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Cannabinoids Production by Hairy Root Cultures of *Cannabis sativa* L.

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Abstract

Tetrahydrocannabinol (THC) derivatives are used clinically as analgesic, anti-inflammatory, appetite stimulant, anti-emetic and anti-tumor cannabinoids. THC and its related compounds are at present obtained by extraction from intact *Cannabis* plants or chemical synthesis, but plant cell cultures may be an alternative source of production. In the present study, hairy root cultures of *C. sativa* (Cannabaceae) were induced by incubation of aseptically grown callus culture with solid B5 medium supplemented with 4 mg/l naphthaleneacetic acid in darkness at 25˚C. Hairy root growth profiles in shake flask, increased periodically during 35 days of growth cycle. The cannabinoid contents produced in minor levels and remained below 2.0 µg/g dry weight. The contents of cannabinoid were analyzed by liquid chromatography and confirmed by mass spectrometry.

Keywords

Tetrahydrocannabinol, Cannabinoids, Callus, Hairy Root, *Cannabis sativa*

1. Introduction

Cannabis sativa L. (marijuana; Cannabaceae) is a traditional Central Asia annual dioecious plant that has gained popularity throughout the world due to its psychoactivity. This plant possesses a unique class of compounds, the cannabinoids, which accumulate mainly in the secretory cavity of the glandular trichomes of leaves [1] [2]. Currently, more than 80 known compound have been isolated from *Cannabis* plant [3] [4]. Cannabinoids (**Figure 1**) and in particular the main psychoactive THC are promising substances for the development of new drugs [5]-[7]. It has been used as analgesic, anti-inflammatory, appetite stimulant, and anti-emetic properties [8]. Among the major cannabinoids, CBD has been reported as neuroprotective antioxidants and a wide range of pharmacologi-

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cal activities [7]-[10]. Pentacyclic triterpenes [11], steroids [12], and alkaloids [13] have been isolated from the root of *C. sativa*. However, the commercial production of *Cannabis* has been limited by a range of issues including illegality of cultivation and breeding of *Cannabis* [14], variability of active components [15]. To address these issues, *in vitro* tissue culture techniques have been exploited to obtain a standardized method for secondary metabolite production [16] [17]. Hairy root cultures are a promising *in vitro* source for production of valuable phytochemicals as compared with plant suspension cultures due to their biochemical and genetic stability and morphological differentiation [18]. Very few reports on Agrobacterium-mediated transformation of *C. sativa* are available [19]-[21], but none of them are related to establishment of hairy root cultures with the capacity to produce cannabinoids. However, rhizogenic callus of *C. sativa* was induced on MS supplemented with different concentrations of NAA [19]. Also, Feeney and Punja studied the effect of different combination of PGRs on initiation rhizogenic callus of *C. sativa*. They found that, MS medium modified with B5 vitamins and supplemented with different combination of 2, 4-D, NAA, IBA, KIN, and BA induced roots after 4 weeks in darkness [22]. However, secondary metabolite production of hairy roots is genetically stable compared with other types of plant cell culture [23] [24]. Transformed hairy roots of *C. sativa* were obtained by infection callus cultures with *A. tumefaciens* harboring the *pNOV*3635 plasmid conferring resistance to mannose [22]. Moreover, Wahby coworkers investigated the stability of *β*-glucuronidase gene incorporated into the genomic DNA of transformed hairy roots from the seedling of *C. sativa* (variety: Futura 77, Delta-llosa, Delta405, CAN0111 and CAN0221) and *Nicotiana tabacum* cv. Burley F.13119 [21]. They used *Agrobacterium* strains (*A. tumefaciens* and *A. rhizogenes* harboring Ti and Ri plasmids, respectively) or disarmed strains of *A. tumefaciens* harboring the *pRi*1855*TL-DNA* genes *rolA*, *rolB*, and *rolC* alone or all together cloned in abinary vector *pBin* 19. They reported that about 10 transformants from different *C. sativa* varieties were screened for desirable traits in growth. Also, they reported that *rolABC* transgenic root cultures were generally characterized by high biosynthetic capacity and biochemical stability. However, to date, there is no report on hairy root induction from callus cultures of *C. sativa*. Keeping these in view, the experiment has been designed as follows: 1) to investigate the role of exogenous auxins on hairy root induction from callus cultures, and 2) to establish an efficient liquid culture system for biosynthesis of cannabinoids, especially THCA.

