Assessment of the Genetic Stability of Micropropagated Plants of *Cannabis sativa* by ISSR Markers

Hemant Lata[1], **Suman Chandra**[1], **Natascha Techen**[1], **Ikhlas A. Khan**[1,2], **Mahmoud A. ElSohly**[1,3]

[1] National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS, USA

[2] Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, MS, USA

[3] Department of Pharmaceutics, School of Pharmacy, The University of Mississippi, University, MS, USA

Abstract

Inter-simple sequence repeat (ISSR) markers were used to evaluate the genetic stability of the micropropagated plants of *Cannabis sativa* over 30 passages in culture and hardening in soil for 8 months. A total of 15 ISSR primers resulted in 115 distinct and reproducible bands. All the ISSR profiles from micropropagated plants were monomorphic and comparable to mother plants, confirming the genetic stability among clones and mother plants. Chemical analysis of cannabinoids, using gas chromatography/flame ionization detection (GC/FID), was done to further confirm whether the qualitative and quantitative differences in the major secondary metabolites exist between the mother plant and micropropagated plants. Six major cannabinoids – Δ^9-THC, THCV, CBD, CBC, CBG, and CBN – were identified and compared with the mother plant. Our results clearly showed a similar cannabinoid profile and insignificant differences in THC content between the two types of plants. These results suggest that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and applicable for clonal mass propagation of *C. sativa*.

Key words

Cannabis sativa · Cannabaceae · gas chromatography · genetic stability · ISSR · micropropagation

Cannabis sativa L. (Cannabaceae) is an important multi-use plant, valued all over the world for yielding fiber and food, as well as a psychoactive drug. Besides its psychoactivity, the major biologically active compound, Δ^9-tetrahydrocannabinol, commonly referred as THC, possesses analgesic, anti-inflammatory, appetite-stimulant, and anti-emetic properties, making this compound a very promising drug for therapeutic purposes, especially for cancer and AIDS patients [1] (**Fig. 1**).

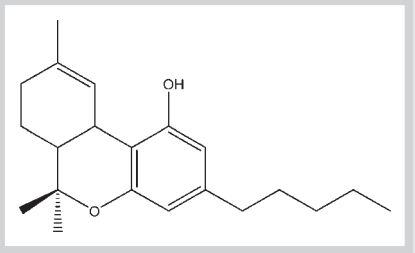

Fig. 1 Chemical structure of Δ^9- tetrahydrocannabinol (THC).

The maintenance of clonal fidelity is an important issue in developing a secure and stable *in vitro* clonal repository of elite *C. sativa* germplasm. *In vitro* culture techniques provide an important means of plant propagation and a tool for crop improvement [2]. However, the occurrence of somaclonal variation is a potential drawback when the propagation of an elite germplasm, where clonal stability is required to maintain the advantages of the desired elite genotypes, is intended. Although plant regeneration protocols have been developed for different *Cannabis* genotypes and explant sources [3–9], considerable variation has been reported in the response of cultures and in the morphogenic pathway.

Our previous work permitted us to establish the optimal conditions for high-frequency plant regeneration of *Cannabis sativa* L. through organogenesis [10]. Of all the cytokinins tested – benzyladenine (BA), kinetin (Kn), and thidiazuron (TDZ), ranging from 0.05 to 9.0 µM or in combination with gibberellic acid (GA_3) – the quality and quantity of regenerants was best using thidiazuron (0.5 µM) with subculturing done every three weeks. Elongated shoots resulted in 95% rooting when transferred to half-strength MS medium supplemented with 500 mg/L activated charcoal and 2.5 µM indole-3-butyric acid. After 30 passages in culture, the rooted plants were successfully transferred, acclimatized, and hardened in soil under controlled growroom conditions (temperature 25 ± 3 °C, relative humidity 60 ± 5%, and light intensity 700 ± 24 µmol/m^2/s at plant canopy level). To date, very little is known about the genetic stability in *Cannabis* culture and, especially, in regenerated plants. Moreover, no study has evaluated the genetic stability of the micropropagated plants of *C. sativa*. Therefore, in the present investigation we report for the first time the results of molecular analysis, based on inter-simple sequence repeat (ISSR) markers, aimed at assessing the effects of *in vitro* culture on the genetic homogeneity or heterogeneity of plants regenerated through organogenesis from a high-yielding *Cannabis sativa* variety (MX-1).

