Cannabinoids production in *Cannabis sativa* L.: An *in vitro* approach

Zur Erlangung des akademischen Grades eines

Dr. rer. nat.

von der Fakultät Bio- und Chemieingenieurwesen
der Technischen Universität Dortmund
genehmigte Dissertation

vorgelegt von

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Tag der mündlichen Prüfung: 26.11.2014

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Dortmund 2014

This thesis is dedicated to my parents, wife and two children

I. Abstract

Cannabis sativa L. (Cannabaceae) is the oldest known medicinal plant. For millennia, the plant has also been used for fibre and oil production.The most prominent feature of *C. sativa* is the psychoactive effect ascribed to its secondary metabolites, cannabinoids (mainly to tetrahydrocannabinol, THC). However, many other pharmacological properties of the aforementioned specialized compounds have been described. Currently, the demand for THC for various medical applications is substantial, while cultivation and breeding of *Cannabis* in most countries is strictly regulated and limited to serving research purposes and meeting therapeutic needs. Therefore, the hereby proposed and discussed production of THC using *in vitro* cultures could be a viable alternative. In the work presented here, *in vitro* organogenesis from callus cultures (undifferentiated plant cell masses grown on solid media) was successfully established, ultimately resulting in regeneration of the complete *C. sativa* plant. Further, production of THC as well as other important cannabinoids was achieved in cell suspension, hairy root and trichome cultures of *Cannabis*. The optimal combination of phytohormones, as applied to the B5 growth medium, fostering the development of meristemoids from callus cultures was: 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA) and adenine hemisulfate salt (AS) in respective concentrations of 0.5, 5 and 40 mg/l. Concurrently, the most favourable augmentation protocols of the B5 medium for the induction and differentiation of shootlets (small plants with leaves but without roots) were: 0.5 mg/l of gibberelic acid (GA3) or 0.25 mg/l of thidiazouron (TDZ) and 3 mg/l of GA3 (8.5 ± 1.73 and 7.25 ±1.03 shootlets/callus, respectively). The subsequent root formation of shootlets was most prominent after supplementation with 1.5 mg/l of indole-3-acetic acid (IAA). *In vitro* acclimatized plants growing in Erlenmeyer flasks formed tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA) and cannabidiolic acid (CBDA), retrieved at respective concentrations of about 0.33, 0.45 and 157.1 mg/g fresh weight. In contrast, *ex vitro* acclimatized plants (grown hydroponically for 8 weeks) synthesized THCA, THC, CBGA, cannabigerol (CBG) and CBDA at corresponding concentrations of 1.54, 28.30, 6.0, 0.125 and 1121.4 mg/g fresh weight. The obtained results confirmed the generation of pharmacologically important cannabinoids; however, the biosynthetic abilities of the investigated cell and hairy root cultures did not provide sufficient levels of the valuable metabolites to warrant scaling-up of the proposed *in vitro* production platform.

II. Zusammenfassung

Cannabis sativa L. (Cannabaceae) ist die älteste bekannte medizinisch genutzte Pflanze. Seit Jahrtausenden wird die Pflanze auch für die Produktion von Fasern und Öl genutzt. Die bekannteste Eigenschaft von *C. sativa* ist die psychoaktive Wirkung, aber auch zahlreiche weitere pharmakologische Eigenschaften sind beschrieben, die auf die Cannabinoide zurückzuführen sind, besonders auf Tetrahydrocannabinol (THC). Gegenwärtig gibt es einen hohen Bedarf an THC für diverse medizinische Anwendungen. Die Kultivierung und Züchtung von Cannabis ist in den meisten Ländern verboten, mit Ausnahme für Forschungs- und pharmazeutische Zwecke. Daher könnte die Produktion von THC mit *in vitro*-Kulturen eine Alternative sein. In der vorliegenden Arbeit wird die Regeneration ganzer Pflanzen ausgehend von Kallus-Kulturen (undifferenziertes Zellwachstum auf festen Medien) und die Bildung von THC und anderen Cannabinoiden in Zellsupsensionen, Haarwurzel-Kulturen und Trichomkulturen beschrieben. Die optimale Kombination zur Stimulierung der Bildung von Vorstufen zur Blattbildung (Meristemoide) ausgehend von Kalluskulturen wurde auf festem B5-Medium erzielt, welches mit den Pflanzenhormonen 1-Naphthalensäure (NAA, 0,5 g/Liter Medium)), 6-Benzylaminopurin (BA, 5 mg/Liter) und Adenein-Hemisulfat (AS, 40 mg/Liter). Das optimale Medium für die Induktion der Bildung kleiner Pflanzen mit Blättern ohne Wurzeln („Shootlets“) war B5 + 0,5 mg Gibberellinsäure (GA_3)/Liter mit 8,5 ± 1,73 Shootlets pro Kallus und B5-Medium + 0,25 mg Thidiazuron/Liter + 3 mg GA_3/Liter (7,25 ± 1,03 Shootlets pro Kallus). Die beste Wurzelbildung der Shootlets wurde mit 1,5 mg Indol-3-Essigsäure (IAA) erzielt. Die *in vitro* in Erlenmeyerkolben angezogenen Pflanzen bildeten THCA, CBGA und CBDA mit einer Konzentration von 033, 0,45 und 157,1 mg/g Frischgewicht. Werden die Pflanzen außerhalb von Erlenmeyerkolben in hydroponischer Kultur über acht Wochen angezogen, werden THCA, THC, CBGA, CBG und CBDA mit Konzentrationen von 1,54, 28,3, 6,0, 0,125 und 1121,4 mg/g Frischgewicht gebildet. Die Untersuchungen zeigten die Bildung pharmakologisch wichtiger Cannabinoide durch *C. sativa*, aber die Biosynthese-Leistung der Zellen ergab keine ausreichenden Konzentrationen, die eine Maßstabsvergrößerung sinnvoll erscheinen lassen würde.

III. Contents

1. Introduction

1.1. Brief history of *Cannabis*

The genus *Cannabis* (family Cannabaceae) is an annual flowering plant. According to Li (1974), the first historical reference to *Cannabis* dates back to 5000 BCE and places its origins in Central Asia, while its medicinal uses were recorded in stone and papyrus documents of ancient Egipt circa 1700-1600 BCE (Ebbell, 1937; Russo, 2007; Russo *et al.*, 2008, Figure 1B). Subsequently, the ancient Greeks and Romans noted the therapeutic value of *Cannabis* (Figure 1C). In the 2nd century BCE, Bausanius and Glen documented it in Roman records (Brunner, 1973), while Diocorides, a Greek physician, in his work *De Materia Medica*, published in the 1st century BCE, recommended *Cannabis* seeds for the treatment of otalgia (Dioscorides, 1968). By the early 10th century, hashish and its medicinal properties were widely known to Arabic physicians (Nahas, 1982). During the Industrial Revolution, marijuana became a popular commodity serving both commercial and medicinal purposes (Fankhauser, 2002; Waldo, 2006). In the following two decades, *Cannabis* was grown extensively in various parts of Europe and the USA (Gaoni and Mechoulam, 1964; Russo, 2011).

The current systematic classification of *Cannabis* is as follows (Sytsma *et al.,* 2002):

Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Hamamelidae
Order	Rosales
Family	Cannabaceae
Genus	*Cannabis* L.
Species	*sativa*

Figure 1: Early records on *Cannabis sativa.* A: Chinese wood print of a *Cannabis* plant with descriptive text from 1234 CE, Edition of *Chêng-lêi pên-ts'ao* (Rätsch, 2001); B: Fragment of the Ebers papyrus depicting *Cannabis* prescription (Ebers, 1875); C: Dioscorides' manuscript *De Materia Medica* (Dioscorides, 1968).

1.2. Botanical description of *Cannabis*

1.2.1. Macroscopic features

Cannabis sativa herb (Figure 2) is characterized by an erect stem up to 1-6 m high, depending on the phenotype and chemotype. The whole plant is covered with trichomes. Its root system is laterally branched, reaching about 30-60 cm up to 2.5 m deep in loose soils or developing very near to the surface under wet soil conditions. Leaves are alternately arranged, palmate in shape and composed of seven lobes with long petioles (2-7 cm) and serrate margins. The leaf surfaces is scattered with white to yellowish-brown, resinous glands. Inflorescences are staminate (the male flower) or pistillate (female) (Stearn, 1970; Gossop, 1981; Clarke, 1981).

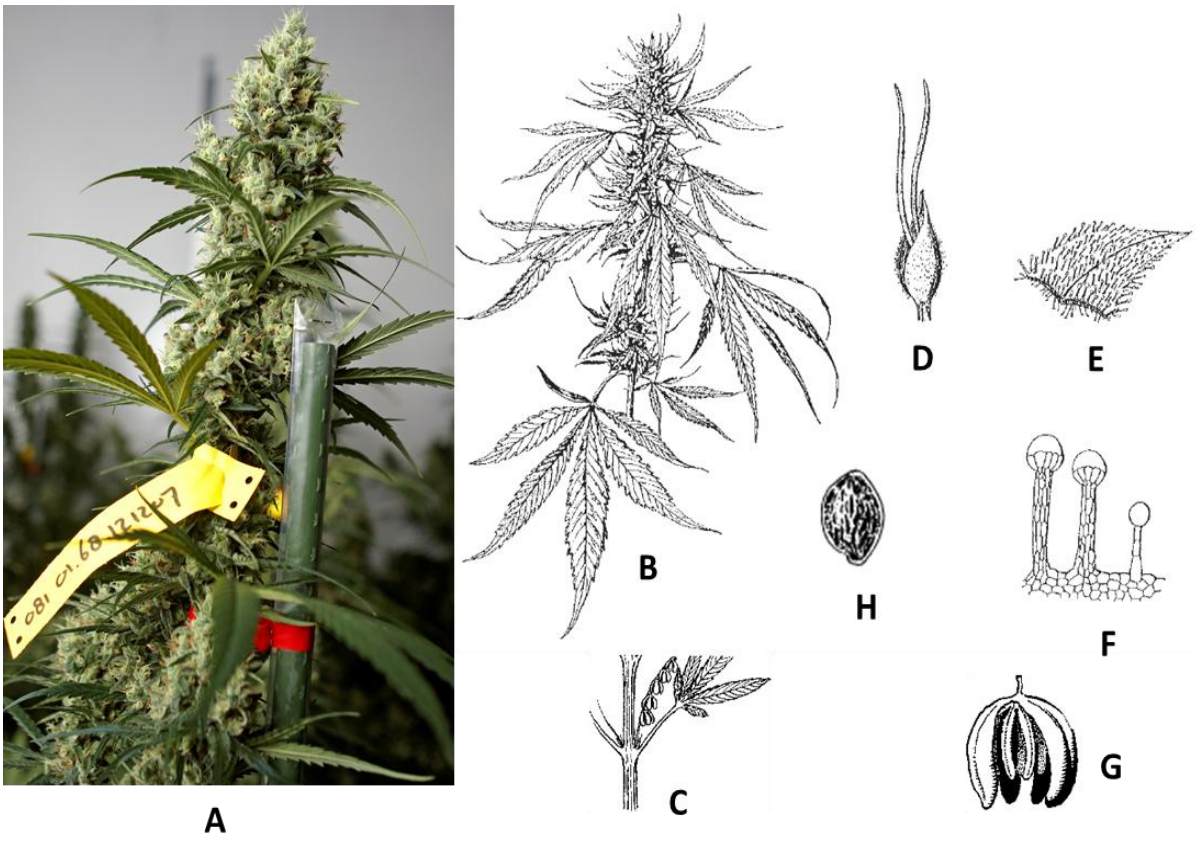


Figure 2: A: Female bud of medicinal *C. sativa* grown commercially by Bedrocan BV (the Netherlands); B: Inflorescence; C: Part of a male inflorescence; D: Female flower; E: Bracts of a female flower; F: Trichomes on epidermal surface; G: Male flower; H: Seed (reprinted with permission of Ed Rosenthal).

1.2.2. Microscopic features

Cannabis glands (Figure 3) are epidermal in origin (Szymanski *et al.,* 2000) and can be subsumed into diverse morphotypes, while each one consists of a stalk and secretory cells (Fahn, 1979; Kim and Mahlberg, 1991; Szymanski *et al.,* 2000; Gershenzon *et al.,* 1992). Further specifications are summarized in Table 1.

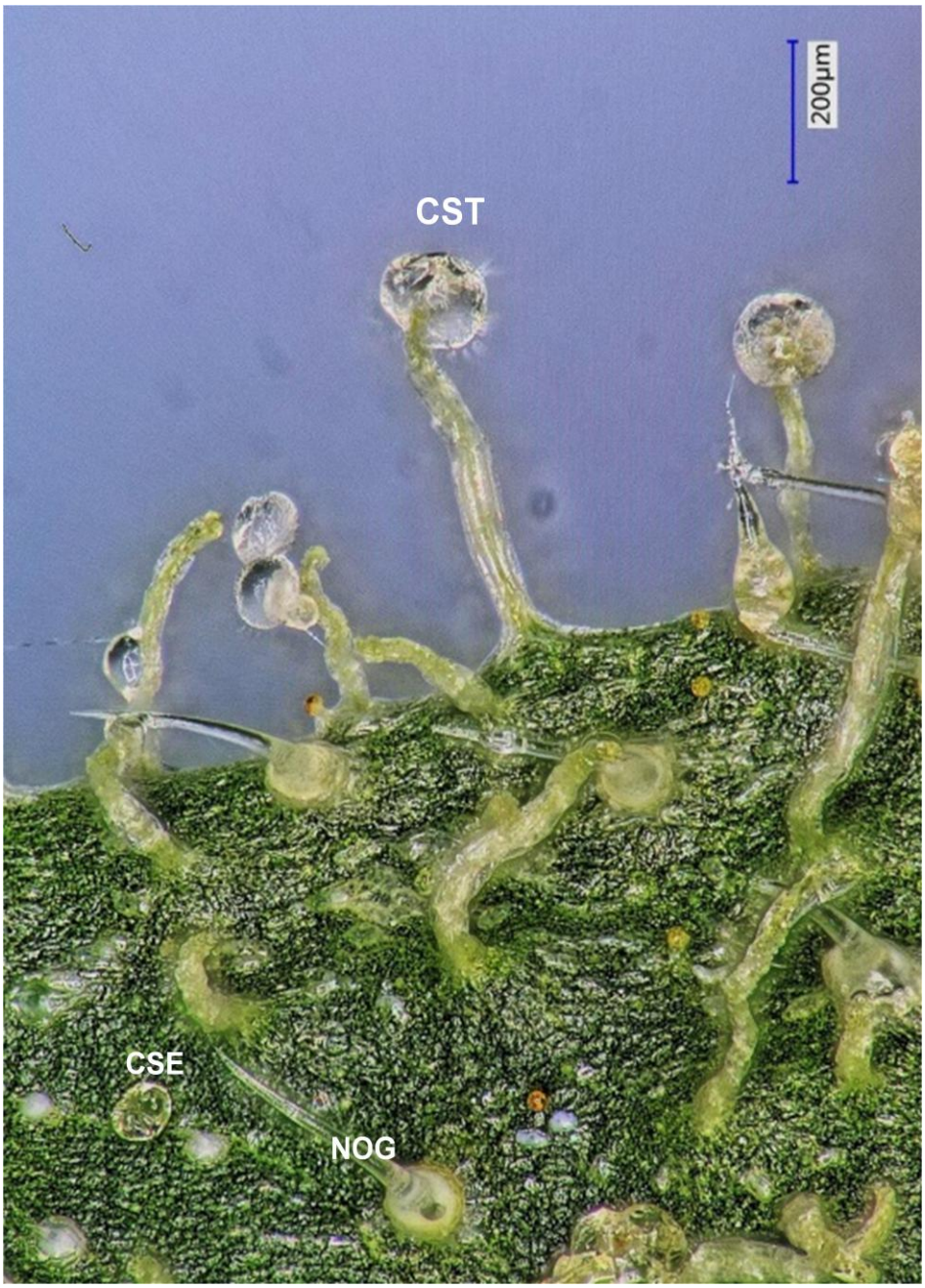


Figure 3: Microscopic photograph of *C. sativa* leaf trichomes. CST: Capitate-stalked trichome; CSE: Capitate-sessile trichome; NOG: Non-glandular trichome.

Table 1: A summary of *Cannabis* trichomes classification, structure, distribution, timing of development, and life span.

Trichomes					
Classification	**Structure**	**Distribution**	**Timing of Development/ density**	**Life Span**	**Referances**
Non-glandular trichomes[a]	a) *non-cystolithic trichomes*: long, unicellular, smooth and curved	lower side of vegetative leaves and pistillate bracts	decreases with age	the viability and functionality of secretion is correlated with senescence of epidermal cells	(Fairbairn 1972; Hammond and Mahlberg 1977; Turner *et al.* 1977, 1980b, 1981; Croteau 1988; Werker 2000; Guy and Stott 2005; Happyana *et al.* 2013)
	b) *cystolithic trichomes*: more squat, unicellular, claw shape, cystolith and containing calcium carbonate				
Glandular trichomes[b]	a) *bulbous*: smallest gland	vegetative leaves and pistillate bracts			
	b) *capitate-sessile (unstalked)*: commonly simple structure, head connected directly to the mesophyll cells				
	c) *capitate-stalked*: more complex structure, their developed resin head (glandular head) resembles a golf ball sitting on a tee (the trachome stalk)	bracts and floral leaves	increases with age		
Antherial sessile trichomes[c]	larg size diameter of approximately 70-80 *µ*m	underside of the anther lobes			

[a]non-glandular trichomes are devoid of cannabinoids;[b]glandular trichomes are the principal or sole storage site of most cannabinoids;[c]male plants develop few glandular trichomes and, consequently, produce marginal amounts of cannabinoids or terpenes.

