# Chapter 19

# In Vitro Propagation of *Cannabis sativa* L. and Evaluation of Regenerated Plants for Genetic Fidelity and Cannabinoids Content for Quality Assurance

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## Abstract

*Cannabis sativa* L. (Marijuana; Cannabaceae), one of the oldest medicinal plants in the world, has been used throughout history for fiber, food, as well as for its psychoactive properties. The dioecious and allogamous nature of *C. sativa* is the major constraint to maintain the consistency in chemical profile and overall efficacy if grown from seed. Therefore, the present optimized in vitro propagation protocol of the selected elite germplasm via direct organogenesis and quality assurance protocols using genetic and chemical profiling provide an ideal pathway for ensuring the efficacy of micropropagated *Cannabis sativa* germplasm. A high frequency shoot organogenesis of *C. sativa* was obtained from nodal segments in 0.5 μM thidiazuron medium and 95 % in vitro rhizogenesis is obtained on half-strength MS medium supplemented with 500 mg/L activated charcoal and 2.5 μM indole-3-butyric acid. Inter Simple Sequence Repeats (ISSR) and Gas Chromatography-Flame Ionization Detection (GC-FID) are successfully used to monitor the genetic stability in micropropagated plants up to 30 passages in culture and hardened in soil for 8 months.

**Key words** *Cannabis sativa* L., Micropropagation, Nodal explants, Genetic fidelity, ISSR markers, GC-FID

## 1 Introduction

*Cannabis sativa* L. is an open pollinated crop belonging to the family Cannabaceae. It is an important medicinal plant well known for its pharmacologic and therapeutic potency that contains a unique class of terpenophenolic compounds (the cannabinoids), which accumulates mainly in the glandular trichomes of the female plant [1]. At present, 104 cannabinoids have been identified from this plant but most of the biological and pharmacological activities are attributed to the psychoactive compound, $\Delta^9$-tetrahydrocannabinol (THC) [2]. Medicinally, THC possesses analgesic, anti-inflammatory, appetite stimulant, and antiemetic properties, making this cannabinoid a very promising drug for therapeutic purposes [3].

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_19, 

Due to the dioecious (male and female flowers on different plants) and allogamous (cross fertilization) nature of *C. sativa*, it is difficult to maintain the consistency in its chemical profile and cannabinoid content if grown from seeds. Since the seeds of *C. sativa* are highly heterozygous and are thus of limited interest for the conservation of particular genotypes, the use of plant tissue culture techniques offer advantages for multiplication and large-scale production of true-to-type *Cannabis* plants. Several medicinal plants such as *Rauvolfia tetraphylla* [4]; *Picrorhiza kurroa* [5]; *Withania somnifera* [6]; *Pterocarpus marsupium* [7]; *Acacia sinuata* [8]; *Cimicifuga racemosa* [9], and *Veronica anagallis-aquatica* [10] have been multiplied through various strategies of micropropagation. In addition to being an alternative means of plant improvement, micropropagation techniques can also potentially overcome many factors limiting traditional approaches to *C. sativa* improvement [11]. Research on in vitro regeneration of Cannabis has resulted in several protocols [12–16]; however, considerable variation has been reported in the response of cultures and in the morphogenic pathway. Since our goal is to develop a secure and stable *in vitro* clonal repository of elite medicinal plants germplasm that will assure future availability of desirable pharmacological active chemotypes, the present protocol has been developed covering the following objectives: (a) propagation of the quality plant material that will allow large-scale clonal production, and (b) comparison of micropropagated plants with the mother plant for consistency in terms of genetic and chemical profiles. This approach of propagation of *C. sativa* would provide a rapid and reliable system for the production of large number of genetically uniform disease-free plantlets and would be a promising tool for commercial level propagation of high-yielding elite varieties for the pharmaceutical use.

## 2 Materials

### 2.1 Equipment and Apparatus

1. Autoclaves.
2. Laminar flow hoods (*see* **Notes 1** and **2**).
3. Water demineralizer.
4. Water distilling unit.
5. Refrigerator.
6. Analytical balance to weigh from 0.1 or 0.01 mg to a few grams.
7. pH meter.
8. Stirrer with hot plate.
9. Water bath.
10. Vacuum pump.
11. Filter units, 0.22 μm.