2. Materials and Methods

2.1. Initiation of Adventitious Root Cultures

Cannabis sativa callus cultures (1 g) cultivated onB5 medium [25] modified with 50 mg/l myo-inositol, 10 mg/l

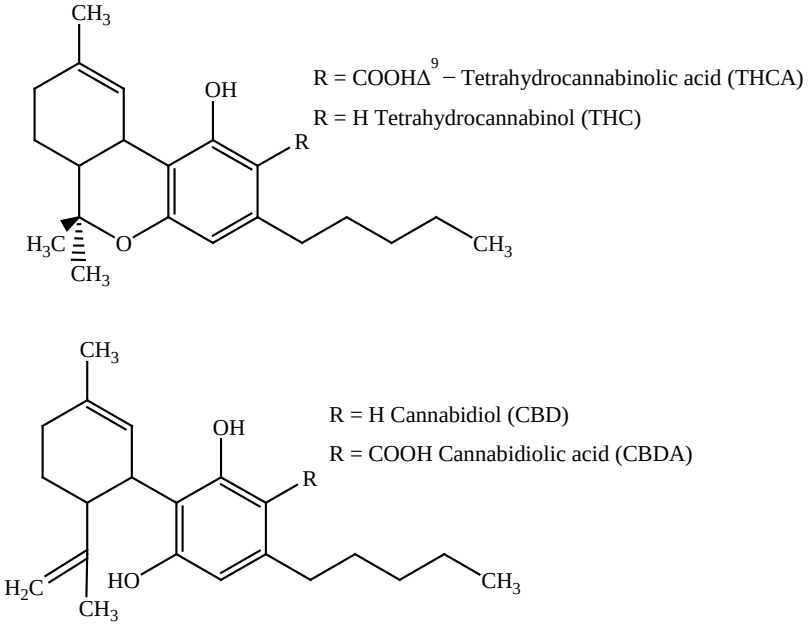

Figure 1. Structures of identified cannabinoids.

thiamine HCl, 1000 mg/l casein hydrolysate and supplemented with 3% sucrose and solidified with 0.4% gelrite, had initiated into adventitious roots on full strength B5 medium supplemented with various concentrations of auxins (1.5, 2.5 and 4 mg/l) of NAA, IBA and IAA (**Table 1**). The emerging hairy root cultures were isolated from callus cultures and subsequently transferred to solid B_5 medium with 4 mg/l NAA for their further proliferation. Hairy rooting cultures were subcultured every 30 days intervals and maintained in the "Grow Bank" system (Plant Master BB. XXL4 CLF Plant Climatics GmbH, Wertingen, Germany) and cultivation was accomplished in the dark condition at 25˚C. Initiation of adventitious root cultures was evaluated after 30 days of cultivation.

2.2. Optimization Hairy Root Cultures

Once established, the adventitious root cultures were cuttransversely and placed on Petri dishes (2 - 3 mm, 4 explants/treatment). Half solid B5 medium with various concentrations of auxins (0.25, 0.5 and 1.5 mg/l) of NAA, IBA and IAA in the dark at 25˚C were applied (**Table 2**). The morphology of hairy roots grown on the cultured media was evaluated using light microscope (Nikon SMZ800) and images were captured by a microscope digital camera system Nikon type-104 (Nikon, Japan). A liquid culture was obtained by incubating the root tips to B5 medium (100:250 ml Erlenmeyer flask) supplemented with 4 mg/l NAA. Roots were then grown in the dark at 25˚C on a rotary shaker (INFORS. HT, Bottmingen, Switzerland) at about 110 rpm. These roots were subcultured every 30 days and maintained for about 3 years in the liquid medium.