Molecular studies using ISSR markers are well accepted in *Cannabis* research. In a preliminary work conducted with three strains of *C. sativa* from different sources, Kojoma et al. [11] reported that different samples were identified by means of ISSR. Furthermore, Hakki et al. [12] used ISSR to separate and discriminate the hemp from *C. sativa*. However, no reports exist for monitoring the genetic stability of the *in vitro* regenerated plants of *C. sativa*. DNA extraction by the DNeasy Plant Mini Kit from Qiagen permitted us to obtain DNA of a quality and quantity that was adequate for ISSR analysis. A total of 18 ISSR primers were initially screened with the DNA of a single donor plant of *C. sativa* and 11 daughter plants as templates. Based on a criterion of the generation of distinct bands that were completely reproducible between the samples, 15 of the 18 primers were selected as suitable primers for *C. sativa* and used for the present study. Three primers generated either no bands or non-scorable bands (**Table 1**).

We carried out ISSR analysis on the DNA of 11 micropropagated and hardened plants, along with the donor mother plant of *C. sativa* with each of the 15 selected primers (**Table 1**). Under growroom conditions, the 11 selected plants showed no discernible differences from the donor plant in general morphology based on several traits, including plant height, branching pattern, and leaf shape. Each tested primer produced clear and scorable amplification products in all the plants. Each primer produced a unique set of amplification products ranging in size from about 177 bp in

Primer	Sequence	Range of amplicons (bp)	Total no. of bands
UBC 807	5′-AGAGAGAGAGAGAGAGT-3′	202–781	4
UBC 808	5′-AGAGAGAGAGAGAGAGC-3′	243–1364	9
UBC 811	5′-GAGAGAGAGAGAGAGAC-3′	269–1823	10
UBC 812	5′-GAGAGAGAGAGAGAGAA-3′	231–1306	9
UBC 817	5′-CACACACACACACACAA-3′	334–1347	8
UBC 826	5′-ACACACACACACACACC-3′	408–2751	9
UBC 834	5′-AGAGAGAGAGAGAGAGYT-3′	**177**–1478	12
(GGC)6 W	5′-GGCGGCGGCGGCGGCGGCW-3′	272–2295	7
(AAG)6Y	5′-AAGAAGAAGAAGAAGAAGY-3′	305–1095	7
(GGAT)4H	5′-GGATGGATGGATGGATH-3′	350–**2995**	8
(GGGGT)3 M	5′-GGGGTGGGGTGGGGTM-3′	343–2009	10
UBC 836	5′-AGAGAGAGAGAGAGAGYA-3′	218–1296	6
UBC 842	5′-GAGAGAGAGAGAGAGAYG-3′	203–677	4
UBC 845	5′-CTCTCTCTCTCTCTCTRG-3′	182–1501	4
UBC 856	5′-ACACACACACACACACYA-3′	430–1342	8
UBC 810	5′-GAGAGAGAGAGAGAGAT-3′	–	0
UBC 857	5′-ACACACACACACACACYG-3′	–	0
(GTA)6 M	5′-GTAGTAGTAGTAGTAGTAM-3′	–	0
			Total: 115

Note: Numbers highlighted in **bold** represent the minimum and maximum size of base pairs

Table 1 ISSR banding pattern of micropropagated and mother plants of *C. sativa*.

