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ORIGINAL RESEARCH PAPER

Molecular analysis of genetic fidelity in *Cannabis sativa* L. plants grown from synthetic (encapsulated) seeds following in vitro storage

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Abstract The increasing utilization of synthetic (encapsulated) seeds for germplasm conservation and propagation necessitates the assessment of genetic stability of conserved propagules following their plantlet conversion. We have assessed the genetic stability of synthetic seeds of *Cannabis sativa* L. during in vitro multiplication and storage for 6 months at different growth conditions using inter simple sequence repeat (ISSR) DNA fingerprinting. Molecular analysis of randomly selected plants from each batch was conducted using 14 ISSR markers. Of the 14 primers tested, nine produced 40 distinct and reproducible bands. All the ISSR profiles from in vitro stored plants were monomorphic and comparable to the mother plant which confirms the genetic stability among the clones. GC analysis of six major cannabinoids [Δ^9-tetrahydrocannabinol, tetrahydrocannabivarin, cannabidiol, cannabichromene, cannabigerol and cannabinol] showed homogeneity in the re-grown clones and the mother plant with insignificant differences in cannabinoids content, thereby confirming the stability of plants derived from synthetic seeds following 6 months storage.

Keywords *Cannabis sativa* L. · Genetic fidelity · Micropropagation seed encapsulation

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Introduction

Plant tissue culture techniques have been successfully applied for rapid clonal multiplication and conservation of several plant species including *Cannabis* plants (Loh et al. 1983; Richez-Dumanois et al. 1986; Mandolino and Ranalli 1999; Slusarkiewicz-Jarzina et al. 2005; Bing et al. 2007, Lata et al. 2009a, b, 2010a). Recently, alginate encapsulation technology for the production of synthetic seeds in conjunction with micropropagation has become a viable approach for in vitro germplasm conservation (Lata et al. 2009b). However, the occurrence of somaclonal variation is a potential drawback when the propagation of an elite germplasm is intended, where clonal stability is required to maintain the advantages of desired elite genotypes. Thus, it is important to assess the genetic stability of the conserved propagules. Although many reports are available on the utilization of synthetic seeds for micro propagation and conservation of various medicinal plant species (Mandal et al. 2000; Anand and Bansal 2002; Singh et al. 2006; Narula et al. 2007; Faisal and Anis 2007; Ray and Bhattacharyaa 2008; Lata et al. 2009b), there

are very few studies on genetic stability of synthetic seed-derived plantlets exist (Gangopadhyay et al. 2005; Srivastava et al. 2009; Mishra et al. 2011).

Molecular studies are well developed in *Cannabis sativa* for genetic characterization and marker-assisted selection and individualization based on random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) analysis, amplified fragment length polymorphism (AFLP), microsatellite markers, inter simple sequence repeat (ISSR) and short tandem repeat (STR) multiplex (Alghanim and Almirall 2003; Datwyler and Weiblen 2006; Faeti et al. 1996; Gilmore and Peakall 2003; Hakki et al. 2003; Kojoma et al. 2002; Mendoza et al. 2009; Lata et al. 2010b).

In continuation of our previous work (Lata et al. 2009), we have developed an efficient conservation protocol to store high-yielding *C. sativa* elite clones at low temperature using synthetic seed technology (unpublished work). Since our goal is to develop a secure and stable in vitro clonal repository of elite *C. sativa* germplasm that will ensure future availability of desirable pharmacologically active chemotypes, the importance of maintaining stability of in vitro conserved plants cannot be ignored. To ensure that synthetic seed technology will indeed conserve the micropropagated propagules of *C. sativa*, following their conversion from encapsulated nodal segment, the genetic fidelity of the synthetic seed grown in vitro conserved *C. sativa* germplasm is assessed using ISSR markers. Furthermore, biomass samples taken from mature synthetic seed raised plants and mother plant were also compared for their major cannabinoids profile and cannabinoids content using GC-FID to assess differences, if any, between the two types of plants.