2.3. Growth Kinetics and Cannabinoids Production

The kinetics of root growth were investigated by transferring (1 g fresh mass of inoculum; 4 - 5 cm of root tips) of two-week-old to liquid B_5 medium (100:250 ml Erlenmeyer flask; pH adjusted to 5.8 prior to autoclave), supplemented with various concentrations of auxins (**Table 3**). During the culture period of 35 days, hairy growth kinetics was evaluated every 7 days to determine the fresh and dry weights and the content of cannabinoids. The fresh weight was obtained by removal of three independent biological replicates induction flasks and washing with distilled water by suction filtration, then the cells were lyophilized for 24 h in freeze dryer (Alpha I-4 LSc Christ GmbH, Osterode am Harz, Germany) at −55˚C and 0.6 mbar until constant dry weight was obtained.

2.4. Extraction and Determination of Cannabinoids

Dry biomass samples were pulverized and homogenized with methanol (70%, 3 × 10 ml: g roots) at 25˚C for 1 h.

Table 1. Induction media for hairy root (IHR) growth on callus cultures.

Nutrient medium	Phytohromone (mg/l)			No. of roots/callus $(\bar{x} \pm SE)^{\dagger}$	Length of roots (cm)/callus $(\bar{x} \pm SE)^{\dagger}$	% Root formation
	Auxins					
	NAA	IBA	IAA			
PGR free	0.0	0.0	0.0	0.0	0.0	0.0
IHR0[a]	4.0			9.3 ± 0.3	1.33 ± 0.3	83.3
IHR1[b]	2.5			0.0	0.0	0.0
IHR2[b]	1.5			0.0	0.0	0.0
IHR3[b]		4.0		0.0	0.0	0.0
IHR4[b]		2.5		0.0	0.0	0.0
IHR5[b]		1.5		0.0	0.0	0.0
IHR6[b]			4.0	0.0	0.0	0.0
IHR7[b]			2.5	0.0	0.0	0.0
IHR8[b]			1.5	0.0	0.0	0.0

Full solid B5 medium with plant growth regulators (PGR free). [a]Fullsolid B5 medium. [b]Various treatments on solid half strength B5. [†]All data the mean 3 replicates with two calli pieces/treatment; repeated three times; mean ± SE; 0 = no response. Induction of hairy roots from callus cultures was observed after 4 subcultures. NAA, naphthaleneacetic acid; IBA, Indolebutyric acid; IAA, indole-3-acetic acid.

Table 2. Effects of different concentrations of auxins on hairy root differentiation on solid medium (SHR).

Nutrient medium	Phytohromone (mg/l)		
	Auxins		
	NAA	IBA	IAA
PGR free	0.0	0.0	0.0
SHR0[a]	4.0		
SHR1[b]	0.25		
SHR2[b]	0.5		
SHR3[b]	1.0		
SHR4[b]		0.25	
SHR5[b]		0.5	
SHR6[b]		1.0	
SHR7[b]			0.25
SHR8[b]			0.5
SHR9[b]			1.0

[a]Wild type on full solid B5 medium (wild type). [b]Various treatments on solid half strength B5. NAA, naphthaleneacetic acid; IBA, Indolebutyric acid; IAA, indole-3-acetic acid.

Table 3. Effects of different concentrations of auxins on growth cannabinoid formation in shake flask culture of hairy roots (LHR).

Nutrient medium	Phytohromone (mg/l)		
	Auxins		
	NAA	IBA	IAA
PGR free	0.0	0.0	0.0
LHR0[a]	4.0		
LHR1[b]	0.25		
LHR2[b]	0.5		
LHR3[b]	1.0		
LHR4[b]		0.25	
LHR5[b]		0.5	
LHR6[b]		1.0	
LHR7[b]			0.25
LHR8[b]			0.5
LHR9[b]			1.0

[a]Wild type on full liquid B5 medium (wild type). [b]Various treatments on liquid halfstrength B5. NAA, naphthaleneacetic acid; IBA, Indolebutyric acid. IAA, indole-3-acetic acid.