1.3. *In vivo* cultivation and breeding of *Cannabis*

1.3.1. Indoor cultivation

Currently, cultivation and breeding of highly potent varieties of *C. sativa* is illegal all over the world. However, select pharmaceutical companies (e.g., Bedrocan BV and GW Pharmaceuticals Ltd.) have been granted a license to grow the plant under strictly controlled conditions, for research and medicinal purposes. The main advantage of indoor cultivation is the ability to control culture conditions facilitating yield improvement, life cycle regulation, pest control and prevention of self- and cross-pollination (Rosenthal, 1984; Stamler *et al.*, 1985; Chandra *et al.*, 2010). Moreover, there are a number of techniques of growing *Cannabis* hydroponically. The most important facet in hydroponics is the nutrient solution consisting of all the essential elements for plant growth and development in appropriate amounts and proportions. The crucial macronutrients in the solution are three cations (potassium, calcium and magnesium) and three anions (nitrate, dihydrogen phosphate and sulphate). Relative proportions of the aforementioned ions should be equilibrated to avoid ionic imbalances by monitoring the pH within a certain range (5.5-6.5) for maximum uptake and optimal plant growth (Steiner, 1961; Argo and Fischer, 2002). On the other hand, many hydroponic growers use special nutrient solution formulations (“recipes”) based on established plant and crop management conditions (Le Bot *et al.*, 1998). Oxygenation of either the hydroponic nutrient solution or rooting medium is another important aspect affecting root function, particularly the rate of water and nutrient uptake (Porterfield and Musgrave, 1998). Indoor *Cannabis* crop cultivation requires artificial light to facilitate and regulate photosynthetic (optimal plant growth) and photoperiodic processes (controlling flowering and plant shape) (Coene, 1995). Types of light sources available for that purpose were described by van Patten (Van Patten, 1992), with a special emphasis placed on high intensity discharge (HID), metal halide (MH) and high pressure sodium (HPS) lamps (Parker, 1994; Jones, 1997; Zheng *et al.*, 2005).

However, the photosynthetic rate is not only light-dependent but also positively correlated to carbon dioxide (CO_2) concentration surrounding the plant (in ambient atmosphere, 300-400 ppm) (Jones, 1997; Sicher and Bunce, 1997). Therefore, delivery of compressed CO_2 gas is required for indoor cultivation, increasing plant size and speed of growth up to 100 % (Jones, 1997). Nowadays, numerous controlled environment agriculture (CEA) protocols are available for the regulation of

air and root temperature, atmospheric humidity and gas composition, light intensity and wavelength composition as well as photoperiod duration, water supply and quality, growth medium composition and nutrient supplementation (Kubota and Thomson, 2006; Van Os *et al.*, 2008).

1.3.2. Seed selection and germination

High potency *Cannabis* seeds should be planted in small jiffy pots to standardize hydroponic indoor cultivation. Germination is initiated in course of three days. Well-developed seedlings are then transferred to small pots for optimal stimulation of their vegetative growth. After sufficient development of roots and biomass, the plants are transferred into a multi-flow hydroponic system equipped with a nutrient reservoir and an electronic timer to control the flow of nutrients from the reservoir to the plant pots (Chandra *et al.*, 2010).

1.3.3. Selection of mother plants and cloning

Selection of *Cannabis* female specimens, exhibiting vitality required to become mother plants, should be performed under constant vegetative light (HID lamps, 24 h/day) and with the continuous supply of the hydroponic nutrient solution, in order to foster the generation of the needed propagation stock. To maintain the genotype of the mother plant throughout subsequent generations, a method relying on the use of clones is applied (Potter, 2004). Rooted cuttings, termed “clones”, are the result of asexual or vegetative propagation. These are transferred into a seedling tray containing a small amount of culture medium and fed with hydroponic solution nutrients. Initially, all clones are exposed to uniform vegetative light (usually HID lamps, 24 h/day), 95-100 % relative humidity (rH) and a temperature of 27 °C for one month. Clones are rooted after 2-3 weeks and, once the roots are of sufficient size, the plants can be transferred into a larger hydroponic system operating under modified conditions fostering accelerated growth (Cervantes, 2006; Chandra *et al.*, 2010).

1.3.4. Vegetative period

At this stage and throughout the growing period, all clones are kept under similar environmental conditions (light, temperature, rH and CO_2 concentration) in a cultivation room. In course of this research project, full-spectrum light was provided

by means of 1000 W HID lamps; a hot air suction fan was attached to the lambs and about 3-4 ft distances between plants and bulbs were always maintained to avoid heating. By adjusting the distance between the plant and the light source, photosynthetically active radiation (PAR) of about 700 ± 25 μmol $m^{-2}s^{-1}$ was sustained at the pot level. Photoperiod (18 h/day) was regulated using an automatic electric timerto preserve the vegetative stage. Growth room temperature and humidity were kept nearly constant at ~60 °C and ~60 %, respectively. Plants were supplemented with a vegetative fertilizer formula for their acclimatization and vegetative growth.Within this period, the plants reach the height of 50 cm and exhibit a healthy root system (Potter, 2004; Chandra *et al.*, 2010).

1.3.5. Flowering period

When the desired growth of the plants is attained, they are fortified with a flowering fertilizer formula and exposed to a 12 h photoperiod to induce flowering. At this stage, the number of forming inflorescences (especially the female pistillate flowers) and the cannabinoid content increase gradually. After eight weeks of flowering, the color of stigmas and trichomes changes into orange/brown and the production of cannabinoids decreases (Potter, 2004; Chandra *et al.*, 2010).

1.4. Secondary metabolites of *Cannabis sativa*

The chemistry of *C. sativa* has received considerable attention, especially due to the psychoactivity of tetrahydrocannabinol (THC). More than 500 compounds have been isolated from different *Cannabis* organs (Zulfiqar *et al.*, 2012), including a wide variety of secondary metabolites such as polyketides, trepenoids, polyphenolics, alkaloids, flavonoids, stilbenoids, quinones and terpenophenolics (Formukong *et al.*, 1989; Appendino *et al.*, 2011).

1.4 1. Cannabinoids

Cannabinoids, a class of terpenophenolic C_{21} (or C_{22} for neutral forms) compounds, have been found, until now, uniquely in *C. sativa* (Page and Nagel, 2006). Currently, about 100 meroterpenoids (prenylated polyketides), accumulating mostly in glandular trichomes and the moss *Radula marginata,* are known (Appendino *et al.*, 2011). According to their core structure, cannabinoids are classified into different types:

- CBG type, like cannabigerol
- CBC type, like cannabichromene
- CBD type, like cannabidiol
- THC type, like Δ^9-tetrahydrocannabinol
- Δ^8-THC type, like cannabicyclol
- CBL type, like cannabielsoin
- CBE type, like cannabinol and cannabinodiol
- CBND and CBT type, like cannabitriol

and miscellaneous cannabinoids (Mechoulam and Shvo, 1963). Representative metabolites are shown in Figure 4. Cannabinoids have been detected in *Cannabis* leaves, flowers, stem, pollen, seeds and roots by immunoassays and chemical analysis (Ross *et al.* 2000; Appendino *et al.* 2011).

1.4 1.1. Biosynthetic pathway

Biochemical and chemical assays confirmed that leaf-glandular trichomes are the main site of cannabinoid biosynthesis (Andre and Vercruysse, 1976; Petri *et al*., 1988; Kim and Mahlberg, 1997; Happyana *et al.*, 2013). The generally accepted metabolic pathway leading to the formation of cannabinoids is outlined in Figure 5. The biosynthesis is initiated by prenylation of olivetolic acid (OA) resulting in the generation of cannabigerolic acid (CBGA). CBGA is then converted into different types of cannabinoid carboxylic acid forms, tetrahydrocannabinoic acid (THCA; Fellermeier *et al.*, 2001), cannabidiolic acid (CBDA; Taura *et al.*, 1996 and 2007) and cannabichromenic acid (CBCA; Gaoni and Mechoula, 1966), in course of specific enzymatic reactions catalysed by THCA synthase, CBDA synthase and CBCA synthase, respectively (Taura *et al.*, 1995; Morimoto *et al.*, 1998; Taguchi *et al.*, 2008). Despite recent advances in cannabinoid pathway elucidation, the process and enzymes involved therein still lack definitive characterization. While Thakur and coworkers (2005) found that the acidic cannabinoids were broken down *via* decarboxylation to their neutral forms *in planta*, further degradation of the metabolies of interest was reported to occur through exposure to illumination, UV-B irradiation, oxidative agents and high temperatures or during long-term storage (Turner and Elsohly, 1979; Razdan *et al.*, 1972; Trofin *et al.*, 2012) (Figure 6).

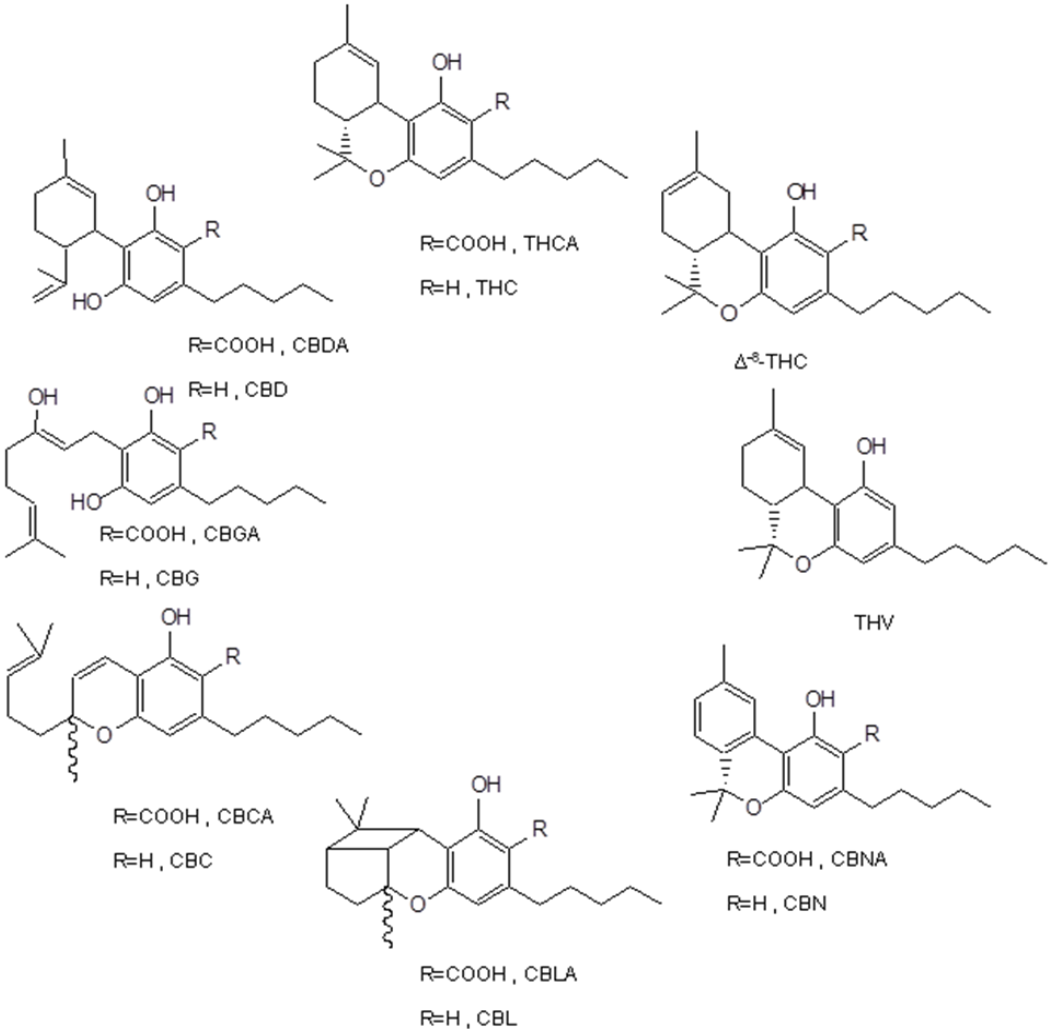


Figure 4: Chemical structures of various cannabinoids found in *Cannabis* extracts.

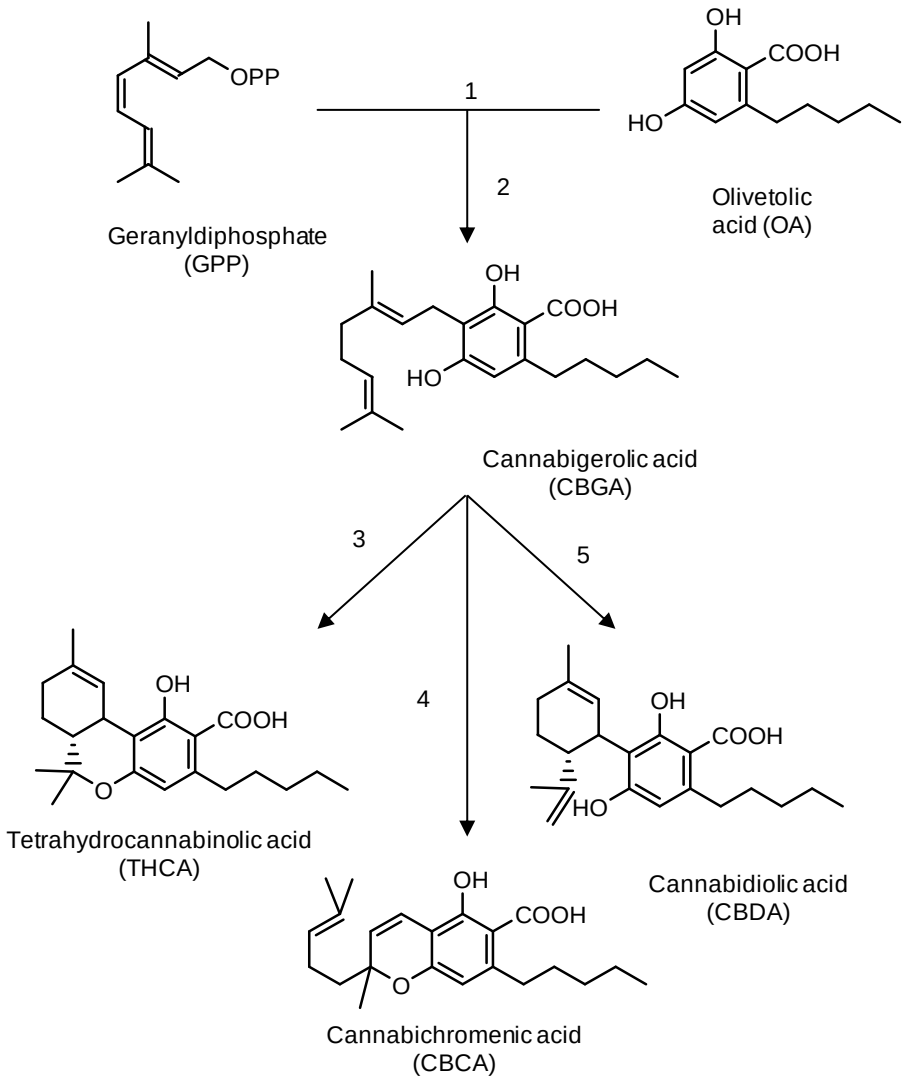


Figure 5: General pathway for biosynthesis of cannabinoids.1: polyketide synthase (PKS); 2: geranyl diphosphate:olivetolate geranyltransferase (GOT); 3: THCA synthase; 4: CBGA synthase, 5: CBDA synthase.

1.4 1.2. Changes in cannabinoid profile over time

From sequential harvesting studies on *C. sativa* chemotypes, it is evident that the concentration of cannabinoids in the flowers increases as the plant enters the full flowering phase (Barnicomparini *et al.*, 1984; Vogelmann *et al.*, 1988; Pacifico *et al.*, 2008; Muntendam *et al.*, 2012). The potency of representatives of the genus *Cannabis* is significantly influenced by the pollination (Fetterma *et al.*, 1971; Fairbairn and Rowan, 1977; Mandolino *et al.*, 2003), phytogeographic region (Hillig and Mahlberg, 2004), illumnation conditions (Mahlberg and Hemphill, 1983), UV-B radiation (Zhang and Bjorn, 2009), temepature and humidity, fertilization and the applied (in- vs. outdoor) breeding method (Latta and Easton, 1975; Staquet *et al.*,

1978; Vanhove *et al.*, 2011). Some reseachers follow up the changes in cannabinoid levels throughout the entire cultivation period, from the stage of developing seedlings till the harvest (Vogelmann *et al.*, 1988; Muntendam *et al.*, 2009; Muntendam *et al.*, 2012), as depicted in Figure 7 and Table 2.

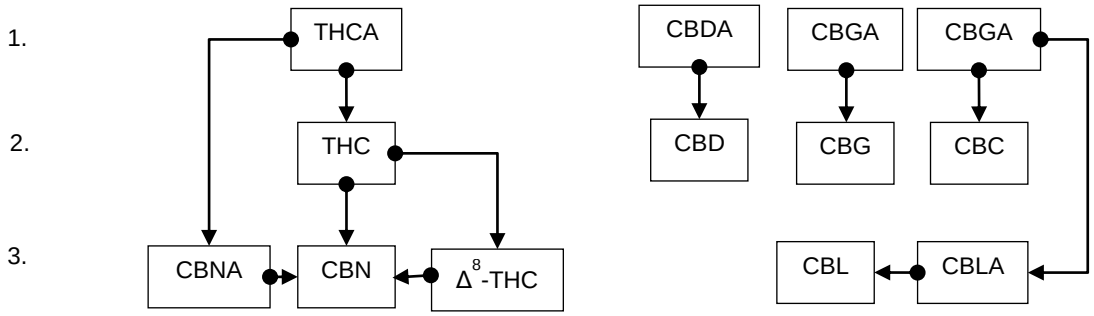


Figure 6: Cannabinoid degradation products (adapted from Hazekamp *et al.*, 2010). 1: product of biosynthesis; 2: product of decarboxylation; 3: product of degradation.

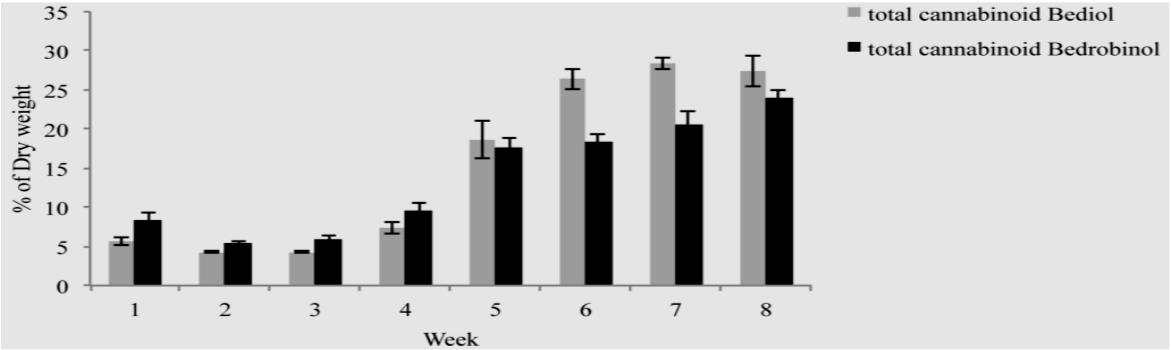


Figure 7: Total cannabinoid accumulation measured over the entire cultivation period (per week). Grey bars represent data characteristic of chemotype II *C. sativa* L. cv. *Bediol* and black – of chemotype I *C. sativa* L. cv. *Bedrobinol*. The percentages were calculated as mg total cannabinoids per 100 mg ± SD of dried *C. sativa* L. flower material. (adapted from Muntendam *et al*., 2012).

