and placed in growth chambers until the plants reached the mature flowering stage. The growth chambers were set at 12 hrs photoperiod for the first six weeks and eight hours of photoperiod for three weeks at 27/25^{o}C (Day/Night) temperature. For approximately ten weeks old female plants were used for insect herbivory tests in the laboratory experiment.

Greenhouse: Cherry Blossom and The Wife seeds were sown in a sterilized potting soil (ProMIX BXM) and grown for three weeks at 27/25oC (Day/Night) temperature in a greenhouse. Three weeks old seedlings transplanted to a raised bed covered with black plastics to suppress weeds development on the Duck Wood Landings farm, Princess Anne, Maryland. When the plant reaches the mature flowering stage (10 weeks old), randomly selected hemp plants were used for insect herbivory tests in the field.

Experimental Insects

Corn earworm second instar larvae were purchased from Benzone research LLC. The larvae were reared in an artificial pinto bean diet in a laboratory at room temperature. Fourth instar larvae were used for the lab and field trials. The larvae pre-starved for at least five hours before use for the experiments.

Lab trial

The hemp plants were housed in 61 x 61 x 61 cm^3 bugdorme cages and acclimated overnight before the experiment starts. The plants from each variety were subjected to the herbivore damage (HD), Mechanical damage (MD), and Control. Twenty-five pre-starved fourth instar larvae were introduced to each hemp flowers and leaves near them in a cage to inflect herbivore damage for 24 hrs. Plants were clamped with paper clamber to inflect mechanical damage. And the Control plants were kept in a cage with no HD and MD. After 24 hours, 10-12 cm flower inflorescences with some leaves were collected from HD, MD, and Control plants for cannabinoids extraction. Each treatment was replicated at least three times using the two varieties of hemp.

Field trial

Randomly 18 plants were selected from each hemp variety planted in the field by putting environmental effect into the considerations. Twelve plants from each variety housed in a 61 x 61 x 61 cm^3 bugdorme cages (Figure S2). In six of these cages, twenty-five, pre-starved fourth instar larvae were introduced to each hemp flowers and leaves near them in a cage to inflect herbivore damage for 24 hrs (HDC). After 24 hrs, the larvae were removed and frozen and discarded in a biological trash bin. The other six plants in a cage were kept as a control cage (CC) without insects' introduction. Moreover, six plants from each variety were selected and flagged as Control without a cage (CWC). After 24 hours, 10-12 cm flower inflorescences with some leaves were collected from HDC, CC, and CWC plants for cannabinoids extraction.

Cannabinoids extraction

The cannabinoids extracted, as described in [20] for cannabinoid extraction and analysis. The flowers were dried at 40^{o}C with forced air ventilation for two hours and pulverized to through in a 1 mm sieve. One hundred milligrams of the ground samples dissolved in 30ml of isopropanol and sonicated for 30 minutes. The samples were centrifuged at 6000 rpm for 3 minutes, and the supernatant was recovered in a glass vial. 1ml of the samples decarboxylated (convert THCA to D9THC/ CBDA to CBD) by evaporating to dryness at 80 °C for 20 minutes. The samples are reconstituted in 500µl of isopropanol and stored at -20^{o}C freezer until injected into GC-FID.

Chemical analysis

The extracted samples were analyzed using Gas Chromatograph equipped with Flame Ion detector (Shimadzu GC-2030 with AOC-20i Autoinjector) using SH-Rxi-5Sil MS Column, 15m x 0.25mm x 0.25µm (part no. 227-36036-01), the GC oven temperature was 200°C ramp at 15°C/min to 300°C, hold 5min. The injector temperature was 250°C; Hydrogen carrier gas, constant inlet pressure at 48.3kPa, split ratio = 10, and the FID temperature was set at 300°C with Hydrogen flow rate 32mL/min, airflow rate 200mL/min, and nitrogen was used as a makeup gas flow rate 24mL/min.For the potency test, three cannabinoids mixture was purchased from Sigma-Millipore (CBD, D9THC and CBN) each at 1000 ug/ml. As described in [20], based on the sample preparation, 100 mg dried plant material dissolved in 30 ml solvent. As per the literature, CBD's expected concentration in hemp ranges from 15% to 21 %, and less than 1% D9THC and other cannabinoids [20]. If we assume the maximium CBD level, that is 21%, it calculates to 21 mg CBD in a 30 ml solution or 700 ug/ml, which was the same order of magnitude as the standard. The external standard was prepared 990 ul of CBD (1000 ug/ml) + 10 ul of two cannabinoid standards (1000 ug/ml) D9THC, and CBN were made a suitable calibration standard for CBD hemp that was 10ug/ml to 1000ug/ml. Data were extracted and analyzed using Microsoft Excel (Microsoft Corporation, USA) and SPSS windows version 4.0 (2014) (SPSS Institute Inc., Cary, NC, USA). Tukey Kramer's honestly significant difference (HSD) test were used to determine significant differences between mean values (± standard error) of the percent concentration of the cannabinoids at α = 0.05 level of significance.

