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Variations in Δ^9-THC and other Major Cannabinoids Content during Developmental Growth Stages in Seed Raised, Field Cultivated Plants of *Cannabis sativa* L.

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ABSTRACT: *Plants of Cannabis sativa L. were grown from seeds in jiffy pots and planted outdoor in the field conditions. At the onset of flowering, male plants were removed from the field to avoid pollination and only female plants were kept for further cultivation. Among these plants, few randomly selected healthy female plants from different plots were selected and periodically analyzed for Δ^9-tetrahydrocannabinol (Δ^9-THC) and other phytocannabinoids content throughout the growing season (from seedling to harvest) using gas chromatography - flame ionization detection (GC/FID). A significant, plant to plant variation in phytocannabinoids content was observed in these plants. Based upon the chromatographic analysis at the budding stage, plants were divided into four different groups i.e. plants having very high THC (> 12%), high THC (~8-12%), intermediate THC (~5-8%) and low THC (< 5%). In general, THC content increase with plant age up to a highest level during budding stage where the THC content reached a plateau for about a week before the plants were harvested. The change in the concentration of other cannabinoids follow a similar pattern in some cases but show more variability depending on the individual plant. Our results, in general, show a significant plant to plant variation in cannabinoids content in the Cannabis plants grown through seeds. The results of the study are critical in selecting specific clones for further propagation depending on their chemical profile.*

Key words: *Cannabidiol, Cannabinoids, Δ^9-tetrahydrocannabinol, GC/FID*

INTRODUCTION

Cannabis sativa L. is an annual herbaceous plant belongs to family Cannabaceae. It is one of the most ancient cultivated plant, growing in different habitats ranging from sea level to alpine foot hills. It is considered as a single, highly polymorphic species, *Cannabis sativa* L. (Small and Cronquist, 1976; Klimko, 1980; Gilmore *et al*., 2003; Wu and Raven, 2003). It is mostly a dioecious (male and female flowers on different plants) species however, occasionally shows hermaphrodite inflorescences leading it to monoecious phenotype. In general, female plants, due to the presence of higher concentration of THC as compared to male and hermaphrodite plants, are used for the cultivation of drug type varieties.

Based on its chemical constituents, *C. sativa* is considered a very complex species (ElSohly and Slade, 2005). *Cannabis sativa* L. contains a unique class of terpenophenolic compounds called cannabinoids. Till date 546 compounds are isolated from *C. sativa*. Out of 546 compounds, 104 are phytocannabinoids (ElSohly and Gul, 2014). Tetrahydrocannabinol (Δ^9-THC), the main constituent responsible for the psychoactive effects of cannabis, is mainly found in the glandular trichomes of the plant. Other major cannabinoids include cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), tetrahydrocannabivarin (THCV) and cannabinol (CBN). Δ^8-THC, a regioisomer of Δ^9-THC, is less abundant and potent than Δ^9-THC, and is thought to be an artifact (Small and Marcus, 2003).

The concentration of cannabinoids in the dried inflorescence (leaves and buds) is considered to be the most objective measure to classify the plant according to psychoactive potency since most of the THC and other cannabinoids are produced in buds and leaves of the plant. Based on the presence of most abundant cannabinoids, THC and CBD, in its leaves and buds, *C. sativa* was initially divided in two distinct categories namely drug type and fiber types

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(Fetterman *et al.*, 1971). According to their classification, if THC/CBD ratio exceeded one, plants were classified as 'drug phenotype' otherwise as 'fiber phenotype'. Small and Beckstead (1973a and 1973b) distinguished *C. sativa* into three phenotypes, namely drug type (THC/CBD ratio>>1), intermediate type (THC/CBD ratio close to 1.0) and fiber type (THC/CBD ratio <<1). A rare, additional chemotype, characterized by a very low content of both THC and CBD and with CBG as the predominant constituent, was later identified by Fournier *et al.* (1987).

