Assessment of Cannabinoids Content in Micropropagated Plants of *Cannabis sativa* and Their Comparison with Conventionally Propagated Plants and Mother Plant during Developmental Stages of Growth

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Key words
- *Cannabis sativa*
- Cannabaceae
- cannabinoids
- Δ^9-tetrahydrocannabinol
- gas chromatography-flame ionization detection
- micropropagation

Abstract

Gas chromatography-flame ionization detection (GC-FID) was used to assess the chemical profile and quantification of cannabinoids to identify the differences, if existing, in the chemical constituents of *in vitro* propagated plants (IVP), conventionally grown plants (VP) and indoor grown mother plants (MP-Indoor) of a high THC yielding variety of *Cannabis sativa* L. during different developmental stages of growth. In general, THC content in all groups increased with plant age up to a highest level during the budding stage where the THC content reached a plateau before the onset of senescence. The pattern of changes observed in the concentration of other cannabinoids content with plants age has followed a similar trend in all groups of plants. Qualitatively, cannabinoids profiles obtained using GC-FID, in MP-indoor, VP and IVP plants were found to be similar to each other and to that of the field grown mother plant (MP field) of *C. sativa*. Minor differences observed in cannabinoids concentration within and among the groups were not found to be statistically significant. Our results confirm the clonal fidelity of IVP plants of *C. sativa* and suggest that the biochemical mechanism used in this study to produce the micropropagated plants does not affect the metabolic content and can be used for the mass propagation of true to type plants of this species for commercial pharmaceutical use.

Abbreviations

GA_3:	gibberellic acid
GC-FID:	gas chromatography-flame ionization detection
IAA:	indole-3-acetic acid
IBA:	indole-3-butyric acid
NAA:	naphthaleneacetic acid
TDZ:	thidiazuron
THC:	Δ^9-tetrahydrocannabinol

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Introduction

Cannabis sativa L. (Cannabaceae) has a long history of use for medicinal purposes [1]. This species contains cannabinoids, a unique class of terpenophenolic compounds, which accumulate mainly in glandular trichomes of the plant [2]. Over 70 cannabinoids have been isolated from *Cannabis sativa*, the major biologically active compound being Δ^9- tetrahydrocannabinol, commonly referred as Δ^9-THC [3]. Chemical structures of six major cannabinoids from *Cannabis sativa* including Δ^9-THC are shown in **Fig. 1**. Besides its psychoactivity, Δ^9-THC possesses analgesic, anti-inflammatory, appetite stimulant and antiemetic properties making this cannabinoid a very promising drug for therapeutic purposes [4]. The pharmacological and therapeutic potency of preparations of *Cannabis sativa* L. and Δ^9-THC have been extensively reviewed [5–10]. Δ^9-THC is currently available on the market in the form of soft gelatin capsules for oral intake known as Marinol®. The supply of the bulk active Δ^9-THC has been restricted to the manufacture of Marinol®, making this difficult to develop the formulations, such as suppositories containing the product Δ^9-THC [8, 11]. Therefore, we have considered making Δ^9-THC available by isolation from the plant material. Finding a quality starting material for natural Δ^9-THC production, which is relatively inexpensive as compared to the synthetic route, has always been a key issue. However, studies are on-

