High Frequency Plant Regeneration from Leaf Derived Callus of High Δ^9-Tetrahydrocannabinol Yielding *Cannabis sativa* L.

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Key words
- *Cannabis sativa*
- Cannabaceae
- callus induction
- Δ^9-tetrahydrocannabinol
- GC-FID
- organogenesis

Abstract

An efficient *in vitro* propagation protocol for rapidly producing *Cannabis sativa* plantlets from young leaf tissue was developed. Using gas chromatography-flame ionization detection (GC-FID), high THC yielding elite female clone of a drug-type *Cannabis* variety (MX) was screened and its vegetatively propagated clones were used for micropropagation. Calli were induced from leaf explant on Murashige and Skoog medium supplemented with different concentrations (0.5, 1.0, 1.5, and 2.0 µM) of indole- 3-acetic acid (IAA), indole- 3- butyric acid (IBA), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxy–acetic acid (2,4-D) in combination with 1.0 µM of thidiazuron (TDZ) for the production of callus. The optimum callus growth and maintenance was in 0.5 µM NAA plus 1.0 µM TDZ. The two-month-old calli were subcultured to MS media containing different concentrations of cytokinins (BAP, KN, TDZ). The rate of shoot induction and proliferation was highest in 0.5 µM TDZ. Of the various auxins (IAA, IBA, and NAA) tested, regenerated shoots rooted best on half strength MS medium (1/2 – MS) supplemented with 2.5 µM IBA. The rooted plantlets were successfully established in soil and grown to maturity with no gross variations in morphology and cannabinoids content at a survival rate of 95% in the indoor growroom.

Abbreviations

2,4-D:	2,4 dichlorophenoxyacetic acid
Δ^9-THC:	Δ^9-tetrahydrocannabinol
BAP:	6-benzylamino-purine
GC-FID:	gas chromatography-flame ionization detection
IAA:	indole- 3-acetic acid
IBA:	indole- 3- butyric acid
KN:	kinetin
MS:	Murashige and Skoog
NAA:	α-naphthaleneacetic acid
TDZ:	thidiazuron

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Cannabis sativa L. (marijuana; Cannabaceae) is a plant with worldwide distribution, yielding fiber and food, as well as a psychoactive drug. The flowers have been used as a medicine for millennia. Investigation of its major chemical components has revealed their utility for the treatment of a wide variety of diseases. Δ^9-tetrahydrocannabinol is currently approved as an oral prescription drug for the treatment of the nausea and vomiting associated with cancer chemotherapy as well as for appetite stimulation in cases of the anorexia associated with AIDS and has a tremendous commercial value in the pharmaceutical area [1]. Micropropagation techniques, in addition to being an alternative means of plant propagation, can potentially overcome many factors limiting traditional approaches to *Cannabis sativa* improvement. These techniques can be further used for the rapid propagation of chemical profile based elite plants of this species. Plant regeneration *in vitro* in different *Cannabis* genotypes and explant sources has been reported but with limited success [2–6]. However, a previous report on organogenic pathway bypassing calli by our group dealt with the successful establishment of a protocol for rapid propagation of identical *Cannabis sativa* plants using axillary shoot buds [7–9]. Furthermore, in a recent report, we also reported the

propagation of *Cannabis sativa* through alginate encapsulation [10]. In the present study we report an alternative approach for the efficient plant regeneration of *Cannabis sativa* plants from leaf derived callus.