**Table 1**
**Plant growth regulators used**

| Plant growth regulators | Solubility | Sterilization | Storage |
|---|---|---|---|
| *Auxin* | | | |
| Indole-3-butyric acid (IBA) | 1 N NaOH | Filter sterilize | 4 °C |
| *Cytokinin* | | | |
| Thidiazuron (TDZ) | 1 N NaOH | Filter sterilize | 4 °C |

### 2.2 Media Preparation

1. Murashige and Skoog (MS) Basal Salts Mixture.
2. Phytohormones (*see* Table 1).
3. pH buffers, 4.0, 7.0, and 10.0.
4. 1 N NaOH.
5. 1 N HCl.
6. Sterile beakers, 250 ml and 1000 ml (*see* **Note 3**).
7. Measuring cylinders 100, 250, and 1000 ml.
8. Micropipettes 20–1000 μl.

### 2.3 Sterilization and Establishment of In Vitro Cultures

1. 0.1 % Tween 20.
2. 0.5 % NaOCl (15 % w/v bleach).
3. Sterile distilled water.
4. Stainless steel forceps 8–12″.
5. Razor blade # 10 and scalpel.
6. Sterile petri dishes, 100 × 25 mm.
7. Culture sealant, Parafilm.
8. Culture vessels, baby food jars with Magenta B caps (4 cm diameter × 9.5 cm high).
9. Thermocol cups.
10. Coco Natural growth medium.
11. Sterile potting mix—Fertilome (Canna Continental).
12. Plastic pots 2″, 5″, 10″.
13. Incubator/tissue culture room with light and temperature control.

### 2.4 Quality Assurance of Micropropagated Plants: Assessing Genetic Fidelity

#### 2.4.1 Isolation of Plant Genomic DNA

1. Eppendorf tubes 2 ml.
2. Mixer Mill MM 2000.
3. DNeasy plant mini kit.
4. NanoDrop 1000 spectrophotometer.
5. Liquid nitrogen.

**Table 2**
**Nucleotides sequences of primers used for ISSR analysis**

| Primer | Sequence |
|---|---|
| UBC 807 | 5′-AGAGAGAGAGAGAGAGT-3′ |
| UBC 808 | 5′-AGAGAGAGAGAGAGAGC-3′ |
| UBC 811 | 5′-GAGAGAGAGAGAGAGAC-3′ |
| UBC 812 | 5′-GAGAGAGAGAGAGAGAC-3′ |
| UBC 817 | 5′-CACACACACACACACAA-3′ |
| UBC 826 | 5′-ACACACACACACACACC-3′ |
| UBC 834 | 5′-AGAGAGAGAGAGAGAGYT-3′ |
| (GGC)6W | 5′-GGCGGCGGCGGCGGCGGCW-3′ |
| (AAG)6Y | 5′-AAGAAGAAGAAGAAGAAGY-3′ |
| (GGAT)4H | 5′-GGATGGATGGATGGATH-3′ |
| (GGGGT)3M | 5′-GGGGTGGGGTGGGGTM-3′ |
| UBC 836 | 5′-AGAGAGAGAGAGAGAGYA-3′ |
| UBC 842 | 5′-GAGAGAGAGAGAGAGAYG-3′ |
| UBC 845 | 5′-CTCTCTCTCTCTCTCTRG-3′ |

6. 5 mg/ml ethidium bromide (EB) stock in water.
7. Agarose.
8. 6× loading dye.

*2.4.2 PCR Amplification*

1. Template DNA.
2. Taq DNA polymerase.
3. Deoxyribonucleoside triphosphates (dNTPs) mix (dATP; dTTP and dGTP; dCTP).
4. 10× polymerase buffer.
5. 1.5 mm $MgCl_2$.
6. Primers (*see* Table 2).
7. Polymerase chain reaction (PCR) tubes.
8. Milli-Q water.
9. Ice.
10. DNA ladder 1 Kb plus.
11. 2 % TAE agarose gel.
12. Bio-Rad gel imaging system.

*2.4.3 Assessment of the Cannabinoids Profile and Content*

1. Chloroform.
2. Methanol.
3. 4-androstene-3,17-dione.
4. Erlenmeyer flask 25 ml.
5. Metal sieve No. 14 (opening 0.0555 in.).
6. Screw cap vial, 3.7 ml.
7. GC vials 2 ml.
8. Pipettes 5–10 ml.
9. Micropipettes.