Table 2: Mean Cannabinoid content of *C. sativa* materials of different age analyzed by HPLC (Adapted from Vogelmann *et al.*, 1988).

Material/Age	Cannabinoid (*µ*g/100 mg dry wt ± SD)				Ratio: CBC:THC :CBG	No. of samples
	CBC	THC	CBG	Total		
Seedlings						
48-50 h	ND	ND	ND	ND	ND	11
52-54 h	0.004 ±	ND	ND	0.004 ± 0.009	–	12
56-58 h	0.36 ± 0.49	ND	ND	0.36 ± 0.49	–	13
60-62 h	1.1 ± 1.2	0.3 ± 0.6	0.3 ± 0.6	1.7 ± 2.3	4.1:1.1:1	12
66-68 h	10.6 ± 9.1	4.5 ± 3.4	7.0 ± 7.0	22.2 ± 19.1	2.3:1:1.6	13
72-74 h	20.9 ± 13.6	5.4 ± 4.6	14.9 ± 10.1	41.1 ± 27.8	3.9:1:2.8	30
96-98 h	102.3 ±	21.8 ± 10.0	47.3 ± 20.3	172.9 ± 62.1	4.7:1:2.2	22
120-122 h	191.0 ±	42.0± 13.0	54.6 ± 12.8	288.2 ± 48.7	4.5:1:1.3	24
144-146 h	197.7 ±	40.6± 10.0	30.6 ± 8.4	269.2 ± 54.6	6.5:1.3:1	15
Vegetative,18-20						
Primary leaves	67.1± 8.5	24.46 ± 3.7	19.2 ± 7.9	110.8 ± 17.5	3.5:1.3:1	3
Secondary	353.3 ±	501.00 ± 7.8	110.33 ± 19.9	968.7± 32.3	3.2:4.5:1	3
Vegetative, 29 days						
Primary leaves	57.2 ± 9.6	16.9 ± 1.6	6.32 ± 1.8	80.4 ± 9.8	9.0:2.7:1	2
Secondary leaves	213.1± 0.3	166.42 ± 5.6	1.67± 0.1	381.2± 5.4	127.6:99.7	3
Adult vegetative						
Controlled lighting	513.1 ± 106	1704.9 ± 407	133.53 ± 139	2354.0 ± 608	3.8:12.8:1	12
Greenhouse	251.8 ± 93	1331.3 ± 968	143.47± 57	1731.8 ± 1.094	1.8:9.3:1	12
Flowering						
Greenhouse	127.2 ± 17	3575.3 ± 1.07	101.8 ± 62	3820.6 ± 1.142	1.2:35.1:1	6

ND, none detected.

–, no data

1.4 1.3. Harvest and processing

1.4 1.3.1. Harvesting and drying

To maximize the retrieval of the main active cannabinoid, Δ^9-THC, plant harvesting should be performed in full flowering period (Vogelmann *et al.*, 1988; Pacifico *et al.*, 2008; De Backer *et al.*, 2012). Currently, there are no published reports concerning the methods of harvesting of *Cannabis.*

Figure 8: A: *C. sativa* vegetative cuttings; B: 2-week old clones; C: Female flowers in full bloo; D: Drying racks, E: Trimming buds; F: Bagging buds. (photos courtesy of Bedrocan BV, the Netherlands).

The protocol adopted at Bedrocan BV (the Netherlands) involves initial trichome assessment of herbs that attained the flowering period followed by cutting the plants at the base and leaving them to dry in the dark with continuous dehumidified air flow with CO_2 supplementation for 7 days. Afterwards, leaves and inflorescences are removed manually and bagged (O. Kayser, personal communication) (Figure 8D-F). According to the GW Pharmaceuticals records, the dried flower yields reach 400 g/m^2 per crop cycle. Multiple harvesting rounds (4-5 times/year) could, therefore, result in total yields of up to 2 kg/m^2 per year (Clarke and Watson, 2002).

1.4 1.3.2. Processing

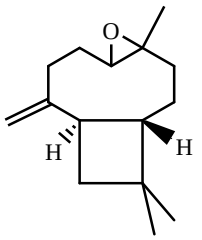
There are many methods of extraction of acidic and neutral cannabinoids. The various protocols involve application of solvents of diverse polarity, from highly polar, such as methanol and ethanol, to less polar, like benzene, petroleum ether and n-hexane (Veress *et al.*, 1990; Raharjo and Verpoorte, 2004). At GW Pharmaceuticals, the standardized crude extracts are subjected to complex processing to remove unwanted ingredients. The method is effective in reducing the costs of extraction and enriching the final crude extract that is then transferred to sealed stainless steel

containers and stored at -20 ± 5 °C to maintain stability and for further use (Guy and Stott, 2005).

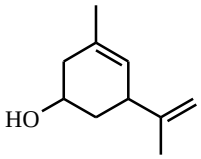
1.4.2. Non-cannabinoid constituents

1.4.2.1. Terpenoids

Plants of the genus *Cannabis* synthesize a variety of terpenoids. At least 200 structurally different terpenoid compounds have been isolated and characterized from their flowers (Ross and ElSohly, 1996), roots (Slatkin *et al.*, 1971), leaves (Hendriks *et al.*, 1975) and trichomes (Kim and Mahlberg, 2003). The most abundant representatives are *β*-myrcene, *trans*-caryophyllene, *α*-pinene, *trans*-ocimene and *α*-terpinolene (Malingre *et al.*, 1975). In durg type strains, the most prominent and unique terpenoids are *β*-caryophyllene-epoxide (the compound sensed by the drug searching dogs) and *m*-mentha-1,8(9)-dien-5-ol (Stahl and Kunde, 1973; Russo, 2011), depicted in Figure 9. The level of terpenoid production *in planta* depends on the applied cultivation and breeding methods as well as the harvest time and the mode of processing (Brenneisen, 2007; Fischedick *et al.*, 2010b).



β-Caryophllene-epoxide



m-Mentha-1,8(9)-dien-5-ol

Figure 9: Two unique terpenoid constituents of *Cannabis*.

1.4.2.2. Flavonoids

At least 20 structurally diverse flavonoids (free and conjugated) have been isolated from the flowers, leaves and pollen grains of *Cannabis* (Paris *et al.*, 1975; Barrett *et al.*, 1986; Vanhoenacker *et al.*, 2002; Choi *et al.*, 2004; Ross *et al.*, 2005). The identified compounds can be classified into three categories: 1) *O*-glycosides of apigenin, luteolin, quercetin and kaempferol, described by McPartland and Mediavilla (2002), 2) *C*-glycosides of orientin and vitexin (Vanhoenacker *et al.*, 2002) and 3) prenylated flavonoids of cannaflavin A and B (Barrett *et al.*, 1986).

1.4.2.3 Alkaloids

Nitrogenous compounds of *C. sativa* have been investigated and only a small number of ten alkaloids have been identified, including some interesting pseudo-alkaloids and related precursors, such as choline, trigonelline (a pyridine), muscarine (a protoalkaloid), isoleucine betaine and neurine (Turner *et al.*, 1980a; Ross and ElSohly, 1995). The aforementioned components have been isolated from *Cannabis* leaves, stems, pollen, roots and seeds (ElSohly *et al.*, 1978; Mechoulam, 1988).

1.4.2.4 Other compounds

In addition to the secondary metabolites mentioned above, phenols, steroidal glycosides, esters and other *Cannabis* constituents have been reported (ElSohly and Slade, 2005; Brenneisen, 2007).

1.5. Approved medicines and therapeutic potential

In pharmaceutical industry, cannabinoids (THC and CBD) have become increasingly important as valuable starting compounds for the development of new drugs. Sativex® (oral spray: 27 mg/ml Δ^9-THC and 25 mg/ml CBD; GW GW Pharmaceuticals) was approved in Canada, United Kingdom, Germany and Spain to treat muscle pain and stiffness in multiple sclerosis (MS) and cancer patients (Whittle and Guy, 2004; Whittle, 2007), while Cannador® (oral capsules: THC/CBD ratio not stated; Society for Clinical Research, Germany) was reported to reduce MS-related tremors (Fox *et al.*, 2004; Holdcroft *et al.*, 2006; Rahn and Hohmann, 2009). Bedrocan®, Bedrobinol® and Bediol® (Bedrocan BV) are dried flower bud preparations sanctioned for medicinal uses. Moreover, several synthetic cannabinoid-based drugs have been approved for the alleviation of nausea and vomiting associated with cancer chemotherapy (Marinol®, Dronabinol, Solvay Pharmaceuticals and Cesamet®, Nabilone, Valeant Pharmaceuticals International) (Stott and Guy, 2004; Davis *et al.*, 2007). A number of new cannabinoid-based products are currently under development and expected to be introduced to the market in the near future.

1.6 *In vitro* culture studies

The term "plant tissue culture" is commonly used to describe the maintenance of all types of plant cells, tissues, organs or whole plants on artificial media, *in vitro*, under

aseptic conditions (Gamborg and Phillips, 1995). A defined nutrient medium contains a large number of inorganic salts (macro- and micronutrients), a carbon source, myo-inositol, glycine and vitamins. Sometimes, growth regulators (auxins, cytokinins, gibberellins, abscisic acid or ethylene) are added. Other components, such as organic nitrogen compounds, organic acids and plant extracts may be supplied for specific purposes. Gelling agents, like agar, gelatin, silica or acrylamide gels, agarose, alginate or gelrite, are used to obtain a solidified medium. The pH of the medium should be adjusted to 5.7-5.8 before or after supplementation with the gelling agent (Gamborg and Phillips, 1995; Robert, 1999).

Plant tissue culture research is a multi-dimensional science with numerous applications:

– Micropropagation of selected genotypes (Phillips and Hubstenberger, 1995).

– Production of pathogen-free plants by meristematic culturing (Wang and Charles, 1991).

–Germplasm preservation *in vitro* using cryopreservation techniques (Withers, 1990).

–Vetrification, which is a result of high humidity in the growth-tube, low agar concentration in the solid medium or high growth in the liquid medium (Sakai and Engelmann, 2007; Kim *et al.*, 2010).

–Genetic manipulation through: a) somatic hybridization, b) cytoplasmic hybridization, c) transplantation and uptake of isolated nuclei, chromosomes, chromosome fragments or organelles (plastids and mitochondria), and d) transformation (Pierik, 1989).

– Plant regeneration through somatic embryogenesis, directly on an explant or from a callus (Fransz and Schel, 1994; Choi *et al.*, 1999).

–Organogenesis (the direct or indirect *de novo* organ formation) (Schwarz and Beaty,1996).

– Regeneration of haploid, sterile and seedless plants through application of pollen or microspore cultures (Reed, 1996).

– Isolation of variants, clones and mutant plants with enhanced resistance to biotic and abiotic stresses (Nabors *et al.*, 1975).

– Overcoming seed dormancy and embryo sterility (Hu and Zanettini, 1995).

– Production of secondary metabolites, such as alkaloids (Verpoorte *et al.*, 1994), terpenoids (Kummritz *et al.*, 2014), lignans (Elfahmi *et al.*, 2006), etc.

1.6.1. Nutrients and requirements of growth

The requirements of plant tissues grown *in vitro* are, in general, similar to those of the intact plants growing in nature (Vasil, 1985b). The success rate of any technology employing plant cell, tissue or organ cultures depends on several factors. Selection of nutritional components and growth regulators is a significant one (Street and Schillito, 1977; Gosal and Kang, 2012), as the isolated cells, tissues and organs lack the capacity to synthesize their own supply of carbohydrates, most vitamins and intrinsic growth substances. Accordingly, all the components utilized by plants in nature must be provided artificially to their *in vitro* cultures to achieve the desired results (Murashige and Skoog, 1962; Gamborg *et al.*, 1968a; Schenk and Hildebrandt, 1972).

1.6.1.1. Nutrient media composition

An ideal nutrient medium consists of inorganic salts, a carbon source, vitamins, growth regulators and other components serving specific purposes (Hunez-Palenius and Ochoa-Alejo, 1999). These include organic nitrogen compounds, complex extracts (casein hydrolysate, yeast extract or coconut milk) and organic acids (Gamborg, 1986). Murashige and Skoog (MS), Linsmaier and Skoog (LS) or Gamborg B5 are the most widely used salt compositions, especially in callus induction or plant regeneration (Sathyanarayana and Verghese, 2007). In general, the choice of plant tissue culture medium largely depends on the purpose of the undertaken *in vitro* cultivation process (Binding, 1986).

1.6.1.2. Inorganic nutrients

The same essential elements that support growth of intact plants are necessary for the sustained growth and development of *in vitro* cells and tissues (Ozias-Akins and Vasil, 1985). The indispensible mineral nutrients have been divided by Clarkson and Hanson (1980) into two major groups: 1) elements that are convalently bound witin carbon compounds and are vital constituents of macromolecules, like DNA, RNA and proteins (nitrogen (N), phosphorus (P),sulfur (S)), and 2) all other elements like boron (B), chlorine (Cl), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo), nickel (Ni), magnesium (Mg), potassium (K) calcium (Ca) that participate in a variety of often overlapping functions, including control of somatic and

electrochemical gradients, regulation of protein conformation and oxidation-reduction reactions of metalloproteins.

1.6.1.3. Macroelements

Deficiencies of macroelements N, P, S, K, Mg and Ca manifest more prominently when cells are cultured in liquid rather than on solid media, since impurities present in agar (most commonly used gelling agent) are considerable (Heller, 1953). Nitrogen, generally supplied in the form of NH_4^+ with NO_3^-, stimulates prolific formation of somatic embryos (as reported for *Atropa belladonna*, Gamborg *et al.*, 1968a; Thomas and Street, 1972 and *Digitalis lanata*, Kuberski *et al.*, 1984). Phosphorus and sulfur are usually supplied as phosphates and sulfates (Vasil, 1985a). Murashige and Skoog (1962) found that P levels greater than 2 mM were often inhibitory to the growth of tobacco pith tissues; therefore, they selected 1.25 mM as the near-optimal quantity. This concentration, however, was suboptimal for *Haplopappus gracilis* suspension cultures, which showed an increase of 50 % in growth rate when the amount was doubled (Eriksson, 1965). The reported level of potassium required in *in vitro* cell culturing is about 20 mM. Its supplementation in combination with low N concentrations (about 1 mM) or addition of KNO_3 with NH_4NO_3 stimulate somatic embryogensis. (Brown *et al.*, 1976).

1.6.1.4. Microelements

Microelements usually included in plant culture media are Fe, Mn, B, Zn, Mo, Cu, I and Co and have a profound effect on cell and tissue growth *in vitro*. Of all the microelements, the deficiency of iron (supplied as Fe-EDTA chelate to avoid precipitation at high pH) was reported to reduce growth most dramatically, while Zn, Cu, B, Mn and Mo deficits also resulted in an inhibitory effect (Nabors *et al.*, 1975). The ideal growth of plant cells and tissues was achieved when the concentration of micronutrients was reduced to 10 % of the originally proposed levels (Murashige and Skoog, 1962; Vasil and Hildebrandt, 1966). Two additional microelements, not proven essential to the whole plant nutrition, are, nevertheless, included in most tissue culture media; they are Co and I. Cobalt was introduced by Murashige and Skoog (1962) because of its documented effect on plant metabolism (Salisbury, 1959).

1.6.1.5. Organic nutrients

Three groups of organic nutrients are required by virtually all tissues cultured *in vitro*, i.e., carbohydrates, plant growth regulators and vitamins. In addition, numerous complex natural extracts and liquid endosperm preparations have been included in culture media.

1.6.1.5.1. Carbohydrates

Carbohydrates are used as carbon sources. The disaccharide, sucrose, at concentrations of 2-3 %, is the most commonly utilised representative in plant tissue culture media (Schaeffer, 1981; Neumann *et al.*, 1999). Carbohydrate concentration may have a pronounced effect on growth and morphogenesis (Negrutiu and Jacobs, 1978; Debnath, 2005). Five other disaccharides (trehalose, maltose, cellobiose, turanose and gentiobiose) may be utilized, depending on the cultured plant species (Verma and Dougall, 1977; Blanc *et al.*, 1999).The trisaccharide, raffinose, tetrasaccharide, stachyose and polysaccharide, starch can be metabolized by some tissues (Verma and Dougall, 1977). Sucrose, however, is the best source of carbon, since it is hydrolysed during the heat-sterilization process into more efficiently utilizable sugars, such as fructose and glucose (Ball, 1953; Wolter and Skoog, 1966). Notably, *in vitro* cultures of *Helianthus tuberosus* tuber proliferated on appropriate media containing sucrose (Minocha and Halperin, 1974), while embryo formation from *Petunia* anther- (Raquin, 1983) and *Digitalis lanata* suspension cultures (Kuberski *et al.*, 1984) was reportedly enhanced by of its substitution with maltose.

1.6.1.5.2. Vitamins

Thiamine, pyridoxine, nicotinic acid and calcium pantothanate are considered the most essential vitamins in *in vitro* growth systems (Gamborg *et al.*, 1981ab).

1.6.1.5.3. Plant growth regulators (PGRs)

In addition to mineral salts, carbohydrates and vitamins, most tissue cultures require exogenous supply of plant growth regulators. In general, differentiation of the cultured tissue depends on the content ratio of auxin to cytokinin (Skoog and Miller, 1957). Cytokinins are 6-substituted purine compounds (Skoog and Armstrong, 1970). These phytohormones are incorporated into the culture medium mainly for the stimulation of cell division, differentiation of adventitious shoots from callus and organ

proliferation (Klee, 1994). Commercially available synthetic cytokinins are kinetin (KIN) and 6-benzylaminopurine (BA), while thoseoccurring naturally in plants are zeatin and 6-(γ,γ-dimethylallylamino)purine (2iP), and their respective ribosides (Skoog and Armstrong, 1970).