Larval feeding bioassay

Exposure experiments were carried out in at least four replicates for each of the cannabinoids. One third-instar larva was transferred into petri dish plates and provided with 1.6 g of the artificial diet spiked with 1 mL of 300 ug/ml of CBD and 55 ug/ml D9THC. The control group larvae were fed with diet spiked with reverse osmosis water and the solvent methanol. The cannabinoid concentrations were within the range of the cannabinoids detected in the HD Cherry blossom plants. The insect larvae were starved for four hours prior to the commencement of the feeding bioassay. Weights of the insect larvae were measured before introduced to the diet and every 24-hour intervals until they metamorphosed to pupa, using a Mettler Toledo weighing balance (Columbus, Ohio). Analysis of the data was performed using Microsoft Excel (Microsoft Corporation, USA) and SPSS windows version 4.0 (2014) (SPSS Institute Inc., Cary, NC, USA). Least Significant Difference(LSD) were used to determine significant differences between mean values (± standard error) of larval weight at α = 0.05 level of significance.

Declarations

Ethics approval and consent to participate: Not applicable
Consent for publication: Not applicable
Availability of data and materials: The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Competing interests: The authors have no conflict of interest in this research

Funding: Not applicable

Authors' contributions: S.Z and T.T conceived and designed the project. S.Z, T.T, B.J, L.G, S.H, and V.V undertook the experiments and wrote the manuscript. S.Z, T.T, L.G, SH, and BJ participated in preparing and analyzing the data. S.Z, T.T, B.J, and V.V participated in cannabinoids analysis. All authors have read and approved the manuscript.

