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In vitro mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants

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ABSTRACT

The present study describes a simple, efficient and one step regeneration system for rapid shoot proliferation and *in vitro* rooting of *Cannabis sativa* nodal explants using meta-topolin (mT), an aromatic natural cytokinin. The best response in terms of explants producing maximum number of shoots with maximum shoot length and percent explants producing shoots was recorded on Murashige and Skoog (MS) medium supplemented with 2 μM mT. Shoots multiplied on the same medium for two sub-cultures were able to induce healthy roots within 4–6 weeks. A separate medium containing auxin was not required for root induction. Regenerated plantlets were successfully acclimatized and hardened off in the climatic controlled grow room with 100% survival rate. Genetic fidelity of *in vitro* propagated plants was tested using inter simple sequence repeat (ISSR) markers. Our results show that all the ISSR profiles from *in vitro* propagated plants were monomorphic and comparable to that of the mother plant, thereby confirming the genetic fidelity. Qualitatively and quantitatively, cannabinoid profiles and the content, using gas chromatography-flame ionization detector (GC–FID), in mother plant and *in vitro* propagated plants were found to be similar to each other. Furthermore, regenerated plants were eco-physiologically and functionally comparable to that of the mother plant. The maximized regeneration protocol using mT is thus effective and safe for large scale production of true to type *C. sativa* plants.

1. Introduction

Cannabis sativa L. a member of family Cannabaceae, is a valuable plant with both fiber and medicinal potential (Pertwee, 2014). Among 104 cannabinoids isolated from the plant till date (ElSohly and Gul, 2014), the major biologically active compounds are Δ^9 tetrahydrocannabinol (Δ^9-THC, known for psycho-activity) and cannabidiol (CBD, a non- psychoactive compound with the potential for the treatment of epileptic seizures in children). Other major cannabinoids are tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabigerol (CBG) and cannabinol (CBN).

Cannabis is generally dioceous and occasionally a hermarphodite, wind pollinated plant. For the production of cannabinoids (or phytocannabinoids) female plants are preferred over male plants for several reasons; firstly, in comparison to male plants, female plants produce higher amount of cannabinoids; secondly, being highly wind pollinated, in presence of males, female plants produce lots of seeds at maturity whereas, seed free plants (sinsemilla, a Spanish word) are preferred to produce higher yield of secondary metabolites and thirdly, if several cannabis varieties are being grown together, cross pollination among the varieties would affect the quality (chemical profile) of the final product. To avoid these situations, removing male plants as they appear, screening of female clones for higher metabolite content and, their conservation and multiplication using biotechnological tools is a suitable way to ensure the consistency in chemical profile of a cannabis crop for the pharmaceutical interest.

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In vitro propagation of plants can be influenced by a variety of factors that are categorized either as environmental or hormonal/plant growth regulators (PGRs). As a vital chemical component, PGRs regulate various physiological and developmental processes during the micropropagation (George et al., 2008). There is a continuous effort aimed to identify new compounds with the ability to stimulate better growth, and to alleviate *in vitro*-induced physiological disorders (Tarkowska et al., 2003). The recent biotechnological advances in the field of phytohormones have significantly facilitated the search for new compounds (Strnad, 1997; Tarkowski et al., 2010). Thus, a new group of aromatic cytokines commonly referred as topolins has been identified (Strnad, 1997). Meta-topolin {6-(3-hydroxylbenzylamino) purine}, first isolated from poplar leaves, is an aromatic cytokinin, differing from isoprenoid cytokinins, such as zeatin and 2-iP, in its biochemistry and biological activity (Strnad, 1997). Topolins have been demonstrated to enhance shoot proliferation, maintain histogenic stability, improve rooting efficiency and alleviate various physiological disorders in micropropagation (Aremu et al., 2012a,b). In *C. sativa*, reports have been available on the shoot regeneration, proliferation and in few cases root formation using various growth regulators (Loh et al., 1983; Richez-Dumanois et al., 1986; Mandolino and Ranalli, 1999; Slusarkiewicz-Jarzina et al., 2005; Bing et al., 2007; Fisse et al., 1981; Feeney and Punja, 2003; Lata et al., 2009a,b, 2010). In the present communication, we report an efficient regeneration protocol for *C. sativa* using novel aromatic cytokinin, meta-topolin. Furthermore, the performance of *in vitro* propagated and hardened plants were evaluated and compared to that of mother plant based upon ecophysiology, photosynthetic pigment content, genetic integrity using ISSR markers and phytocannabinoids content using GC–FID.

2. Material and methods

A step by step schematic diagram of experimental design for one step *in vitro* propagation of *C. sativa* is shown in Fig. 1.

2.1. Plant material

Plants of *C. sativa* were grown from seeds of a high THC yielding Mexican variety in a climatic controlled indoor cultivation facility at Coy–Waller laboratory, School of Pharmacy, University of Mississippi. On flowering, male plants were removed and vegetatively propagated cuttings of selected (based on chemical profile) elite female plants were used for study. This was achieved by a systematic screening process. After a desirable growth, cuttings from seedlings were made at vegetative growth stage. Seedlings were than subjected 12 h photoperiod for flowering till maturity while maintaining cuttings at the vegetative stage under the 18 h photoperiod. On flowering, male plants and their related vegetative cuttings were removed. Samples of flowering female plants were collected and analyzed for their THC concentration using GC/FID. Based on chemical analysis, the elite, highest THC yielding female plant was identified and its related vegetative cuttings were used as high yielding elite 'mother plants' for the current study.