Materials and methods

In vitro propagated *C. sativa* plantlets were produced according to a protocol described by Lata et al. (2009a). Nodal segments (3–5 mm) excised from in vitro proliferated shoots were encapsulated (Lata et al. 2009b) and kept for germplasm conservation at either 5, 15 or 25°C (Lata et al. 2011). After 6 months the seeds were re-grown in MS + 0.5 µM TDZ (for shoot induction) and ½ MS + 2.5 µM IBA (for rooting) media under in vitro conditions for 8 weeks followed by their hardening and propagation in soil under grow-room condition (Lata et al. 2009a). About 43, 60 and 47% of the seeds had survived at the respective temperatures over 6 months. After 3 months of vegetative growth (18 h photoperiod) followed by 7 weeks of reproductive growth (12 h photoperiod) under controlled growing conditions (25 ± 3°C, 55 ± 5% RH and PAR 700 ± 24 µmol m^{-2} s^{-1} at plant canopy level), biomass samples were collected from the mature mother plant and 11 randomly selected clones, representing all three different storage conditions (5, 15 and 25° C), and all samples were subjected to ISSR analysis.

DNA extraction

A fresh leaf sample (20 mg) was frozen in liquid N_2 and ground in a 2 ml micro-centrifuge tube using Mixer Mill MM 2000 (Retsch, Newtown PA). The total genomic DNA was extracted using a DNeasy plant mini kit (Qiagen) and resuspended in 50 µl elution buffer. The purified total DNA was quantified and its quality verified by using a nano-drop 1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). In the initial screening 14 primers were used for ISSR analysis.

PCR amplifications

PCR amplifications were performed in 25 µl. Each PCR reaction contained 0.1 µM of each primer, 1 unit Platinum Taq DNA polymerase (Invitrogen), 200 µM of each dNTP (Promega), 1.5 mM $MgCl_2$, 20 ng template DNA, and PCR buffer. Amplifications were carried out 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 followed by a final step of extension 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. Gels were scanned with the Bio-Rad Gel Imaging System and analyzed using Quantity One analysis software version 4.3.0 (Bio-Rad Laboratories Inc., Hercules, CA). The sizes of the PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA).

Amplified DNA marker scoring

Only well-separated bands from 0.1 to 3 kb with high intensity were scored as being present or absent for

Table 1 ISSR banding pattern of *C. sativa* mother plant and the plants grown from synthetic seeds stored under slow growth condition at different temperatures (5, 15 and 25°C) for 8, 16 and 24 weeks

Primer	Sequence	Range of amplicons (bp)	Total no. of bands
UBC 807	5′-AGAGAGAGAGAGAGAGT-3′	184–738	4
UBC 808	5′-AGAGAGAGAGAGAGAGC-3′	223–832	5
UBC 817	5′-CACACACACACACACAA-3′	318–767	4
UBC 825	5′-ACACACACACACACACT-3′	473–**2093**	6
UBC 834	5′-AGAGAGAGAGAGAGAGYT-3′	161–1134	5
(AAG)6Y	5′-AAGAAGAAGAAGAAGAAGY-3′	152–856	6
(GGAT)4H	5′-GGATGGATGGATGGATH-3′	137–554	2
UBC 842	5′-GAGAGAGAGAGAGAGAYG-3′	**74**–1054	3
UBC 845	5′-CTCTCTCTCTCTCTCTRG-3′	731–879	5
		Total	40

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

ISSR markers. Data was scored as 1 for being present and 0 for the absence of DNA band in each micropropagated and mother plant.

GC analysis

For comparison of phytocannabinoids among the mother plant and synthetic seeds, biomass samples were collected at their peak reproductive stage and were extracted and analyzed (see Ross et al. 1996). Six major cannabinoids, i.e. Δ^9-tetrahydrocannabinol (THC), tetrahydrocannabivarin (THCV), cannabidiol (CBD), cannabichromene (CBC), cannabigerol (CBG) and cannabinol (CBN) were identified and quantified by GC (column DB-1; 15 m × 0.25 mm, 0.25 μm film thickness; initially at 170°C for 1 min then programmed to 250°C at 10°C/min; injection at 240°C; detector at 260°C; with a flame ionization detector. The concentration of each cannabinoid was calculated using an internal standard (IS) of 4-androstene-3,17-dione.