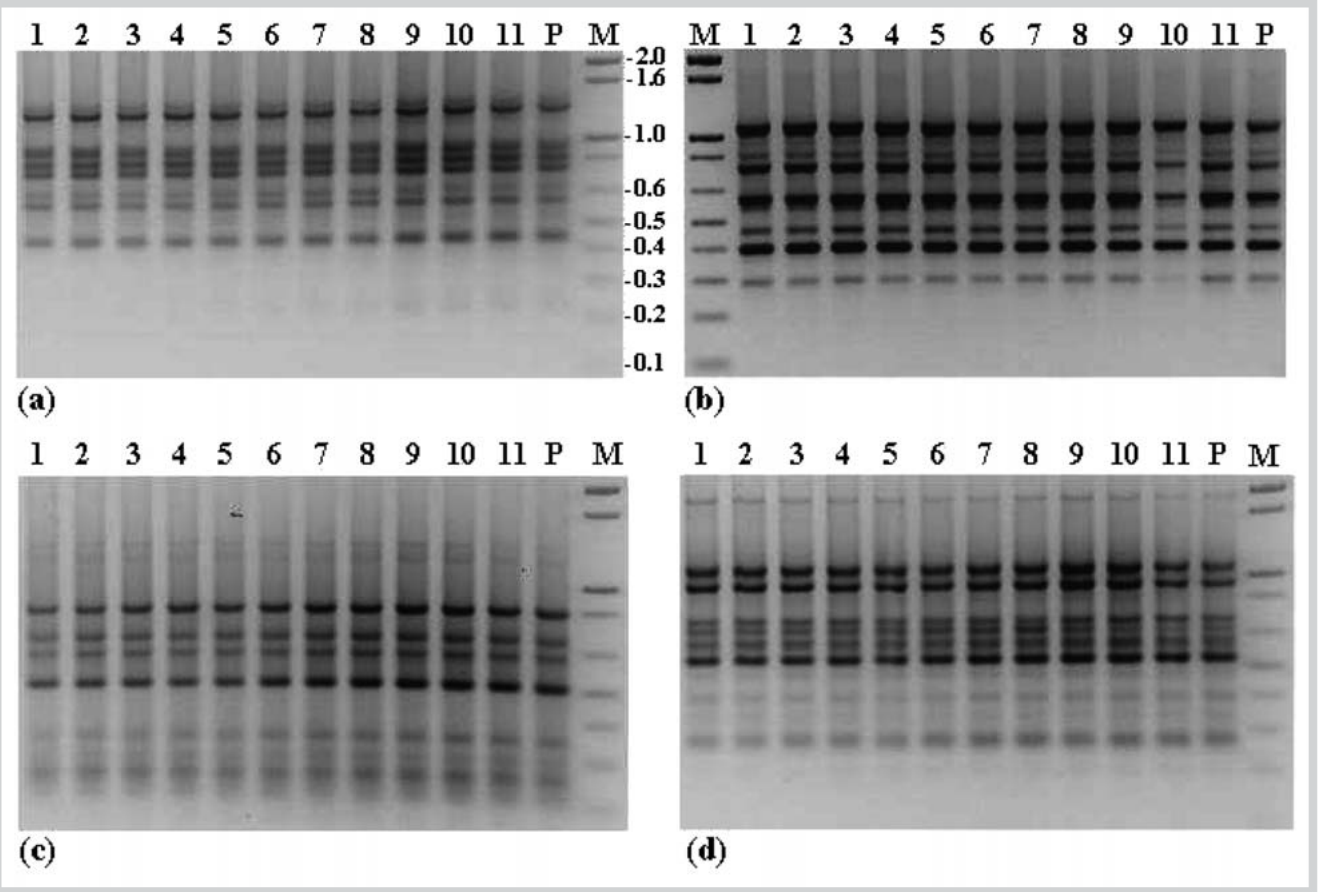

Fig. 2 Inter-simple sequence repeat (ISSR) amplification pattern obtained for mother plant (P) and daughter/micropropagated plants (1–11) using primer UBC 856 (**a**), primer (AAG)6Y (**b**), primer UBC 808 (**c**), and primer UBC 811 (**d**).

UBC 834 to 2995 bp in $(GGAT)_4H$ (**Table 1**), with an average of 7.7 bands per primer. A total of 1380 bands (the number of plantlets analyzed multiplied by the number of bands with all primers) were generated by the ISSR method, giving rise to monomorphic patterns across all 12 plantlets analyzed. No ISSR polymorphism was observed in the micropropagated plants (**Fig. 2**).