Auxins, in turn, are included in plant tissue culture media in order to promote cell growth and division (Perrot-Rechenmann, 2010). Indole-3-acetic acid (IAA) is the only natural auxin. Its inherent tendency to be decomposed by heat, light and oxygen (Kefeli and Kalevitch, 2003) precludes its wide application. In contrast, synthetic auxins, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D, especially effective in the induction of somatic embryogenesis; Skoog and Miller, 1957), are stable and commonly used.. Other auxins, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxyacetic acid (MCPA), Dicamba and Picloram (Diazcolo *et al.*, 1972) are also available. Moreover, TDZ has been used, with considerable success, in the promotion of plant regeneration (Lu, 1993; Jones *et al.*, 2007). Whereas high auxin:cytokinin ratios stimulate the formation of roots, the opposite phytohormone proportions instigate shoot development. At intermediate ratio levels, the tissue grows as an undifferentiated callus (Akiyoshi *et al.*, 1983). There are three more classes of plant growth regulators: gibberelins (GAs), abscisic acid (ABA, influencing the maturation of somatic embryos; Calic *et al.*, 2012) and ethylene. While the effects of ABA, GA_3 (gibberellic acid) and zeatin on embryogenesis and organogenesis have been examined by several investigators (George and Eapen, 1994; Gaspar *et al.*, 1996), only a few studies concerning the impact of the gaseous ethylene have been carried out (Biddington, 1992; Kumar *et al.*, 1998).

1.6.1.5.4. Other organic supplements

Addition of amino acids to the culture medium may enhance cell growth and facilitate plant regeneration (Channarayappa, 2007). L-glutamine, for example, can serve as the sole source of nitrogen (Saunders *et al.*, 1997). Enzymatically hydrolyzed proteins, such as N-Z-Amine® Type A (Sigma-Aldrich), which is a casein hydrolyzate, are effectively used at concentrations of up to 2 g/l (Phillips and Hubstenberger, 1995). Supplementation with malate, citrate, pyruvate and similar organic acids proved beneficial in protoplast culturing, alleviating salt toxicity salts (Gamborg and Shyluk, 1970). Moreover, plant extracts, such as coconut milk, can be very effective

in providing an undefined mixture of organic nutrients and growth factors (Letham, 1974).

1.7. Phytohormone-regulated cell cycle control

Plant cell cycle can be divided into four phases: the mitosis stage (M), the phase of DNA synthesis (S) and two gap periods (G1 and G2) (Inze and De Veylder, 2006; Kuijt and Schnittger, 2007). For the development to proceed, the timing and rate of cell division and, consequently, entry into the cell cycle must be precisely controlled. This is achieved through complex interplay of various kinases, phosphatases and proteases. The key enzymes that control the transitions between the different stages of the cell cycle (G1 to S and G2 to M), and the entry of non-dividing cells into it, are cyclin-dependent protein kinases (CDKs) (Mironov *et al.*, 1999; Umeda *et al.*, 2005).It has been reported that auxins and cytokinins are implicated in the regulation of the cell cycle by controlling the activity of CDKs (Zhang *et al.*, 2005). The gene that encodes the major CDK, *cdc2* (cell division cycle 2), is regulated by auxin. However,its auxin-induced expression results in enzymatically inactive kinase and high levels of CDK alone are not sufficient to promote cell division. Thus, the process is arrested at the end of either G1 or G2 (Koens *et al.*, 1995). Concurrently, Zhang *et al.* (1996) documented the G2-arrest of cultured cells in the absence of cytokinin, coinciding with the reduction in CDK activity caused by enhanced phosphorylation of the tyrosine residues of the enzyme. When such cultures were resupplied with cytokinin, tyrosine moieties were dephosphorylated, the kinase reactivated, and cell division resumed. The described functionality of cytokinin provides an insight into the concerted cytokinin and auxin involvement in cell cycle regulation. Moreover, cytokinins elevate the expression of the cyclin D3 gene. As D-type cyclins are key players in the regulation of cell proliferation, the cytokinin-mediated boost in their level (and thus, the activity of CDKs) is considered the major regulatory function of the phytohormone (Riou-Khamlichi *et al.*, 1999; Richard *et al.*, 2002).

1.8. Establishment of callus and cell suspension cultures

Callus cultures are clumps of actively dividing undifferentiated cells derived from plant tissue (often arising from its injury), usually sustained on gel medium (Pierik, 1987). The nature of the callus tissue, its texture, compactness, friability and coloration, depends on the genotype and age of the primary explant (Sen *et al.*,

2014). Callus derived from the original explant can be established and maintained in an actively growing state through transfer of its fragments to fresh medium at regular four-week intervals (Remotti and Loffler, 1995). Growth of the callus culture can be monitored via measurement of its fresh and dry weight or packed cell volume as well as determination of its growth, cell number or mitotic indices (Dung *et al.*, 1981; Kittipongpatana *et al.*, 1998; Mustafa *et al.*, 2011). When callus is suspended in liquid growth medium, its cells disperse producing cell suspension culture characterized by faster and uniform growth (Mustafa *et al.*, 2011). Many investigators established callus cultures from explants of different *C. sativa* organs (roots, hypocotyls, epicotyls, cotyledons, petioles, leaves and immature flower buds) (Itokawa *et al.*, 1975; John *et al.*, 1978; Francoise and Vincent, 1981; Fisse *et al.*, 1981; Heitrich and Binder, 1982; Verzar-Petri *et al.*, 1982; Loh *et al.*, 1983; Braut-Boucher *et al.*, 1985; Fisse and Andres, 1985). In addition, callus could be derived from seed explants of numerous hemp varieties, such as *Carmagnola, Fibranova*, *Uniko* and *Kompolti* (Mandolino and Ranalli, 1999b), *Uniko-B*, *Kompolti Anka* and *Felina-34* (Feeney and Punja, 2003), *Sileia*, *Fibriman-24*, *Novosadska*, *Juso-15* and *Fedrina-74* (Slusarkiewicz-Jarzina *et al.*, 2005), *Carmagnola* (Pacifico *et al.*, 2008), *Beniko*, *Silesia* and *Bialobrzeskie* (Wielgus *et al.*, 2008). While numerous publications describing *in vitro* studies of many medicinal plants are currently available, scientific records on cell suspension cultures of *Cannabis*, established for extraction of secondary metabolites and analysis of their biotransformation (Figure 10), are relatively limited. The first relevant report was published by Veliky and Genest in 1972. The authors investigated the accumulation of cannabinoids and phenolic compounds in cell suspension cultures of *C. sativa*, concluding that the former were produced in trace amounts and the latter reached content levels of 0.19 % (as compared to 2.15 % in the whole plant leaves). In contrast, Itokawa *et al.* (1975) examined the constituents of *Cannabis* callus cultures induced from different expalnts (roots, hypocotyls, leaves of seedlings and male and female floral axes) and cultured on MS agar medium supplemented with 0.1-0.01 ppm KIN and 1.0 ppm 2,4-D. While cannabinoids were not detected, other compounds, such as methyl palmitate, methyl oleate, methyl stearate, 5*α*-ergostan-3-one, 5*α*-stigmastan-3-one, campesterol, stigmasterol, *β*-sitosterol, 5α-stigmast-22-en-3-one as well as $\Delta^{5,24(28)}$-unsaturated sterol and fatty acid esters were successfully detected . Subsequently, the aforementioned group of researchers (Itokawa *et al.*, 1977) investigated

biotransformation of selected natual products in cell cultures geraniol, nerol, *trans*-cinnamyl alcohol, isophorol, *trans*-verbenol and *cis*-verbenol were transformed into their corresponding aldehydes. Further on, Hartsel *et al.* (1983) reported the biotransformation of CBD to CBE in cell cultures of *C. sativa* and *Saccharum officinarum* grown on 1.5 % agar MS medium containing the vitamins of B5 medium, with 3 ppm 2,4,5-T as the sole hormone. The suspensions were shaken on a rotary shaker (100 rpm) at 27 °C, with an eight-hour daily photoperiod. The authors also found that incubation of the cultures with olivetol resulted in the generation of an unidentified cannabinoid characterized by a molecular ion of m/z 210 in mass spectrometry (MS) analysis. Braemer *et al.* (1987) investigated bioconversion of flavonoids into their glucosides in suspension cultures of *C. sativa*. The cells were grown in B5 medium supplemented with 0.5 mg/l KIN and 1 mg/l 2,4-D on a rotary shaker (120 rpm), with a 16 h daily photoperiod at 25 °C. They reported that quercetin was completely transformed into quercetin 3-*O*-glucoside, quercetin 3-*O*-diglucoside, isorhamnetin 3-*O*-glucoside and isorhamnetin 3-*O*-diglucoside, while apigenin was converted to apigenin 7-*O*-glucoside and 7-*O*-glucuronide as well as vitexin. Concurrently, the group studied cannabinoid biotransformations in *Cannabis* cultures obtained from calli of leaf explants. The cell suspensions were grown under the aforementioned conditions; however, they were kept in total darkness. Subcultures were transferred to fresh medium every three weeks. In effect, CBD was converted to bound CBE ((*R*)- and (*S*)-cannabielsoin) and THC to CBC, as determined by gas-liquid chromatography (GLC). Unfortunately, quantitative analysis was not feasible due to insufficient accumulation of the produced compounds (Braemer and Paris, 1987). More recently, Flores-Sanchez *et al*. (2009) studied the influence of biotic and abiotic elicitors on cannabinoid production in *C. sativa* cultures. Cell suspensions were obtained from leaf-derived calli and grown in MS basal medium supplied with B5-specific vitamins, 1mg/l 2,4-D and 1mg/l KIN on an orbital shaker (110 rpm), under light of 1000-1700 lx intensity and at 25 °C. Subcultures were transferred to fresh medium every two weeks. The authors reported that cannabinoid biosynthesis was not stimulated or induced by biotic and abiotic elicitors. They also noted low levels of THCA synthase gene expression. Further elicitation studies focused on the influence of jasmonic acid and pectin on metabolism of two cell lines of *C. sativa*. The applied nuclear magnetic resonance

(NMR) spectroscopy and multivariate data analyses resulted in the detection of tyrosol, a naturasl phenolic antioxidant (Pec *et al*., 2010).

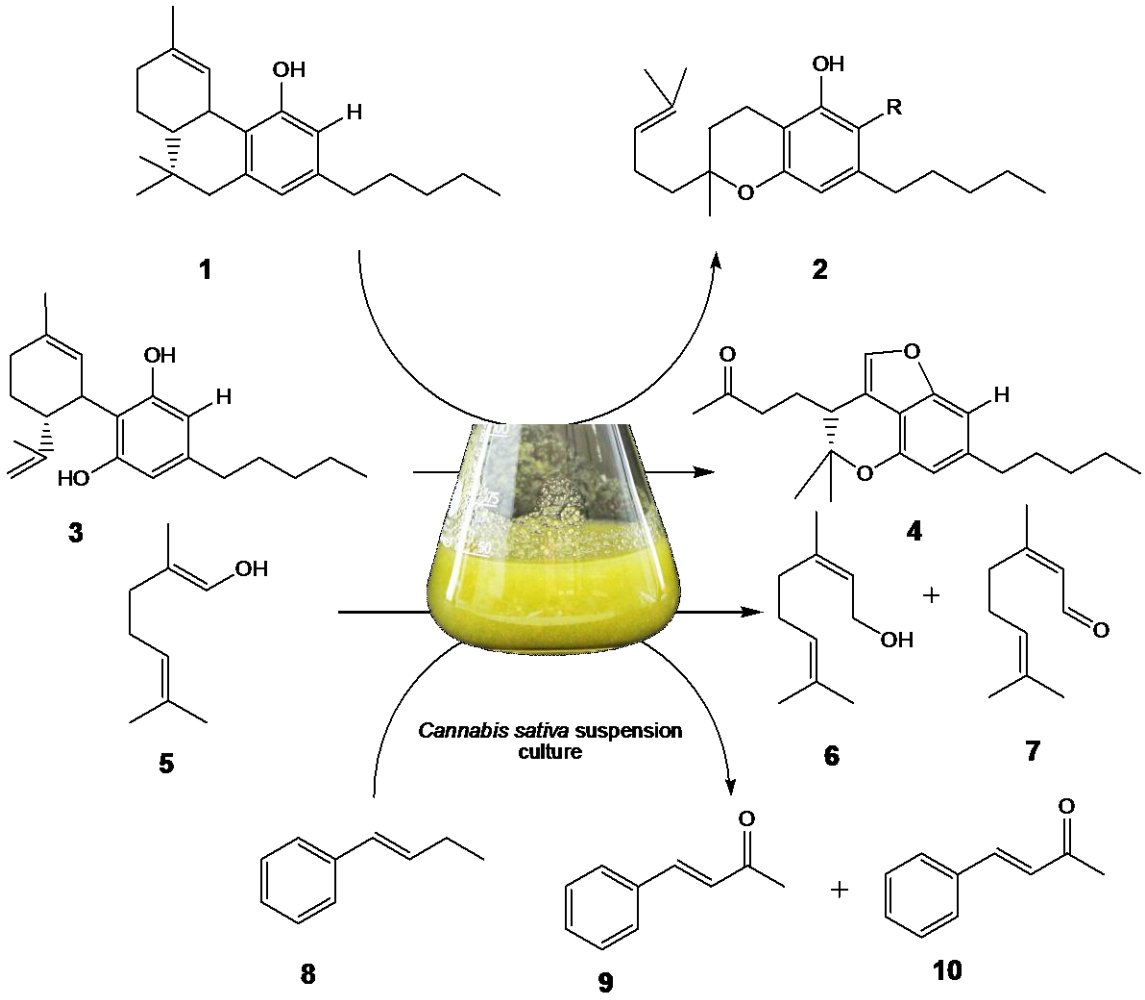


Figure 10: Selected biotransformations occurring in suspension cultures of *C. sativa*. 1: THC; 2: CBC; 3: CBD; 4: CBE; 5: geraniol; 6: nerol; 7: citrol; 8: *trans*-cinnamyl alcohol; 9: *trans*-cinnamylaldehyde; 10: cinnamic acid.

1.9.Regeneration and plantlet adaptation

Organogenesis is the ability of plant tissues to form various or gans *de novo* in *in vitro* culture, either indirectly (with an intervening callus stage) or directly (without the intermediary proliferative step). This is achieved by altering the concentration of growth regulators, mainly auxins and cytokinins, in the culture medium (Trigiano and May, 1996; Singh and Chikara, 2013). Micropropagation, a well-established technique of plant regeneration and breeding, offers a number of clear advantages, including 1) fast propagation, due to potentially high multiplication rates, under strictly controlled conditions, 2) independence from the seasonal and geographic factors and

3) reliable protection from microorganism-borne diseases (Zafar *et al.*, 1992; Debnath *et al.*, 2006). On the other hand, *in vitro* propagation of *C. sativa* through seeds is possible for most cultivars. This method, however, entails high level of heterozygosity, which could lead to rapid and dramatic shifts in secondary metabolite profiles from one generation to the next (Chandra *et al.*, 2010). Many *in vitro* propagation protocols (using explants or through organogenesis of callus cultures or somatic embryogenesis) have been reported for conservation of several medicinal plants (Sharma *et al.*, 1993; Hosoki *et al.*, 1995; Sudha and Seeni, 1996; Lata *et al.*, 2002; Bobak *et al.*, 2004; Kanwar and Kumar, 2008).

Despite considerable progress in the field of plant biotechnology, the efficient method of *C. sativa* regeneration is still lacking. First reports on *de novo* organogensis of *C. sativa* were published in early 1980s (Fisse *et al.*, 1981). Subsequent studies started emerging only after nearly two decades; these dealt with the generation of micropropagation-derived calli from different *Cannabis* genotypes and explant sources, including roots (Ranalli and Mandolino, 1999), stems (Mandolino and Ranalli, 1999a; Wielgus *et al.*, 2008), internodes and axillary buds as well as petioles (Slusarkiewicz-Jarzina *et al.*, 2005), cotyledons (Wielgus *et al.*, 2008), and young leaves (Lata *et al.*, 2010). Alternatively, the use of meristematic calli for micropropagation was investigated (Te-chato and Lim, 1999 and 2000), resulting in the attainment of genetic stability of elite germplasm. Lata *et al.* (2010) demonstrated that *in vitro* rooting of *C. sativa* was extremely difficult and the response to shoot elongation efforts, poor (2-3 cm). The highest proportion of root differentiation (95 %) was observed by the authors within ten days on half-strength MS medium supplemented with 2.5 *μ*M indole-3-butyric acid (IBA). Concurrently, Wang *et al.* (2009) reported that the proliferated hemp buds were successfully rooted on MS medium supplemented with 0.1 mg/l IBA and 0.05 mg/l NAA, resulting in 85 % rate of plantlet rooting. However, to our knowledge, *Cannabis* regeneration through direct or indirect somatic embryogenesis has not been reported.

1.10. Hairy root cultures

The possibilities of induction and enhancement of secondary metabolite production in hairy root systems using phytohormones or through *Agrobacterium tumefaciens* transformation have been studied extensively (Brown, 1995; Gelvin 2000; Balvanyos *et al.*, 2001; Shi *et al.*, 2011).

In early reports on the induction of rhizogenic calli of *C. sativa*, the hairy root cultures were established on MS supplemented with different concentrations of NAA (Fisse *et al*., 1981). Later on, Feeney and Punja (2003) studied the effect of various PGR combinations on the *Cannabis* rhizogenic callus. They found that MS medium, with a modified, B5-specific, vitamin spectrum and supplemented with different blends of 2,4-D, NAA, IBA, KIN and BA, induced root formation after four weeks in darkness. The roots were obtained following the infection of the callus cultures with *A. tumefaciens* harboring the pNOV3635 plasmid conferring resistance to mannose. Moreover, Wahby *et al*. (2013) investigated hairy root induction in several *C. sativa* varieties (*Futura 77*, *Delta-llosa*, *Delta405*, *CAN0111* and *CAN0221*) and *Nicotiana tabacum* cv. Burley F.13119. The hairy root cultures were established through infection of seedlings with *Agrobacterium* strains to investigate the stability of the *β*-glucuronidase gene incorported into the genomic DNA of transfomed tissues. About ten transformants of different *C. sativa* varities were then screened for desirable traits in growth. In conclusion, the authors reported that the *rolABC* transgenic root cultures were generally characterized by high biosynthetic capacity and biochemical stability.