The extracted solution for each treatment were combined, filtered and evaporated *in vacuo* at 35˚C using a Büchi Rotavapor (R-210, Switzerland) and the resulting residue dissolved in chloroform (2 × 10 ml). The chloroform extracts were combined dehydrated over sodium sulfate anhydrous, filtered and evaporated *in vacuo* at 35˚C to give total cannabinoids mixture. The total mixture was then re-dissolved again in 0.5 ml methanol, centrifuged at 13,000 × g for 4 min, at 4˚C. Then the clear supernatant was removed, filtrated by 0.2 µm filter and kept in a 2 ml clean amber vial, deoxygenated with CO_2, and stored at −20˚C for further qualitative and fingerprinting studies. A calibration curve was established using CBDA, THCA, and THC (THC-Pharm, Frankfurt, Germany) as a standard.

2.5. High-Performance Liquid Chromatography

Chromatographic analysis of residue obtained after extraction and evaporation (see above) was carried out in an

Agilent HPLC 1260 infinity (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model no. G1312B), an auto-sampler (model no. G1367D), a degasser (model no. G1379B) and a photo-diode array detector (model no. G4212B). The HPLC system was controlled by the ChemStation software (Agilent, v. 04.03). The separation of cannabinoids was achieved on a Gemini C18 column (250 × 4.60 mm, 5 µm particle size; Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of two solvents: 0.1% aqueous trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The compounds were eluted with a step gradient as follows: 0 - 10 min (65% B), 10 - 13 min (95% B), 13 min (65% B); 13 - 15 min (65% B). The flow rate was 1.5 ml/min and the injection volume was 10 µl. Peaks of cannabinoids were detected at 224 nm and quantified on the basis of authentic compounds.

2.6. Liquid Chromatography/Mass Spectrometry

Cannabinoids in the Methanol crude extract of hairy roots were analyzed by LC/(+)ESI-MS LTQ-Orbitrap spectrometer (Thermo Scientific GmbH, Schwerte, Germany). The mass spectrometer was equipped with HPLC system (Agilent 1200) consisting of pump (model no. G1312), and autosampler (model no. G1367 HIP-ALS). Nitrogen was used as protective gas (60 arbitrary units), and helium served as the collision gas. The separations were performed by using a Luna C18 column (50 × 3 mm, 3 µm particle size; Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of two solvents: 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The compounds were eluted with a step gradient as follows: 0 - 1 min (20% B), 6 - 10 min (100% B), and 10 - 15 min (20% B). The flow rate was 0.5 ml/min and the injection volume was 5 µl. Peaks of cannabinoids were detected at 224 nm and identified on the basis of authentic compounds. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 150 - 450) with nominal mass resolving power of 60,000 at *m*/*z* 400 with a scan rate of 1 Hz, with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass; *m*/*z* 391.284286; bis-(2-ethylhexyl)-phthalate. MS/MS experiments were performed by CID (collision induced decay, 35 eV) mode. LC/MS^2 was sufficient for identification THC and CBDA. However, THCA in hairy root samples (time course) were produced in a mount that were two small to be identify by LC/MS^2. Subsequently, the protonated molecular ion *m*/*z* for THCA were used for CID to identify their fragmentation by LC/MS^3 with CID (collision induced decay, 40 eV). Following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260˚C, tube lens 70 V.

2.7. Chemicals and Reagents

B5 medium was supplied by Sigma-Aldrich Company (Germany). THCA, THC and CBDA were purchased from THC-Pharm, (Germany). All other plant growth regulators and chemicals involved in this study were reagent grade.