Cannabis plant, over the last few decades has acquired much public interest and a lot of scientific attention, not only because of the problems associated with its abuse but also because of its potential for therapeutics, food, fuel and fiber. *Cannabis sativa* has a long history of medicinal use with references as far back as the 6th century B.C (Doyle and Spence, 1995; Zuardi, 2006). As a plant it is valued for its hallucinogenic and medicinal properties and has also been used to treat a variety of ailments including pain, glaucoma, nausea, asthma, depression, insomnia and neuralgia (Mechoulam *et al.*, 1976; Duke and Wain, 1981). The therapeutic values of Cannabis derivatives have also been highlighted against HIV/AIDS (Abrams *et al.*, 2007) and multiple sclerosis (Pryce and Baker, 2005). The pharmacologic and therapeutic potency of preparations of *Cannabis sativa* L. and its two primary cannabinoids Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) has been extensively reviewed (Mattes *et al.*, 1994; Brenneisen *et al.*, 1996; Mechoulam *et al.*, 2002; Zuardi *et al.*, 2002; Long *et al.*, 2005; Sirikantaramas *et al.*, 2007).

Cannabis in general is a dioceous plant, growing from seeds, results a portion of crop being male plants. Furthermore, due to highly allogamous nature of this species a significant amount of plant to plant variation in its cannabinoids profile and content is observed, even though the crop is propagated through a single seed variety. Consistency in secondary metabolite composition and content in starting biomass material, however, is of utmost importance if the substance is subjected to be used in pharmaceuticals. Therefore, understanding the seasonal and plant to plant variation in the cannabinoids content in growing *C. sativa* plants is important for screening and selecting appropriate clones for the clonal mass propagation and to determine the time of harvest of such materials (Chandra *et al.* 2010). The current study therefore aims to discuss firstly, the accumulation of cannabinoids content in field grown *C. sativa* plants with the developmental stages to determine the optimal time of plant harvest and secondly, to assess the range of variations in Δ^9-THC and other cannabinoids content in the outdoor grown plants of *C. sativa* grown from a single seed variety.

MATERIAL AND METHODS

Plant Material

Plants of a drug type Mexican variety of *C. sativa* were raised from seeds in 2 inch jiffy pots. Well rooted healthy seedlings (~8-10 cm tall) were planted and cultivated in open fields located at medicinal plant garden at the University of Mississippi (weather data of growing location during the growing season is shown in Fig. 1). Outdoor planting normally starts during late March or early April depending upon the weather conditions and could last till late November depending on the variety. For this study, plantation of seedlings were started during first week of June. To avoid pollination, male plants were removed from the field at the onset of flowering and only female plants were kept for further cultivation (Fig. 2). Sixteen randomly selected healthy plants from different plots were periodically sampled for the analysis of cannabinoids content at different developmental stages of growth before they were harvested in October. Samples were taken from 60 days (vegetative stage), 75 days (early flowering stage), 90 days (flowering stage), 105 days (flowering stage) days and 120 days old plants (budding stage) and gas chromatography-flame ionization detection (GC/FID) was used to assess the chemical profile and cannabinoids content.

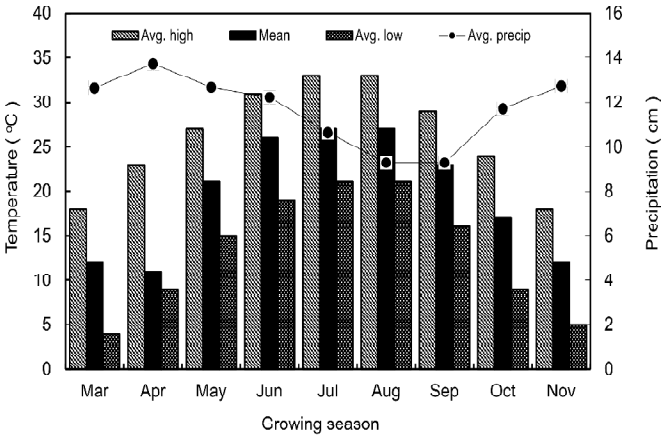

Figure 1: Climatic conditions (average monthly temperature and average precipitation) of the growing field at The University of Mississippi during the growing season (March till November)

Figure 2: Field cultivation of *Cannabis sativa* L. crop. Cannabis plants at vegetative stage (A and B), Flowering female plants (C and D)

Analysis of Phytocannabinoids

Sample Preparation

Biomass samples taken at each developmental stages were dried at 120 ^{o}F and individually manicured by hand using a 14 mesh (0.0555 in. opening) metal sieve to remove seeds (if any) and stems. Triplicate of each sample were used for the cannabinoids analysis. Following Ross *et al.* (1996), three 0.1g samples were each extracted with 3 mL of internal standard (ISTD)/ extracting solution (100 mg of 4-androstene-3, 17-dione + 10 mL chloroform + 90 mL methanol) at room temperature for 1 hr. The extracts were withdrawn into disposable transfer pipettes through cotton plugs for filtration and are transferred into GC vials, which are then capped and placed, on the auto sampler. One µL aliquots were injected.