*2.4.4 Gas Chromatographic Analysis*

1. Column: DB-1, 15 m × 0.25 mm, with 0.25 μm film thickness.
2. Helium gas as carrier gas.
3. Varian CP-3380 Gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler.

## 3 Methods

Different steps involved in the micropropagation of *Cannabis sativa* through direct organogenesis are shown in Figs. 1 and 2.

***3.1 Media Preparation***

1. Prepare the Murashige and Skoog's medium [17] as per the directions on the bottle.
2. Add 3 % (w/v) sucrose. Make the final volume of culture medium as required. Set the pH 5.7.
3. Add 0.8 % (w/v) type E agar.
4. Autoclave the media at 121 °C at 105 kPa for 30–45 min (*see* **Notes 4, 5** and **6**).
5. Cool the medium and swirl the solution as it cools.
6. Add Murashige and Skoog vitamins.
7. For shoot multiplication semisolid medium (SM), add Thidiazuron (TDZ) at a concentration ranging from 0.05 to 9.0 μM.
8. For root induction, semisolid medium (RI), add the auxin indole-3-butyric acid (IBA) at 2.5–5.0 μM concentration supplemented with 500 mg/L activated charcoal.
9. Dispense the sterile medium (25 ml) in glass culture vessel (4 cm diameter × 9.5 cm height baby food jars with magenta B caps).

***3.2 Explant Preparation***

1. Prepare the explants by cutting 1.0–1.5 cm long nodal segments containing at least one axillary bud with the help of a sharp blade.
2. Thoroughly wash the explants in continuous flow of tap water for 1 h, followed by soaking in solution of 0.1 % Tween 20, then rinsing in tap water for 10 min.