1.11. Synthetic seed technology and aspects of cryopreservation

The main disadvantage of *in vitro* cell and tissue cultivation is the necessity of continuous subculturing which is time-consuming and involves a high risk of microbial contamination and subsequent loss of original cultures (Grout, 1995; Gangopadhyay *et al.*, 2011). Synthetic seed technology constitutes a viable alternative, affording sustainable management and circumventing the aforementioned difficulties. Despite the progress that has been made in the last decade concerning cryopreservation of plant material (Ganapathi *et al.*, 2001; Sicurani *et al.*, 2001; Brischia *et al.*, 2002; Hao and Deng, 2003), there are only few reports on cryoconservation of hairy root cultures (Lambert *et al.*, 2009). To date, no *Cannabis*-specific protocols for *in vitro* propagation using synthetic seeds or slow freezing of hairy roots are available.

1.12.Scope of the thesis

The main goals of this study were 1) to establish an efficient and reliable protocol for *in vitro* micropropagation of *C. sativa* leaf-derived callus using different combinations of phytohormones and 2) to select the desirable, clonally propagated, plants according to their cannabinoid profiles and production efficiencies. Moreover, the growth kinetics and cannabinoid content in shake flask cell suspension and hairy root cultures were to be analysed and documented. In this context, several questions regarding relevant technical challenges were addressed.

In order to fulfill the objectives of this thesis, appropriate experiments have been carried out between 2011 and 2014 under controlled environmental conditions (temperature, air humidity and light intensity) in growth chambers. To secure high-cannabinoid genotype of *C. sativa*, the starting leaf material was obtained from Bedrocan BV (the Netherlands).

The hereby proposed study addresses the following aspects:

1) *In vitro* regeneration of leaf-derived *C. sativa* callus.
   - i. Formation of meristemiods.
   - ii. Multiplication of shootlets.
   - iii. *In vitro* root formation.
   - iv. Acclimatization of rooted plantlets to *ex vitro* conditions in a hydroponic system followed by stimulation of flowering.
   - v. Analysis of cannabinoid content in *in vitro* and *ex vitro* cultured plants, callus, hairy roots and trichomes.

2) Characterization of cannabinoids in *C. sativa* cell suspension cultures.
   - i. Measurement of growth kinetics.
   - ii. Analysis of cannabinoid content.
   - iii. Fingerprinting and profiling of cannabinoids.

3) Characterization of cannabinoids in *C. sativa* hairy root cultures.
   - i. Measurement of growth kinetics.
   - ii. Analysis of cannabinoid content.
   - iii. Fingerprinting and profiling of cannabinoids.
   - iv. Cryopreservation of hairy root cultures.

4) Induction of trichome formation from callus cultures of *C. sativa*.
   - i. Influence of plant growth regulators on trichome differentiation.
   - ii. MALDI-TOF imaging of cannabinoids.

2. Material and methods

2.1 Material

2.1.1. Plant material

Juvenile leaves of high THC yielding medicinal *C. sativa* L. cv. Bedrobinol (Δ^9-THC, approx. 11 % and CBD, approx. < 1 % of plant dry weight) were used for the preparation of explants. The leaf specimens were obtained from plants grown under standardized conditions and kindly supplied by Bedrocan BV (Veendam, the Netherlands).

2.1.2. Solvents and chemicals

Table 3: Solvents and chemicals

Substance	**Manufacturer/Supplier**
Methanol	Carl Roth GmbH, Karlsruhe
n- hexane	Carl Roth GmbH, Karlsruhe
Formic acid	Carl Roth GmbH, Karlsruhe
2,5-dihydrozybenzoic	Carl Roth GmbH, Karlsruhe
Acetone	Sigma Aldrich GmbH, München
Deuterochloroform	Sigma Aldrich GmbH, München
Acetonitrile	VWR Chemie, Langenfeld
Tetrahydrocannabinolic acid	THC-Pharm, Frankfurt
Tetrahydrocannabinol	THC-Pharm, Frankfurt
Cannabidiolic acid	THC-Pharm, Frankfurt
Cannabidiol	THC-Pharm, Frankfurt
Cannabigerolic acid	THC-Pharm, Frankfurt
Cannabigerol	THC-Pharm, Frankfurt
Cannabinol	THC-Pharm, Frankfurt
Trifluoroacetic acid	Sigma Aldrich GmbH, München
Gamborg's basal medium	Sigma Aldrich GmbH, München
2,4-dichlorophenoxyacetic acid	Sigma Aldrich GmbH, München
1-naphthaleneacetic acid	Sigma Aldrich GmbH, München
Indolebutyric acid	Sigma Aldrich GmbH, München
Zeatin	Sigma Aldrich GmbH, München
Indoleacetic acid	Sigma Aldrich GmbH, München
Gibberellic acid	Sigma Aldrich GmbH, München
Adenine hemisulfate salt	Sigma Aldrich GmbH, München

Substance	Manufacturer/Supplier
Benzylaminopurine	AppliChem GmbH, Darmstadt
Thiamine-HCl	AppliChem GmbH, Darmstadt
Thidiazuron	Dr. Ehrenstorfer GmbH, Augsburg
Kinetin	Carl Roth GmbH, Karlsruhe
Casein hydrolysate	Carl Roth GmbH, Karlsruhe
Myo-Inositol	Carl Roth GmbH, Karlsruhe
Sodium Alginate	Carl Roth GmbH, Karlsruhe
Sucrose	Carl Roth GmbH, Karlsruhe
Sodium sulfate anhydrous	Carl Roth GmbH, Karlsruhe
Gelrite	Duchefa Biochemie BV, Haarlem
Flora Series	General Hydroponics Europe, Fleurance

2.1.3. Plant culture media

Modified B5 medium (Gamborg *et al*., 1968; Table 4) with 50 mg/l myo-inositol, 10 mg/l thiamine HCl, 1 g/l casein hydrolysate and 3 % sucrose, solidified with 0.4 % gelrite (solid medium) and fortified with diverse concentrations of cytokinins (kinetin (KIN), 6-benzylaminopurine (BA), gibberellic acid (GA_3), zeatin (Z), thidiazouron (TDZ), adenine hemisulfate (AS) and/or auxins (2,4-dichlorophenoxyacetic acid, 2,4-D; indole-3-acetic acid, IAA; indole-3-butyric acid IBA; 1-naphthaleneacetic acid, NAA), corresponding to the various aspects of the project, was used.

Table 4: Composition of Gamborg's B5 medium.

Ingredient	mg/l
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	3.00
KI	0.75
$MnSO_4$.H2O	10.00
$NaMoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	2.00
CaCl	113.23
KNO_3	2500.00
$MgSO_4$	121.56
NaH_2PO_4	130.44
$(NH_4)_2SO_4$	134.00

Ingredient	mg/l
i-Inositol	100.00
nicotinic acid	1.00
Pyridoxine HCl	1.00
Thiamine HCl	10.00
Sucrose	30000.00

The pH of the medium was adjusted to 5.8 before autoclaving.

2.1.4. Equipment

Table 5: List of equipment

Device	Manufacturer/Supplier
Analytical balance, KBBA 100	Sartorius AG
DMR microscope-DC200	Leica GmbH
Freeze dryer, Alpha I-4LSc	Christ GmbH
HPLC 1260 infinity	Agilent Technologies , Inc.
Linear ion trap mass spectrometer, MALDI LTQ XL	Thermo Scientific Inc.
LC-ESI-MS/MS …	Thermo Scientific GmbH
NMR spectrometer, Bruker Avance DRX 500	Bruker GmbH
Pilot shaker, Kühner JRC 1-T	Adolf Kühner AG
pH meter, inoLab pH level 1	WTW GmbH
Rotary shaker, Minitron	INFORS HT
Scanning electron microscope,s4500	Hitachi Ltd.
Stereomicroscope, SMZ800	Nikon Instruments Inc.
Vacuum rota vapor, R-210	Büchi Labortechnik GmbH
Water bath, WNB7	Memmert GmbH

2.1.5. Culture vessels

Explants as well as callus or hairy root cultures on solid media were maintained either in sterile Petri dishes (Diagonal GmbH, Germany) or 180 ml platstic jars (Fleischhacker GmbH, Germany; Figure 13A). Micropropagation (solid medium) as well as growth and maintenance of liquid cultures took place in Erlenmeyer flasks of various sizes (250, 300 and 500 ml).

2.1.6. Sterilisation

2.1.6.1. Culture instruments and glassware

The aseptic processing of plant cell and tissue cultures was conducted under a laminar flow hood (decontaminated by wiping all the surfaces with 70 % ethanol and UV-irradiation for 10 min). Glassware (cylinders, beakers and Erlenmeyer flasks) and consumables (e.g., paper filters, pipette tips, aluminium foil, etc.) were autoclaved at 121 °C for 20 min.

Figure 12: Plant growth banks at TU Dortmund A: CLF PlantMaster; B: BB.XXL4 plant tissue culture chambers.

Figure 13: A view inside plant growth chambers. A: Callus cultures; B: Micropropagated shootlets; C: Hydroponically grown plants.Shake flask cultures. D: Suspension cultures; E: Hairy root cultures

Other instruments, such as scalpels, strainers and forceps, were rinsed 2 or 3 times with 70 % ethanol, flamed and allowed to cool completely before handling.

2.1.6.2. Tissue culture media and other materials

The media and water were autoclaved at 121 °C for 20 min. Materials aiding acclimatization of micropropagated plants, such as rockwool (GrodanExpert®, Landgraaf, the Netherlands) and hydroton spheres (Dortmund, Germany), were washed three times with tap water and then autoclaved at 121 °C for 20 min.

2.1.6.3. Plant material

Solutions of 0.5 % Sodium hypochlorite (NaOCl) (12 % (v/v) bleach) and 0.1 % (v/v) Tween 20 were used for surface disinfection. The treatment duration was 10 min.

2.1.7. Plant growth chambers

2.1.7.1. Standard culture conditions

C. sativa cultures, on solid and in liquid media, were grown under different culture conditions.

- Chamber I (Figures 12B and 13A-B): Cultures of calli and micropropagated shootlets were maintained in the growth bank system (PlantMaster BB.XXL4, CLF PlantClimatics GmbH, Wertingen, Germany) under light/dark regime of 16/8 h, at 25 °C and rH of 55 %. Chamber illumination: Philips TL-D Reflex lamps 58W/840 and 18W/840, ~300 µmol m^{-2} s^{-1}.
- Chamber II (Figures 12B and 13B): Root formation in the shootlets was achieved in the growth bank" system described above, under reduced illumination of 139 µmol m^{-2} s^{-1}.
- Chamber III (Figure 13C): Hydroponically grown acclimatized plantlets were maintained in an indoor cultivation chamber (CLF PlantClimatics GmbH, Wertingen, Germany) under light/dark regime of 12/12 h (to induce flowering), at 25 °C and rH of 70 %. Chamber illumination for flowering: Philips PAR-HID bulbs 3500 K, ~ 260 µmol m^{-2} s^{-1}.
- Shake flask cell suspension cultures (Figure 13D) were agitated on the Kühner JRC 1-T pilot shaker (Adolf Kühner AG, Birsfelden, Switzerland) at 110 rpm and 27 ± 1 °C, under continuous illumination (Philips PL-L 55W/865/4P and 55W/830/4P, 92.59 µmol m^{-2} s^{-1}).

- Hairy root cultures (Figure 13E) were agitated on a rotary shaker (INFORS HT, Bottmingen, Switzerland) at 110 rpm and 25 °C, in the dark.

2.2. Methods

2.2.1. In vitro micropropagation of C. sativa leaf-derived calli

2.2.1.1. Sterilization and explant preparation

Juvenile leaves of *C. sativa* were washed three times in sterile distilled water and treated as described in section (2.1.6.3.). Subsequently, they were again washed in sterile distilled water (three times for 5 min) prior to inoculation on the culture medium.

2.2.1.2. Callogenesis

The sterilized juvenile leaves were cut into several small pieces (explants) of about 2 mm and aseptically cultured on modified B5 medium, fortified with various concentrations of 2,4-D, NAA, KIN and BA for callus induction. 15 ml of sterile medium was poured into sterile Petri dishes. Growth, amount and percentage of callogenesis in each treatment were recorded monthly. The calli were subcultured every 20 days under the conditions described above.

2.2.1.3. Meristemoid initiation

In order to initiate meristemoid formation from callus cultures, green, healthy and friable calli were transferred onto solid modified B5 basal medium (as described above) with phytohormone augmentation. Morphological changes of the calli in each treatment were recorded monthly. The calli were subcultured in 20-day intervals under the conditions described above. After 20 days, meristemoid-forming calli were cut into small pieces and subcultured on the same medium.

2.2.1.4. Shootlet induction and multiplication

Well-grown calli treated for meristemoid formation were used for shootlet induction after five months of growth, within seven subcultures. Calli of about 2 g and 1-2 cm in diameter were incubated on modified B5 medium supplemented with various concentrations and blends of cytokinins (BA, Z, KIN and TDZ) in combination with GA_3. The shoot cultures were observed weekly. After one month, the first shootles appeared. The evaluated parameters were: average number of shootlets per callus,

average shootlet height (cm) and perecnetage of callogensis (g). After one month, shootlet-forming calli were cut into small pieces (each with about five shootlets) and subcultured on the same medium.

2.2.1.5. Rooting of shootlets

To obtain whole *Cannabis* plantlets, single shootlets showing normal development (about 3 cm in length after three months of growth and subculturing) were separated and cultivated in 500 ml beakers containing 100 ml solidified B5 medium supplemented with various concentrations of auxins (0.5, 1.0 and 1.5 mg/l of NAA, IBA and IAA) for root formation. All shoot cultures were incubated for one week in darkness at 20 ºC, after which they were transferred back into the light and grown for four weeks as described in section (2.1.7.1.) The shootlets were observed monthly for growth and appearerence, shoot height, number of roots per shootlet, root length per shootlet and percentage of root formation.

2.2.1.6. Ex vitro acclimatization

To obtain whole plants of *C. sativa*, plantlets were removed from the solid medium and their root system was rinsed carefully with sterilized water. The plants were then transferred into flower pots (10×10×10.5 cm) containing sterilized rockwool and covered with hydroton spheres of about 1 cm diameter. The flower pots were submerged into the nutrient solution 0.25 ml/l Flora Series® (General Hydroponics Europe, Fleurance, France). The plants, covered with translucent plastic bags (31×19.5 cm) to maintain humidity and avoid light stress, were grown for 20 days under controlled environmental conditions. After 15 days, the plastic bags were removed daily for 30 min. Gradually, the daily exposure time was increased by 30 min; the bags were removed completely after 20 days. Subsequently, the plantlets were transferred into an aeroponic system for indoor cultivation. The aeroponic system culture was performed by placing the plants in bigger pots (15×15×20 cm) filled with hydroton spheres. The hydroponic solution was circulated regularly to maintain a sufficient moisture level under the plants (flow rate, 0.5 l/h) and consisted of tap water supplemented with micro- and macroelements of Flora Series® at pH 6.0. The stock solution and feeding program were applied according to the manufactuerer's specifications (GHE, 2014). During different stages of growth, all *Cannabis* plants were kept under strictly controlled, indoor environmental conditions.

Data concerning the average percentage of survival, plant height (cm), numbers of true leaves and nodes per acclimatized plant were recorded.

2.2.2. Shake flask suspension cultures

Cell suspension cultures were established from calli (after 20 passages) by transferring 0.5 g fresh-weight inocula of two-week-old, friable, green and homogenized (sieving with a stainless-steel mesh, pore size of 1mm) cell clusters to the modified B5 liquid medium (50:100 ml Erlenmeyer flasks), supplemented with various concentrations of cytokinines (BA and TDZ) in combination with GA_3 (Table 6).

Table 6: Induction media for the stimulation of cannabinoid formation in *C. sativa* suspension cultures.

Medium	**Phytohormone (mg/l)**	
	CKs	**GA_3**
PGR free [a]	0.0	0.0
CSM0 [b]	0.0	0.0
CSM1	0.5 BA	0.0
CSM2	1.0 BA	0.0
CSM3	1.5 BA	0.0
CSM4	0.5 TDZ	0.0
CSM5	1.0 TDZ	0.0
CSM6	1.5 TDZ	0.0
CSM7	0.0	1.5
CSM8	0.5 BA	1.5
CSM9	1.0 BA	1.5
CSM10	1.5 BA	1.5
CSM11	0.5 TDZ	1.5
CSM12	1.0 TDZ	1.5
CSM13	1.5 TDZ	1.5

[a] Gamborg B5 basal medium.

[b] modified Gamborg B5 basal medium.

2.2.2.1. Determination of growth kinetics

During the cultivation period of 35 days, cell growth (fresh and dry weight) was evaluated every seven days. The fresh weight (determine the weight of water retained by the membrane separately and subtract this amount from the measured

fresh weight). The cells were then lyophilized for 24 h in a freeze dryer (Alpha I-4LSc Christ GmbH, Osterode am Harz, Germany), at -55 °C and 0.6 mbar, until constant dry weight was obtained. Morphological changes in cell suspension cultures of *C. sativa* during different time courses were evaluated and documented using the Leica DMR-DC200 light microscope equipped with Leica DC200 digital camera (Leica GmbH, Dortmund, Germany).

2.2.3. Hairy root cultures

2.2.3.1. Initiation of adventitious root cultures

Callus cultures (1 g) of *C. sativa* incubated on modified B5 medium were 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). The emerging hairy root cultures were isolated from the callus cultures and subsequently transferred to the solid B5 medium with 4 mg/l NAA for their further proliferation. Hairy root cultures were subcultured in 30-day intervals and maintained in the growth bank system (PlantMaster BB.XXL4; CLF PlantClimatics GmbH, Wertingen, Germany). The cultivation was accomplished in the dark at 25 °C The initiation process was evaluated after 30 days.

2.2.3.2. Shake flask hairy root cultures

Once established, the adventitious root cultures were cut transversely and placed on Petri dishes (2-3 mm explants/treatment) containing half-solid B5 medium with various concentrations of auxins (0.25, 0.5 and 1.5 mg/l of NAA, IBA and IAA; Table 7 & 8) and cultivated in the dark at 25 °C. Morphology of the hairy roots was evaluated using a light microscope (Nikon SMZ800) and the images were captured using a Nikon type 104 projection microscope (Nikon Instruments Inc., Japan). Liquid cultures were obtained by inoculating root tips into B5 medium (100:250 ml Erlenmeyer flasks) supplemented with 4 mg/l NAA. The roots were then grown in the dark at 25 °C on a rotary shaker at about 110 rpm, subcultured every 30 days and maintained for about three years in the liquid medium.