Assessment of cannabinoids using GC-FID

Six major cannabinoids (Δ^9-THC, THCV, CBD, CBC, CBG and CBN, Fig. 3) were identified and quantified using GC-FID. GC analysis was performed using Varian CP-3380 gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler, a capillary injector and dual flame ionization detectors. The column was a 15 m x 0.25 mm DB-1, 0.25 µ film (J&W Scientific, Inc.). Data are recorded using a Dell Optiplex GX1 computer and Varian Star (version 6.41) workstation software. Helium is used as the carrier gas. An indicating moisture trap and an indicating oxygen trap located in the helium line from upstream to downstream, respectively, were used. Helium was used as the "make-up" gas at the detector. Hydrogen and compressed air were used as the combustion gases. The instrument parameters used for monitoring samples are: Air - 30 psi (400 mL/min); Hydrogen - 30 psi (30 mL/min); column head pressure - 14 psi (1.0 mL/min); split flow rate - 50 mL/min; split ratio - 50:1; septum purge flow rate - 5 mL/min; make up gas pressure - 20 psi (20 mL/min); injector temp - 240 ^{0}C; detector temp - 260°C; initial oven temp- 170 ^{0}C; initial temperature hold time - 1 min; temperature rate - 10 ^{0}C/min; final oven temperature - 250 ^{0}C and final temperature holds time - 3 min. The concentration of a specific cannabinoid is calculated as follows:

Cannabinoids (%) =
{GC area (cannabinoid) / GC area (ISTD)} ×
{Volume (ISTD) / Amount (sample)} × 100

Biomass yield

On maturity, plants used in the study were harvested and used for the determination of biomass yield. After harvest, dead leaves and stems are removed from the mature buds. Plant material is air dried at 40^{o}C for 12-15 hours using a commercial tobacco drying barn (BulkTobac, Gas fired Products, In., US). Plant material is then hand manicured and sticks and branches are removed from buds and leaves. Biomass yield is determined by accumulated weights of buds and leaves. Average plant yield is determined by total biomass yield divided by number of plants harvested.

Statistical Analysis

Statistical analysis of the data was done by agricolae module using statistical software "R" version 2.2.1 (2005).

RESULTS AND DISCUSSION

Variations in climatic conditions throughout the growing season (March till November) are shown in Fig. 1. Ambient air temperature increased from March till July. Average temperature during the months of July and August was recorded around 33^{o}C. A gradual decreased in the air temperature was recorded from August till the end of growing season. For the present study, cannabis plants were raised from seeds in jiffy pots and planted outdoor for field cultivation. On initiation of flowering, the male plants were removed and only female plants were kept for biomass production. Sixteen randomly selected healthy female plants were used for analysis of

cannabinoids content at different developmental growth stages from vegetative till harvest. Six major cannabinoids Δ^9-THC, THCV, CBD, CBC, CBG and CBN were analyzed using GC-FID. Considerable plant to plant variations were observed in cannabinoids content in seed raised *Cannabis sativa* plants. Based on their potency Δ^9-THC content, at the budding (ready to harvest) stage, plants were categorized in four different groups, very high THC group, THC > 12%; high THC group, THC > ~8 - 12%; intermediate THC group – THC ~5 - 8% and low THC group - THC < 5%.

Variations in Δ^9-THC content in different Cannabis groups at different developmental stages are shown in Fig. 4. Δ^9-THC is the principal psychoactive constituent (phytocannabinoid) of *Cannabis sativa*. It has been used as an antiemetic in chemotherapy-associated nausea and emesis, as an appetite promoter, especially for AIDS and cancer patients who are prone to severe weight loss due to anorexia and anorexia-cachexia, respectively, as an analgesic, e.g., for cancer, migraine, post-operative, spinal cord injury, dental and phantom limb pain, for treatment and symptom management of neurological disorders such as multiple sclerosis and for the management of glaucoma (Nahas *et al.,* 2002; Carlini, 2004).