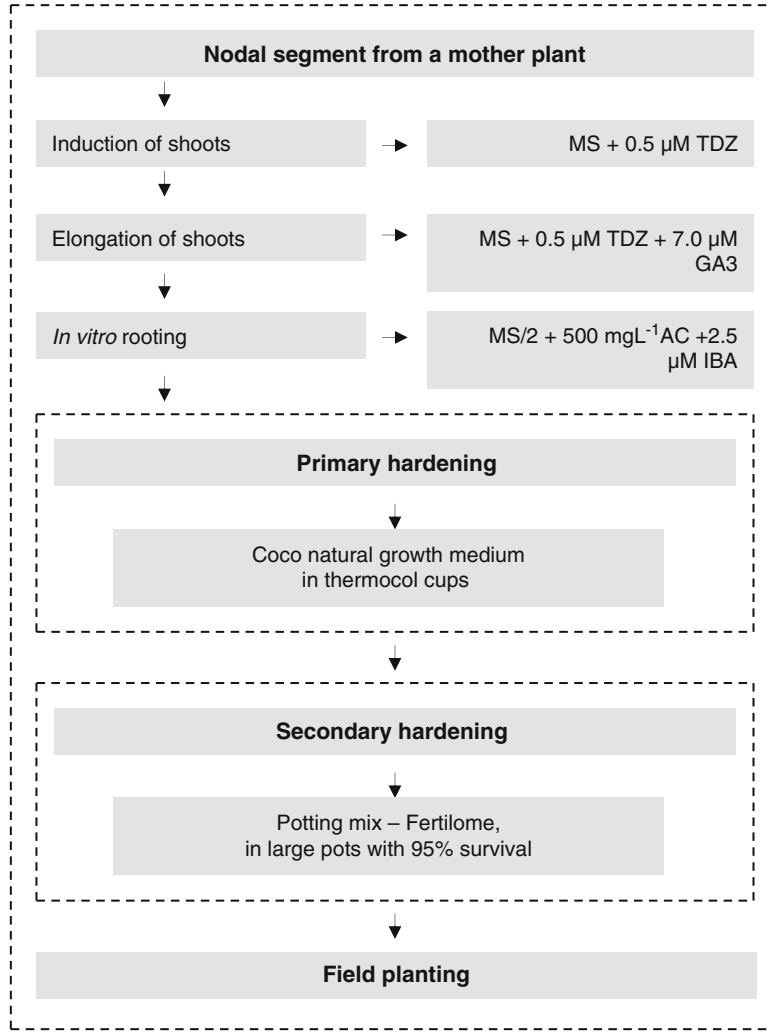


**Fig. 1** Schematic diagram of in vitro propagation of *Cannabis sativa*

### *3.3 Surface Sterilization and Establishment of In Vitro Cultures*

1. Sterilize the explants surface using 0.5 % NaOCl (15 % v/v bleach) and 0.1 % Tween 20 for 20 min under a laminar hood.
2. Wash the explants in sterile distilled water by rinsing 3 times for 5 min each.
3. Soak the explants in 0.1 % mercuric chloride for 2 min, followed by distilled water (*see* Subheading 4) (*see* **Note 7**).
4. Slice off the exposed end slightly using a sterilized blade prior to inoculation on the culture medium (*see* **Note 8**).
5. Inoculate single nodal explants in the culture vessel containing the SM media and incubate in the culture room at 25 ± 2 °C with 16-h photoperiod under fluorescent light with a photon flux of 52 μmol/$m^2$/s and 60 % relative humidity.

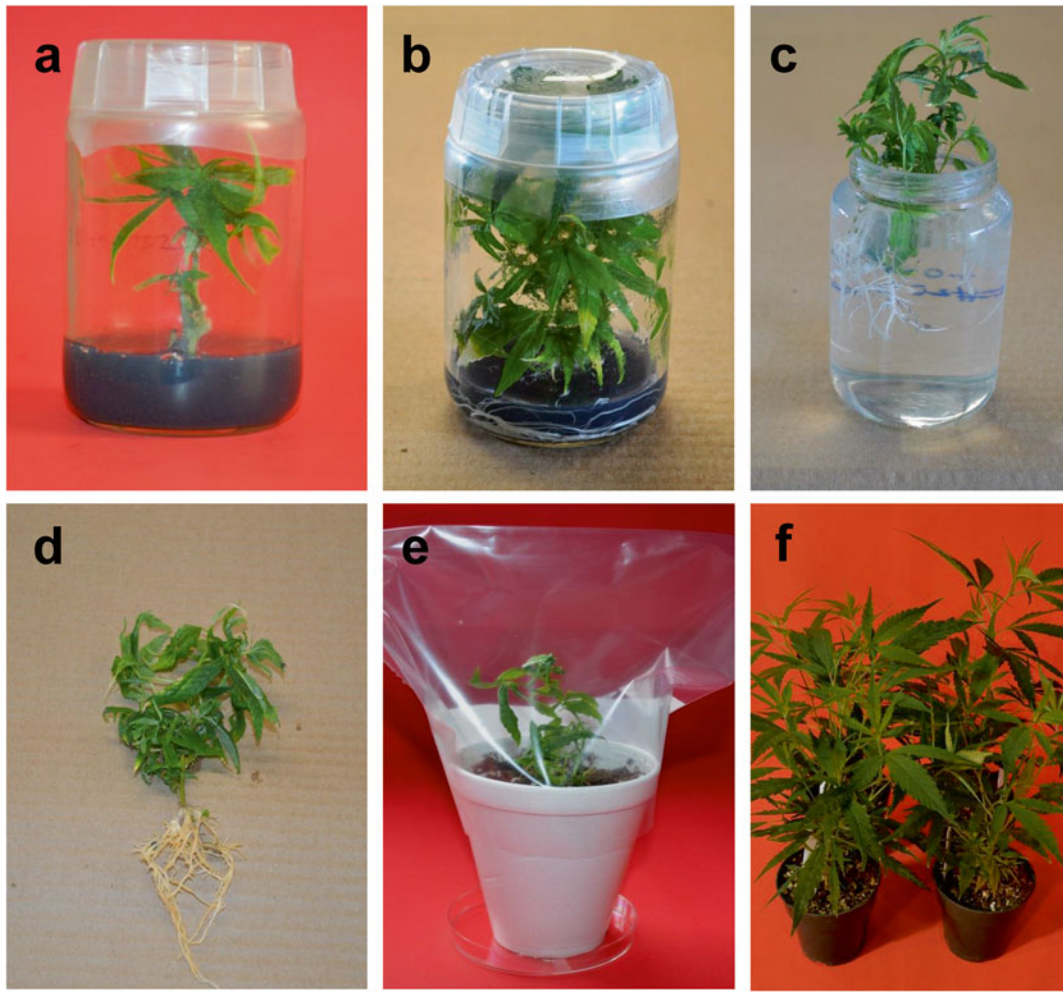


**Fig. 2** Micropropagation of *Cannabis sativa*. (**a**) Shoot formation on MS + 0.5 μM TDZ (**b**) Rooting on ½ MS medium supplemented with 500 mg/L activated charcoal and 2.5 μM IBA (**c**, **d**) Well-rooted plantlet (**e**) A well-rooted plant at soil acclimatization stage (**f**) Fully acclimatized *Cannabis sativa* plants