Table 7: Optmaization of hairy root growth on solid B5 medium supplemented with various concentrations of auxins.

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

[a]Wild type on full B5 medium (wild type).

[b]Various treatments on half strength B5.

Table 8: Optimization of hairy root growth and cannabinoid formation in shake flask cultures.

Medium	Phytohromone (mg/l)		
	Auxins		
	NAA	IBA	IAA
PGR free	0.0	0.0	0.0
LHR0 [a]	4.0	0.0	0l.0
LHR [b]	0.25	0.0	0.0
LHR2 [b]	0.5	0.0	0.0
LHR3 [b]	1.0	0.0	0.0
LHR4 [b]	0.0	0.25	0.0
LHR5 [b]	0.0	0.5	0.0
LHR6 [b]	0.0	1.0	0.0
LHR7 [b]	0.0	0.0	0.25
LHR8 [b]	0.0	0.0	0.5
LHR9 [b]	0.0	0.0	1.0

[a]Wild type on full B5 medium (wild type).

[b]Various treatments on half strength B5.

2.2.3.3. Determination of growth kinetics

The kinetics of root growth was investigated by transferring 1 g fresh-mass inocula of 4-5 cm two-week-old root tips to liquid B5 medium (100:250 ml Erlenmeyer flasks), supplemented with various concentrations of auxins. During the culture period of 35 days, hairy growth kinetics was evaluated every 7 days by fresh and dry weight measurement, as described previously in section (2.2.2.1.).

2.2.4. Conservation of hairy root cultures

2.2.4.1. Preparation of encapsulation matrix

Sodium alginate, at a concentration of 5 % (w/v), was added to the B5 medium supplemented with 4 mg/l NAA. For complexation (an ion exchange reaction between Na^+ and Ca^{2+} resulting in the formation of insoluble calcium alginate, Figure 14A), the medium was further augmented with 50 mM of a complexing agent ($CaCl_2 \times 2H_2O$) (Lata *et al*., 2009b).

2.2.4.2. Formation of beads

The beads (spherical gel) were formed by dropping hairy roots (2-3 mm in length, two weeks old) of *C. sativa*, mixed with the sodium alginate solution, into the calcium chloride solution in a beaker (Figure 14B). The ensuing complexation was performed for 30 min. The beads were then retrieved using a stainless mesh and the traces of the complexing agent were removed by washing with sterilized distilled water. The encapsulated root tips, now called synthetic seeds/ beads, were dehydrated by laminar hood flow for 0-4 h. The initial and the attained bead weight values were recorded (Sartorius KB BA 100, Göttingen, Germany). The dehydrated beads were placed in cryovials (10 beads/cryovial at each time point) and stored at 4 °C for 7, 14, 30 and 180 days. Afterwords, the beads were thawed rapidly in a water bath at 40 °C for 1 min and placed in the liquid B5 medium. Their viability was documented as the percentage of hairy roots growing out of the beads after incubation in the dark at 25 °C.

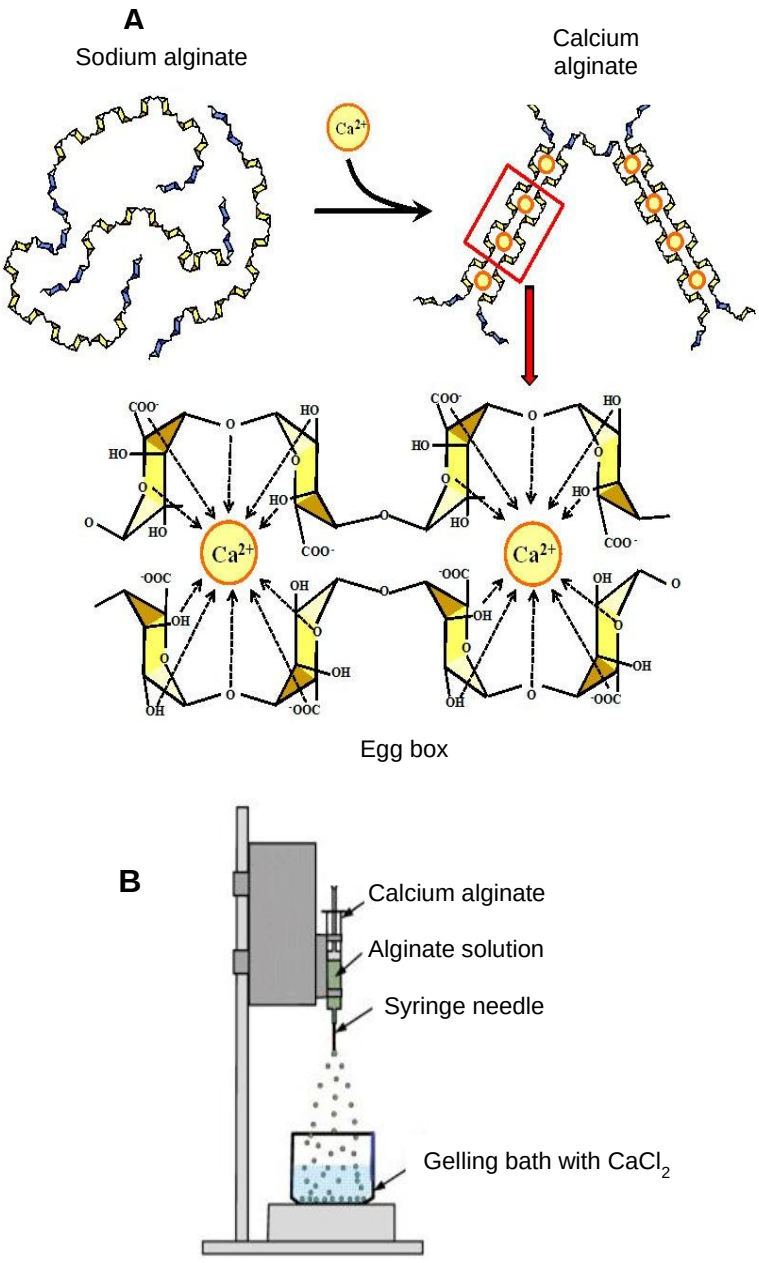


Figure 14: A: Schematic model of alginate gel formation (adapted from Keita and Masanao, 2012); B: Apparatus for encapsulation of hairy root tips in alginate.

2.2.4.3. Reestablishment of shake flask cultures

The fresh biomass of root growth was investigated by transferring two-week old bead-developed root samples (1 g) to liquid LHR0 medium (100: 250 ml Erlenmeyer flask; Table 8; sub-section 2.2.3.2.). After 21 days, the fresh weight was determined.

2.2.5. Induction of trichome formation

2.2.5.1. Effect of phytohormones on trichome induction

In order to initiate trichome formation from *C. sativa* callus cultures, green, healthy and friable calli (50 mg, 14 days old) were transferred aseptically onto the solid B5 basal medium supplemented with various concentrations of TDZ and GA_3. Trichome cultures were incubated under the same conditions as those utilized for callus induction. All experiments were carried out in at least three replicates. After 7, 14 and 21 days of incubation, the morphology and morphotypes of trichomes were evaluated using a light microscope (Nikon SMZ800) and the images were captured by a digital camera system (Nikon type-104,Tokyo, Japan).

2.2.5.2. Trichome analysis

Freeze-dried 21-day old callus cultures, grown on B5 supplemented with 1 mg/l TDZ and 3 mg/l GA_3, were used for scanning electron microscopy (SEM) of the induced trichomes. Square fragments were excised, using a razor blade, from the surface of the callus (underlying tissue thickness, ~1 mm). The samples were then mounted on aluminum stubs using two-sided adhesive carbon tape and examined with the Hitachi s4500 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 1 kV. Size and types of trichomes per unit of callus surface area were determined.

2.2.6. Analytical methods

2.2.6.1. Extraction of cannabinoids and sample preparation

2.2.6.1.1. Callus cultures, plantlets grown in solid media and hydroponic plants

plants grown *in vitro* (IV) and the flower tops of *ex vitro* in a growth room (IVH) as well as control plants (sample fresh weight, 1 g) were ground into a fine powder in the presence of liquid nitrogen with a mortar and a pestle, then transferred to test tubes. The powdered samples were extracted by vortexting at room temperature for 1 min with 3× 5 ml of *n*-hexane followed by sonication for 5 min. After filtration the

extracting solvent, then it was evaporated under vacuum,at 35 ºC and 31 mar Vacuum rota vapor R-210 (Büchi Labortechnik GmbH, Essen, Germany), and the dry residue dissolved in 1 ml *n*-hexane. The obtained hexane crude extracts (HCEs) were used for cannabinoid quantification by liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS). Four extract replicates were used.

2.2.6.1.2. Cell suspension and hairy root cultures

One gram of dry cell suspension or hairy root samples were pulverized and homogenized with methanol (70 %, 3× 10 ml) at 25 ºC for 1 h. The combined extracts were filtered, solvents evaporated at 35 ºC using a rotavapor R-210 (Büchi Labortechnik GmbH, Essen, Germany) and the resulting residues dissolved in chloroform (2× 10 ml). The chloroform extracts were combined, dehydrated over anhydrous sodium sulfate, then extracting solvent filtered and evaporated *in vacuo* at 35 ºC to give total cannabinoid mixtures. These were then re-dissolved in 0.5 ml of methanol and centrifuged at 13000 ×*g* and 4 °C for 4 min. The clear supernatants were removed and transferred to 2 ml clean amber vials, deoxygenated with CO_2 and stored at -20 ºC for further qualitative and fingerprinting studies. Calibration curves were established using CBDA, THCA and THC (THC-Pharm, Frankfurt, Germany) as standards.

2.2.6.2. Fingerprinting of cannabinoids

2.2.6.2.1. LC-ESI-MS (callus cultures, in vitro plantlets and hydroponic plants)

Cannabinoids in the HCEs were analyzed by LC-ESI-MS. The LTQ-Orbitrap mass spectrometer (Thermo Scientific GmbH, Schwerte, Germany) was coupled to the Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with the G1312 pump and the G1367 HIP-ALS autosampler). Nitrogen (N_2) was used as the protective gas (60 arbitrary units) and helium (He) served as the collision gas. The separations were performed using the Luna C18 column (50×3 mm, 3 *µ*m 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 using the following step gradient: 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. Cannabinoid-corresponding HPLC peaks were detected at 224 nm and identified through comparison with those

characteristic of authentic compounds. The spectrometer was operated in the positive mode (1 spectrum/s, mass range: 150-450) with the nominal mass resolving power of 60000 at m/z 400 and the 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.

2.2.6.2.1.1. Standard curves

Calibration curves were derived from six independent injections of six concentrations (2, 10, 50, 200, 1000 and 5000 ng/ml) of THCA, THC, CBGA, CBG, CBDA and CBD. Linearity in peak area versus injected amount for all six compounds was found in the concentration range between 10 and 1000 ng/ml with high reproducibility and accuracy. Regression analysis of the calibration curve data points showed linear relationships for THCA, THC, CBGA, CBG, CBDA and CBD, with excellent correlation coefficients (r^2) of 0.9984, 0.9966, 0.9969, 0.9838, 0.9996 and 0.9987, respectively.

2.2.6.2.2. LC-ESI-MS/MS (cell suspension and hairy root cultures)

Cannabinoids in the crude extracts (prepared on the 7th, 21st and 35th day of the culture cycle) of cell cultures grown in B5 medium supplemented with (0.5 mg/l TDZ; Table 6) were analyzed by liquid chromatography - electrospray ionization - tandem mass spectrometry (LC-ESI-MS/MS). The LTQ-Orbitrap mass spectrometer was coupled to the Agilent 1200 HPLC system. The separations were performed using the YMC-Triart C8 column (50×2 mm, 1.9 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 using the following step gradient: 0-1 min (30 % B), 11-15 min (100 % B) and 16-23 min (50 % B). The flow rate was 0.3 ml/min and the injection volume was 5 µl. Cannabinoid-corresponding HPLC peaks were detected at 224 nm and identified through comparison with those characteristic of authentic compounds. The spectrometer was operated in the positive mode (1 spectrum/s, mass range: 200-800) with the nominal mass resolving power of 60000 at m/z 400 and the 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 using

the collision-induced dissociation (CID) technique. LC/MS^2 (CID, 35 eV) was sufficient for the identification of THC and CBDA. However, THCA in hairy root samples (Table 8) was produced in too low amounts. Therefore, the protonated molecular ion of THCA was used to identify its fragamentation by LC/MS^3 with the CID of 40 eV. Further parameters in the performed experiments were: spray voltage, 5 kV; capillary temperature, 260 °C; tube lens, 70 V.

2.2.6.2.3. HPLC analysis

Chromatographic analysis of the cell suspension and hairy root culture (sub-section 2.2.6.2.2.) derived was carried out using the Agilent HPLC 1260 Infinity system (Agilent Technologies, Waldbronn, Germany). The system consisted of a binary pump (model no. G1312B), an auto-sampler (model no. G1367D), a degasser (model no. G1379B) and a photodiode array detector (model no. G4212B), and was controlled by the ChemStation software (Agilent, v. 04.03). The separation of cannabinoids was performed using the 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 using the following step gradient: 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 corresponding to cannabinoids were detected at 224 nm and quantified through standard curve-based extrapolation.

2.2.6.2.3.1. Standard curves

A stock methanolic solution of pure authentic CBDA, CBD, CBGA, OA, THC and THCA standard compounds was prepared (100 µM/ml) Calibration curves (Figure 15) were derived from four independent concentrations of 0.5, 2.0, 10, 50 µM; the volume of each standard sample was readjusted to 0.5 ml with methanol. The absorbance values were determined at 224 nm. Regression analysis of the calibration curve data points showed linear relationships for CBDA, CBD, CBGA, OA, THC and THCA, with excellent correlation coefficients (r^2) of 0.9978, 0.9972, 0.9981, 0.9886, 0.9985 and 0.9987, respectively.

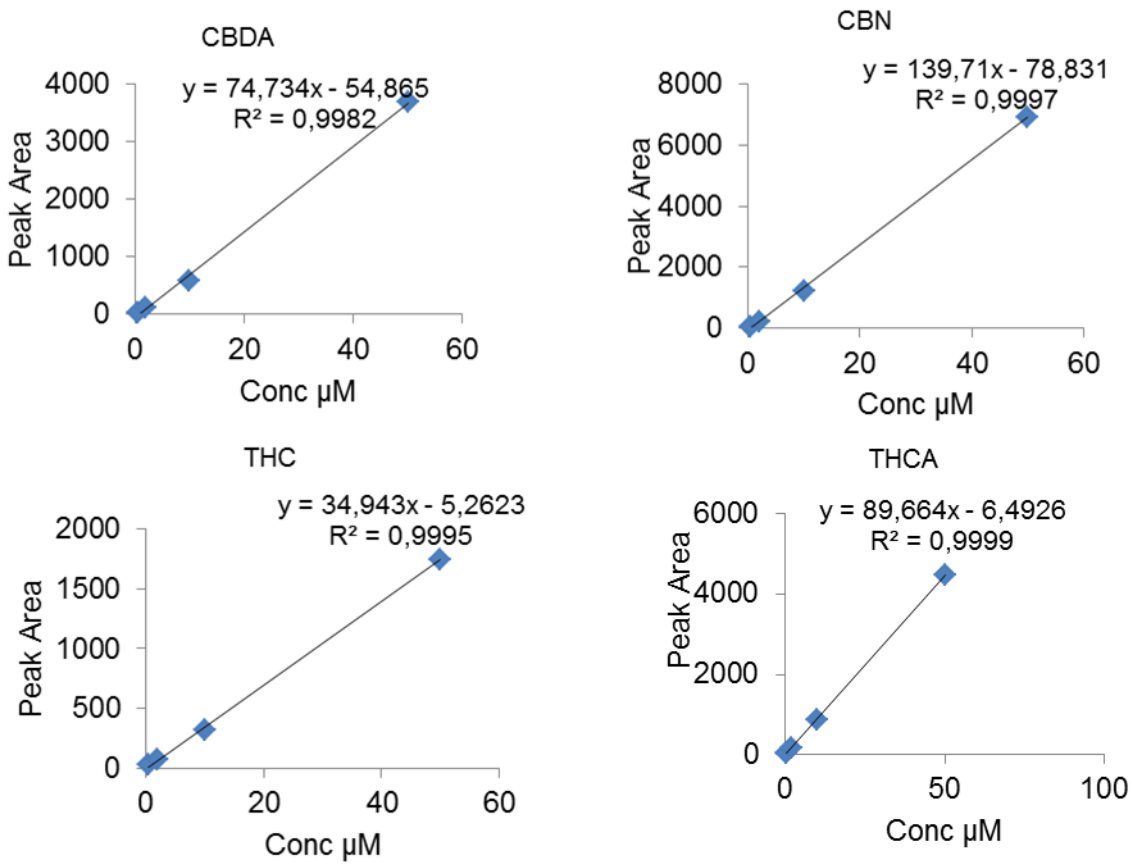


Figure 15: Standard curves of cannabinoids.

2.2.6.2.4. ^{1}H NMR

Dry chloroform extracts of slected cell cultures in section (2.2.2), prepared on the 7th, 14th, 21st, 28th and 35th day of the growth cycle, were dissolved in deuterated chloroform ($CDCl_3$) containing tetramethylsilane (TMS) as an internal standard. The nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Varian Inova 500 spectrometer (North Carolina, USA) operating at 499.78 MHz. The following parameters were applied: acquisition time, 4.09 s; relaxation delay, 5 s; pulse width, 4.75 µs; FID (free induction decay) data points, 90122; spectral width, 11001.10 Hz and number of scans, 16. ACD/Labs software was used for data processing. The proton signals of the target cannabinoids were identified by comparison to the authentic singlet peaks.

2.2.6.2.5. MALDI imaging MS

The aim of this study was to use the capabilities of matrix-assisted laser desorption ionization imaging mass spectromtetry (MALDI imaging MS) to determine the spatial distribution of cannabinoids in capitate-stalked trichomes induced from *C. sativa* callus.