Nodal segments containing axillary buds from selected mother plants were used as an explant for initiation of shoot cultures. Explants were surface-disinfected using 0.5% NaOCl (15% v/v bleach) and 0.1% Tween 20 for 20 min. and washed in sterile distilled water three times for 5 min each, prior to inoculation on the culture medium.

2.2. Shoot regeneration, elongation and rooting

In our previous study, we have reported the effect of different growth hormones on shoot formation and rooting of *C. sativa*. Different concentrations of BA, Kn, TDZ and GA_3 were tested for shoot formation, and IAA, IBA and NAA for rooting. Out of all the growth regulators, best shoot formation were obtained in MS + TDZ whereas, the best rooting were obtained in ½MS + IBA (Lata et al., 2009a,b). Therefore, in the present study, the effect of different concentrations of TDZ (0.05, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 μM) with MS medium and IBA (0.05, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 μM) with ½MS were tested and compared with similar concentrations (ranging from 0.05 μM to 5.0 μM) of meta-topolin (mT).

Disinfected explants were inoculated on Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) type B agar (Sigma Chemical Co., St. Louis, MO) and 500 mg L^{-1} activated charcoal supplemented with various concentrations of MS + thidiazuron (TDZ, a cytokinin) and ½ MS + Indole-3-butyric acid (IBA, an auxin) and compared with MS + mT ranging from 0.05 to 5.0 μM adjusted to pH 5.7. Sterile medium was dispensed (25 ml) in glass culture vessels (4-cm diameter × 9.5-cm height) with magenta B caps.

All cultures were incubated at 25 ± 2 °C with 16-h photoperiod under fluorescent light with a photon flux of ~52 μmol m^{-2} s^{-1}. All the experiments were repeated three times with ten explants, with one explant per jar. The parameters evaluated were the average number of shoots per explant, average shoot length, percentage of explants producing shoots, average number of roots per explant, average root length, and percentage of explants producing rooted plantlets.

2.3. Acclimatization and ex-vitro propagation

Well-rooted plants were carefully taken out of the medium, washed thoroughly in running tap water to remove all traces of medium attached to the roots. These plants were pre-incubated in coco natural growth medium (Canna Continental, Los Angeles, CA) in thermocol cups for 10 days before transferring in sterile potting mix-fertilome (Canna Continental) in large pots. All these plantlets were kept under controlled environmental conditions (light, ~700 μmmol m^{-2} s^{-1} with 16-h photoperiod, temperature 25–30 °C and relative humidity ~60%) in an indoor cultivation facility. Mother plants and well acclimatized tissue culture raised plants were transferred *ex vitro* for further cultivation.

2.4. Comparison of tissue culture raised plants with mother plants

2.4.1. Eco-physiology

Plants regenerated from tissue culture and their mother plant were compared for photosynthetic characteristics at peak vegetative stage. Measurements were carried out on three upper undamaged, fully expanded and healthy leaves of five randomly selected *in vitro* propagated plants (IVP) and mother plant (MP) using a climatic controlled portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, Nebraska, USA). Throughout the experiment, cuvette temperature and light level were maintained at 25 °C and 1600 μmol m^{-2} s^{-1} photon flux densities (PPFD), respectively, since these conditions were found optimum for the photosynthesis and growth of *C. sativa* following our previous studies (Chandra et al., 2008). Four gas exchange parameters *viz.* photosynthetic rate (P_n), transpirational water loss (E), stomatal conductance (gCO_2) and intercellular CO_2 concentration (C_i) were measured simultaneously at steady state condition. Water use efficiency (WUE) of plants was calculated as a ratio of the rate of photosynthesis (P_n) and transpiration (E).

2.4.2. Pigments content

Leaves used for the photosynthetic gas exchange measurements were later harvested for the determination of pigments content. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid

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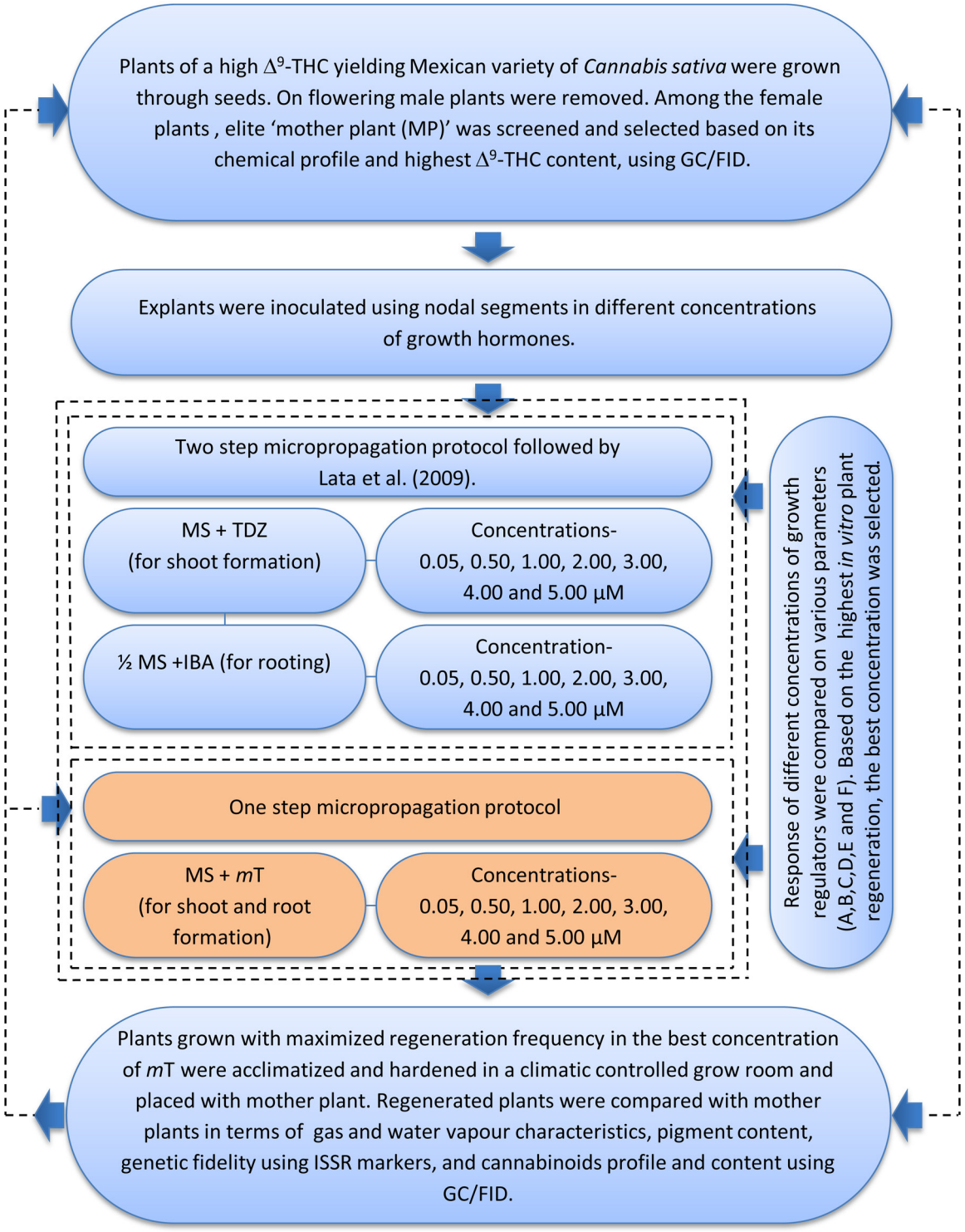

Fig. 1. A schematic diagram of experimental setup for one step micropropagation protocol for *C. sativa* L. (A) average number of shoot formation, (B) average shoot length, (C) percent explants producing shoots, (D) average number of roots produced, (E) average root length and (F) percent explants producing roots.

(Car) content were measured spectrophotometrically following the method of Holm (1954).

2.4.3. Molecular analysis

Genomic DNA was extracted from nine randomly selected tissue culture raised plants and mother plant for the molecular analysis of genetic fidelity. After harvest, leaf samples (20 mg each) were immediately frozen in liquid nitrogen and later ground in a 2.0 ml micro-centrifuge tube using Mixer Mill, MM 2000 (Retsch, USA). The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, USA) and re-suspended in 50 µl dilution buffer. The purified total DNA was quantified and its quality was verified using Nano DropTM 1000 Spectrophotometer (Thermo Scientific, USA).

Fifteen arbitrary were used to screen polymorphism among mother and tissue culture raised plants through PCR amplification. The reactions were performed in a 25 µl final volume

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comprising 0.1 μM each primer, 1 unit of Platinum Taq DNA Polymerase (Invitrogen, USA), 200 μM of each dNTP (Promega Corporation, USA), 1.5 mM of MgCl2, 20 ng of template DNA, and 1 × PCR buffer. Amplifications were carried out in heated lid M&J Research Gradient Cycler PTC-225 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 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 (Tris-Acetate-EDTA) agarose gel and visualized under UV light. The sizes of the PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen, USA). Gels were also scanned with the Bio-Rad Gel Imaging System and ana-lyzed using Quantity One analysis software version 4.3.0 (Bio-Rad Laboratories Inc., USA).

Only clear and well-marked bands in the size ranging from 0.1 to 3.0 kb were scored as being present or absent for ISSR markers. Data for DNA band in each micropropagated and mother plant were scored in binary character matrices as 1 and 0 being present and absent, respectively.

2.4.4. Cannabinoids profile and content

Biomass samples taken from fully matured mother plant ($n = 3$) and tissue culture raised plants ($n = 9$) were compared for cannabinoids profile and content. Triplicate of each sample were used for the analysis of six major cannabinoids (Δ^9-tetrahydrocannabinol, Δ^9-THC; tetrahydrocannabivarin, THCV; cannabidiol, CBD; cannabichromene, CBC; cannabigerol, CBG and cannabinol, CBN) using GC-FID following the method described by Ross et al. (1996) and ElSohly et al. (2000).