Statistical analysis was performed to assess the differences, if any, in the chemical constituents between mother plant (T12) and synthetic seed raised clones (T1–T11) using SYSTAT software package (SYSTAT software Inc. Chicago, IL).

Results and discussion

DNA from *C. sativa* leaves was extracted using DNeasy plant mini kit from Qiagen. A total of 14 ISSR primers were initially screened with the DNA of 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, 9 of the 14 primers were selected as suitable primers for *C. sativa*, and thus used for the present study (Table 1).

Eleven randomly selected plants regenerated from synthetic seeds stored under different storage conditions (5°C—three plants, 15°C—five plants and 25°C—three plants) derived from encapsulated nodal segments along with the mother plant were subjected to ISSR analysis at maturity. These plants showed no discernible differences among them and as compared to the mother plant in the ISSR analysis. The general morphology of the plants grown under the controlled growroom conditions was also similar. 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 74 bp in UBC 842 to 2093 bp in UBC 825 (Table 1) with an average of 4.4 bands per primer. A total of 480 (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 among MP and the plantlets regenerated from synthetic seeds after 24 weeks of storage under different slow growth conditions (Fig. 1).

The number of the primers (9) used in this study, as well as total number of bands (480) together with the observed normality and homogeneity of the plants generated in this study, strongly suggest that the

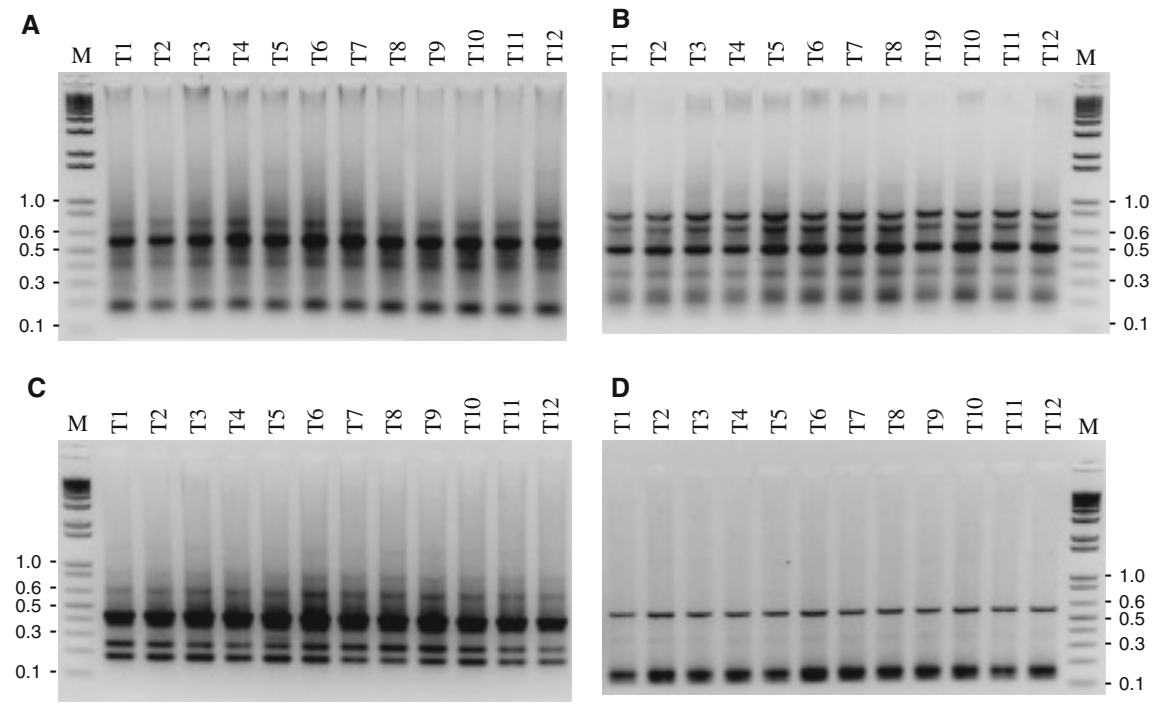

Fig. 1 ISSR amplification pattern obtained for the mother plant (T12) and randomly selected plants raised from synthetic seeds (1–11) after 24 weeks of storage under slow growth conditions. *Lane* T1–T3: represents the plants grown from the synthetic seed stored at 5°C, T4–T8: stored at 15°C and T9–T11: stored at 25°C. **a** primer UBC 807, **b** primer UBC 808, **c** primer UBC 842 and **d** primer UBC 845