The structure of Δ^9-THC was first reported by Gaoni and Mechoulam, two pioneers of Cannabis research (Gaoni and Mechoulam, 1964, Fig. 3A). Gaoni and Mechoulam not only determined its absolute configuration of Δ^9-THC but also discussed its psychotropic properties. In the plant, THC is present in the form of Δ^9-THC A (acid). Our data reveal that the concentration of Δ^9-THC, in general, increase with plant age up to a highest level during budding stage where the THC content reached a plateau for about a week before the plants were harvested. A considerable variation in Δ^9-THC content was observed in plants/ plant-groups. Average Δ^9-THC concentration of plants from very high THC group was ~14.86% (ranging from 13.79 to 16.94%) followed by high THC group, 11.30% (10.00 to 12.32%), intermediate THC, 7.31%, (6.95 to 7.52%) and low THC group, 2.97% (2.52 to 3.91%) at the harvesting stage. About 800%, 500%, 400% and 200% increase in Δ^9-THC content was observed at budding stage, as compared to the vegetative stage in very high THC, high THC, intermediate THC and low THC groups, respectively. Over all, lowest Δ^9-THC content was observed in plant ID-3124 (low THC group) and highest in plant ID-3124 (very high THC group) ranging from 2.52% to 16.94% at the harvesting stage. These data represent a significant plant to plant variation in Δ^9-THC content ($p < 0.05$), even though plants were grown from a single seed lot. Similarly, according to Potter, a large degree of natural variations in sibling plants are expected even if they are grown from seeds derived from just two parent plants, no data however was presented (Potter, 2015).

Tetrahydrocannabivarin (THCV, Fig. 3B, Table 1), a homologue of tetrahydrocannabinol (THC) having a propyl (3-carbon) side chain instead of a pentyl (5-carbon) group on the molecule, makes it produce very different effects from THC. This terpeno-phenolic compound is found naturally in cannabis plant, sometimes in significant amount, depending on the variety. Similar to Δ^9-THC, an increasing trend in THCV content was observed in cannabis with plant age. Average tetrahydrocannabivarin (THCV) content was highest in very high THC group (~0.12%, ranging from 0.09 to 0.15%) followed by high THC group (0.08%, ranging from 0.05 to 0.11%), intermediate THC (0.07% ranging from 0.05 to 0.09%) and low THC group (0.03% ranging from 0.03 to 0.04%) at the harvesting stage. An increase of about 250%, 280%, 150% and 150% was found in THCV concentration in very high THC, high THC, intermediate THC and low THC groups, respectively, at budding stage as compared to vegetative growth stage.

The other important cannabinoid in Cannabis of current interest is Cannabidiol (CBD, Fig. 3C, Table 1). There has been a significant interest in CBD over the last few years and in cannabis preparations of high CBD content because of its reported activity as an antiepileptic agent, particularly its promise for the treatment of intractable pediatric epilepsy (Mechoulam and Carlini, 1978; Cunha *et al.*, 1980). Cannabidiol (CBD) is a non-psychotropic compound. It was first isolated from Mexican marijuana (Adams *et al.,* 1940a) and its structure was determined by Mechoulam and Shvo (1963). In the plant, the lowest CBD content was observed at vegetative stage whereas, the highest CBD content was observed at budding stage. Although there was no certain trend observed, it was interesting to note that the lowest THC yielding group of plants has exhibited the highest amount of CBD (1.88%) at the budding stage. The average CBD content was 0.06%, 0.05% and 0.02% in very high THC, high THC, and intermediate THC groups, respectively, at the budding/harvesting stage. These data represent a high degree of variation in the CBD concentration ($p < 0.05$) in the plants grown from the seeds.

Cannabichromene (CBC, Fig. 3D, Table 1) was concurrently discovered by two different research

Table 1
Variation in THCV, CBD, CBC, CBG and CBN content in different groups of *C. sativa* plants at different stages of developmental growth.