2.5. Statistical analysis

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

3. Results and discussion

3.1. In vitro *regeneration using meta-topolin*

The main aim of this work was to refine the existing protocol for the mass propagation of *C. sativa.* In our previous reports, we have reported two separate growth regulators for *in vitro* shoot formation (TDZ, 0.5 μM) and rooting (IBA 2.5 μM) in *C. sativa* (Lata et al., 2009a,b, 2010). However, in the present study, a simple one step protocol was established using MS with an aromatic cytokinin meta-topolin *m* T (Fig. 2). Table 1 shows the effects of different growth regulators used for shoot and root growth. Overall, the highest average number of shoots (13.44 ± 1.38 cm) was obtained in the treatment with MS + 2.0 μM *m* T with maximum shoot length (11.44 ± 0.80 cm). All the explants (100%) inoculated *in vitro* with 2.0 μM *m* T were capable of producing shoots (Table 1). In TDZ, on the other hand, of the different concentrations tested, highest average number of shoots (11.89 ± 0.93 cm) was obtained in MS + 0.5 μM TDZ with average shoot length of 7.35 ± 0.58 cm (similar to our previous report, Lata et al., 2009a,b). The average number of shoot production and shoot length was higher using mT as compared to TDZ (Table 1). Similar to our results, the superiority of using aromatic cytokinin meta-topolin for shoot proliferation has been emphasized been by many researchers (Roels et al., 2005; Wojtania et al., 2004; Bairu et al., 2007, 2009Amoo et al., 2012). (Staden et al., 2010) have emphasized the role of meta-topolin in the production of morphogenically competent cells. Till date, there is no report on the use of topolins in the micropropagation of Cannabaceae species. To the best of our knowledge, this is the first report on application of mT on *C. sativa.*

Due to the vigorous growth in MS + mT medium (especially mT concentration ranging from 1 μM to 4 μM) plants were subcultured on the same fresh medium after 3 weeks. Roots started appearing within 3–4 weeks and fully developed healthy roots were formed by 6 weeks in culture. A separate medium for root initiation was not needed. Of various concentrations of mT tested, the best concentration for rooting was MS medium supplemented with 2.0 μM mT (Table 1). About 96% of the regenerated shoots were able to develop roots with an average of 14 roots per shoot and 18.68 ± 1.07 cm shoot length, within 4 weeks of transfer to fresh (MS + 2.0 μM mT) medium. Roots induced at this concentration were thicker, robust and with lot of branches. Meta topolin concentrations higher than 4 μM were inhibitory to rooting. Although, auxin alone has been a growth regulator for rooting (Gaspar et al., 1996), and specifically IBA has been used in plant propagation because of its efficacy in the stimulation of adventitious roots (Staden et al., 2010), however, the superiority of mT as compared to IBA was remarkably observed in the present study, especially at the lower concentrations. Of different concentrations of IBA tested, the best rooting (about 5 roots per shoot with an average of 5.38 ± 0.33 cm root length) was observed in ½ MS + 3 μM IBA. These results are consistent to those reported in our previous report (Lata et al., 2009a,b). However, the number of roots produced and average root length was significantly higher ($p < 0.05$) in MS + 2 μM mT as compared to those in ½MS + 3 μM IBA (Table 1). Furthermore, plants produced with mT rooted much better during the acclimatization after transfer to soil in the indoor climate controlled growing facility than shoots produced with TDZ. Similar effects of mT have been observed by Werbrouck et al. (1996) in *Spathiphyllum florigbundum* . Working on *Prunus* (*Prunus domestica* L. and *Prunus insititia* × *domestica*), Gentile et al. (2014) have reported a higher rooting percentage, root number and length in mT as compared to BA. Similarly, Westhuizen (2014) working on Eucalyptus species reported less vitrified shoots and better *in vitro* rooting in 0.2 mg L^{-1} meta topolin as compared to other concentration of cytokinins tested. In the present study, plantlets regenerated in MS supplemented with 2.0 μM mT medium were successfully acclimatized and hardened off with 100% survival rate.

3.2. Comparison of in vitro *propagated plants with mother plant*

3.2.1. Photosynthetic characteristics and pigment content

A comparison of photosynthetic characteristics between mother plant (MP) and *in vitro* propagated plants (IVP) are shown in Fig. 3. Photosynthesis, rate of transpiration and consequently, water use efficiency play an important role in the logistics of survival, growth and development of plants. Most of the dry matter in live plants is reported to be derived from photosynthetic CO_2 assimilation (Zelitch, 1975). Variations in photosynthesis in different plants species reflects their ability to grow and survive at a particular environmental condition. In the present study, gas and water vapour exchange parameters are used as a tool to compare physiological functioning of mother and tissue culture raised plants. In general, average P_n (MP = 22.17 and IVP = 23.17 μmol m^{-2} s−1), gCO_2 (MP = 207.79 and IVP = 213.82 mmol m^{-2} s^{-1}) and WUE (MP = 3.65 and IVP = 4.05) were slightly higher and, average E (MP = 6.19, IVP = 5.74 mmol m^{-2} s^{-1}), C_i (MP = 234.33, IVP = 232.50 μmol mol^{-1}) and C_i/C_a (MP = 0.67, IVP = 0.66) were slightly lower in tissue culture raised plants as compare to the mother plant. These differences between MP and IVP plants, however, were statistically insignificant ($P_n = 1.23$, $gCO_2 = 8.65$, $E = 0.46$, $C_i = 6.01$, $C_i/C_a = 0.04$ and WUE = 0.41; data represents LSD between MP and IVP at $p < 0.05$). In a similar experiment Bag et al. (2000) have reported at par or slightly better performance of tissue culture raised plants than mother plant in terms of their photosynthetic characteristics and morphological parameters.