The number of primers (15) used in this study as well as the total number of bands (1380), together with the observed normality and homogeneity of plantlets after 30 passages of *in vitro* multiplication and hardening in soil for up to 8 months, strongly suggest that propagation through axillary buds ensures maintenance of genomic integrity in *C. sativa* clonal propagation. These

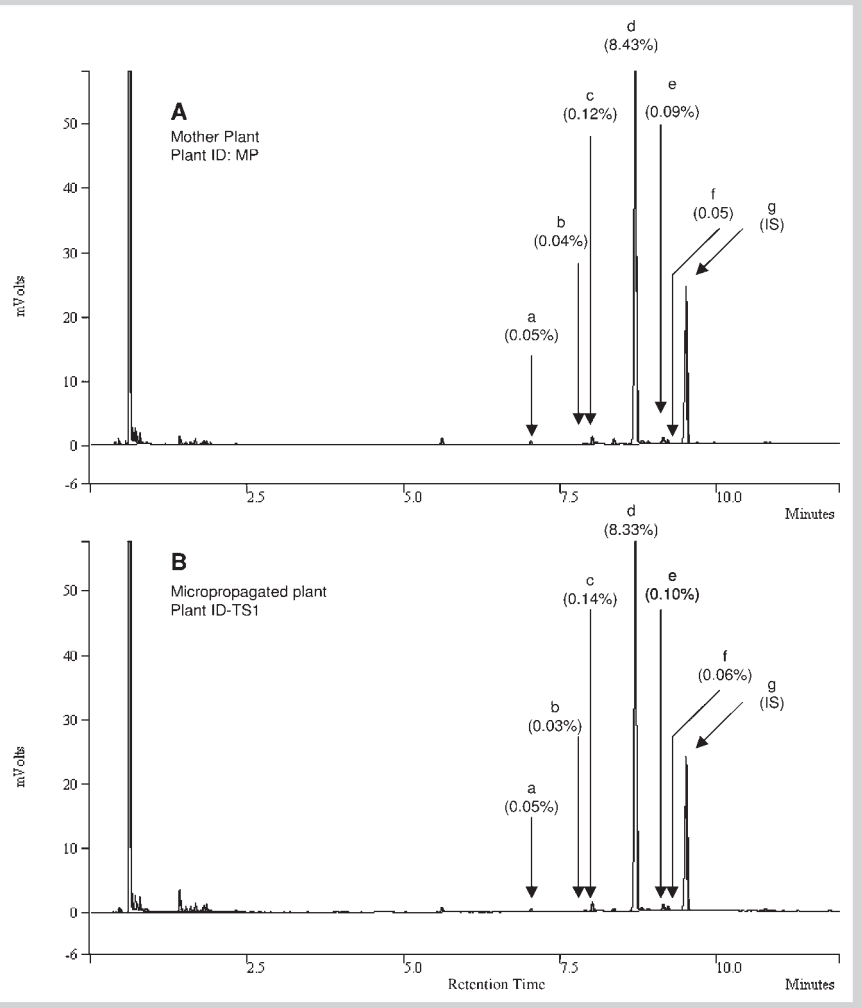

Fig. 3 GC-FID analysis (using 15 m × 0.25 mm DB-1, 0.25-mm film column at 170 °C for 1 min and then increased to 250 °C by 10 °C/min) of mother plant (**A**, plant ID: MP) and a representative *in vitro* propagated plant (**B**, plant ID: TS1) of *Cannabis sativa* at maturity stage. a: Tetrahydrocannabivarin (THCV); C19H26O21; MW, 286; retention time (RT), 7.022 min. b: Cannabidiol (CBD); C21H30O2; MW, 314; RT, 7.914 min. c: Cannabichromene (CBC); C21H30O2; MW, 314; RT, 8.008 min. d: D9-tetrahydrocannabinol (D9-TCH); C21H30O21; MW, 314; RT, 8.700 min. e: Cannabigerol (CBG); C21H32O2; MW, 316; RT, 9.140 min. f: Cannabinol (CBN); C21H26O2; MW, 310; RT, 9.212. g: Internal standard (IS). Chemical profiles of other micropropagated plants (plant IDs TS2–TS11) were also found to be identical to that of the mother plant.