Material and Methods

Screening of high Δ^9-THC yielding elite clones

Plants of a high THC yielding drug type *Cannabis sativa* variety (MX) were grown through seeds in an indoor growroom, under a controlled environmental condition (25 ± 3 °C temperature, 55 ± 5% RH and ~ 700 ± 24 µmol · m^{-2} · s^{-1} photosynthetically active radiation at plant canopy level) for their optimum growth [11]. On flowering, male plants were removed and only female plants were considered for further propagation. Among these plants, ten randomly selected healthy female mother clones were used for periodic GC-FID analysis. Five cuttings from vegetative nodes were made from each clone. These cuttings were well marked and kept separately at vegetative stage under an 18-hour photoperiod. Mother clones were kept growing under a 12-hour photoperiod until maturity. Biomass samples were taken from apical segments of mother clones at various stages (vegetative, reproductive, and senescence) of growth of the plants. These samples were dried at 48 °C and analyzed (GC-FID) following Ross et al. [12]. Based on the GC-FID analysis, the highest THC yielding mother clone was identified and its three randomly selected healthy clones, among five representative vegetative cuttings, were used for micropropagation. It is important here to make a note that the aim of the study was to screen and propagate high yielding *Cannabis sativa* for the isolation of natural Δ^9-THC. Therefore, plants were first screened and propagated through high Δ^9-THC yielding female clones, since the female plants contain more THC as compared to male plants. However, the methodology described in this study may also be used for the propagation of male plants and any other genotypes of this species.

Plant material for micropropagation

Young leaves (0.5–10.0 mm) were used as an explant for initiation of shoot cultures. Explants were obtained from vegetatively propagated plants of a screened and selected (based on its chemical profile using GC-FID) high Δ^9-THC yielding elite female plant (MX_{E-1}) of *Cannabis sativa* variety (MX) grown under controlled environmental conditions in an indoor cultivation facility at Coy-Waller Laboratory, University of Mississippi, where a voucher specimen (SHP4678) is deposited.

Sterilization

Explants were surface disinfected using 0.5% NaOCl (15% v/v bleach) and 0.1% Tween 20 for 20 minutes. The explants were washed in sterile distilled water three times for 5 minutes prior to inoculation on the culture medium.

Inoculation

Disinfected explants were inoculated on Murashige and Skoog's medium [13] containing 3% (w/v) sucrose, 0.8% (w/v) Type E agar (Sigma Chemical Co.) supplemented with various concentrations (0.5, 1.0, 1.5, and 2.0 µM) of IAA, IBA, NAA (all from Sigma) and 2,4-D in combination with 1.0 µM of TDZ for callus induction. The medium was adjusted to pH 5.7. For shoot initiation, two-month-old calli were transferred to MS media containing different concentrations (0.5, 1.0, 2.5, 5.0, and 10.0 µM) of cytokinins (BAP, KN, TDZ; Sigma). Sterile medium was dispensed (25 mL) in glass culture vessels (4 cm diameter × 9.5 cm high, baby food jars with magenta B caps). Shoots taller than 2.5 cm were transferred to half strength MS medium supplemented with different auxins (IAA, IBA, and NAA) at 0.5, 1.0, 2.5, 5.0, and 10.0 µM concentrations for root induction. All cultures were incubated at 25 ± 2 °C with a 16-h photoperiod under fluorescent light with a photon flux of ≈ 52 µmol · m^{-2} · s^{-1}. All experiments were repeated at least three times with 10 explants, with one explant per jar. The cultures were observed constantly for any response. The parameters evaluated were the average number of shoots per explant, average shoot length, % of explants producing shoots, average number of roots per explant, average root length, and % of rooted plantlets.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) followed by the Tukey test with the level of significance set at 5% using SAS version 9.1 (SAS Institute).

Acclimatization

Rooted shoots were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces of medium attached. Plantlets were preincubated in coco natural growth medium (Canna Continental) in thermocol cups (Walmart Stores, Inc.) for ten days. The cups were covered with polythene bags to maintain humidity, kept in a growroom and later acclimatized in sterile potting mix (fertilome; Canna Continental) in large pots. All the plants were kept under strict controlled environmental conditions (25 ± 3 °C temperature and 55 ± 5% RH). Initially, plants were kept under cool fluorescent light for 10 days and later exposed to full spectrum grow lights (18-hour photoperiod, ~ 700 ± 24 µmol · m^{-2} · s^{-1} at plant canopy level, measured by LI-COR quantum meter, model LI-189) provided with seven full spectrum 1000 Watt HID (high density discharge) lamps in combination with seven 1000 Watt high pressure sodium bulbs (Sun Systems), covering a 335 square foot area. A hot air suction fan was attached and about a 3 to 4 feet distance between plants and bulb was maintained to avoid heating due to HID bulbs. Plants were watered regularly and individually to maintain sufficient moisture content in the pots.