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Fig. 2. Micropropagation of *C. sativa* using meta-topolin. (A) mother plant, (B) shoot formation and (C–E) rooting in MS + 2 μM meta-topolin, (F) and (G) well established plants in soil and (H) field plantation of *C. sativa* plants.

Table 1
Response of different growth regulators on *in vitro* propagation of *C. sativa* L. data represent mean ± SD.

Growth regulator	Con. (μM)	Shoot formation			Rooting		
		Average no. of shoots	Average shoot length (cm)	% explants producing shoots	Average no. of roots	Average root length (cm)	% explants producing roots
½ MS + IBA	0.05	–	–	–	2.63 ± 0.33	2.99 ± 0.18	18.52
	0.50	–	–	–	2.59 ± 0.23	3.16 ± 0.33	37.04
	1.00	–	–	–	3.33 ± 0.22	3.38 ± 0.37	51.85
	2.00	–	–	–	4.37 ± 0.35	3.76 ± 0.31	77.80
	3.00	–	–	–	4.63 ± 0.34	5.38 ± 0.33	92.59
	4.00	–	–	–	4.30 ± 0.35	4.90 ± 0.29	81.48
	5.00	–	–	–	3.70 ± 0.27	4.76 ± 0.36	77.78
MS + mT	0.05	3.15 ± 0.32	3.96 ± 0.37	44.15	4.26 ± 0.34	3.70 ± 0.28	33.33
	0.50	5.44 ± 0.34	4.47 ± 0.31	55.56	4.70 ± 0.28	5.26 ± 0.33	66.67
	1.00	6.19 ± 0.58	6.10 ± 0.69	66.67	6.48 ± 0.56	7.17 ± 0.38	88.89
	2.00	13.44 ± 1.38	11.44 ± 0.80	100.00	13.78 ± 1.14	18.68 ± 1.07	96.30
	3.00	12.67 ± 1.11	7.41 ± 0.68	77.78	10.56 ± 0.89	16.27 ± 1.02	77.78
	4.00	6.22 ± 0.70	4.39 ± 0.34	74.07	5.44 ± 0.44	12.16 ± 1.09	66.67
	5.00	5.89 ± 0.52	3.29 ± 0.32	70.37	4.41 ± 0.37	10.11 ± 1.26	59.26
MS + TDZ	0.05	4.33 ± 0.38	4.45 ± 0.44	92.59	–	–	–
	0.50	11.89 ± 0.93	7.35 ± 0.58	100.00	–	–	–
	1.00	11.41 ± 0.64	4.66 ± 0.49	88.89	–	–	–
	2.00	9.96 ± 0.76	5.35 ± 0.42	85.19	–	–	–
	3.00	9.85 ± 0.66	6.51 ± 0.55	81.48	–	–	–
	4.00	6.19 ± 0.51	3.58 ± 0.35	77.78	–	–	–
	5.00	5.59 ± 0.64	3.03 ± 0.25	74.06	–	–	–

Magnitude of photosynthetic and respiratory characteristics of plants is strongly related to variations in its pigment content (Todaria, 1990). In a plant species, variation in pigments and therefore, in photosynthetic characteristics while grown in different environmental conditions reflect its adjustment to the climatic rigors (Anjum et al., 2011). Application of cytokinins is also reported to promote photosynthesis by enhancing total Chl content (Caers and Vendrig, 1986). In a dose response study on *Raphanus sativus* L., Unsal et al. (2002) reported an increase in total Chl content with the application of mT as compared to those of control plants. In the present study, *in vitro* propagated plants were grown in the same soil composition and in similar environmental conditions *ex vitro* as the mother plant. Our result shows no statistically significant difference in Chl a (LSD = 0.45, $p < 0.05$), Chl b (LSD = 0.21, $p < 0.05$), total Chl (LSD = 0.58, $p < 0.05$), Chl a/Chl b ratio (LSD = 0.88, $p < 0.05$), Car (LSD = 0.12, $p < 0.05$) and total Chl/Car ratio (LSD = 0.21, $p < 0.05$) between mother and tissue culture raised plants. The data,