2.8. Data Processing and Statistical Analysis

Thermo Electron's Xcalibur (version 2.2) was used as instrument control and data processing platform for LC-MS data collection, peak identification, and measurement. All other intermediate data manipulation was carried out using. The data were analyzed statistically using Microsoft Excel 2010, and the data of each sample (means ± SE) was analyzed in triplicate.

3. Results and Discussion

3.1. Initiation of Adventitious Hairy Roots from Calli

In general, all the testedauxins (**Table 1**) did not stimulate root differentiation except full strength B5 medium with 4 mg/l NAA (IHR0). After calli were incubated on IHR0 for 16 weeks in darkness at 25˚C, numerous hairy roots began to emerge vigorously from the surface of calli with 83.3% root formation. Eight weeks later, hairy roots showed the characteristics of the typical adventitious roots, having numerous branching covered with root hairs. However, the ability of callus cultures of other systems to proliferate hairy roots in response to PGRs was studied [26] [27]. Similar results were reported that NAA initiated the growth of hairy roots from callus cultures of *C. sativa* [19].

3.2. Optimization Hairy Root Cultures

The auxins type influenced the root growth and multiplication (**Table 2**). Hairy roots on solid media SHR5, and SHR8 gave high significance of numbers of hairy roots with an average (37 ± 4.7 and 27.25 ± 4.9) root per culture, respectively. On the other hand, media SHR2, and SHR8 gave high significance of lateral hairy roots with an average (6.3 ± 0.14 and 5.2 ± 0.28 cm) root length per culture, respectively. The auxin concentration was affected on root phenotypes. While phenotypes of roots hairs formed on SHR0, SHR2, and SHR5 were rather different than on SHR8. From morphologic perspective, hairy roots (Figure 1S) are divided into three zones [28] [29]. The meristematic zone (MZ) where cell division and formation of a lateral branch takes place, the elongation zone (EZ) where cells expand along the longitudinal axis and the differentiation zone (DZ) where root hairs develop. The obtained results are in agreement with those reported that auxins NAA, IBA, and IAA are more efficient in initiation and phenotypic variation of hairy root [30].

3.3. Growth Kinetics and Cannabinoids Production of Hairy Roots in Shake Flask

Effects of exogenous auxins on the growth kinetics and cannabinoids formation of hairy root was determined and illustrated in Figure 1S.

3.3.1. Hairy Root Growth

A representative growth curves in shake flasks achieved after the 35 days. As shown in **Table 3**, auxins had positive influence on biomass and almost increased linearly. The hairy root cultured in LHR1 (1/2 B5 + 0.25 mg/l NAA) gave the highest biomass (fresh weight 5.31 ± 0.15 g/flask and dry weight 0.77 ± 0.008 g/flask at day 35 of the growth cycle) as compared with that of wild typeLHR0, (B5 + 4 mg/l NAA) culture which was (fresh weight 4.46 ± 0.05 g/flask and dry weight 0.62 ± 0.03 g/flask at day 35 of the growth cycle).

3.3.2. Cannabinoids Fingerprinting of Hairy Root Cultures in Shake Flask

1) HPLC analysis

The HPLC results, showed that the highest levels of THCA was noted with LHR3 (1/2 B5+ 1.0 mg/l IAA) was 1.04 µg/g dry weight (0.32 ± 0.01 g/flask), at day 28 of the growth cycle, respectively. Interestingly, the highest CBGA levels was noted with LHR9 (1/2 B5+ 0.5 mg/l IBA) was 1.63 µg/g dry weight (0.23 ± 0.02 g/flask), at day 28 of the growth cycle. The highest CBDA levels was recorded with wild type (LRH0) was 1.67 µg/g dry weight (0.42 ± 0.01 g/flask), at day 28 of the growth cycle. Also, it was noted that the formation of cannabinoids decreased after 28 days of the growth cycle. On the other hand, *de novo* biosynthesis and formation of cannabinoids in dark-grown hairy root cultures are not surprising, and could be many enzymes in pathway up-regulated in dark. From these results we conclude that cannabinoids biosynthesis is not dependent on light, as in our case the hairy root cultures were maintained in darkness.