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Figures

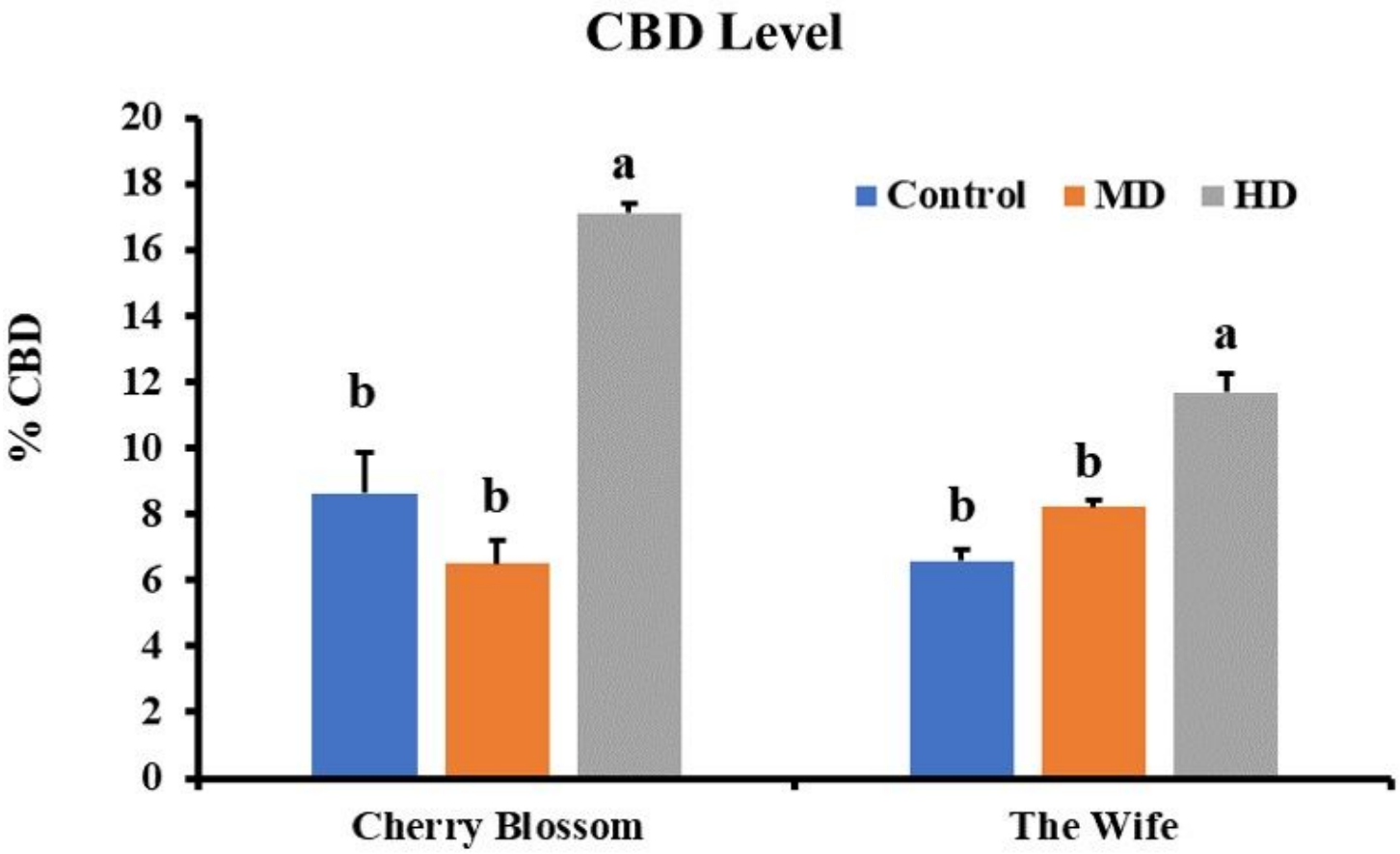

Figure 1

The level of CBD in Cherry Blossom and the Wife plants in a laboratory trial. Herbivore damage (HD), Mechanical Damage (MD), and Control. Data are means ± standard error. Bars labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