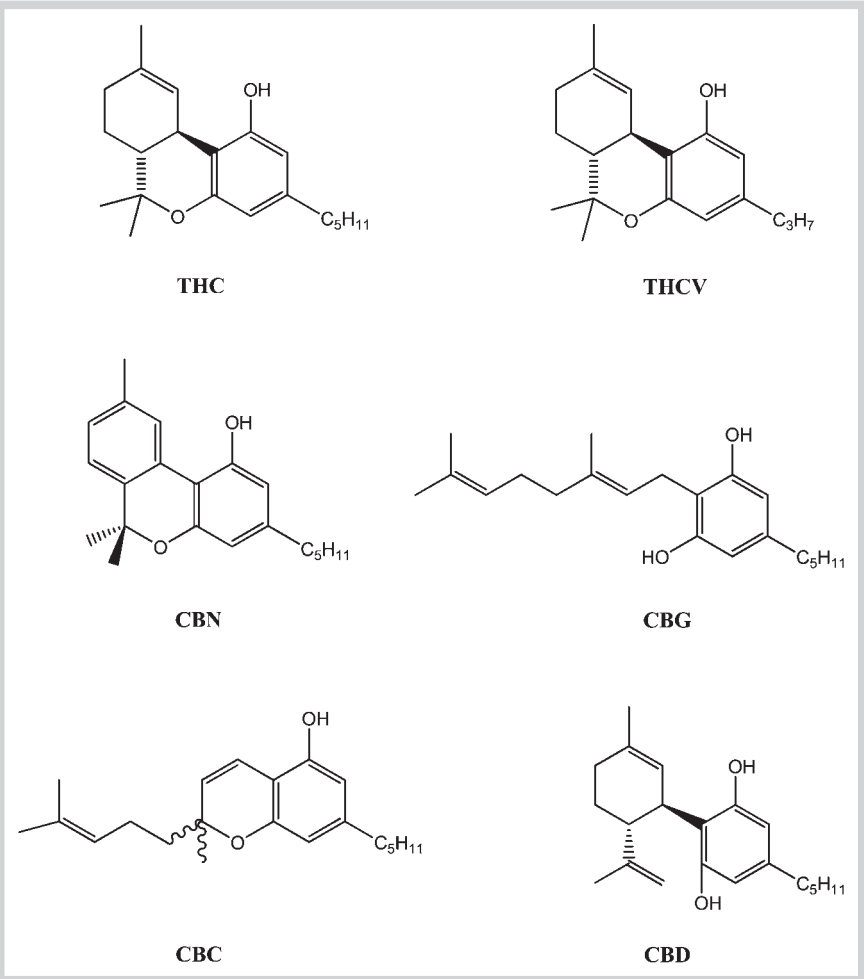

Fig. 1 Chemical structures of major cannabinoids present in *Cannabis sativa.* THC: tetrahydrocannabinol, THCV: tetrahydrocannabivarin, CBN: cannabinol, CBG: cannabigerol, CBC: cannabichromene, CBD: cannabidiol.

going to screen improved high THC yielding elite *Cannabis* varieties for production of quality biomass as a starting material for the isolation of natural THC for the purpose of commercial pharmaceutical use. The increasing potency trend in confiscated marijuana in the USA since 1980 has been reported by ElSohly et al. and Mehmedic et al. [11, 12]. Due to the allogamous (cross-fertilization) nature of *Cannabis sativa* it is very difficult to maintain the efficacy of selected high THC yielding elite varieties grown from seeds under field or greenhouse conditions. Thus, advanced biotechnological approaches, including tissue culture can be used as a tool for rapid multiplication of selected female high yielding clones of this species. Few plant regeneration protocols have been developed for different *Cannabis* genotypes and explant sources [13–18], and considerable variation has been reported in the response of cultures and in the morphogenic pathway.

Since, it is very important to have consistency in starting/raw material (plant biomass in this case) to maintain efficacy of any product of pharmaceutical interest, we have successfully developed an efficient protocol for *in vitro* propagation of nodal segments containing axillary buds [19]. In order to test the micropropagation protocol and to ensure the quality and stability of useful chemical constituents, the *in vitro* propagated plants were further compared with vegetatively propagated plants of the same age raised from the same female mother plant, throughout their growth and developmental stages using gas chromatographic analysis and comparison of their cannabinoids content.

Material and Methods

Plant material

Plants of *Cannabis sativa* were grown through high potency Mexican seeds in the marijuana plant garden at the University of Mississippi during the year 2006. A crop of ~ 31 000 plants was started and each plant was tagged with a unique barcode to construct an accurate inventory of plants and to ensure the identity of each and every plant for further research. On flowering, male plants were removed from the field to avoid cross-pollinations and only female plants were kept for further cultivation. Among these plants, 50 randomly selected healthy female plants from different plots were periodically analyzed for their cannabinoids content (Δ^9-tetrahydrocannabinol, Δ^9-THC; tetrahydrocannabivarin, THCV; cannabidiol, CBD; cannabichromen, CBC; cannabigerol, CBG; cannabinol, CBN) during different stages of growth and development (from seedling to harvest). On the basis of initial GC analysis at the vegetative stage, cuttings were made from a few selected female plants for further cultivation. These cuttings were grown indoors under the combination of 1000 Watts full spectrum metal highlight and sodium bulbs and maintained under vegetative growth conditions. The indoor grown high yielding clones were identified based upon the THC and other cannabinoids content of their related field grown mother plants at the end of annual growth season in the field. Plants were carefully marked and categorized based upon the information on their field grown mother plants. These selected female plants

served as indoor mother plants for future propagation. For the present study a clone from a field grown high yielding elite plant (ID: P1–2714) was selected as an indoor mother plant.

After 6 months of acclimatization and growth under indoor vegetative lights cycle (18-h light and 6-h dark), fresh nodal segments were taken from a well marked mother plant (ID #: P1–2714) for *in vitro* propagation (IVP). After six weeks, well established and enough proliferated shoots were transplanted in rooting medium. At the same time, to compare *in vitro* propagated plants with vegetatively propagated plants, ~ 6–10 cm long nodal cuttings were taken from the same mother plant and planted in 2-in jiffy pots for vegetative propagation. After five weeks, well rooted micropropagated and vegetatively grown plants were transplanted in same size (4-inch diameter) pots containing similar soil (coco natural growth medium mixed with fertilome potting mix in 1 : 1 ratio; Canna Continental) and kept side-by-side under similar environmental conditions (25 ± 3 °C temperature and 55 ± 5 % RH). Indoor light (~ 700 ± 24 µmol m^{-2} s^{-1} at plant canopy level measured by LI-COR quantum meter, model LI-189) was provided with seven full spectrum 1000 Watts HID (high density discharge) lamps in combination with seven 1000 Watt high pressure sodium bulbs (Sun Systems), hung on the top of plants covering a 350-square feet area. A hot air suction fan was attached and about 3 to 4 feet distance between plants and bulb was maintained to avoid heating due to HID bulbs. These plants were thereafter transplanted in 12-inch pots after four weeks of growth.