6. Observe the cultures weekly. Note any morphological changes and growth responses in the cultured tissue. Record the following data:
   (a) The time of emergence of shoots from axillary buds.
   (b) The frequency of axillary branches per responding culture.
   (c) The number of shoots per culture.
7. After in vitro establishment, these cultures can be multiplied by inoculating 2–3 nodal segments per vessel. The culture may be used as a source of inocula for further multiplication.

### 3.4 Root Induction

1. Transfer isolated or two shoots to semisolid root induction medium.
2. Observe the cultures weekly for root initiation. Record the following data:
   (a) The time of root emergence.
   (b) Frequency of shoot developing from roots derived from each explants.
   (c) The number of roots per shoot.
3. Carefully remove the in vitro-developed plantlets (Fig. 2) after 6 weeks and clean the roots very gently removing agar using tap water (*see* **Note 9**).

### 3.5 Acclimatization

1. Wash the rooted plantlets thoroughly in running water to remove all traces of medium.
2. Preincubate the plantlets in Coco Natural growth medium in Thermocol cups.
3. Cover the cups with polyethylene bags to maintain humidity and keep in the indoor growth room for at least 1 week (Fig. 2).
4. Transfer the in vitro-hardened plantlets in large pots containing sterile potting mix Fertilome. Keep the plantlets under similar environmental conditions maintaining 16-h photoperiod, 25–30 °C temperature, and 60 % relative humidity.
5. After 30–40 days as new leaves start appearing and the plants appear robust, transfer the plants to the field conditions (*see* **Note 10**).

### 3.6 Quality Assurance of Micropropagated Plants: Assessing Genetic Fidelity

#### 3.6.1 Isolation of Plant Genomic DNA

Total genomic DNA is extracted using a DNeasy Plant Mini Kit.

*Protocol for DNA Extraction using DNeasy Plant Mini Kit*

1. Finely powder the plant sample.
2. Weigh 20 mg (0.02 g) in a preweighed 2 ml sterile tube. Immediately put the tube on ice (crushed). Powder sample in a mixer mill (30 s., amplitude max = 100).
3. Add 600 μl API buffer into powder sample tube. Vortex upside and down (both sides) 15 s. Add 4 μl RNase A and vortex for 20–30 s.
4. Incubate the mixture for 10 min at 65 °C. Mix 2 or 3 times during incubation by inverting tubes.
5. Add 195 μl AP2 Buffer to the lysate and vortex for 20–30 s. Incubate on ice for 5 min.
6. Centrifuge the lysate for 5 min at 18,000 × *g*.
7. Pipette the lysate into QIA shredder mini spin column (purple/lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at 14,000 rpm.

8. Transfer the flow-through from the previous step into a new 2 ml tube without disturbing the cell debris pellet. (At this point check the amount of lysate obtained after centrifugation).
9. For 500 µl lysate obtained, add 750 µl AP3 Buffer. Mix by pipetting.
10. Use the white DNeasy Mini Spin column for this step. Pipette 650 µl of the mixture (may include any precipitate), into the DNeasy Mini Spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 800 rpm. Discard the flow-through. Reuse the collection tube for the next step.
11. Repeat the previous step with the remaining sample. Discard the flow-through and collection tube.
12. Place the DNeasy Mini Spin column into a new 2 ml collection tube.
13. Add 500 µl AW Buffer. Centrifuge for 1 min at 800 rpm. Discard the flow-through and reuse the collection tube for the next step.
14. Add 500 µl AW Buffer to DNeasy Mini Spin columns. Centrifuge for 2 min at 14,000 rpm (to dry the membrane). Discard the flow-through and reuse the collection tube.
15. Add 500 µl AW Buffer to DNeasy Mini Spin columns. Centrifuge for 2 min at 14,000 rpm (to dry the membrane). Discard the flow-through and reuse the collection tube.
16. Transfer the DNeasy Mini Spin column to a 1.5 ml microcentrifuge tube (let the tube be open). Pipette 50 µl AE Buffer onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25 °C), centrifuge for 1 min at 8000 rpm to elute.
17. Again pipette 50 µl AE Buffer to the membrane. Incubate for 5 min at room temperature. Centrifuge for 1 min at 8000 rpm to elute.
18. DNA is obtained (about 100 µl) in an eppendorf tube. Store on ice and switch to –80 °C.