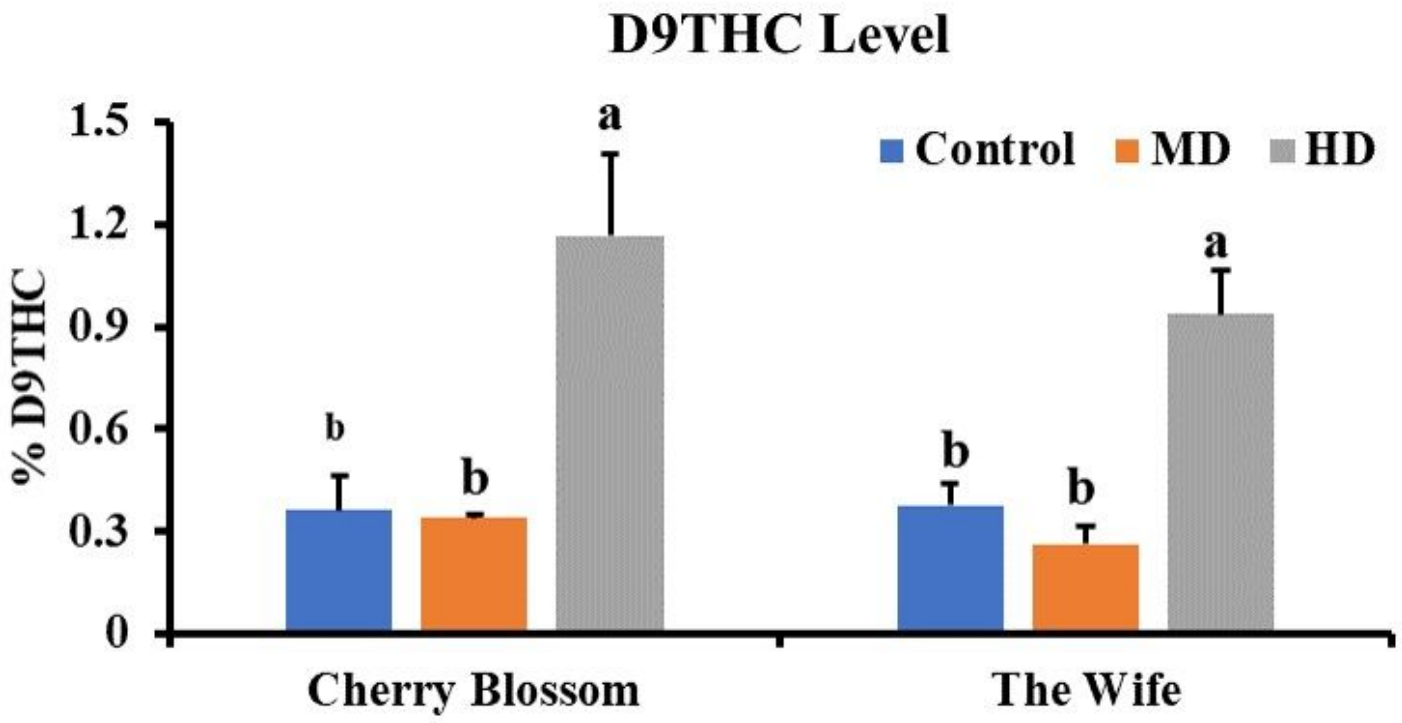

Figure 2

The level of D9THC in Cherry blossom and The Wife plants in a laboratory trail. Herbivore damage (HD), Mechanical Damage (MD), and control (C). Data are means ± standard error. Bars labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