After six weeks in 12-inch pots, samples from the apical part were taken for cannabinoids content at the peak vegetative stage. After that, both sets of plants along with MP-Indoor were exposed to a flowering light cycle (12-h light and 12-h dark). Onset of flowering was seen within 15 days. Periodic sampling was done in both sets of plants and MP-Indoor at the same time at different growth stages (vegetative to onset of senescence) and compared for cannabinoids content. Nine IVP and nine VP plants from the same mother plant were used for the comparison. It is important to make a note here that periodic sampling for the analysis of secondary metabolites (cannabinoids) for all three sets of plants was done at several stages (vegetative to onset of senescence) of the growth cycle in 15-day intervals but the data on peak vegetative (15 week/105-day-old plant), peak reproductive (24-week-old plant, nine weeks under flowering light), late reproductive (26-week-old plant) and onset of senescence stage (28-week-old plant) only were used for comparison (**Fig. 2**). The age of the plant was calculated from the day cuttings were taken from MP-Indoor for vegetative propagation or micropropagated shoots were transplanted in rooting medium. This study has three important segments; first, micropropagation and hardening of the *Cannabis* plants, second, vegetative propagation and third, chemical analysis of plant samples taken from MP-Outdoor, MP-Indoor, IVP plants and VP plants and at different growth stages.

Micropropagation and hardening

Apical nodal segments containing axillary buds (~ 1 cm in length) were used as an explant for initiation of shoot cultures. Explants were obtained from healthy branches of a screened and selected high yielding *Cannabis sativa* clone (MP-Indoor) grown in an indoor cultivation facility housed at Coy-Waller laboratory, University of Mississippi. Explants were surface disinfected using 0.5 % NaOCl (15 % v/v bleach) and 0.1 % Tween 20 for 20 minutes. The explants were washed in sterile distilled water three times for 5 minutes prior to inoculation on the culture medium. Micropropagation and hardening of micropropagated plants was done by following the protocol described by Lata et al. [19].

Vegetative propagation

A fresh nodal segment about 6–10 cm in length containing at least two nodes was taken from the same mother plant for vegetative propagation. Using a sterile tissue culture blade, a soft apical branch was cut at a 45 degree angle just below the node and dipped immediately in distilled water to avoid formation of any air bubble in the stem which might block fluid uptake later. About 2 cm of base of the cuttings were dipped in green light rooting hormone (Green Light Co.) for better rooting. Similar to IVP plants, cuttings were also placed in same size, i.e., 2-in jiffy pots containing coco natural growth medium and sterile potting mix-fertilome (Canna Continental) mixed in 1 : 1 ratio. At least one node was dipped in the soil for efficient rooting. All these plantlets were kept side-by-side with IVP plant under fluorescent light to provide similar environmental conditions. Although rooting was initiated in 2–3 weeks, most of the cuttings raised plants were kept in this environment for six weeks for better vegetative growth, similar to IVP plants.

Chemical analysis

Biomass samples were taken from apical segments of MP-Indoor and their IVP clones and VP clones at several stages during growth and development of plants. These samples were dried at 120 °F and individually manicured by hand. Triplicates of each sample were used for the cannabinoids analysis.

Following Ross et al. [20], all *Cannabis* samples were manicured in a 14 mesh (0.0555 in. opening) metal sieve to remove seeds and stems. Triplicated 0.1 g samples were each extracted with 3 mL of internal standard/extracting solution (100 mg of 4-androstene-3, 17-dione + 10 mL chloroform + 90 mL methanol) at room temperature for 1 h. 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.

Six major cannabinoids content Δ^9-THC, THCV, CBD, CBC, CBG and CBN were identified and quantified using GC-FID. Gas chromatography analysis was performed following Ross et al. [20], using a 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 × 0.25 mm DB-1, 0.25 µ film (J&W Scientific, Inc.). Data were recorded using a Dell Optiplex GX1 computer and Varian Star (version 6.41) workstation software. Helium was 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 were: 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 temperature – 240 °C; detector temperature – 260 °C; initial oven temperature – 170 °C; initial temperature hold time – 1 min; temperature rate – 10 °C/min; final oven temperature – 250 °C and final temperature hold time – 3 min. The concentration of a specific cannabinoid is calculated as follows:

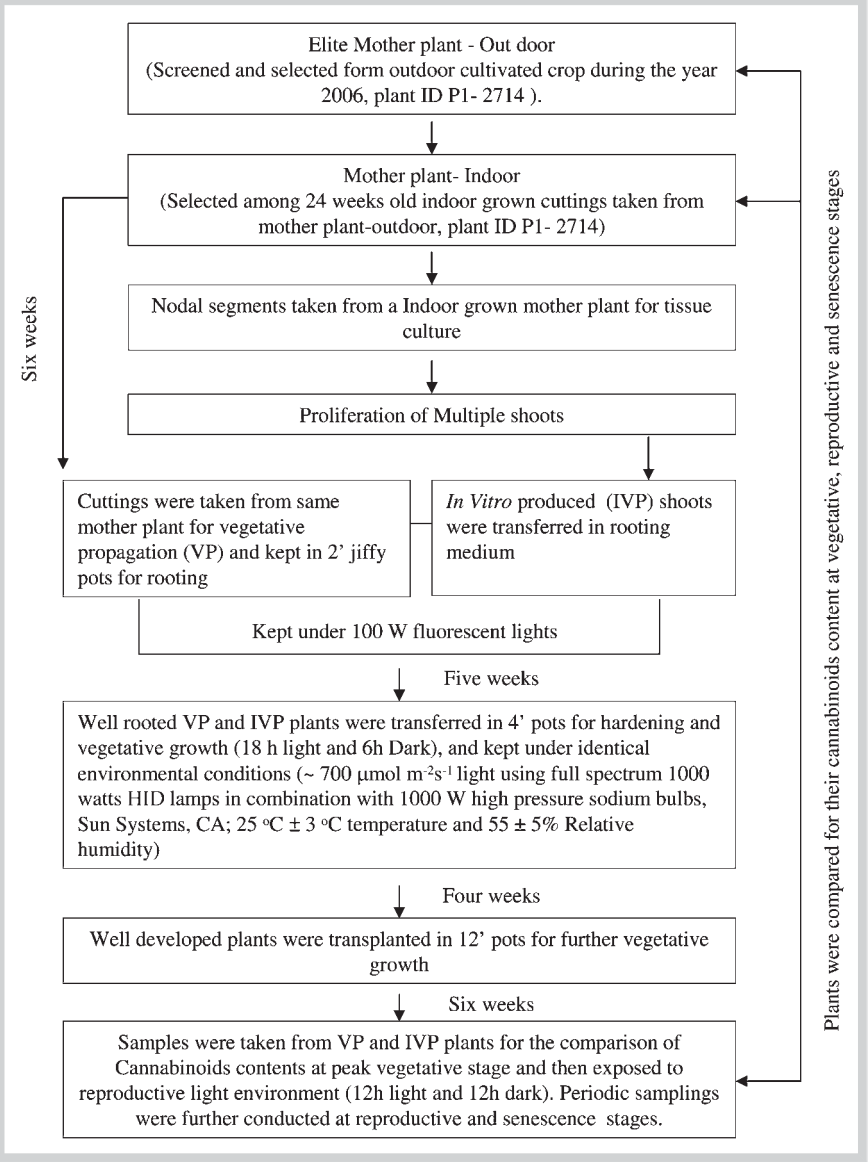

Fig. 2 Schematic diagram for the comparison of cannabinoids contents among *in vitro* propagated (IVP) plants, vegetatively propagated (VP) plants, indoor grown mother plant (MP-Indoor) and outdoor grown mother plant (MP-Outdoor) of *Cannabis sativa* at different stages of growth.

$$\text{cannabinoid } \% = \frac{\text{GC [area] (cannabinoid)}}{\text{GC [area] (ISTD)}} \times \frac{\text{volume (ISTD)}}{\text{amount (sample)}} \times 100$$

Currently, this method is being used by our group to analyze the confiscated marijuana samples submitted by the US Drug Enforcement Agency (DEA) and other US enforcement agencies under National Institute of Drug Abuse (NIDA) Marijuana Project, Potency Monitoring Program at the University of Mississippi [11].

Statistical analysis

Statistical analysis was conducted using SAS version 9.1 (SAS Institute). Data on micropropagation and variation in secondary metabolites (cannabinoids) content with growth in three different types of plants (MP-Indoor, VP and IVP) was evaluated by one-way, fixed effect ANOVA and Tukey's post hoc tests. Analysis of variance for cannabinoids content during different growth stages of VP and IVP plants was conducted on data obtained from 9 plants from each group. Each sample for MP-Indoor, VP and IVP plants at every growth stage was analyzed in triplicate and the data were used for statistical analysis.

Supporting information

Indoor grown mother plant, *in vitro* raised plants, vegetatively propagated plants, and fully grown *in vitro* propagated and vegetatively propagated plants of *Cannabis sativa* are shown in **Fig. 1S** as Supporting Information.