Cannabinoids(%)	*THC Groups*	*60 days old plants (V)*	*75 days old plants (F)*	*90 days old plants (F)*	*105 days old plants (F)*	*120 days old plants (F)*
THCV	Very high THC	0.035 ± 0.029	0.068 ± 0.026	0.098 ± 0.031	0.120 ± 0.027	0.123 ± 0.025
	High THC	0.021 ± 0.017	0.023 ± 0.013	0.075 ± 0.031	0.075 ± 0.031	0.080 ± 0.026
	Intermediate THC	0.026 ± 0.022	0.033 ± 0.022	0.053 ± 0.034	0.065 ± 0.017	0.065 ± 0.019
	Low THC	0.013 ± 0.010	0.013 ± 0.004	0.020 ± 0.008	0.028 ± 0.004	0.033 ± 0.004
CBD	Very high THC	0.028 ± 0.015	0.023 ± 0.010	0.039 ± 0.027	0.038 ± 0.015	0.055 ± 0.010
	High THC	0.010 ± 0.007	0.010 ± 0.003	0.030 ± 0.008	0.025 ± 0.013	0.048 ± 0.005
	Intermediate THC	0.008 ± 0.003	0.005 ± 0.001	0.038 ± 0.013	0.020 ± 0.008	0.024 ± 0.013
	Low THC	0.364 ± 0.704	1.263± 1.1448	1.835 ± 2.124	1.953 ± 2.298	1.878 ± 2.146
CBC	Very high THC	0.098 ± 0.114	0.165 ± 0.065	0.153 ± 0.029	0.168 ±0.042	0.215 ± 0.042
	High THC	0.105 ± 0.070	0.163 ± 0.108	0.180 ± 0.041	0.175 ± 0.044	0.190 ± 0.041
	Intermediate THC	0.078 ± 0.033	0.090 ± 0.062	0.118 ± 0.031	0.115 ± 0.024	0.113 ± 0.028
	Low THC	0.160 ± 0.130	0.180 ± 0.137	0.160 ± 0.076	0.210 ± 0.132	0.190 ± 0.103
CBG	Very high THC	0.130 ± 0.070	0.600 ± 0.342	1.063 ± 0.022	0.723 ± 0.167	0.763 ± 0.271
	High THC	0.148 ± 0.111	0.200 ± 0.160	0.728 ± 0.363	0.405 ± 0.195	0.458 ± 0.269
	Intermediate THC	0.090 ± 0.069	0.178 ± 0.064	0.903 ± 0.584	0.583 ± 0.481	0.645 ± 0.584
	Low THC	0.095 ± 0.109	0.198 ± 0.133	0.278 ± 172	0.380 ± 0.289	0.428 ± 0.266
CBN	Very high THC	0.009 ± 0.008	0.013 ± 0.005	0.088 ± 0.056	0.070 ± 0.041	0.078 ± 0.024
	High THC	0.012 ± 0.012	0.015 ± 0.017	0.055 ± 0.026	0.075 ± 0.017	0.110 ± 0.047
	Intermediate THC	0.083 ± 0.127	0.070 ± 0.001	0.060 ± 0.067	0.068 ± 0.049	0.088 ± 0.038
	Low THC	0.015 ± 0.007	0.015 ± 0.006	0.038 ± 0.055	0.013 ± 0.005	0.028 ± 0.017