### *3.6.2 Quantification of DNA*

1. The DNA can be quantified by running on 1 % agarose gel and checking the absorbance at 260 nm (*see* **Note 11**).
2. Agarose gel: Mix 2 ml 50× TAE Buffer to a final volume of 100 ml. Add 1 g agarose, boil in a microwave, and cool to 50–60 °C. Carefully add EB. Seal the free ends of gel tray, fix the combs, and dispense the molten gel. Allow it to solidify. Remove the comb and put the gel tray in the gel reservoir containing 1× TAE Buffer. Make sure the gel is fully submerged (*see* **Note 12**).

3. Mix the DNA sample, loading 6× dye, and Milli-Q water (1 + 2 + 9 μl) by repeated pipetting. Load in the wells of gel carefully.
4. Close the lid of the gel reservoir and turn on the power supply. The gel runs from the negative pole (black) towards positive pole (red). Check after few minutes if the gel is running. Check the gel regularly to prevent the samples from running off the gel (*see* **Note 13**).
5. For PCR, 20 ng amount of DNA is sufficient per reaction and therefore dilution of DNA should be made with sterile Milli-Q water in such a way that 1 μl contains approximately 20–25 ng of DNA (*see* **Notes 14** and **15**).

*3.6.3 PCR for DNA Amplification*

1. Amplification reaction is performed using MJ Research PTC-225 gradient cycler.
2. PCR is carried out in a total volume of 25 μl for each reaction in 0.2 ml PCR tube.
3. Set up the PCR reaction mixture using 14 primers (*see* Table 2), based on the criterion of the generation of distinct bands that are reproducible between samples. Each PCR reaction contains 0.1 μM of each primer, 1 unit Platinum Taq DNA polymerase, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 20 ng template DNA, and PCR buffer (*see* **Note 16**).
4. Taq polymerase should be added at the end.
5. All this should be carried out in ice.
6. Transfer this reaction mixture in PCR tube and spin it for few seconds for uniform mixing.
7. Carry out the PCR in thermal cycler using the following conditions:
   (a) Initial denaturation at 94 °C for 3 min.
   (b) Followed by 45 cycles each consisting of a denaturation step at 94 °C for 30 s, primer annealing step at 50 °C for 30 s, and amplification at 72 °C for 3 min.
   (c) Final extension at 72 °C for 7 min followed by arresting the reaction at 4 °C for infinite period.
8. Load the amplified DNA on 2 % agarose gel in 1× TAE Buffer stained with 0.5 μg/ml EB. Scan the gel with Bio-Rad Gel Imaging System and analyze with Quantity One Analysis Software Version 4.3.0 (*see* **Notes 17** and **18**).
9. Run the amplified products on the gel with molecular size standard 1 kb plus DNA ladder.
10. Score only the well separated bands in size range of 0.1–3.0 kb as present or absent for ISSR markers.

### 3.7 Quality Assurance of Micropropagated Plants: Cannabinoids profile and Content

#### 3.7.1 Harvesting

1. Take the biomass samples from apical segments (flowering buds) of the mother plant, in vitro-propagated and vegetatively propagated plants in triplicates.
2. Dry the samples at 120 °F in oven overnight and individually manicure by hand.

#### 3.7.2 Extraction

1. Manicure all the samples in a 14 mesh (0.0555 in. opening) metal sieve to remove seeds and stems.
2. Extract the powdered/sieved material (triplicated samples 0.1 g sample each) with 3 ml of internal standard/extracting solution (100 mg of 4-androstene-3, 17-dione + 10 ml chloroform + 90 ml ethanol) at room temperature for 1 h.
3. Withdraw the extracts into disposable transfer pipettes through cotton plugs for filtration and transfer into GC vials, cap the vials, and place on auto sampler. This extract can be used for quantitative analysis of the cannabinoids through GC/FID.