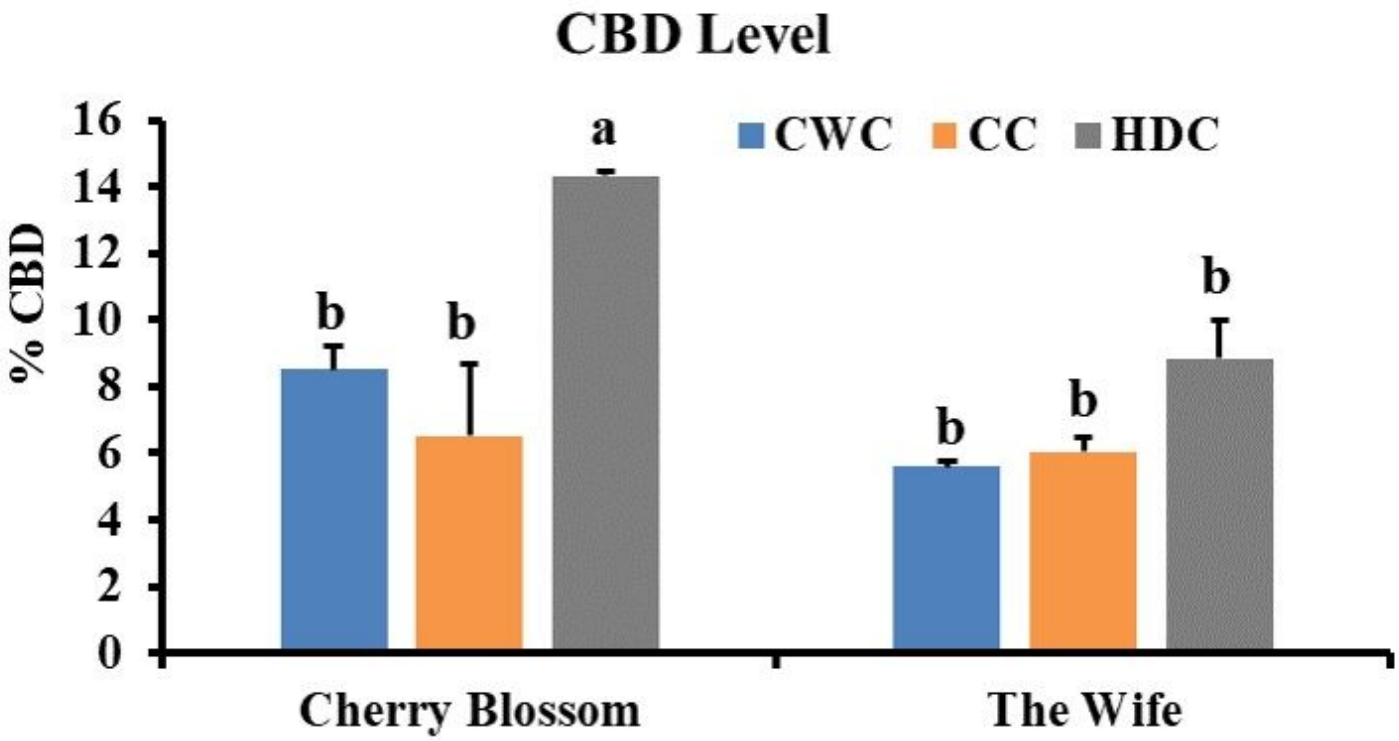

Figure 3

The level of CBD in Cherry blossom and The Wife plants in a field trail. Herbivore damage with cage (HDC), Control with cage (CC), and Control without cage (CWC). Data are means ± standard error. Bars labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

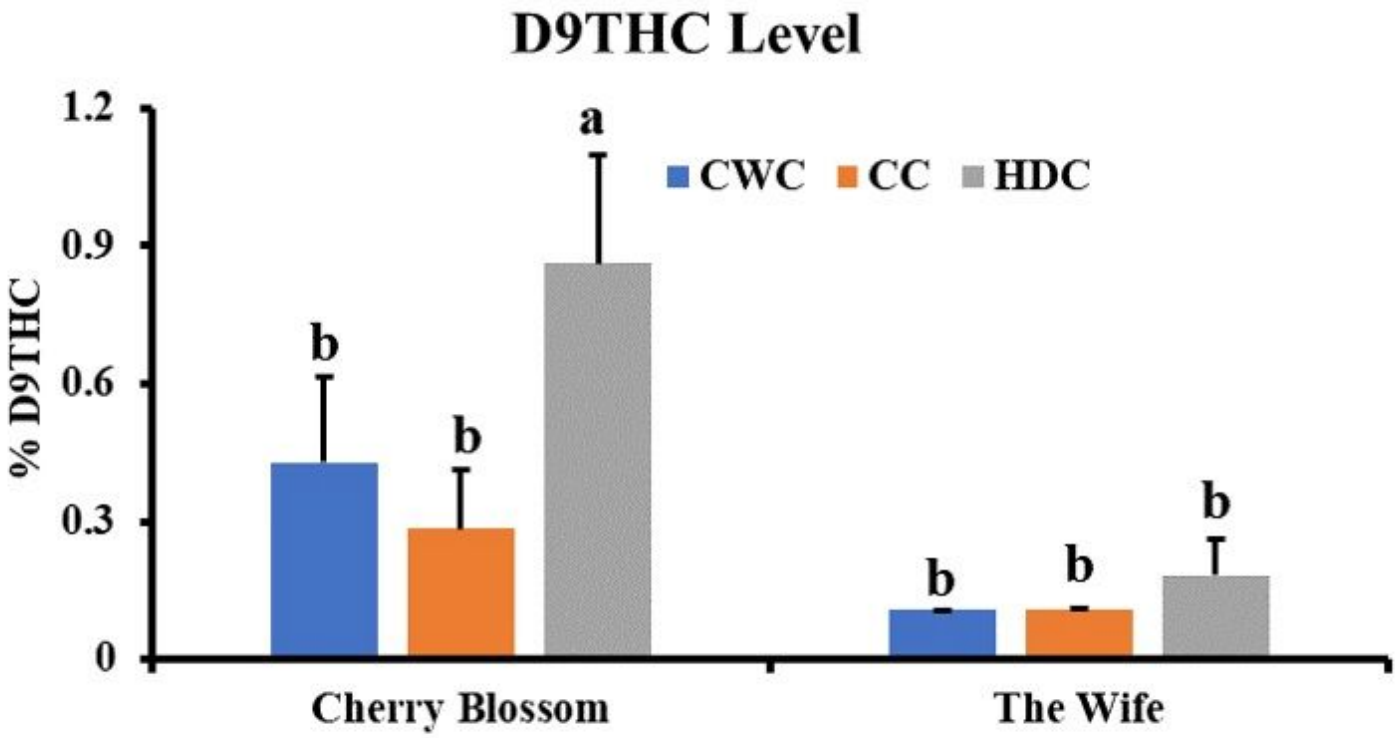

Figure 4

The level of D9THC in Cherry blossom and The Wife plants in a field trail. Herbivore damage with cage (HDC), Control with cage (CC), and Control without cage (CWC). Data are means ± standard error. Bars labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