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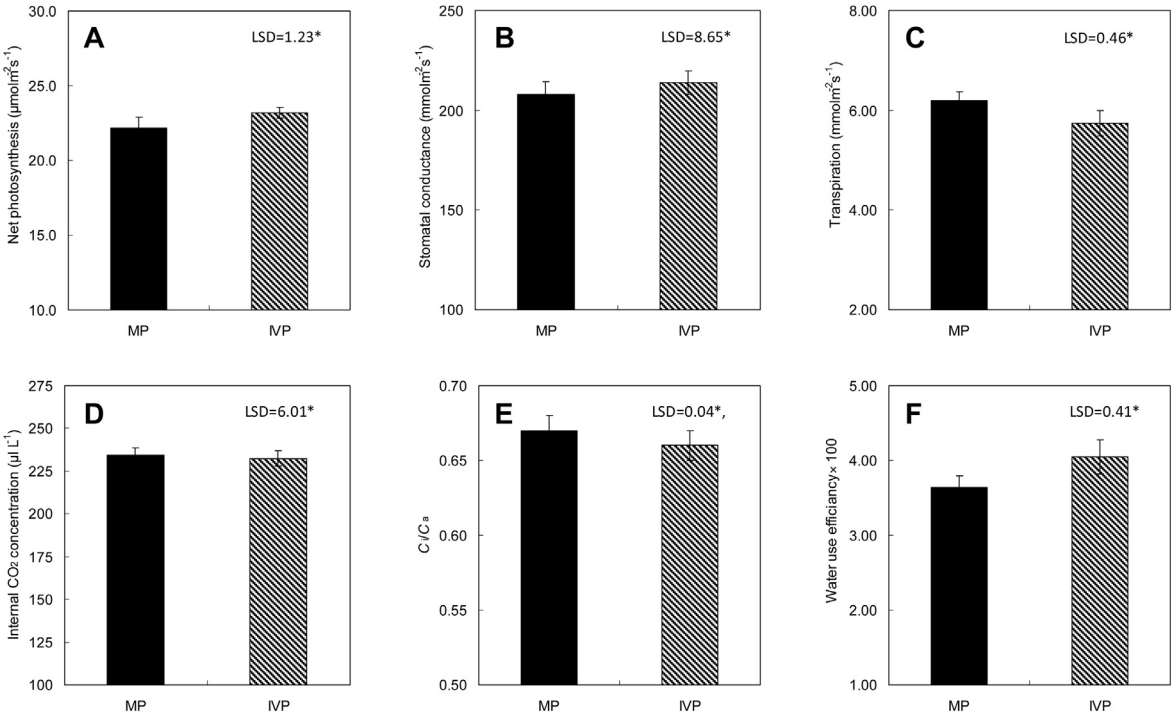

Fig. 3. Comparison of eco-physiological characteristics (A–F) between mother plant and *in vitro* propagated plants. C_i/C_a, the ratio of intercellular to ambient CO_2 concentration. Data represents mean ± SD, $n = 9$, LSD = least significant difference, level of significance * = $P < 0.05$.

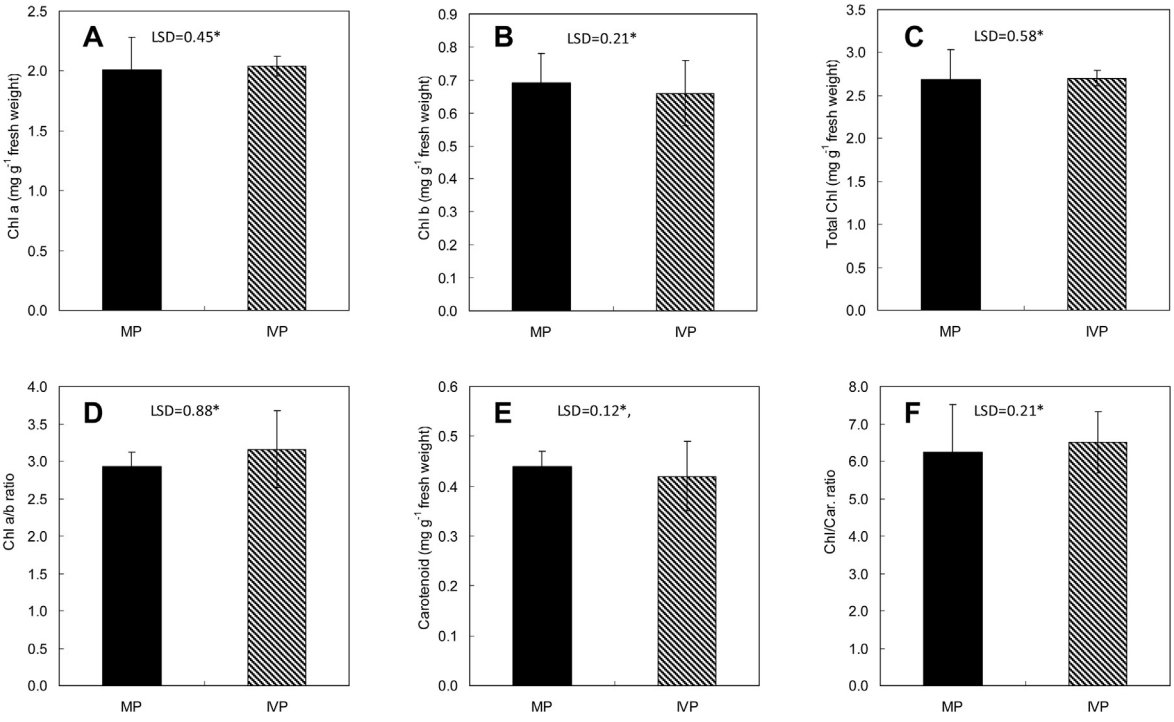

Fig. 4. Comparison of photosynthetic pigments content (A–F) between mother plant and *in vitro* propagated plants. Data represents mean ± SD, $n = 9$, LSD = Least significant difference, Level of significance * = $P < 0.05$, Chl = Chlorophyll and Car. = Carotenoid.

therefore, suggest high level of comparability of IVP plants with the mother plant (Fig. 4).