2) LC-MS/MS analysis

Identification of cannabinoids in the extracted samples of LHR0at different time points (7, 14, 21, 28 and 35 day of the growth cycle) was detected by LC/(+)ESIMS/MS (**Figure 2** and **Figure 3**). **Table 3** summarizes the following: 1) Compound and formula, 2) t_R (min), 3) and *m*/*z* ratios for the protonated $[M+H]^+$ molecules (**Table 4**) and 4) detected cannabinoids at 14, 28 and 35 days of the growth cycle. The detailed MS/MS spectra of some cannabinoids were found in crude metabolites of LHR0 are illustrated in **Figure 2**. The high resolution mass spectra showed the presence of ions with characteristics peaks of cannabinoids: *m*/*z* 315.12 (THC), and *m*/*z* 355.19 (CBDA). Intensity peaks of THCA, THC, and CBDA were found in higher levels after 7 days of culturing. After 21 days the intensity peaks of those compounds were not detected. The retention times and LC-MS/MS spectra of the detected compounds were identical to the data obtained for the standards without interfering. The

Table 4. Summary of detected peaks by LC-MS/MS.

No	Compound and formula	Theoretic mass	t_R [min]	Major product ions $[m/z]^+$	Cannabinoids/day				
					7	14	21	28	35
1	THCA-$C_{22}H_{30}O_4$	358.47	5.90	358.35 $[M + H]^+$, 341.17, 331.12, 301.14, 207.10	+	+	+	-	-
2	THC-$C_{21}H_{30}O_2$	314.45	5.80	315.12 $[M + H]^+$, 297.22, 272.17, 255.21, 207.14	+	+	+	+	-
8	CBDA-$C_{21}H_{30}O_4$	358.47	5.06	355.19 $[M + H]^+$, 341.17, 327.21, 301.14, 207.10	+	+	+	-	-