2.2.6.2.5.1. Callus handling and sample preparation

2.2.6.2.5.1.1. Cryosectioning of callus-trichome tissue

Fresh callus cultures (1-3 cm in diameter) were stored at -26 ˚C. Before analysis, the calli were fixed with a polymeric mixture (polyvinyl alcohol, polyethylene glycol, potassium formiate, 7: 5: 2) on sample plates for cutting in a cryostate (Microm HM 550, Thermo Scientific Inc., Bremen, Germany). The cutting temperature was optimized at -15 to -18 ˚C and slice thickness was adjusted to 15-20 µm then kept on glass slides. Prior to matrix application, a photograph Leica DMR-DC200 (Leica Camera AG, Solms, Germany) of the plate was taken to use as a reference for MALDI imaging MS. The glass slides with the samples were transferred and fixed onto round stainless steel MALDI plates.

2.2.6.2.5.1.2. Matrix application

Matrix application was performed with a sprayer (TransMIT GmbH, Giessen, Germany) suitable for the generation of matrix layers for high imaging scan resolution (≤ 10 µm). The applied matrix solution comprized 2,5-dihydroxybenzoic acid (DHB) in acetone/water 1:1 (v/v) and 0.1 % formic acid. The spray application was performed with the following parameters: matrix flow rate, 5 µl/min; N_2; spray cycle duration, 1× 30 min and sample plate revolution speed, 255 rpm. Afterwards, the sprayed sample was examined using Leica DMR-DC200 (Leica Camera AG, Solms, Germany) and the regions of interest were marked to facilitate their location under measurement conditions.

2.2.6.2.5.1.3. MALDI and imaging

The-ion mobility spectrometry (IMS) experiments were performed with the atmospheric pressure scanning microprobe matrix-assisted laser desorption/ionization source, AP-SMALDI (TransMIT GmbH, Giessen, Germany) coupled with the Q Exactive HRMS (Thermo Scientific Inc., Bremen, Germany). A N_2

pulsed laser (MNL 100 series, LTB Lasertechnik GmbH, Berlin, and Germany) was used (60 Hz, 2-5 ns). The laser spot was adjusted to ~6-7 µm. Scan resolution was adjusted to ~6.25 µm (50 steps; slight oversampling). The scans were taken from a rectangle raster of ~375×344 µm and performed in full scan positive mode at m/z 150-600 with internal lock mass of DHB, m/z 379.09246 $[2M\text{-}2H_2O+H]^+$. Further mass spectrometric parameters (Q-Exactive): mass resolution, 70 at m/z 200; S-lens level, 65; spray voltage, 4000 V; injection time, 300 ms. Further source parameters (AP-SMALDI): attenuator temperature, 20 ˚C.

2.2.6.2.5.1.4. Further software

Marathon control, v. 1.60 (LTB Lasertechnik GmbH, Berlin, Germany): laser software for activation and deactivation of the laser. MCP, v. 3.9.32.15 (TransMIT GmbH, Giessen, Germany): adjustment of scan raster, resolution and attenuator. IDS Falcon/Eagle DEMO, v. 4.61.0000.0 (IMS Imaging Development Systems GmbH, Obersulm, Germany): CCD camera image for orientation on sample plate. Thermo Q-Exactive Tune Software, v. 2.3 (Thermo Scientific GmbH, Bremen, Germany): mass spectrometer tune software. Thermo XCalibur, v. 3.0.63: evaluation of MS data files. Matrix Sprayer Control, v. 1.9.2890 (TransMIT GmbH, Giessen, Germany): controlling the spray program and parameters.

2.2.6.2.6. Data processing

Selected MALDI ion images (sub-section 2.2.6.2.5.1.3.) were analyised with software Mirion, v. 2.1.4.411 (TransMIT GmbH, Giessen, Germany).

2.2.7. Experimental design and statistical analysis

All the cultures were examined periodically in replicates. The morphological changes were recorded monthly on the basis of weekly visual observations. The data were analyzed and recorded as mean values ± SE (standard error).

3. Results

3.1. *In vitro* micropropagation of leaf-derived calli

An efficient plant regeneration protocol, starting from leaf-derived calli of *C. sativa*, was developed. The procedure is shown schematically in Figure 16. It comprised the following steps:

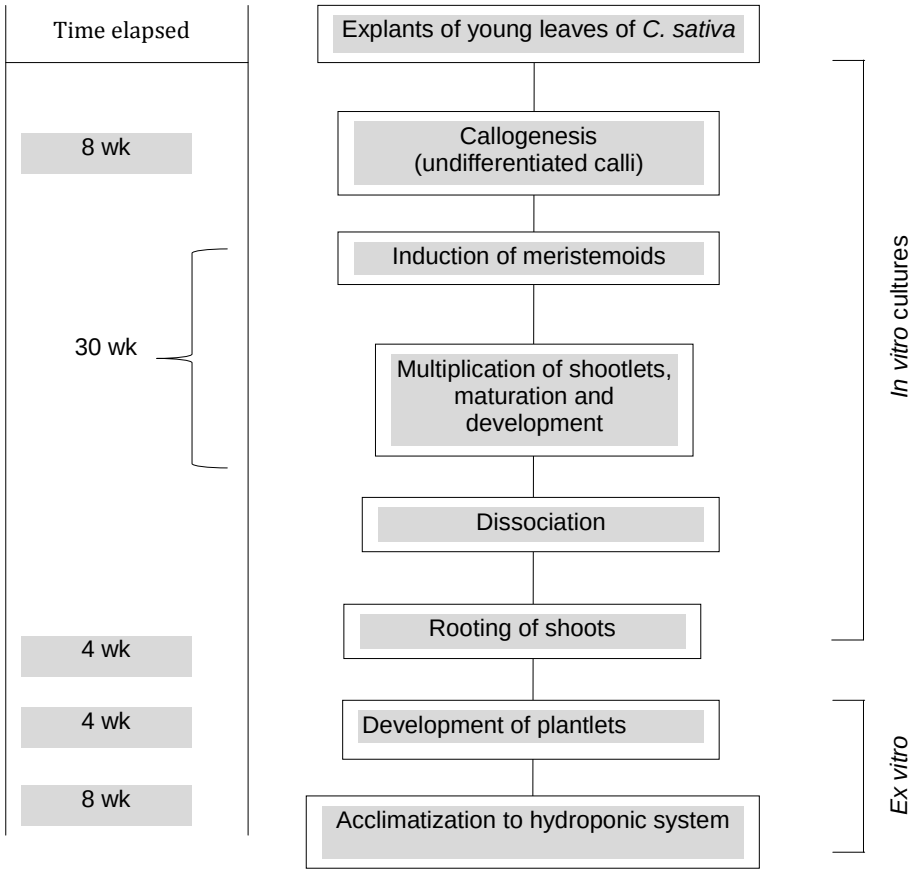


Figure 16: Main steps of plant regeneration procedure from *C. sativa* leaf-derived calli

3.1.1. Callogenesis

Explants of juvenile *C. sativa* leaves were transferred to B5 medum supplemented with various combinations of phytohromones for optimization of callus induction. Callogenesis was remarkably influenced by the concentration of applied growth regulators. Callus formation was achieved after two months of explant incubation. The highest degree of callus formation (95 % of explants yielding calli) was observed with CNm7 medium containing 1mg/l NAA and 1mg/l BA (Table 9, Figure 17A). Exposing leaf explants to the combination of 2,4-D and KIN inhibited callogenesis,

while the leaf explants incubated on medium without any phytohromones (PGR-free, Table 9) were characterized by the lowest callus formation rate (only 15% of explants developed into calli). The colour of the calli ranged from brown or yellow to dark green, while morphologically and they could be described as friable or compact. Two to three additional months of subculturing were required until meristemoid formation could be initiated.

Table 9: Effect of varying concentrations of phytohormones on callogensis of leaf explants from *C. sativa.*

Medium†	Phytohormones (mg/l)				Callogenesis	
	Auxins		Cytokinins			
	2,4-D	NAA	Kin	BA	Amount of callus††	% of callus cultures*
Nm0	0.0	0.0	0.0	0.0	++	15
CNm1		1.0	1.0		_	0
CNm2		1.0	3.0		_	0
CNm3		3.0	1.0		_	0
CNm4	1.0			1.0	+	25
CNm5	1.0			3.0	+	18
CNm6	3.0			1.0	+	22
CNm7		1.0		1.0	+++	95
CNm8		1.0		3.0	++	85
CNm9		3.0		1.0	++	75

† Medium used consisted of modified B5 basal medium and Nm0 served as control.
††- = No response, + = < 130 mg, ++ = 130 to 180 mg, +++ = >180 mg.
* All data recorded after four weeks of inoculation, with numbers of replicates 20 explants.

3.1.2. Meristemoid initiation

To establish the optimal conditions for the development of meristemoids from calli, five media were tested (Table 10). Green, healthy, friable calli, obtained from leaf explants, were transferred to the modified B5 medium supplemented with various combinations of auxins and cytokinins. The most advantageous phytohormone combination was: NAA, BA and AS at respective concentartions of 0.5, 5 and 40 mg/l (Figure 17B). Meristemoids – small, organized clusters of cells arising from the surface of the callus tissue, also termed “nodules” or “growth centers”, could be easily identified by their intensive green colour.

3.1.3. Shoot induction and multiplication

Shootlet induction and multiplication of meristemoids proved to be a function of the

applied CKs/GA_3 ratio. Induction of shootlets was investigated after the meristemoids generated on culture media MNm2 and MNm3 (Table 10) were transferred onto various shootlet induction media (Table 11). Data concerning the average numbers of shootlets per callus, height of shootlets and rate of callogenesis were collected. The obtained results indicated that SNm18 medium, containing 0.5 mg/l GA_3 (Figure 17C-I), and SNm25 medium, supplemented with 0.25 mg/l TDZ and 3 mg/l GA_3, were the most efficient, with the average of 8.5 ± 1.73 and 7.25 ± 1.03 shootlets per callus, respectively. In the absence of the aforementioned plant hormones, no shoot formation was observed. Moreover, GA_3 concentrations of 0.25 mg/l and 0.5 mg/l were optimal for obtaining shootlets of the highest length (3.27 ± 0.41 and 2.78 ± 0.39 cm, respectively), as compared to the hormone-free control. Additionally, shootlet formation was inhibited in meristemoids grown on media containing Z and GA_3 or BA and GA_3 that mostly promoted callogensis (Table 11). On the other hand, the rate of callogensis was variable, with the best growth taking place on SNm31 medium, supplemented with 1 mg/l Z and 3 mg/l GA_3, or SNm10 medium, containing 0.5 mg/l BA, where callus weight values ranged from 2.76 to 2.75 g (Table 12). Figure 17H shows well-developed vegetative, bud-like structures that ultimately gave rise to multiple shootlets formed after three weeks of growth on SNm22 medium containing 0.25 mg/l KIN and 3 mg/l GA_3. Stable rates of shootlet multiplication (more than 20 new shootlets per callus) were observed on SNm18 and SNm25 and did not decrease in continuous subcultures on the same media (Table 11).

Table 10: Phytohormone composition of media used for meristemoids induction

Medium†	Phytohormone (mg/l)			% of Meristemoid formation†††	Morphogenetic responses
	Auxin	CKs			
	NAA	BA	AS*		
Nm0	0.0	0.0	0.0	-	Undifferentiated, compact green calli
MNm1	0.25	10	40.0	-	Undifferentiated, friable green calli
MNm2	0.5	5.0	40.0	50	Numerous meristemoids††
MNm3	1.0	3.0	40.0	16.6	Less meristemoids††
MNm4	1.5	1.0	40.0	-	Undifferentiated, green calli

† modified B5 basal medium; PGR-free medium was used for control experiments

†† media 2 and 3: friable, yellowish callus with dark-green colored cells

††† data recorded after five months (6 Petri dishes in each seven callus pieces; ~1 g fresh biomass, 2 weeks old), represented as: (total numbers of callus / total numbers of meristemoids × 100); -, no response

Table 11: Effects of different concentrations of cytokinins and GA_3 for shootlets induction and multiplication

Medium†	Phytohormone (mg/l)					No. of shootlets/callus	Height of shootlets (cm) /callus	Callogensis rate (g)*
	CKs				Gibberellins			
	Kin	Z	BA	TDZ	GA_3	($\bar{x}$ ± SE)††	($\bar{x}$ ± SE)††	($\bar{x}$ ± SE)††
Nm0	0.0					0.0 ± 0.0	0.0 ± 0.0	2.25 ± 0.07
SNm1	0.25					1.0 ± 0.40	0.2 ± 0.07	1.85 ± 0.17
SNm2	0.5					1.25 ± 0.47	0.22 ± 0.10	2.1 ± 0.19
SNm3	1.0					1.25 ± 0.47	0.22 ± 0.08	2.25 ± 0.24
SNm4	1.5					2.75 ± 0.50	0.3 ± 0.12	1.71 ± 0.18
SNm5		0.25				2.0 ± 0.57	0.22 ± 0.02	1.8 ± 0.15
SNm6		0.5				3.25 ± 0.62	0.25 ± 0.05	2.27 ± 0.11
SNm7		1.0				3.25 ± 0.25	0.27 ± 0.07	2.36 ± 0.22
SNm8		1.5				3.5 ± 0.28	0.25 ± 0.05	2.43 ± 0.11
SNm9			0.25			3.5 ± 0.50	0.22 ± 0.02	2.35 ± 0.1
SNm10			0.5			4.25 ± 0.50	0.27 ± 0.07	2.75 ± 0.09
SNm11			1.0			4.25 ± 0.47	0.3 ± 0.04	2.34 ± 0.14
SNm12			1.5			1.5 ± 0.50	0.27 ± 0.07	1.59 ± 0.04
SNm13				0.25		1.5 ± 0.50	0.27 ± 0.07	1.59 ± 0.04
SNm14				0.5		1.25 ± 0.5	0.27 ± 1.83	1.83 ± 0.36
SNm15				1.0		3.00 ± 1.32	0.37 ± 1.75	1.75 ± 0.16
SNm16				1.5		3.5 ± 0.9	0.27 ± 1.7	1.7 ± 0.26
SNm17					0.25	5.25 ± 1.65	0.35 ± 1.27	1.27 ± 0.77
SNm18					0.5	8.5 ± 1.73	2.78 ± 0.39	2.0 ± 0.08
SNm19					1.0	1.75 ± 0.50	2.7 ± 0.35	1.79 ± 0.06
SNm20					1.5	1.25 ± 0.50	1.75 ± 0.07	1.49 ± 0.15
SNm21	0.25				3.0	6.5 ± 0.95	0.92 ± 0.39	2.04 ± 0.08
SNm22	0.5				3.0	5.25 ± 0.47	1.14 ± 0.55	2.34 ± 0.23
SNm23	1.0				3.0	4.0 ± 0.70	1.62 ± 0.60	1.99 ± 0.26
SNm24	1.5				3.0	3.5 ± 0.50	0.17 ± 0.02	1.99 ± 0.09
SNm25				0.25	3.0	7.25 ± 1.03	0.9 ± 0.27	2.33 ± 0.12
SNm26				0.5	3.0	2.75 ± 0.25	0.22 ± 0.02	1.67 ± 0.19
SNm27				1.0	3.0	2.5 ± 0.28	0.17 ± 0.07	1.64 ± 0.17
SNm28				1.5	3.0	2.25 ± 0.62	0.27 ± 0.02	1.46 ± 0.16
SNm29		0.25			3.0	0.0	0.0	2.24 ± 0.48
SNm30		0.5			3.0	0.0	0.0	2.44 ± 0.26
SNm31		1.0			3.0	0.0	0.0	2.76 ± 0.29
SNm32		1.5			3.0	0.0	0.0	1.85 ± 0.28
SNm33			0.25		3.0	0.0	0.0	1.51 ± 0.07
SNm34			0.5		3.0	0.0	0.0	2.31 ± 0.16
SNm35			1.0		3.0	0.0	0.0	1.99 ± 0.11
SNm36			1.5		3.0	0.0	0.0	1.4 ± 0.13

† modified B5 medium; PGR-free medium was used for control experiments

†† for each test medium, 5 Erlenmeyer flasks (70: 300 ml) with 4 meristemoid pieces were tested; the experiments were repeated twice and the results represented as means ± SE; 0.0, no response

* * callogensis rate: weight of the induced calli after 4 weeks divided by the initial weight of inoculums (the initial weight was 2 g fr. wt.