Groups are as described in figure 4, V: Vegetative stage, F: Flowering stage

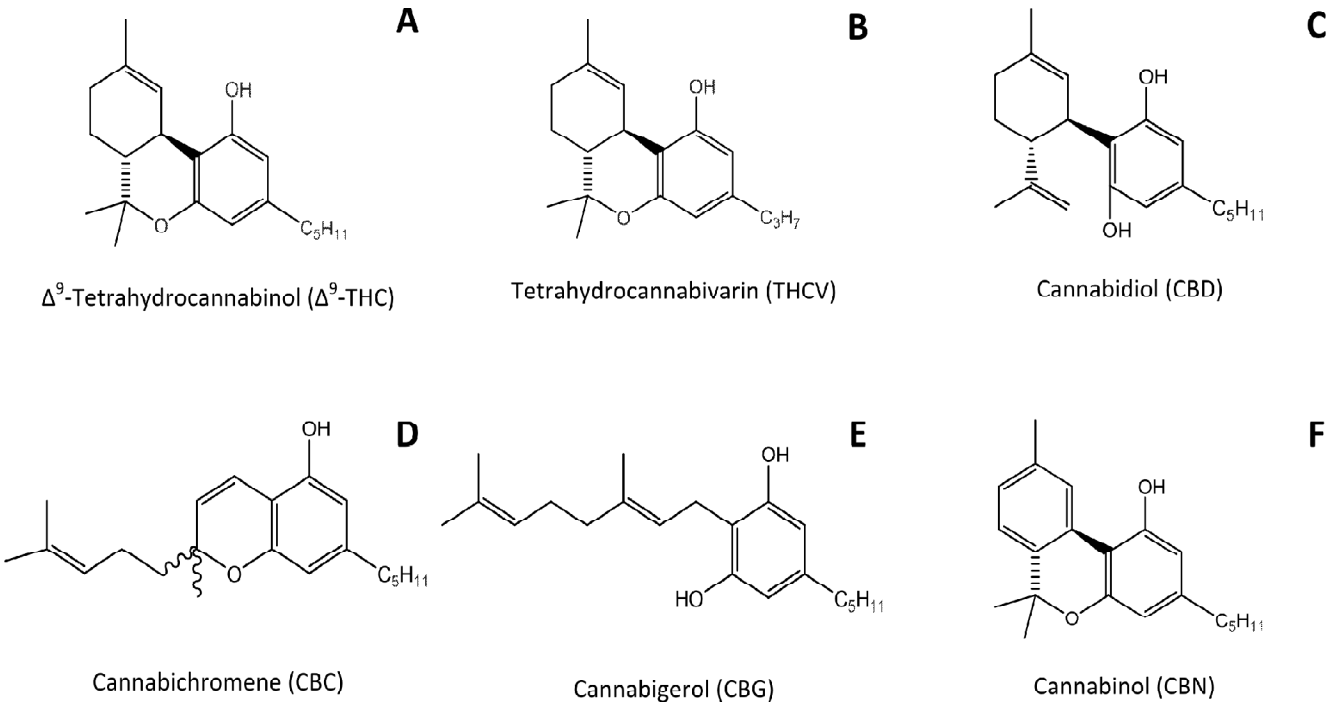

Figure 3: Chemical structures of Δ⁹-THC and other major cannabinoids in *C. sativa* L

groups (1) Claussen *et al.*, 1966 and (2) Gaoni and Mechoulam, 1966 (Fig. 3). Currently, eight CBC-type cannabis constituents are reported in *Cannabis sativa* (ElSohly and Gul, 2014). The antibacterial and antifungal activities of CBC and its homologs and isomers were reported as strong and mild to moderate, respectively, (Turner and ElSohly, 1981), while its anti-inflammatory properties were as effective as those of phenylbutazone (PBZ) at equivalent doses (Wirth *et al.,* 1980). In general, CBC increased with plant age in all the THC groups. However, a significant ($p < 0.05$) plant to plant variation was observed. Average CBC content was highest in very high THC group (0.22%, ranging from 0.17% to 0.27%) followed by high THC group (0.19%, ranging from 0.25 to 0.16%) and low THC group (0.190%, ranging from 0.06 to 0.31%) and, intermediate THC group (0.11% ranging from 0.08 to 0.14%), at the harvesting stage.

Cannabigerol (CBG, Fig. 3E, Table 1) was the first compound isolated from cannabis resin in a pure form through the hexane extraction of hashish (Gaoni and Mechoulam, 1964b). Currently, there are seventeen CBG-type cannabis constituents are reported in *C. sativa* (ElSohly and Gul, 2014). In the present study, a tremendous plant to plant variation was observed in seed raised field grown plants of *C. sativa*. Cannabigerol content was observed highest in the plants from very high THC group (0.76%) followed by intermediate THC group (0.65%), high THC group (0.46%) and low THC group (0.43%) at the budding stage. Cannabigerol content was highest in plant ID-22040 (1.47%, Intermediate THC group) whereas, it was found lowest in plant ID-3378 (0.05%, low THC group) at maturity stage. These data represent a high degree of variation in CBG content in seed grown plants.