Results and Discussion

A systematic diagram of the experimental design for the comparison of secondary metabolites contents between *in vitro* propagated plants and conventionally/vegetatively propagated plants at different stages of growth is depicted in **Fig. 2**. Source mother plant (ID #: P1–2714) for this study was screened among ~ 31 000 plants of field cultivated crop of *Cannabis sativa* during the growing year 2006 at the University of Mississippi. Screening and selection was based upon the chemical/metabolic profiles of 50 healthy randomly selected plants from five different plots using GC/FID at different developmental stages of growth (from vegetative to fruiting and harvesting). Chemical profiles of screened high yielding field grown mother plant, from which cut-

Cannabinoid contents (%)	Stage I	Stage II	Stage III	Stage IV	Stage V
Δ^9-THC	1.00	2.85	11.53	13.64	11.21
THCV	0.02	0.02	0.08	0.10	0.08
CBD	0.04	0.01	0.03	0.04	0.04
CBC	0.01	0.17	0.24	0.32	0.29
CBG	0.03	0.10	0.52	0.45	0.41
CBN	0.01	0.02	0.11	0.12	0.17

Date of field plantation: June 26, 2006; stage I: vegetative stage, 60-day-old plants; stage II: vegetative stage, 75-day- old plant; stage III: reproductive stage, 90-day-old plant; stage IV: reproductive stage, 105-day-old plant; stage V: on set senescence 120-day-old plant. Δ^9-THC: Δ^9-tetrahydrocannabinol, THCV: tetrahydrocannabivarin, CBD: cannabidiol, CBC: cannabichromene, CBG: cannabigerol, CBN: cannabinol

Table 1 Development in Δ^9-THC and other cannabinoids content in a field grown mother plant of *Cannabis sativa* with different stages of growth during 2006.

Growth regulators (GR)	Optimum concentration of GR (µM)	Average number of shoots produced	Average shoot length (cm)	Percent explants producing shoots
BA	0.50	4.04^{hi}	3.94^{efgh}	71.42
	2.50	6.14^{def}	3.92^{fghi}	73.53
	5.00	7.13^{cd}	4.62^{efg}	65.52
KN	0.50	3.72^{i}	3.71^{hi}	76.64
	2.50	6.53^{cde}	4.81^{ef}	84.62
	5.00	5.32^{efghi}	3.42^{fghi}	51.01
TDZ	0.50	13.32^{ab}	8.53^{b}	100.00
	2.50	12.44^{b}	7.20^{c}	95.62
	5.00	6.33^{cde}	4.72^{efgh}	82.53
BA + GA_3	0.50 + 7.00	4.71^{ghi}	3.51^{hi}	5.90
	2.50 + 7.00	4.41^{ghi}	4.41^{ef}	66.92
	5.00 + 7.00	4.92^{efgh}	4.70^{e}	32.50
Kn + GA_3	0.50 + 7.00	3.93^{ghi}	3.80^{hi}	45.61
	2.50 + 7.00	6.52^{de}	3.31^{i}	64.72
	5.00 + 7.00	5.81^{defg}	3.71^{hi}	56.82
TDZ + GA_3	0.50 + 7.00	14.60^{a}	8.93^{a}	84.84
	2.50 + 7.00	12.51^{b}	8.12^{b}	95.62
	5.00 + 7.00	8.61^{c}	6.32^{d}	79.81
		Average number of roots produced	**Average root length (cm)**	**Percent explants producing roots**
IAA	2.50	1.42^{b}	1.50^{b}	33.90
	5.00	1.63^{b}	1.62^{b}	44.64
IBA	2.50	4.70^{a}	4.61^{a}	95.33
	5.00	3.90^{a}	4.84^{a}	81.82
NAA	2.50	2.50^{b}	2.13^{b}	56.31
	5.00	2.62^{b}	2.51^{b}	44.82

Table 2 The effect of the concentration of growth regulators on multiplication, shoot proliferation and root induction of cultures initiated from axillary buds of *Cannabis sativa*. Data represents the mean of six replicates with three explants for each treatment. Means followed by same letter do not differ statistically at p = 0.05 according to the Tukey test.

tings were taken and grown indoors (MP-Indoor) for *in vitro* and vegetatively propagated, is shown in **Table 1**. In general, THC content increased with plant age up to the highest level during the peak reproductive/budding stage where the THC content reached a plateau followed by a decrease during the onset of senescence. Under the field conditions, highest concentration of THC was found to be about 11.53% and 13.64% during the growth stage III and stage IV, respectively, i.e., 90- to 105-day-old plant. A decline in THC concentration was observed in the samples taken on the 120th day of growth from this plant. It was interesting to note that THCV content in these plant samples were < 1.00% of total THC concentration at the harvesting stage. THCV, being a homolog of THC has a very close chemical structure (**Fig. 1**), is therefore very difficult to separate from THC. Higher THCV concentration in *Cannabis* extracts makes the THC purification process more complex and expensive. Therefore, chemical profile-based selection of mother plants for vegetative and micropropagation of this species for the production of pure THC can play a key role in setting the total cost of the production and especially the purification process. In this regard, having high THC and fairly low THCV concentration, plant ID # P1–2714 was selected as the elite mother plant suitable for further propagation.