results are supported by Lata et al. [10], who reported similarities in growth and photosynthetic characteristics among mother and micropropagated plants. Furthermore, our results from GC analysis showed homogeneity in the cannabinoid profile of the mother plant and micropropagated plants (**Fig. 3**). THC content in the mature buds of micropropagated *Cannabis sativa* plants were found to be comparable to each other and to that of the mother plant. An average of 8.46% THC content was found in the mother plant, and 8.34%, 8.93%, 9.02%, 9.02%, 8.12%, 8.46%, 8.54%, 9.08%, 9.05%, 8.53%, and 9.02% THC content was found in micropropagated plants TS1–TS11, respectively. Similar to these results, Ma and Gang [13] have also reported that the micropropagated plants of *Zingiber officinale* produce same metabolites (qualitative) at approximately the same concentration (quantitative) as corresponding non-*in vitro* propagated plants.

Because all the ISSR-based bands were monomorphic and because there were no variations detected in the micropropagated plants compared with the mother plant, indicating high genetic stability among the clones, we concluded that our micropropagation protocol for an elite *Cannabis sativa* variety (MX-1) can be carried out for a considerable length of time without much risk of genetic instability. Furthermore, ISSR can be used as a tool for testing the quality of micropropagated plants. This study is of particularly high significance because these plants have been selected to be used in the mass propagation of *Cannabis sativa* for the production of biomass, as a starting material for the isolation of THC as a high-value bulk active pharmaceutical.

Materials and Methods

High-frequency shoot regeneration using nodal segments containing axillary buds from a one-year-old mother plant of a high-yielding *C. sativa* variety (MX-1) was achieved on Murashige and Skoog (MS) medium [14] containing 3% (w/v) sucrose and 0.8% (w/v) type-E agar supplemented with 0.5 µM thidiazuron (TDZ) and adjusted to pH 5.7, following Lata et al. [10]. Eleven micropropagated, hardened plants and a mother plant grown in an indoor cultivation facility housed at Coy-Waller Laboratory, University of Mississippi, were used for analysis of genetic stability using ISSR markers.

A 20-mg leaf sample was frozen in liquid nitrogen and ground in a 2.0-mL microcentrifuge tube using Mixer Mill MM 2000 (Retsch). Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen; cat no. 659104) and resuspended in 50 µL elution buffer provided by the kit. Purified total DNA was quantified and its quality verified by using a Thermo Scientific NanoDropTM 1000 spectrophotometer. In the initial screening, 18 primers (13 from the University of British Columbia and 5 of our design) were used for ISSR analysis (**Table 1**). PCR amplifications were performed in a volume of 25 µL. Each PCR reaction contained

0.1 µM of each primer, 1 unit of Platinum Taq DNA Polymerase (cat no. 10966–034; Invitrogen), 200 µM of each dNTP (Promega), 1.5 mM $MgCl_2$, 20 ng template DNA, and 1 × PCR buffer. Amplifications were carried out in an M&J Research PTC-225 gradient cycler with a heated lid programmed at 94 °C for 3 min for initial denaturation, followed by 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min for 45 cycles and then a final extension step at 72 °C for 7 min. After amplification, each PCR reaction was analyzed by electrophoresis on a 2% TAE agarose gel and visualized under UV light. Gels were scanned with a Bio-Rad Gel Imaging System and analyzed with Quantity One analysis software version 4.3.0 (Bio-Rad Laboratories, Inc.). The sizes of the PCR products were compared with the molecular size standard 1 kb plus DNA ladder (cat no. 10787–018; Invitrogen). Only well-separated bands in the size range of 0.1–3.0 kb with high intensity were scored as present or absent for ISSR markers. Data were scored as 1 for the presence and 0 for the absence of DNA bands in each micropropagated and mother plant.