encapsulation of nodal segments using synthetic seed technology is a reliable approach for germplasm conservation of *C. sativa*. Our results corroborate with reports of genetic stability of synthetic seed derived plants of *Ananus comosus* (Gangopadhyay et al. 2005), *Cineraria maritime* (Srivastava et al. 2009) and *Picrorhiza kurrooa* (Mishra et al. 2011) after short term storage period.

Furthermore, our results using GC analysis also showed homogeneity in the cannabinoids profile and cannabinoids content of the mother plant and the randomly selected clones propagated through synthetic seeds following storage under slow growth conditions (5, 15 and 25°C) for 6 months (Fig. 2). The level of Δ^9-THC, the major psychoactive compound in the mature buds of the synthetic seed raised clones of *C. sativa* plants was comparable in all the clones ($10.42 \pm 1.33\%$, $n = 33$) and to that of the mother plant ($10.37 \pm 1.14\%$, $n = 3$). Similar to Δ^9-THC, the profile and content of other cannabinoids i.e. THCV, CBD, CBC, CBG and CBN were also identical to be each other and to those of the mother plant. These results confirm that the biochemical mechanism followed to produce the synthetic seeds maintains the same metabolic profile of the mother plant and, therefore, synthetic seeds can be used as a cost effective mechanism for the short term conservation and mass propagation of true-to-type plants of *C. sativa* for commercial pharmaceutical use. Furthermore, short term storage of synthetic seeds had no negative impact on the chemical profile of the plants that survived the storage process.

Since all the ISSR based bands were monomorphic and no gross morphological variation detected in the plantlets regenerated from synthetic seeds after 24 weeks of storage, under different slow growth conditions, as compared with that of the mother plant, indicating high genetic stability among the clones, we conclude that this high THC yielding elite germplasm of *C. sativa* L. can be conserved up to 24 weeks without the risk of genetic instability. This study is particularly of high significance as these clones are