Supporting information

Formation of callus on leaf segment, multiple shoots regeneration, *in vitro* rooting, and regenerated plant establishment in soil (**Fig 1S**), as well as a qualitative and quantitative comparison of cannabinoids contents of the mother plant and a representative micropropagated plant using GC-FID analysis (**Fig 2S**) are shown as Supporting Information.

Results and Discussion

A schematic diagram of the selection of high Δ^9-THC yielding clone is shown in **Fig. 1**. Based on GC-FID analysis, plant ID MX_{E-1} was identified as elite with 11.91% Δ^9-THC on a dry weight basis during the budding stage. Qualitative and quantitative analysis of cannabinoids of this clone are shown in **Fig. 2Sb** (Supporting Information). Since young leaf explant from vegetative cuttings of this clone responded better than leaves from the mature flowering mother plant, leaves from its three randomly selected vegetative cuttings were used for micropropagation.

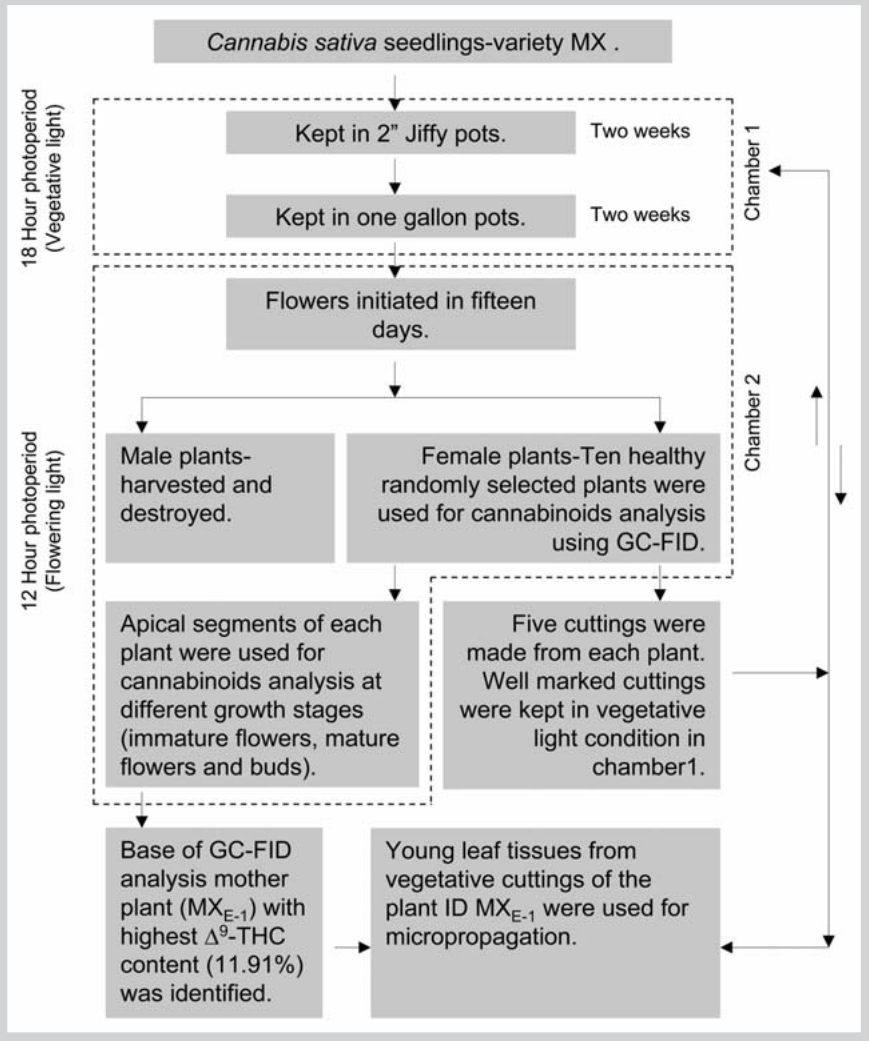

Fig. 1 Schematic diagram for the screening of high Δ^9-THC yielding mother plant of *Cannabis sativa* based on GC-FID analysis.