Cuttings from a selected field grown mother plant (P1–2714) were grown in an indoor grow room under controlled environmental conditions. After 6 months of plant growth under vegetative light conditions, young nodal segments containing axillary buds were used for micropropagation. Among the various methods developed to micropropagate the plants, enhanced axillary branching is advantageous because it is simple and the propagation rate is relatively better [21]. Furthermore, plant tissue culture using axillary buds/organized meristems is generally considered to be a low risk method for genetic instability [20], because the organized meristems are generally more resistant to genetic changes as compared to unorganized callus under *in vitro* conditions [22]. **Table 2** shows the effect of the concentration of growth regulators on shoot proliferation and root induction of cultures initiated from axillary buds. Best response for the shoot induction was observed on Murashige and Skoog (MS) [23] medium containing 0.5 µM thidiazuron (TDZ) in this species. Well developed shoots were then transferred to half strength MS me-

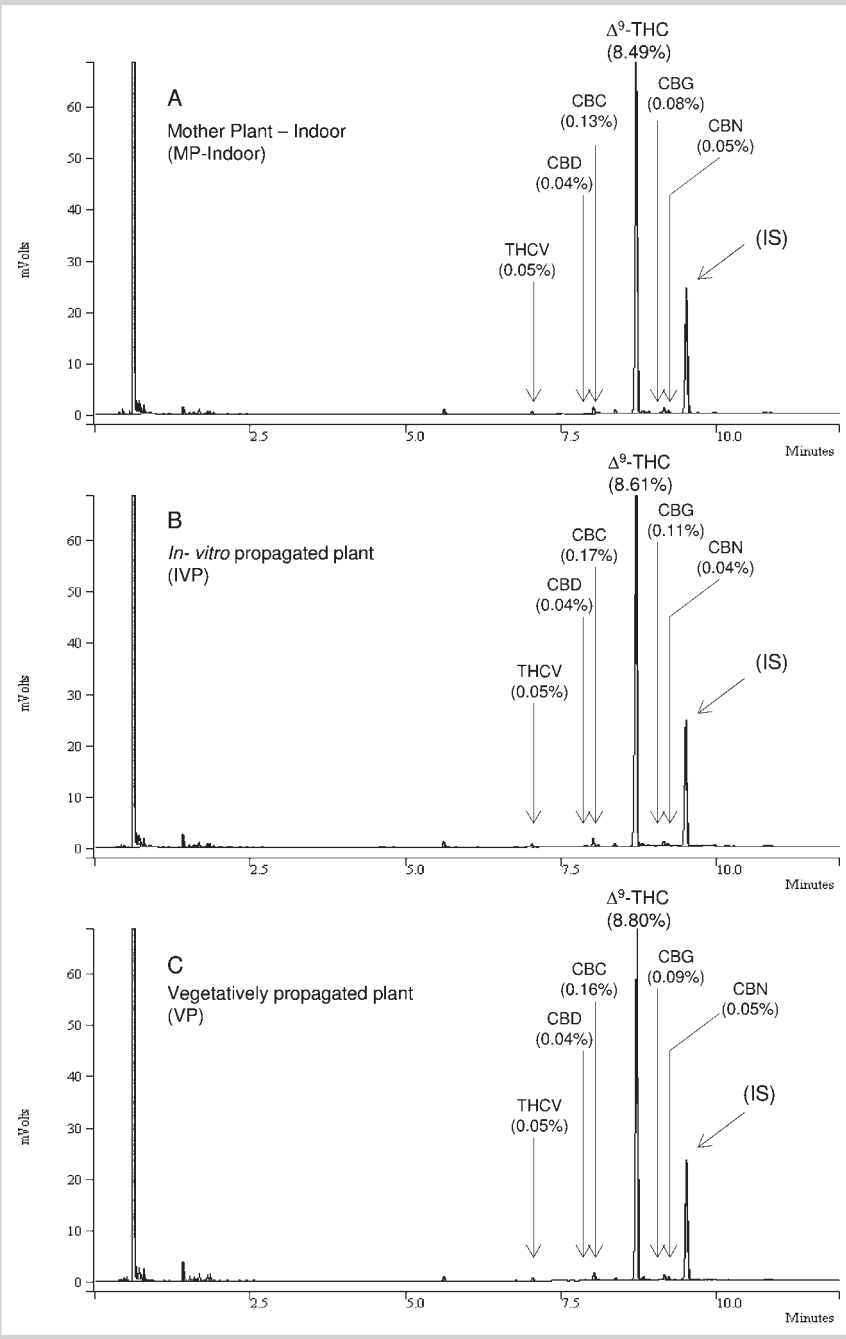

Fig. 3 Chromatograms from the gas chromatography-flame ionization detection (GC-FID) analysis of the mother plant (**A**), an *in vitro* propagated (IVP) plant (**B**) and a vegetatively propagated plant (VP) (**C**) of *Cannabis sativa*.