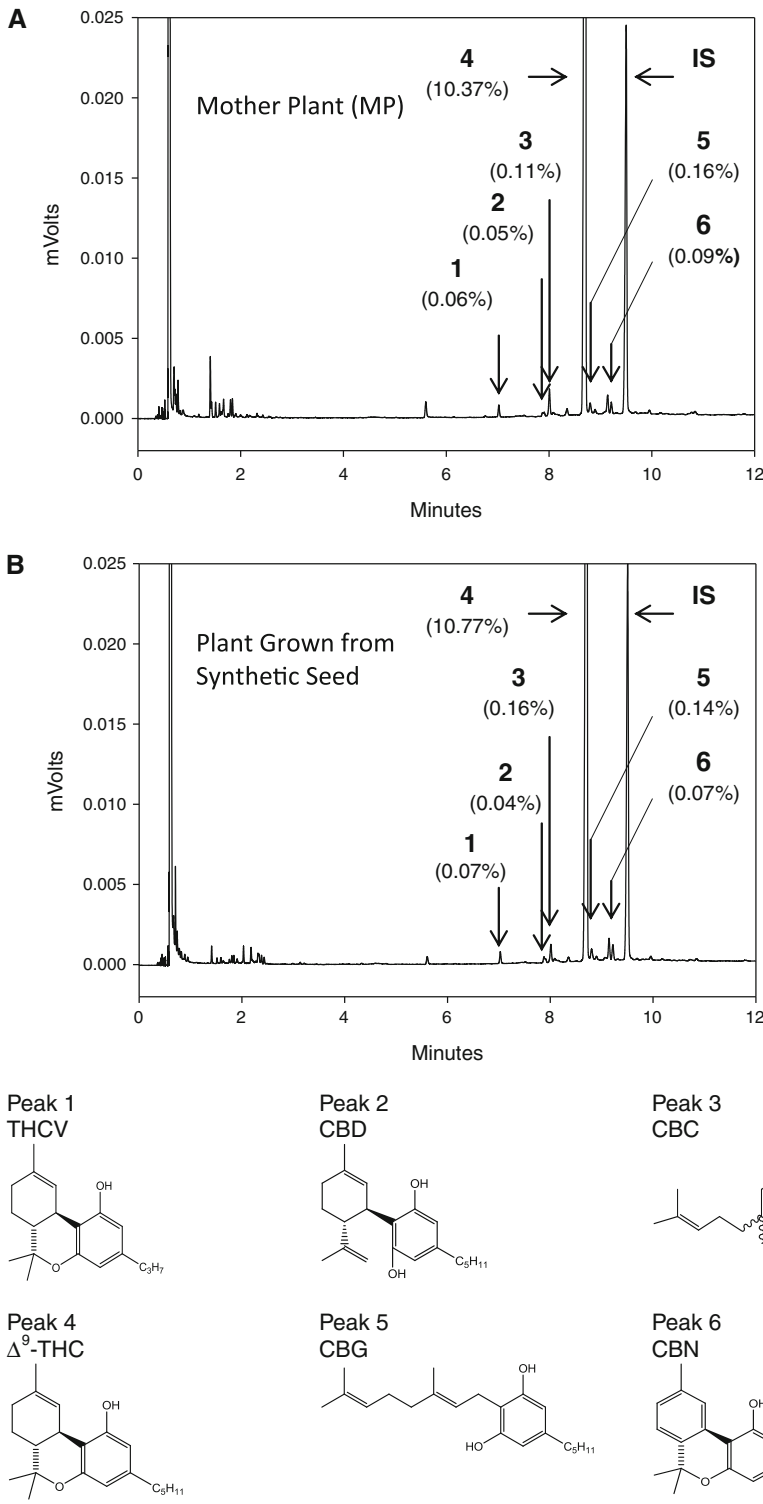

Fig. 2 GC-FID analysis of *C. sativa* mother plant (MP) and a randomly selected representative plant grown from synthetic seed preserved under slow growth condition for 24 weeks. Chemical profiles of other synthetic seed grown plants were also found identical to each other and to that of MP. *IS* Internal standard of 4-androstene-3,17-dione

selected for the isolation of THC, a high value bulk active pharmaceutical.

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References

Alghanim HJ, Almirall JR (2003) Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. Anal Bioanal Chem 376(8): 1225–1233

Anand Y, Bansal YK (2002) Synthetic seeds: a novel approach of in vitro plantlet formation in vasaka (*Adhatoda vasica* Nees). Plant Biotech 19:159–162

Bing X, Ning L, Jinfeng T, Nan G (2007) Rapid tissue culture method of *Cannabis sativa* for industrial uses. CN Patent 1887043 A 20070103

Datwyler SL, Weiblen GD (2006) Genetic variation in hemp and marijuana (*Cannabis sativa* L.) according to amplified fragment length polymorphisms. J Forensic Sci 51:371–375

Faeti V, Mandolino G, Ranalli P (1996) Genetic diversity of *Cannabis sativa* germplasm based on RAPD markers. Plant Breed 115:367–370

Faisal M, Anis M (2007) Regeneration of plants from alginate encapsulated shoots of *Tylophora indica* (Burm.f.) Merrill, an endangered medicinal plant. J Hort Sci Biotechnol 82:351–354

Gangopadhyay G, Bandyopadhyay T, Poddar R, Gangopadhyay SB, Mukherjee KK (2005) Encapsulation of pineapple micro shoots in alginate beads for temporary storage. Curr Sci 88:972–977

Gilmore S, Peakall R (2003) Isolation of microsatellite markers in *Cannabis sativa* L. (marijuana). Mol Ecol Notes 3: 105–107