Among the different concentrations of various growth regulators tested (IAA, IBA, NAA, and 2,4-D in combination with 1.0 µM of TDZ for callus induction; BAP, KN, and TDZ for shoot formation as well as IAA, IBA, and NAA for rooting) the effect of best responding growth regulators (NAA + 1.0 µM TDZ for callus induction, TDZ for shoot formation, and IBA for rooting) is depicted in **Table 1**. Leaf explants were inoculated on MS medium containing 3% (w/v) sucrose, 0.8% (w/v) Type E agar supplemented with various concentrations (0.5, 1.0, 1.5, and 2.0 µM) of IAA, IBA, NAA, and 2,4-D in combination with 1.0 µM of TDZ to test the callus induction. Leaf explants enlarged and developed callus at the cut surface in all media within 1–2 weeks of inoculation depending on auxin type and concentration. The callus formation was seen predominantly on the adaxial surface during the early stages of development as compared to abaxial surface (**Fig. 1Sa** and **b**, Supporting Information).

The formation and growth of the callus was remarkably affected by growth regulator type and concentration. While there was a little extension in growth of the explants, no callus growth was observed during the culture period in the basal medium without a growth regulator (control). Of the various auxins tested, NAA at different concentrations (0.5–2.0 µM) induced the highest average callusing percentage (~ 82.0%) from leaf explant (**Table 1**). However, the interaction of other auxins (2, 4-D, ~ 55.0%; IAA, ~ 21.0% and IBA, ~ 17.0%) with the cytokinin TDZ in the range of the same concentrations tested was found less effective (data not shown in table). Within the different concentrations of NAA (0.5–2.0 µM), MS basal medium supplemented with 0.5 µM NAA in combination with 1.0 µM TDZ promoted rapid growth, the highest amount of callus and produced the highest callusing percentage (93.3%). However, NAA at a higher concentration (2.0 µM) induced the lowest callusing percentage (66.6%). TDZ has proved to be a potent cytokinin for promoting callus from woody plants [14]. Exposing plants to the combination of auxin and TDZ demonstrated that TDZ may possess an auxin-like property or may impinge upon the endogenous auxins by modifying their biosynthesis or metabolism. These results agree with the findings of Al-Juboory et al. [15] and Lata et al. [16] reporting similar effects on callus induction of *Gardenia jasminoides* and *Cimicifuga racemosa* respectively. Two-month-old calli were further subcultured using MS media supplemented with various concentrations of cytokinins for organogenesis. Our results indicated that shoot regeneration and proliferation was better in MS with TDZ supplemented media (**Table 1**) than with any other media containing BAP or KN (data not shown). Our earlier work described a similar effect of TDZ on direct organogenetic shoot regeneration and proliferation of *Cannabis sativa* cultures through nodal segments containing axillary buds [7]. In the present study, the shoot-like structures could be distinguished by the presence of green, opaque, and compact nodules. The optimum response in terms of regen-

Table 1 Effect of growth regulators on callus induction from leaf explant as well as root and shoot regeneration from leaf derived callus of *C. sativa*.