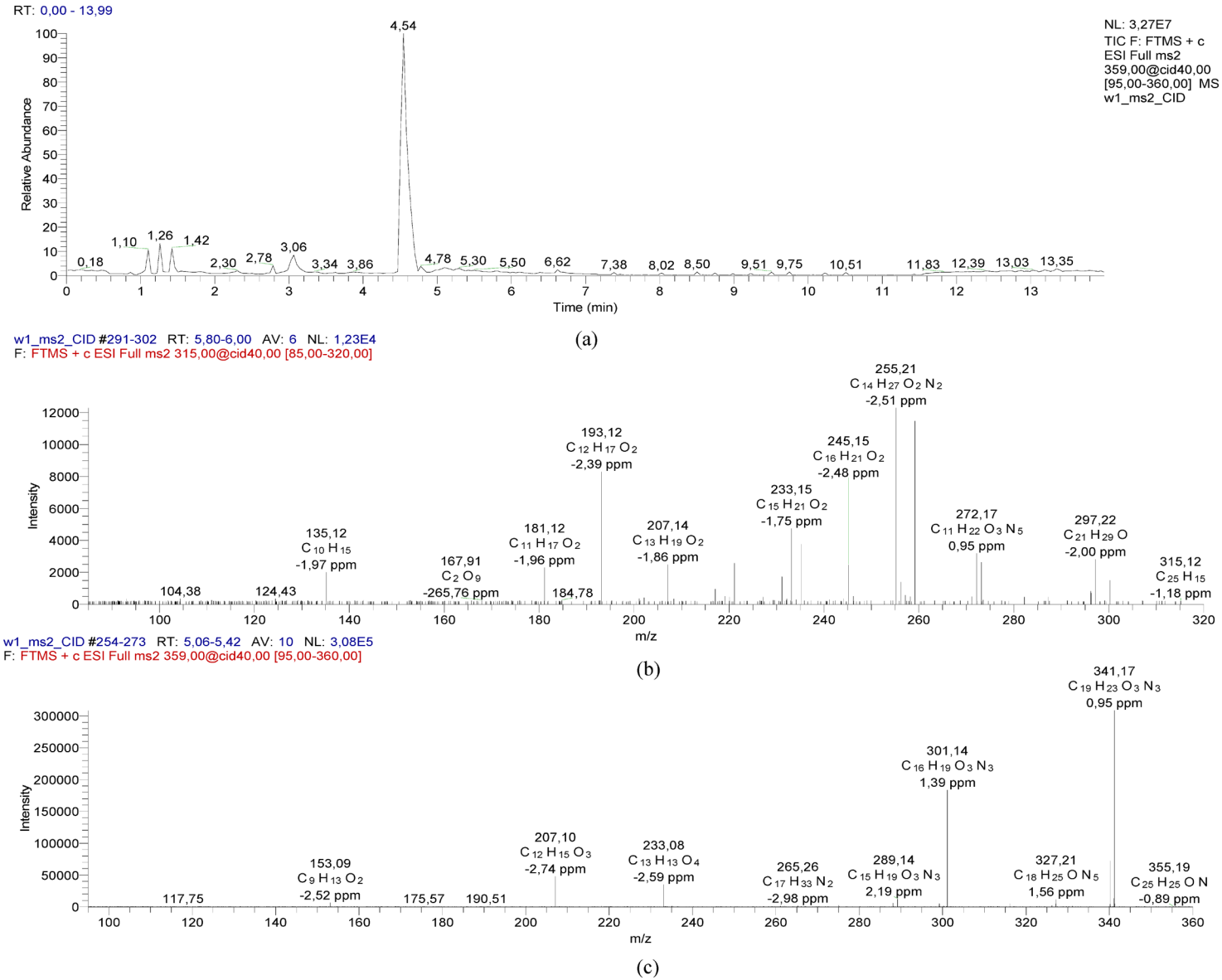

Figure 2. LC-MS/MS spectra of the total ion current (TIC) of shake flask hairy root cultures of LHR0 (4 mg/l NAA, after one week of co-culture) extract (a), MS2 spectra THC (b) and MS2 spectra CBDA (c).