Hakki EE, Uz E, Sag A, Atasoy S, Akkaya SM (2003) Genotyping of *Cannabis sativa* L. accessions from Turkey using RAPD and AFLP markers. Forensic Sci Int 136:31

Kojoma M, Iida O, Makino Y, Sekita S, Satake M (2002) DNA fingerprinting of *Cannabis sativa* using inter-simple sequence repeat (ISSR) amplification. Planta Med 68:60–63

Lata H, Chandra S, Khan I, ElSohly MA (2009a) Thidiazuron induced high frequency direct shoot organogenesis of *Cannabis sativa* L. In Vitro Cell Dev Biol Plant 45:12–19

Lata H, Chandra S, Khan I, ElSohly MA (2009b) Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L.—an important medicinal plant. Physiol Mol Biol Plants 15(1):79–86

Lata H, Chandra S, Khan I, ElSohly MA (2010a) High frequency plant regeneration from leaf derived callus of high Δ^9-tetrahydrocannabinol yielding *Cannabis sativa* L. Planta Med 76:1629–1633

Lata H, Chandra S, Khan I, ElSohly MA (2010b) Assessment of genetic stability of micropropagated *Cannabis sativa* plants by ISSR markers. Planta Med 76:97–100

Lata H, Chandra S, Mehmedic Z, Khan I, ElSohly MA (2011) In vitro germplasm conservation of high Tetrahydocannabinol yielding elite clones of *Cannabis sativa* L. under slow growth conditions. Acta Physiol Plant (communicated)

Loh WHT, Hartsel SC, Robertson W (1983) Tissue culture of *Cannabis sativa* L. and in vitro biotransformation of phenolics. Z Pflanzenphsiol 111:395–400

Mandal J, Patnaik S, Chand PK (2000) Alginate encapsulation of axillary buds of *Ocimum americanum* L. (hoary basil), *O. basilicum* L. (sweet basil), *O. gratissimum* L. (shrubby basil), and *O. sanctum* L. (sacred basil). In Vitro Cell Dev Biol Plant 36:287–292

Mandolino G, Ranalli P (1999) Advances in biotechnological approaches for hemp breeding and industry. In: Ranalli P (ed) Advances in hemp research. Haworth Press, New York, pp 185–208

Mendoza MA, Mills DK, Lata H, Chandra S, ElSohly MA, Almirall JR (2009) Genetic individualization of *Cannabis sativa* by short tandem repeat multiplex system. Anal Bioanal Chem 393:719–726

Mishra J, Singh M, Palni LMS, Nandi SK (2011) Assessment of genetic fidelity of encapsulated microshoots of *Picrorhiza kurrooa*. Plant Cell Tissue Organ Cult 104:181–186

Narula A, Kumar S, Srivastava PS (2007) Genetic fidelity of in vitro regenerants, encapsulation of shoot tips and high diosgenin content in *Dioscorea bulbifera* L., a potential alternative source of diosgenin. Biotechnol Lett 29:623–629

Ray A, Bhattacharyaa S (2008) Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentine*-an effective way of conservation and mass propagation. S Afr J Bot 74:776–779

Richez-Dumanois C, Braut-Boucher F, Cosson L, Paris M (1986) Multiplication vegetative in vitro du chanvre (*Cannabis sativa* L.) application a la conservation des clones selections. Agronomie 6:487–495

Ross SA, Parker M, Arafat R, Lovett K, ElSohly MA (1996) The analysis of confiscated marijuana samples for different cannabinoids using GC/FID. Am Lab 16:16–17

Singh AK, Sharma M, Varshney R, Agarwal SS, Bansal KC (2006) Plant regeneration from alginate to encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, a medicinally important plant species. In Vitro Cell Dev Biol Plant 42:109–113

Slusarkiewicz-Jarzina A, Ponitka A, Kaczmarek Z (2005) Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. Acta Biol Craco Ser Bot 47:145–151

Srivastava V, Khan SA, Banerjee S (2009) An evaluation of genetic fidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following six months of storage. Plant Cell Tissue Organ Cult 99:193–198