All marijuana samples were extracted following Ross et al. [15]. Gas chromatography analysis was performed using a Varian CP-3380 gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler under the following conditions: column: DB-1; 15 m × 0.25 mm, with 0.25 µm film thickness (J&W Scientific, Inc.); temperature: 170 °C for 1 min, then increased to 250 °C by 10 °C/min; injection temperature: 240 °C; detector temperature: 260 °C; carrier gas: helium at approximately 1 mL/min; detector-flame ionization detector (FID) with halogen flow rate of 30 mL/min and air flow rate of 300 mL/min. Statistical analysis was performed with the SYSTAT software package (SYSTAT Software, Inc.) to assess the relationship between studied traits (mother plant; micropropagated plants TS1–TS11 and chemical content).

Acknowledgements

The work was supported in part by the National Institute of Drug Abuse (NIDA) (Contract no. N01DA-0–7707).

References

1 *Sirikantaramas S, Taura F, Morimoto S, Shoyama Y.* Recent advances in *Cannabis sativa* research: biosynthetic studies and its potential in biotechnology. Curr Pharm Biotechnol 2007; 8: 237–243
2 *Vasil IK.* Progress in the regeneration and genetic manipulation of cereal crops. Biotech 1988; 6: 397–402
3 *Fisse J, Braut F, Cosson L, Paris M.* Etude *in vitro* des capacities organogenetiques de tissues de *Cannabis sativa* L. Effet de differentes substances de croissance. Planta Med 1981; 15: 217–223
4 *Loh WHT, Hartsel SC, Robertson W.* Tissue culture of *Cannabis sativa* L. and *in vitro* biotransformation of phenolics. Z Pflanzenphysiol 1983; 111: 395–400
5 *Richez-Dumanois C, Braut-Boucher F, Cosson L, Paris M.* Multiplication vegetative *in vitro* du chanvre (*Cannabis sativa* L.). Application a la conservation des clones selections. Agronomie 1986; 6: 487–495
6 *Mandolino G, Ranalli P.* Advances in biotechnological approaches for hemp breeding and industry. In: Ranalli P, editor. Advances in hemp research. New York: Haworth Press; 1999: 185–208
7 *Feeney M, Punja ZK.* Tissue culture and agrobacterium-mediated transformation of hemp (*Cannabis sativa* L.). In Vitro Cell Dev Biol Plant 2003; 39: 578–585
8 *Slusarkiewicz-Jarzina A, Ponitka A, Kaczmarek Z.* Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. Acta Biol Crac Ser Bot 2005; 47: 145–151
9 *Bing X, Ning L, Jinfeng T, Nan G.* Rapid tissue culture method of *Cannabis sativa* for industrial uses. CN Patent 1887043 A 20070103; 2007
10 *Lata H, Chandra S, Khan I, ElSohly MA.* Thidiazuron induced high frequency direct shoot organogenesis of *Cannabis sativa* L. In Vitro Cell Dev Biol Plant 2009; 45: 12–19
11 *Kojoma M, Iida O, Makino Y, Sekita S, Satake M.* DNA fingerprinting of *Cannabis sativa* using inter-simple sequence repeat (ISSR) amplification. Planta Med 2002; 68: 60–63
12 *Hakki EE, Uz E, Sag A, Atasoy S, Akkaya SM.* Genotyping of *Cannabis sativa* L. accessions from Turkey using RAPD and AFLP markers. Forensic Sci Int 2003; 136: 31
13 *Ma X, Gang DR.* Metabolic profiling of *in vitro* micropropagated and conventionally greenhouse grown ginger (*Zingiber officinale*). Phytochemistry 2006; 67: 2239–2255
14 *Murashige T, Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15: 473–497
15 *Ross SA, Parker M, Arafat R, Lovett K, ElSohly MA.* The analysis of confiscated marijuana samples for different cannabinoids using GC/FID. Am Lab 1996; 16: 16–17

received April 27, 2009
revised May 29, 2009
accepted June 12, 2009

Bibliography
DOI 10.1055/s-0029-1185945
Published online July 27, 2009
Planta Med 2010; 76: 97–100

ISSN 0032-0943

Correspondence
Hemant Lata
National Center for Natural Products Research
School of Pharmacy
The University of Mississippi
University, MS 38677
USA
Phone: + 1 66 29 15 59 28
Fax: + 1 66 29 15 55 87
hlata@olemiss.edu