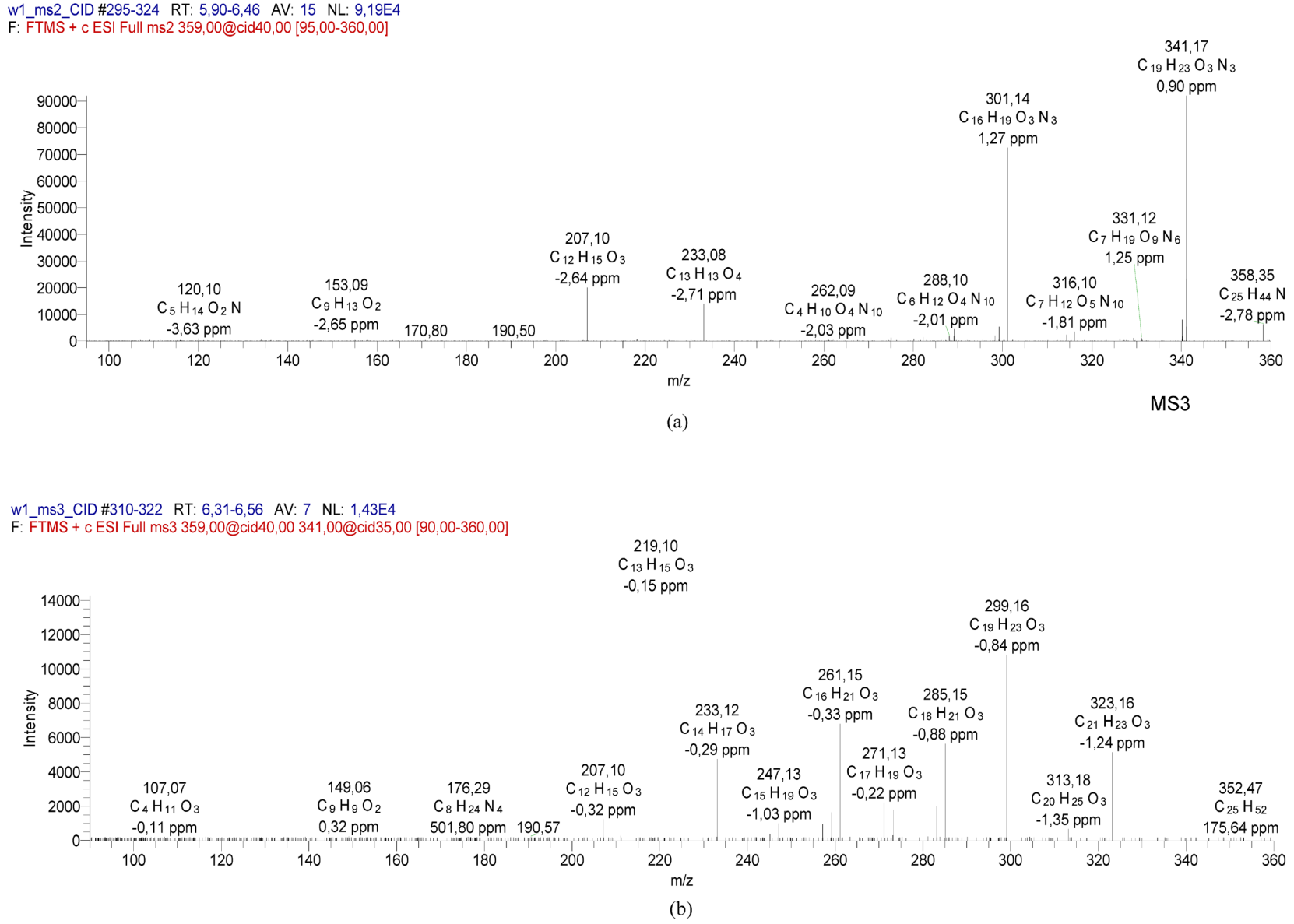

Figure 3. MS2 spectra of THCA (top) and MS3 spectra of THCA of peak m/z 341.17 (bottom).

fragmentation of THCA was confirmed by comparison MS^2 and MS^3 spectra of standard with the spectra of extracted sample. The MS^2 spectrum of $[M + H]^+$ for THCA evidences an intense signal at *m*/*z* 341. Further fragmentation of this ion was verified by MS^3 (**Figure 3**), showing a dominant fragmentation pathway: *m*/*z* 323 → *m*/*z* 285 → *m*/*z* 261 → *m*/*z* 233 → *m*/*z* 219, respectively. These product ions are identical to those of standard.

4. Conclusion

To the best of our knowledge, this work is the first report on the induction of hairy roots and cannabinoids production of *C. sativa*. These induced roots may be served as an alternative source of cannabinoids. The results of HPLC indicated that cannabinoids produced in shake-flask cultures were very low and feasibility of scaling up these cultures will be difficult. Further, LC/(+)ESI/MSMS and LC/(+)ESI/MS^3 analyses conformed the production of THCA, THC, and CBDA compounds in hairy roots.

5. Supporting Information

Induction of adventitious hairy roots on callus cultures, proliferation (Figure 1S).

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Abbreviations

B5: Gamborg's medium
MS: Murashige & Skoog medium
PGRs: plant growth regulators
2,4-D: 2,4-Dichlorophenoxyacetic acid
NAA: naphthaleneacetic acid
IAA: indole-3-acetic acid
IBA: Indolebutyric acid
KIN: kinetin
BA: benzylaminopurine
THC: Δ^9-Tetrahydrocannabinol
CBD: cannabidiol
THCA: tetrahydrocannabinolic acid
CBDA: cannabidiolic acid
HPLC: high-performance liquid chromatography
LC-MS: liquid chromatography-mass spectrometry
CID: collision induced dissociation
TIC: total ion current
m/*z*: mass-to-charge ratio