dium activated charcoal supplemented by different concentrations of IAA, IBA and NAA for rooting. It is important to mention that cuttings from mother plants were made for vegetative propagation (**Fig. 1 SD** Supporting Information) at the same time when the well-developed micropropagated shoots were transferred to rooting medium. Similar to *in vitro* propagated plants (IVP), vegetatively propagated cuttings (VP) were also kept under fluorescent lights (18-hour light cycle) for rooting and early growth. Highest percentage of rooting in micropropagated plants was achieved in half strength Murashige and Skoog (1/2 MS) salts with 500 mg/L activated charcoal supplemented with 2.5 µM indole-3-butyric acid (IBA, **Fig. 1SB** Supporting Information). **Figs. 1SA, 1SE** and **1SF** (Supporting Information) show the mother plant and well-developed *in vitro* propagated and vegetatively propagated plants from the same mother plant, respectively. A sample of apical plant part was taken at the peak vegetative stage from all the three groups of plants before switching lights to a 12-h photoperiod for flowering. Onset of flowering was observed within 15 days. Although no clear trend in early flowering was observed among the groups, MP-Indoor have shown the first few flowers on day 15 followed by IVP and VP propagated plants on day 17.

This study was initiated to determine whether *Cannabis* clones of a screened and selected high Δ^9-tetrahydrocannabinol (THC) yielding variety derived from *in vitro* micropropagated plants obtained following the protocol produced in our laboratory [19] will have similar chemical profile and cannabinoids content as that of vegetatively propagated plants from the same mother plant. In general, a problem that has been observed with the plants produced via *in vitro* propagation regimes is a high rate of

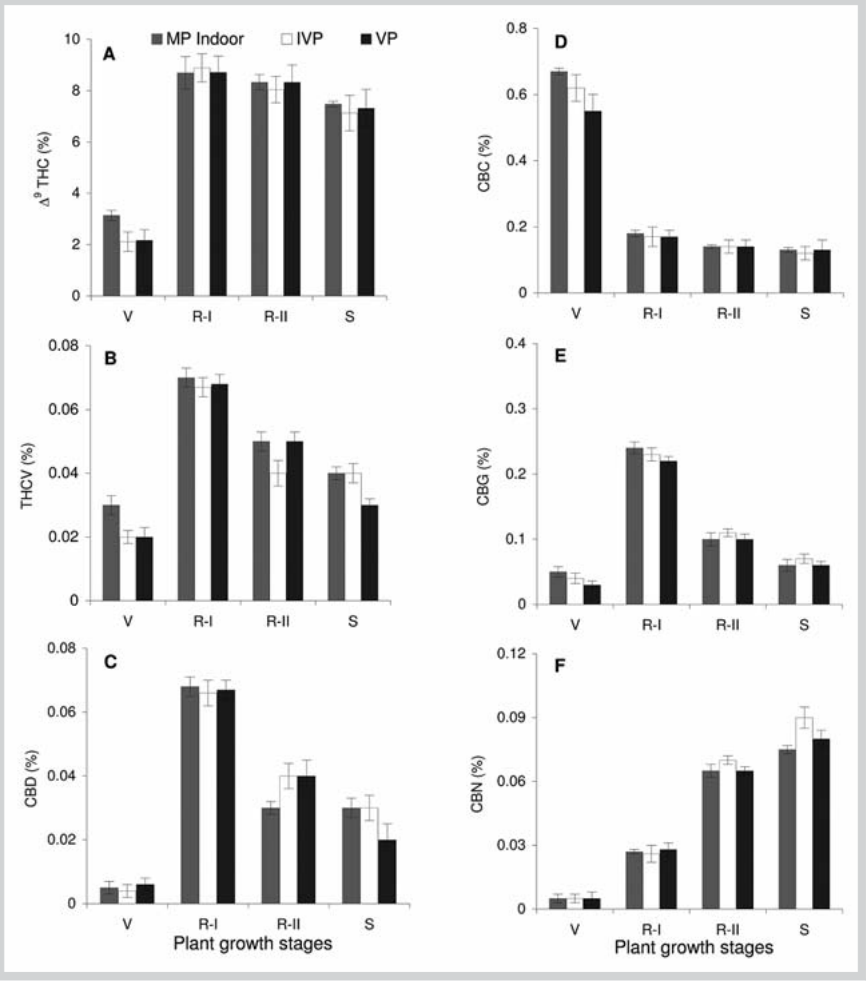

Fig. 4 Delta[9] THC (**A**), THCV (**B**), CBD (**C**), CBC (**D**), CBG (**E**) and CBN (**F**) content in *in vitro* propagated (IVP) plants (± SD, *n* = 27) of *Cannabis sativa* and their comparison with vegetatively propagated (VP) plants (± SD, *n* = 27) and mother (MP-Indoor) plant (± SD, *n* = 9) during different developmental stages of growth. V: peak vegetative stage, 15 weeks (105-day-old) plants; R-I: peak reproductive stage, 24-week-old plant; R-II: late reproductive stage, 26-week-old plant; S: onset of senescence: 28-week-old plant.

somatic mutation [24–27]. Because the major goal of our research was to develop an efficient *in vitro* propagation method for the mass production of high yielding *Cannabis* plants as a consistent source of biomass for the extraction of THC, we wanted to be certain that the method did not introduce mutations that could lead to alterations in metabolism. To evaluate whether such a mutation occurred, we used a genetic stability scheme to evaluate cannabinoids composition and content using GC-FID in the plants produced from *in vitro* propagation compared to plants produced through traditional vegetative propagation method at different stages of growth. **Fig. 3** shows chromatograms from the gas chromatography-flame ionization detection (GC-FID) analysis mother plant, *in vitro* (IVP) and vegetatively propagated (VP) plants of *Cannabis sativa*. Chemical profiles of IVP plants and VP plants were found to be identical to each other and to that of the mother plant. Similar results were reported on micropropagated and conventionally green house grown plants of *Zingiber officinale* (ginger) by Ma and Gang [28]. A report on the assessment of the genetic stability of micropropagated plants of *Cannabis sativa* using ISSR markers has already been reported by our group [29].