3.2.2. Molecular analysis

Somaclonal mutation is a common problem which has often been observed in the plants raised through micropropagation especially, plants grown through indirect organogenesis. In the recent years, different molecular techniques (*i.e.,* restriction fragment length polymorphism, RFLP; Amplified fragment length polymorphism, AFLP; Inter-simple sequence repeat, ISSR; Random Amplified Polymorphic DNA, RAPD etc.) are being used to confirm genetic integrity or somaclonal variations between mother plants and clones produced through *in vitro* techniques (Prasad et al., 2015). Each technique has its own advantages over other depending

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Table 2
ISSR band pattern of micropropagated and mother plant of *C. sativa.*

Primer	Sequence	Range of amplicons(in bp)	Total bands
UBC826	5′-ACACACACACACACC-3′	290–2000	7
UBC834	5′-AGAGAGAGAGAGAGAGYT-3′	171–1894	10
UBC 835	5′-AGAGAGAGAGAGAGAGYC-3′	358–880	5
UBC 845	5′-CTCTCTCTCTCTCTCTRG-3′	155–1337	4
UBC 856	5′-ACACACACACACACACYA-3′	331–1124	6
(GGGGT) 3M	5′-GGGGTGGGGTGGGGTM-3′	340–1837	8
(GGAT) 4H	5′-GGATGGATGGATGGATH-3′	363–1446	6
(AAG) 6Y	5′-AAGAAGAAGAAGAAGAAGY-3′	297–959	7
		Total	53

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

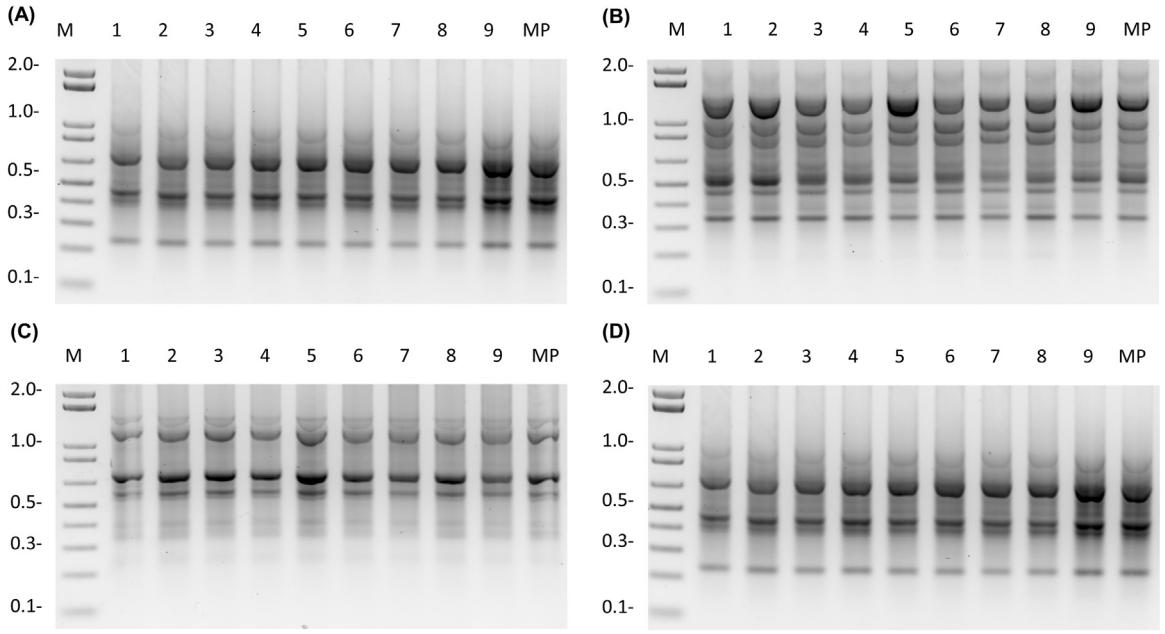

Fig. 5. Inter-simple sequence repeats (ISSR) amplification pattern obtained for mother plant (MP) and *in vitro* propagated plants (1–9); using (A) primer UBC 835, (B) primer (GGGGT) 3 M, (C) primer (GGAT) 4H, and (D) primer (AAG) 6Y.