#### 3.7.3 Gas Chromatography-Flame Ionization Detection (GC-FID)

1. Quantitative estimation of cannabinoids is carried out by gas chromatography analyses following Ross et al. [18] using Varian CP-3380 gas chromatograph, equipped with Varian CP-8400 automatic liquid sampler, a capillary injector, and a dual flame ionization detector (*see* **Note 19**).
2. The column used is 15 mm × 0.25 mm DB-1, 0.25 μ film
3. Data is recorded using a Dell OptiPlex GX1 computer and Varian Star workstation software (version 6).
4. Helium is used as a carrier gas and detector makeup gas, with an upstream indicating moisture trap and a downstream indicating oxygen trap.
5. The following parameters are used:
   (a) Air—30 psi (400 μl/min).
   (b) Hydrogen—30 psi (30 ml/min).
   (c) Column head pressure—14 psi (1.0 ml/min).
   (d) Split flow rate—50 ml/min.
   (e) Split ratio—50:1.
   (f) Septum purge flow rate—5 ml/min.
   (g) Makeup gas pressure—20 psi (20 ml/min).
   (h) Injector temperature—240 °C.
   (i) Detector temperature—260 °C.
   (j) Initial oven temperature—170 °C.
   (k) Initial temperature hold time—1 min.
   (l) Temperature rate—10 °C/min.

(m) Final oven temperature—250 °C and final temperature hold time—3 min.

6. The concentration of a specific cannabinoid is calculated as follows:

$$\text{Cannabinoid}(\%) = \{\text{GC area}(\text{cannabinoid}) / \text{GC area}(\text{ISTD})\} \times \{\text{Volume}(\text{ISTD}) / \text{Amount}(\text{sample})\} \times 100$$

## 4 Notes

1. It is very important to work on a surface that is free of dust and other potential contaminants.
2. Regularly check the air flow gauge of laminar air flow chamber. Switch on the UV light for 30 min. Before starting any activity in the laminar air flow bench, wipe the surface of the laminar air bench with alcohol frequently during any aseptic operation. Fumigate the entire transfer room.
3. Before using, soak all the glassware overnight in Clorox solution and powdered detergent. Thoroughly wash with tap water to remove the last trace of detergent. Finally, rinse glassware with double distilled demineralized water and dry in hot air oven at 150 °C for 2 h.
4. While autoclaving, allow sufficient air volume in the flask to prevent the media from boiling over.
5. Make sure that the volume of the autoclaved medium plus the volume of the added filter-sterilized compound sum to the final volume of the prepared medium.
6. The standard conditions for autoclaving media are 121 °C at 105 kPa for 20 min. For larger volumes the time should be increased: for 500 ml, 30 min; for 1000 ml, 40 min. Prior testing of the 40-min autoclaving is recommended, unless sugars are autoclaved separately.
7. Avoid contact of body parts with $HgCl_2$, β-mercaptoethanol, and EB as all these chemicals are highly mutagenic, carcinogenic, and hazardous. As a precaution, wear gloves while handling and dispose of the chemicals properly!
8. Possible source of danger exists if a person, after flaming an instrument reinserts the hot instrument into the alcohol dip. *Caution*: Ethanol is flammable! One should be very careful.
9. In vitro roots are delicate, so do not let them break.
10. Disposal of the cannabis plant material should be done strictly following DEA regulations.
11. Alternatively, DNA yield may also be checked spectrophotometrically.

12. While preparing the agarose gel, take care that the final volume should never be reduced due to evaporation during boiling. Take care: do not entrap any air bubble.
13. Clean the gel tray, gel reservoir, combs, and other materials with ethanol properly before and after use.
14. While loading the sample, do not let the sample spill out. Wear gloves during the entire operation and prevent contamination.
15. Pay attention to the quality of DNA-band when there are many fragments.
16. To minimize the error and for convenience mix all the dNTPs in equal amounts and make a stock in advance, and then take 1 μl for each PCR reaction.
17. If the DNA does not dissolve at room temperature or at 37 °C, incubate at 65 °C for 1 h.
18. Insufficient denaturing of the target genomic DNA in the initial cycle is a common cause of PCR reaction failure. The incubation time for primer extension at 72 °C varies according to the length of the target DNA sequence to be amplified. For most purposes, 2 min is usually sufficient.
19. The method used for the analysis should be precise, accurate, robust, and validated. The general steps of chromatography should be followed. Carefully record all the observations of the experiment.

## Acknowledgements

This work was supported in part through the National Institute on Drug Abuse (NIDA), National Institute of Health (NIH), Department of Health and Human Services, USA, contract no. N01DA-10-7773.

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