Variation in Δ^9-THC content during growth and developmental stages in IVP, VP and MP is shown in **Fig. 4A**. Δ^9-THC increased with plant growth, reached the highest level during the 24th week (8.70 ± 0.63, 8.89 ± 0.55, 8.71 ± 0.63 % in MP Indoor, IVP and VP plants, respectively) followed by a plateau for about two weeks (optimum harvest time for highest THC content) before the plants started showing a decline in Δ^9-THC content which is considered the onset of senescence of the plants.

Similar to THC, an increase in tetrahydrocannabivarin (THCV) and cannabidiol (CBD) content was observed in all groups of plants with plant growth (**Fig. 4B** and **4C**, respectively). Highest level of THCV (0.068 ± 0.003, 0.064 ± 0.003 and 0.067 ± 0.004 in MP Indoor, IVP and VP plants, respectively) and CBD (0.069 ± 0.003, 0.064 ± 0.004 and 0.063 ± 0.003 in MP Indoor, IVP and VP plants, respectively) content was observed during the peak reproductive stage followed by a sharp decline in all groups of plants. It was interesting to note that the plants from all three groups followed the same trend in THCV and CBD concentration with plant growth. Although few minor differences were observed in THCV and CBD content at vegetative, late-reproductive and onset of senescence stages of plants from the three different groups, no significant difference was found at peak reproductive stage. This shows a high level of homogeneity in these plants in terms of secondary metabolites.

A decline in cannabichromene (CBC) content was observed in all groups (MP, VP and IVP) of *Cannabis sativa* plants with plant age (**Fig. 4D**). However the decline was substantial (~ 73 %, 72 % and 69 % in MP, IVP and VP plants, respectively) during the vegetative to peak reproductive stage and was less pronounced during later growth stages. Variation in the CBG content during different growth stages in all three groups of plants are shown in **Fig. 4E**. The change in the concentration of CBG has followed a pattern similar to that of THC, THCV and CBD. The highest level of CBG

was observed at the peak reproductive period (0.241 ± 0.009, 0.234 ± 0.010 and 0.223 ± 0.007 in MP, IVP and VP plants, respectively) which was generally five times higher than at the peak of the vegetative stage. A reduction in CBG concentration was observed during late reproductive and senescence stages.

Contrary to CBC, a gradual increase in cannabinol (CBN) concentration was observed in all groups of plants with age, from vegetative to onset of senescence (**Fig. 4F**). Among the three groups, no significant difference in CBN content was observed at vegetative and reproductive stages, whereas during senescence a variation in IVP and VP plants was observed ($p = 0.05$). Interestingly, at this stage the difference between MP and other two types of plants was statistically insignificant. CBN content in all three types of plants (0.027 ± 0.001%, 0.026 ± 0.004% and 0.028 ± 0.003% in MP, VP and IVP, respectively) at the peak reproductive (harvest) stage was observed to be very close to each other.

A comparison of the cannabinoids content of the indoor grown plants (MP-Indoor, IVP and VP plants) with the MP-Outdoor (the source mother plant) reveals that MP-Outdoor had maintained a higher level of THC (13.64%) as compared to the indoor plants (MP-Indoor, ~ 8.70% and; IVP and VP plants, ~ 8.89 and 8.71%, respectively). In spite of the fact that the plants grown indoor vs. outdoor had the same genetic makeup, the differences in the concentrations of the secondary metabolites in indoor vs. outdoor plants may be explainable based on the differences in environmental conditions. While the light intensity outdoor during sunny-summer days in Mississippi is ~ 1500 µmolm^{-2} s^{-1}, the light intensity used indoors was only 700 ± 24 µmol m^{-2} s^{-1}. We have previously reported that higher photosynthesis and better growth of *Cannabis sativa* were observed under 30 °C and 1500 µmolm^{-2} s^{-1} light intensity [30].

In conclusion, the *in vitro* propagated plants were found to be comparable to the conventionally grown plants and mother plant, in terms of biochemical profile and yield of cannabinoids content. These results confirm the clonal fidelity of tissue culture raised plants of *Cannabis sativa* and suggest that the biochemical mechanism followed to produce the micropropagated plants does not affect the metabolic content and can be used to mass propagate true-to-type plants of this variety for commercial pharmaceutical use.

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