Growth regulator	Concentration (µM)	Regenerants		Percent of explants producing
		Amount of callus*		**Callus***
0	0	0		0
TDZ + NAA	1.0 + 0.5	+++		93.3
	1.0 + 1.0	+++		86.6
	1.0 + 1.5	++		80.0
	1.0 + 2.0	+		66.6
		Average shoot number†	**Average shoot length** (cm) †	**Shoots**†
TDZ	0.5	12.3[a]	8.0[a]	96.6
	1.0	8.5[b]	6.7[b]	93.3
	2.5	7.5[b]	4.6[c]	90.0
	5.0	3.2[c]	3.5[d]	86.6
	10.0	2.9[c]	2.8[e]	83.3
		Average root number†‡	**Average root length** (cm)†‡	**Roots**†‡
IBA	0.5	5.2[bcde]	4.5[bc]	86.6
	1.0	5.8[bc]	4.2[cd]	83.3
	2.5	10.0[a]	6.6[a]	96.6
	5.0	6.5[b]	5.2[b]	80.0
	10.0	5.3[bcd]	4.1[cd]	80.0

* Number of replicates: 30. +: < 1.0 g, ++: 1.0 to 3.0 g, +++: > 3.0 g. † Data represents the mean of three replicates with ten explants for each treatment; means followed by the same letter do not differ statistically at p = 0.05 according to the Tukey test. ‡ Medium used consisted of half strength Murashige and Skoog salts (1/2-MS)

eration frequency, number of shoots per callus, and proliferation was recorded in MS medium supplemented with 0.5 µM TDZ (**Fig. 1Sc**, Supporting Information). On this medium, 96.6% of the cultures responded with an average of 12 shoots per culture. The regenerated shoots elongated within 2 wk of culture. Thidiazuron (TDZ), a phenylurea compound, is known to exhibit a higher degree of cytokinin activity in callus culture and regeneration than BAP, KN, and zeatin [17,18]. It has also been suggested that the biological activity of TDZ is higher than or comparable to that of the most active adenine type of cytokinins [19,20]. Although, *C. sativa* shoots rooted on all media augmented with different concentrations of auxin, the presence of IBA resulted in a significantly higher rooting percentage (80–96%) than IAA or NAA. The average number of roots and the root length was at a maximum on 1/2-MS media containing 2.5 µM IBA (**Table 1** and **Fig. 1Sd**, Supporting Information). The promoting effect of IBA on the *in vitro* rooting of shoots has also been obtained in different plants [7,17,21]. As an alternative approach to our previous work [7], organogenic calli derived from young leaf tissues of *C. sativa* were used to induce shoot-buds with subsequent regeneration. Regeneration of plantlets via leaf calluses [4] and internode, petiole and axillary bud derived calluses [5] have been reported with other growth hormones like 2,4-D, KN, IBA, and BAP. However, the frequency of the induced shoots with these hormones are not as high as obtained with TDZ as reported in the present work. A report by Fisse et al. [22] assessed organogenesis but did not observe any direct organ formation on explants and reported that *Cannabis* calluses readily produced roots but were unreceptive to shoot formation. Mandolino and Ranalli [4] reported occasional shoot regeneration from calluses. Feeney and Punja [23] failed to regenerate hemp plantlets, either directly or indirectly from callus or suspension cultures. Richez-Dumanois et al. [3] induced direct multiplication of shoots from apical and axillary bud explants, using BAP; however rooting was described to have been extremely difficult and the response poor. In our experiments, the efficiency of shoot regeneration obtained was very high using TDZ (83 to 96%) in comparison to BAP. Rooted plantlets were successfully transferred to soil and new growths were observed after 2 weeks. The plants attained 16–18 cm in height within 6 weeks of transfer. These plants exhibited a 95% survival rate 8 weeks after transfer. Well rooted and acclimatized plants in soil as well as a comparison of cannabinoids profiles of the mother plant and a representative micropropagated plant are shown in **Fig. 1Se** and **f** and **Fig. 2S** (Supporting Information), respectively. The acclimatized tissue culture raised plants exhibited normal development, similar cannabinoids profile, comparable Δ^9-THC content and no gross morphological variation as compared to that of the mother plant. In conclusion, this study describes an efficient and reproducible protocol for regeneration of *C. sativa* plants from leaf derived callus which may be useful for the mass propagation and *in vitro* conservation of chemical profile based elite varieties of this important medicinal plant for pharmaceutical interest.

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