on the purpose (McGregor et al., 2000). ISSR markers are reported to be simple, highly polymorphic and cover all the advantages of AFLP and RAPD markers (Reddy et al., 2002; Phulwaria et al., 2015), therefore, being used to test the genetic fidelity of micropropagated plants in general and thus used in this study. Eight ISSR primers were screened with the DNA of mother plant and nine randomly selected tissue culture raised plants. Each tested primer produced clear and scorable amplification products ranging in size from about 155 bp with UBC 845 to 2000 bp with UBC826 with an average of 6.6 bands per primer generating monomorphic patterns across all 10 plants analyzed (Table 2). No ISSR polymorphism was detected showing genetic integrity of micropropagated plants (Fig. 5). Our results corroborate with earlier reports of genetic fidelity of micropropagated plants based on ISSR analysis of *C. sativa* (Lata et al., 2009a,b, 2010), *Balanites aegyptiaca* (Varshney and Anis, 2013), *Moringa peregrine* (Khateeb et al., 2013), *Psidium guajava* (Rai et al., 2012), *Dendrocalamus strictus* (Goyal et al., 2015), *Alhagi maurorum* (Agarwal et al., 2014), *Ceropegia evansii* (Chavan et al., 2015), *Salvadora oleoides* (Phulwaria et al., 2015).

3.2.3. Phytocannabinoids

Consistency in the chemical profile and useful metabolites content of raw material is of utmost interest in any plant based pharmaceutics. Direct organogenesis from shoot cultures represents an alternative system to vegetative propagation due to its consistency in the production of secondary metabolites and genetically stable plants as compared to indirect organogenesis or other micropropagation methods (Chandra et al., 2010; Amoo et al., 2013). Genetic stability and secondary metabolite production, however, in micropropagated plants can be influenced by several factors such as growth environment, choice and concentration of growth regulator used during micropropagation etc. (Dornenburg and Knorr, 1995). In the present study, micropropagated plants were acclimatized and grown till maturity with mother plant in identical environmental conditions. Biomass samples of mature buds/mature flowers (since buds contain highest amount of phytocannabinoids) of mother and micropropagated plants were compared for cannabinoids profile and content. The average level of Δ^9-THC (7.25 ± 0.15, $n = 27$) in IVP plants were found to be highly comparable to each other and to that of the mother plant (7.52 ± 0.04, $n = 9$). Similarly, CBD, a non-psychologically active compound with high pharmacological value (MP, 0.08 ± 0.01; IVP, 0.07 ± 001; LSD = 0.02 at $p < 0.05$) and other cannabinoid contents (*i.e.* THCV, CBD, CBG and CBN) in micropropagated plants were also comparable to the mother plant. Minor differences in different cannabinoids content between mother plant and micropropagated plants were found to be statistically insignificant ($p < 0.05$, Table 3). These results thus suggest plants propagated through the current *in vitro* protocol appear to be 'normal' and identical to each other and, to that of the mother plant in terms of useful secondary metabolite content.

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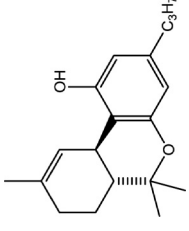
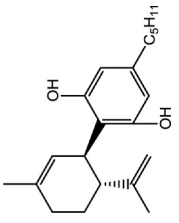
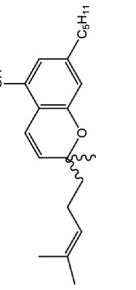
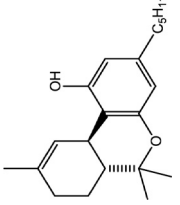
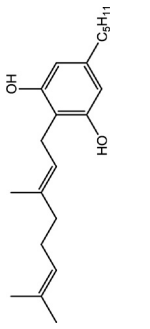
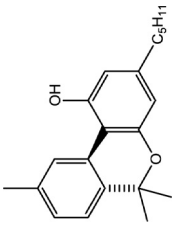
Table 3
Comparison of major cannabinoid contents between fully mature mother plant and *in vitro* propagated plants using meta topolin.

Parameters	THCV	CBD	CBC	Δ^9-THC	CBG	CBN
Chemical structures						
MP	0.05 ± 0.01	0.08 ± 0.01	0.28 ± 0.01	7.52 ± 0.04	0.17 ± 0.01	0.23 ± 0.01
IVP	0.05 ± 0.01	0.07 ± 0.01	0.27 ± 0.02	7.25 ± 0.15	0.16 ± 0.02	0.23 ± 0.02
LSD, $P < 0.05$	0.009	0.013	0.013	0.281	0.021	0.012

MP, mother plant; IVP, *in vitro* propagated plants; THCV, tetrahydrocannabivarin; CBD, cannabidiol; CBC, cannabichromene; Δ^9-THC, Δ^9-tetrahydrocannabinol, CBG, cannabigerol and CBN, cannabinol. Values represent mean ± SD; n (MP) = 9; n (IVP) = 27.

4. Conclusion

In summary, the use of mT (2.0 μM) provides a one-step protocol effective in promoting adventitious shoot formation and root induction in the same medium. The maximized regeneration protocol is simple and cost effective considering the high shoot proliferation rate, the ease of rooting, and the 100% survival frequency of acclimatized plants. To the best of our knowledge, this is the first report on the successful application of mT on *in vitro* propagation of *C. sativa* . Our results confirm the clonal fidelity of micropropagated plants and reveal that current protocol using mT is safe for large-scale production of true-to-type *C. sativa* plants.

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