Wood *et al.,* (1896) reported cannabinol (CBN, Fig. 3F, Table 1), but its correct structure was determined by Adams *et al.*, (1940b). Till date, ten CBN-type cannabinoids are reported in *C. sativa*. In the field grown plants, increasing trend in CBN content was observed with age. However, higher increase in CBN content (from vegetative to budding stage), was observed in the plants from very high THC and high THC groups as compared to those in low and intermediate THC Groups. At the budding stage, a significant ($p < 0.05$) difference in CBN content was observed in seed grown plants.

On maturity, all the plants were harvested for the determination of biomass yield (Fig. 5). Highest biomass yield (leaves and flowers) was observed in intermediate THC group (147.91 ± 46.97g) followed by very high THC group (135.96 ± 55.21g), low THC group (120.89 ± 63.77g) and high THC group (105.26 ± 20.39g). In general, a significant ($p < 0.05$) plant to plant variation in per plant biomass yield was observed, ranging from 74.22g (plant ID-3516, low THC group) to 218.00 g (plant ID-1269, very high THC group).

In conclusion, cannabinoids content in general increased with plant age and reached at the highest levels at budding stage. However, a high degree of plant to plant variations in the per plant useable biomass and cannabinoids content were observed in seed grown plants. These results are critical in selecting specific clones for further propagation depending on their chemical profile. Alternatively, the study suggest that screening of high yielding elite female plants and their mass propagation, using vegetative cutting or biotechnological tools such as micropropagation, is the most suitable way to maintain the uniformity among the plants especially, consistency in their cannabinoids content. This can be achieved by making cuttings from seedlings at vegetative stage and subjecting parent seedling to flowering till maturity while maintaining cuttings at the vegetative stage. On flowering, male plants and their related vegetative cuttings are removed. Based on the chemical analysis of mature female plants, the elite mother plant/plants can be identified and their related vegetative cutting can be used as high yielding elite plants for the future use.

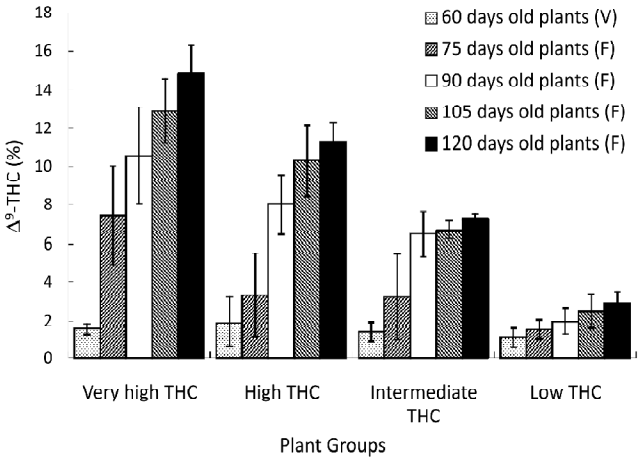

Figure 4: Variation in Δ^9-THC content in field grown *C. sativa* plants, raised from a single seed variety (high potency Mexican), at different stages of developmental growth. Plants categorized in different groups based on their THC content at budding stage using GC-FID analysis. Very high THC group – THC > 12%, high THC group – THC ~8 - 12%, intermediate THC group – THC ~5 -8%) and low THC group – THC < 5%. V: Vegetative stage, F: Flowering stage

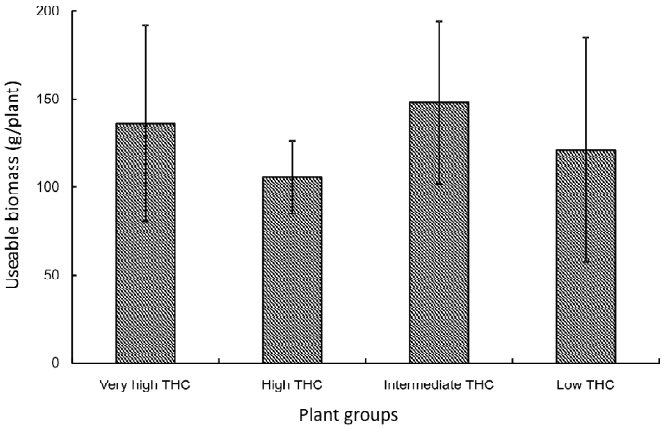

Figure 5: Variation in average useable biomass (leaves and buds) in different groups of *C. sativa* plants. Groups are as described in figure 4

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