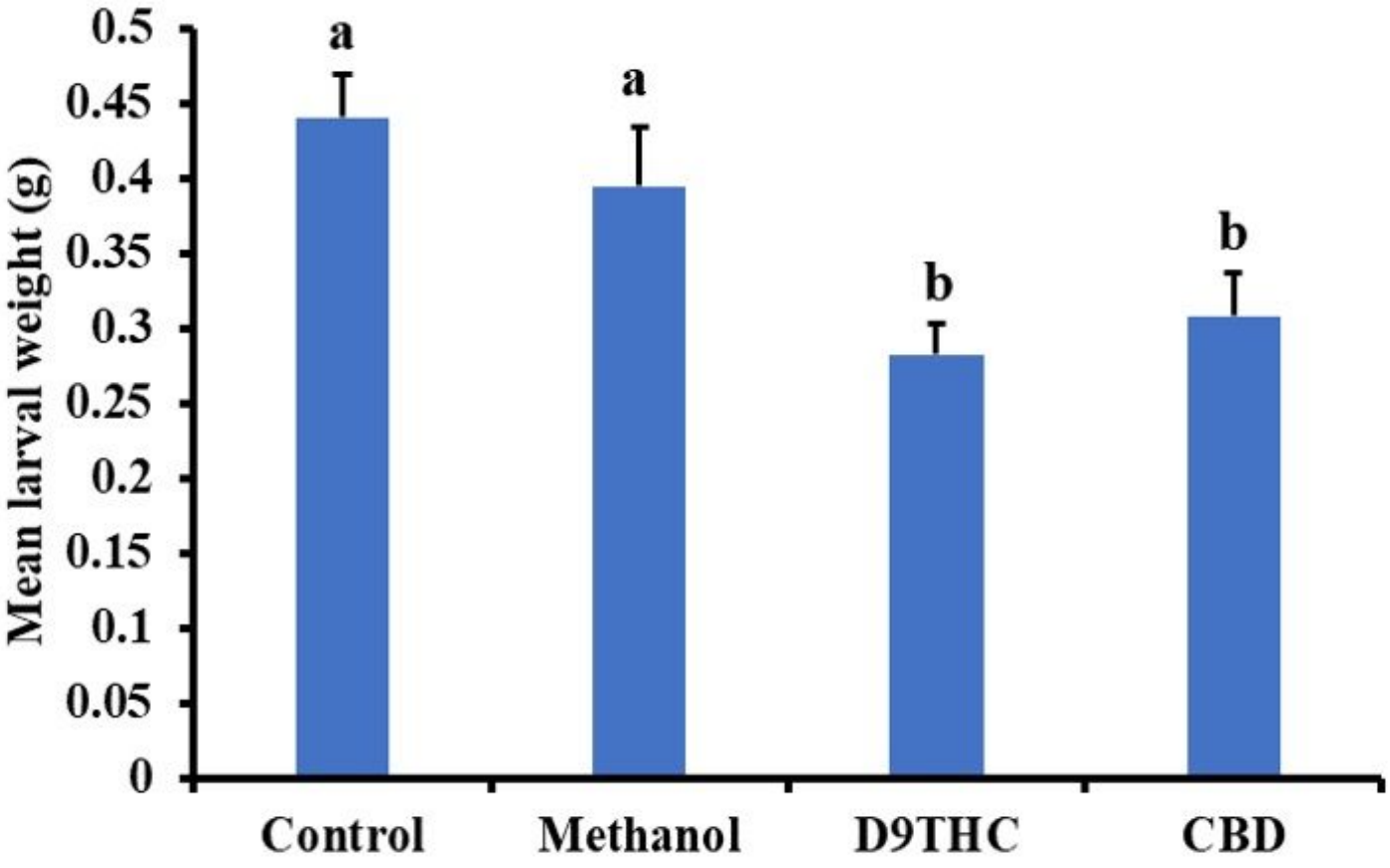

Figure 5

Mean larval weight of Corn earworm fed on CBD, D9THC, methanol spiked artificial diet and control diet. Means with different letters above the bars are significantly different using Student's t-test (P<0.05).

Supplementary Files

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