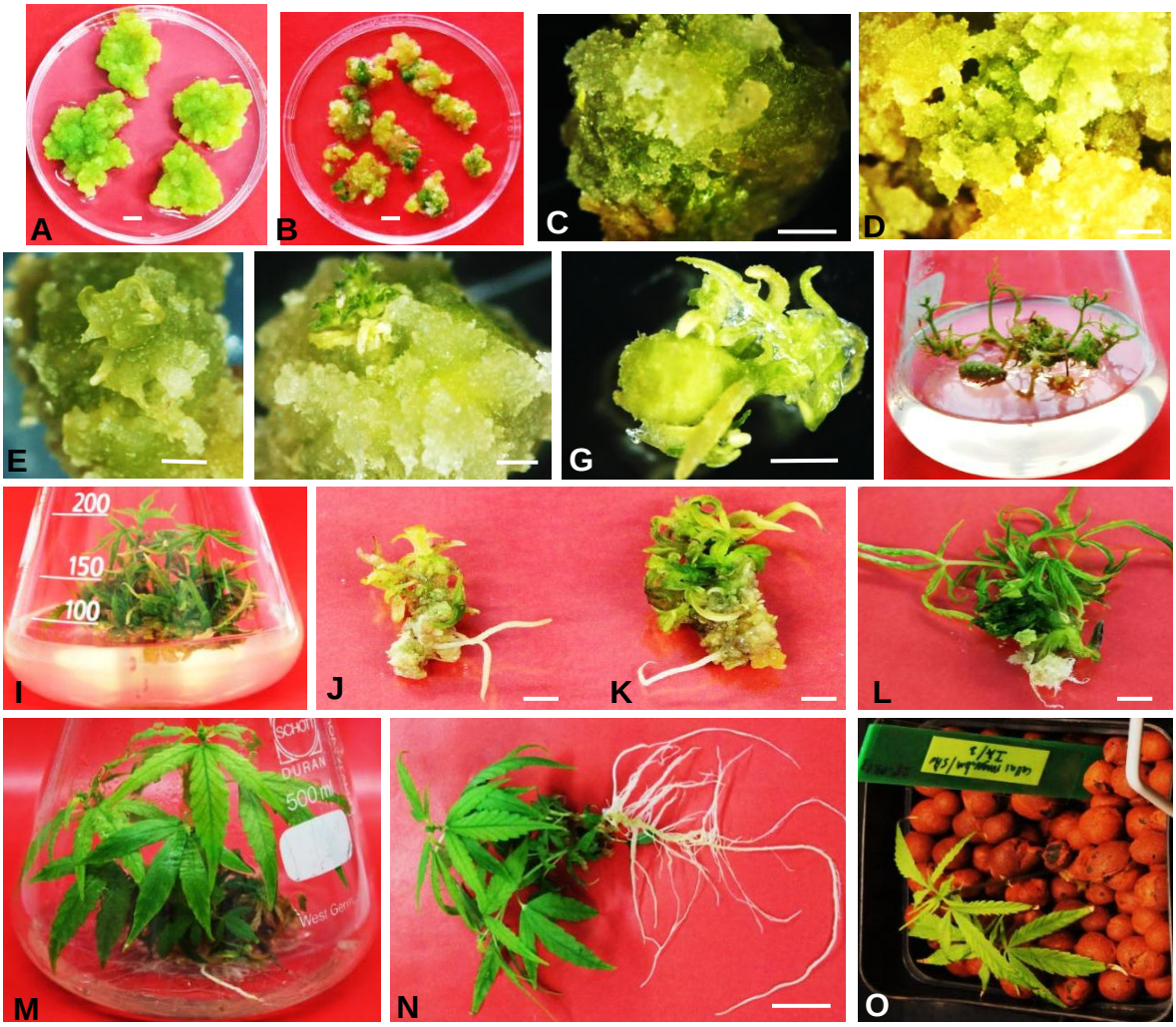


Figure 17: Stages of *in vitro* micropropagation of *C. sativa* leaf-derived calli: A: Callus induced from *C. sativa* explants after 8 weeks of cultivation on modified B5 medium containing 1 mg/l NAA and 1 mg/l BA; B: Meristemoids induced from callus cultures after 5 months on B5 medium containing NAA, BA and AS (0.5, 5 and 40 mg/l, respectively); C-G: Differentiation of meristemoids and shootlets induced from callus after one month on B5 medium containing 0.5 mg/l GA_3; H: Shootlets developing on B5 medium containing 0.25 mg/l KIN and 3 mg/l GA_3; I: Multiple shootlets cultured on B5 medium containing 0.5 mg/l GA_3; J-K: Rooting of shootlets on modified B5 medium containing 0.5 mg/l IAA; L: Rooting of shootlets on modified B5 medium containing 1.0 mg/l IAA; M-N: Rooting of shootlets on modified B5 medium containing 1.5 mg/l IAA; O: *Ex vitro Cannabis* plantlet growing in a 10×10×10.5 cm flower pot with rockwool and covered with hydroton pebbles in plant growth chamber. Scale bars equal: 1 cm (A-B and J-K), 50 µm (C-F), 1 mm (G) and 2 cm (I-N).

3.1.4. Rooting of shootlets

For induction of root formation, different auxins were tested. The separated shootlets (about 3 cm in length) were transferred to root induction medium supplemented with different concentrations of NAA, IBA or IAA, ranging from 0.5 mg/l to 1.5 mg/l (Table 12). After one-week incubation in darkness at 20 °C (dark phase), the shootlets were exposed to light (sub-section 2.2.1.5.). *De novo* generated root tips with fine hairs were observed after four weeks. The most efficient medium for root formation was RNm9 containing 1.5 mg/l IAA: up to 100 % of shootlets developed roots, with 2.75 ± 0.94 roots per shootlet, 8.7 ± 1.65 cm average root length and 5.8 ± 0.77 cm average shootlet length (Figure17J-N).

Table 12 :*In vitro* responses to different concentrations of auxin on root induction of *C. sativa* shoots

Medium†	Phytohormone (mg/l)			No. of roots/shoot ($\overline{\times}$ ± SE) ††	Length of roots (cm) /shoot ($\overline{\times}$ ± SE) ††	Height of shoots (cm) /culture ($\overline{\times}$ ± SE) ††	Root formation (%)
	Auxins						
	NAA	IBA	IAA				
Nm0	0.0	0.0	0.0	0.0	0.0	0.0	0
RNm1	0.5			0.5 ± 0.28	0.2 ± 0.01	3.9 ± 0.38	50
RNm2	1.0			1.0 ± 0.70	0.5 ± 0.08	3.3 ± 0.54	50
RNm3	1.5			1.25 ± 0.75	0.8 ± 0.12	3.7 ± 0.47	50
RNm4		0.5		0.95 ± 0.27	0.12 ± 0.07	2.5 ± 0.36	50
RNm5		1.0		2.0 ± 0.70	0.25 ± 0.1	3.0 ± 0.45	75
RNm6		1.5		2.25 ± 0.75	1.5 ± 0.35	3.1 ± 0.37	75
RNm7			0.5	2.16 ± 0.18	0.3 ± 0.12	3.6 ± 0.31	75
RNm8			1.0	2.75 ± 1.03	1.07 ± 0.15	3.8 ± 0.3	75
RNm9			1.5	2.75 ± 0.94	8.7 ± 1.65	5.8 ± 0.77	100

† modified B5 medium; control experiments were conducted without addition of phytohormones.
†† for each test medium, 4 Erlenmeyer flasks (100: 500 ml) with 4 separate shootlets were investigated; the experiments were repeated twice and the results represented as means ± SE; 0.0, no response.
††All data represent the mean of four replicates

3.1.5. Ex vitro acclimatization (indoor cultivation)

The rooted *C. sativa* plantlets (IV) were transferred from *in vitro* cultures to hydroponic pots and placed in a growth room for *ex vitro* acclimatization. Data concerning the average plant height (cm), numbers of nodes and true leaves per plant and survival quotients were recorded routinely every four weeks (Table 13). The rate of plant survival after transfer to hydroponic cultivation conditions was 80 %. The following 16 weeks of indoor cultivation resulted in plants (IVH) of average height of 53.16 ± 16.34 cm, with 6.2 ± 20.78 true leaves and 9.25 ± 0.47 nodes per plant. Some of the acclimatized *Cannabis* plants possessed abnormal leaves (single or three leaflets instead of the usual seven) and the appearance of some plants was atypical: instead of a single stem, a shrub-like growth with multiple stems was observed (Figure 18A-B).

Table 13: Frequency of *ex vitro* survival and growth of acclimatized propagates of *C. sativa* under controlled environmental conditions

Parameters	**Weeks after transfer***		
	4	**8**	**16**
Survival (%)	100	80	80
Plant height (cm)	4.44 ±0.84	16.7 ± 2.67	53.16 ±16.34
Numbers of true leaves	4.6 ±1.25	15,7 ±4,03	61,2 ±20,78
Numbers of nodes	1.99 ±0.30	3.49 ±0.28	9.25 ±0.47

* all data represent the average values of 4 replicates of 4 hydroponic propagates; mean ± SE.

3.1.6. Quantitative determination of cannabinoid content

Quantification of cannabinoids in the leaves of IV plantlets, flower tops of IVH plants (Figure 18C) and flower top-derived control samples was performed in four replicates by LC-MS. The obtained THC and THCA concentration values characteristic of both test samples were comparable to each other, but highly divergent from those found in the control samples (Table 14 and Figure 19). Cannabinoid levels detected in the leaves of IV plantlets were very low (relative to the control): THCA content was estimated at ~0.3 %, while THC and CBG were not detectable. Surprisingly, while the relative concentration of CBGA was 0.45 %, that of CBDA was 157.1 %, as compared to the control plants. Concurrently, the relative THCA and THC concentrations found in the flower tops of IVH plants were 1.54 % and 28.3 %, respectively. Further, in contrast to the control, a small amount of CBD was detected (0.29 mg/g fr. wt.).

Figure 18: Hydroponically grown of *ex vitro* acclimatized micropropgated cannabis plants (16 weeks old plants) in (15 × 15 × 20 cm) pots under controlled enviromental conditions (20 weeks old plant); A: female plant showing normal growth (note compound leaves); B: female plant showing abnormal growth (white arrow); C: flower tops. *Bars* equal to 10 cm (A-B) and 5 cm (C).

The relative CBGA level was 6 % and that of CBG, only 0.125 %. The highest difference was noted regarding CBDA which was detected at an eleven-fold higher concentration in the flower tops of IVH plants than in the control plants.

Table 14: Quantitative comparison of cannabinoid content in the leaves of IV plantlets and the flower tops of IVH plants relative to mother plant samples (control).

Cannabinoids	*In vitro* propagated				Control*†	
	IV* plantlets		IVH* plants			
	mg/g FW	%	mg/g FW	%	mg/g FW	%
THCA	0.40 ± 0.05	0.33	1.86 ± 0.28	1.547	120.2 ± 15.48	97.89
THC	0.0	0.0	0.15 ± 0.008	28.30	0.53 ± 0.008	0.431
CBGA	0.00763 ± 0.0046	0.45	0.10 ± 0.03	6.024	1.66 ± 0.20	1.351
CBG	0.0	0.0	0.15 ± 0.001	12.5	0.12 ± 0.03	0.097
CBDA	0.44 ± 0.02	157.	3.14 ± 0.61	1121.42	0.28 ± 0.04	0.228
CBD	0.00977 ± 0.02	0.0	0.29 ± 0.04	0.0	0.0	0.0

† contol: flower tops of C. sativa cv. Bedrobinol plants grown in Berdocan VB under controlled environmental conditions

* values of detected cannabinoid levels are given in mg/g fr. wt. and in % (relative to control) and represent the average of 4 replicate samples; mean ± SE; 0.0, no cannabinoids detected

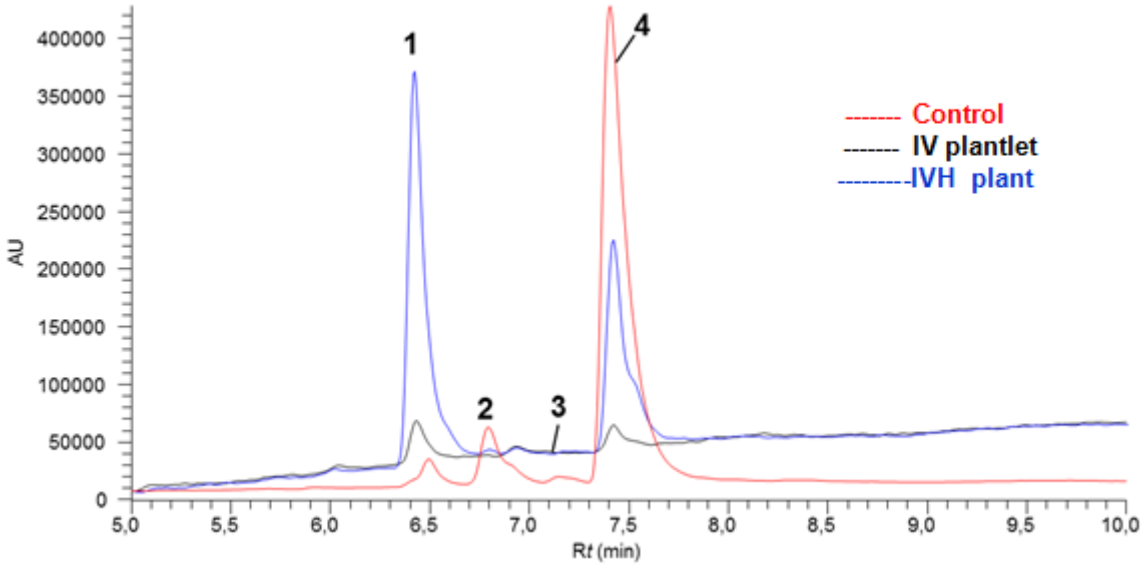


Figure 19: LC-MS total ion chromatograms of extracts derived from *in vitro* plantlets (IV), flower tops of hydroponically grown plants (IVH) and control plant samples illustrating their cannabinoid profiles. 1: CBDA, t_R 6.46 min; 2: CBD, t_R 6.71 min; 3: THC, t_R 7.17 min; 4: THCA, t_R 7.45 min.

3.2. Shake flask cultures

3.2.1. Characterization of cannabinoids in cell suspension cultures

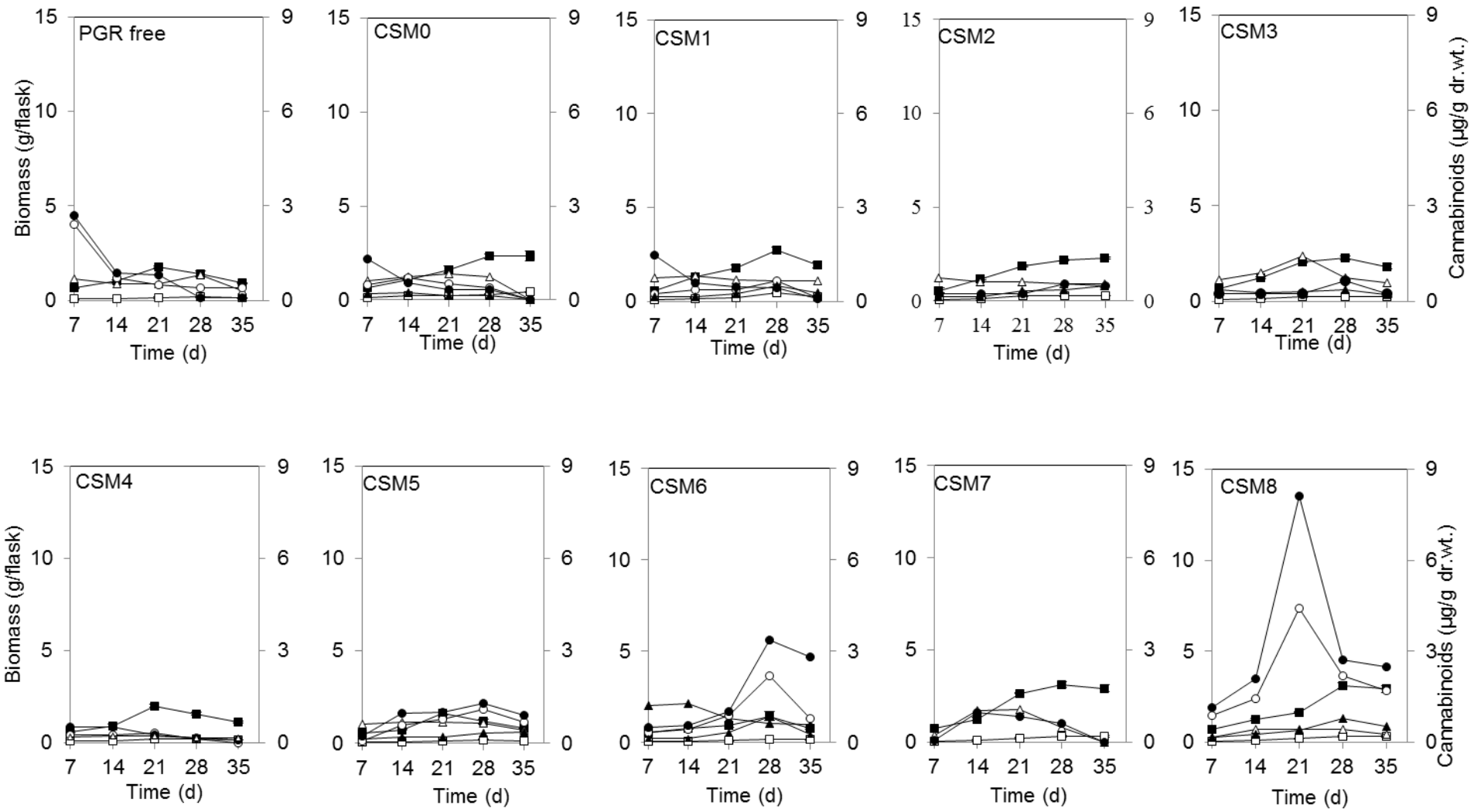
3.2.1.1. Growth rates

Shake flask experiments were performed using green and homogenous cell aggregates of *C. sativa* grown in modified B5 medium supplemented with various combinations of plant growth regulators (Table 6). Growth kinetics, recorded during the culture cycle of 35 days, were variable (Figure 20). Media CSM13 (1 mg/l TDZ and 1.5 mg/l GA_3) and CSM12 (1.5 mg/l TDZ and 1.5 mg/l GA_3) afforded most favourable growth conditions. Doubling of fresh biomass for these cultures occurred after about 28 days, yielding 11.19 ± 0.79 g fr. wt./flask and 0.72 ± 0.03 g dr. wt./flask for CSM12 and 11.55 ± 0.81 g fr. wt./flask and 0.78 ± 0.008 g dr. wt./flask for CSM13, as compared to the control cultures. Subsequently, the fresh and dry weight values decreased.

3.2.1.2. Time course of cannabinoid production

3.2.1.2.1. Characterization of high yielding cell cultures

Cell cultures grown in the media CSM8 (0.5 mg/l BA and 1.5 mg/l GA_3), CSM12 (1mg/l TDZ and 1.5 mg/l GA_3) or CSM13 (1.5 mg/l TDZ and 1.5 mg/l GA_3) yielded the highest cannabinoid levels, as examined by means of HPLC and compared to those grown in other test media listed in Table 6. Figure 20 illustrates the changes in cannabinoid concentration over the course of the culture cycle (as examined on its 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35^{th} day) in all investigated cell suspensions. In cell cultures grown in CSM8 (Figure 20), THCA and THC were detected at highest levels of 8.12 and 4.45 µg/g dr. wt., repectively, on day 21 of the culture cycle. Concurrently, the most pronounced yield of CBDA was obtained using medium CSM12: 1.5 µg/g dr. wt. on day 28 of the culture cycle, while the cells grown in CSM13 (Figure 20 & 22) produced the highest amounts of CBGA: 1.18 µg/g dr. wt. on day 21 of the culture cycle. The obtained results clearly showed drastic reduction in cannabinoid content after 28 days of cultivation, indicating a correlation between the culture age and instability of cannabinoid levels. It is of interest that culture media supplemented with gibberellic acid (GA_3) in combination with 6-benzylaminopurine (BA) or thidiazouron (TDZ) provided a favourable system for enhanement of cannabinoid biosynthesis.


PGR free
CSM0
CSM1
CSM2
CSM3
CSM4
CSM5
CSM6
CSM7
CSM8
Biomass (g/flask)
Cannabinoids (µg/g dr.wt.)
Time (d)
0
5
10
15
3
6
9
7
14
21
28
35

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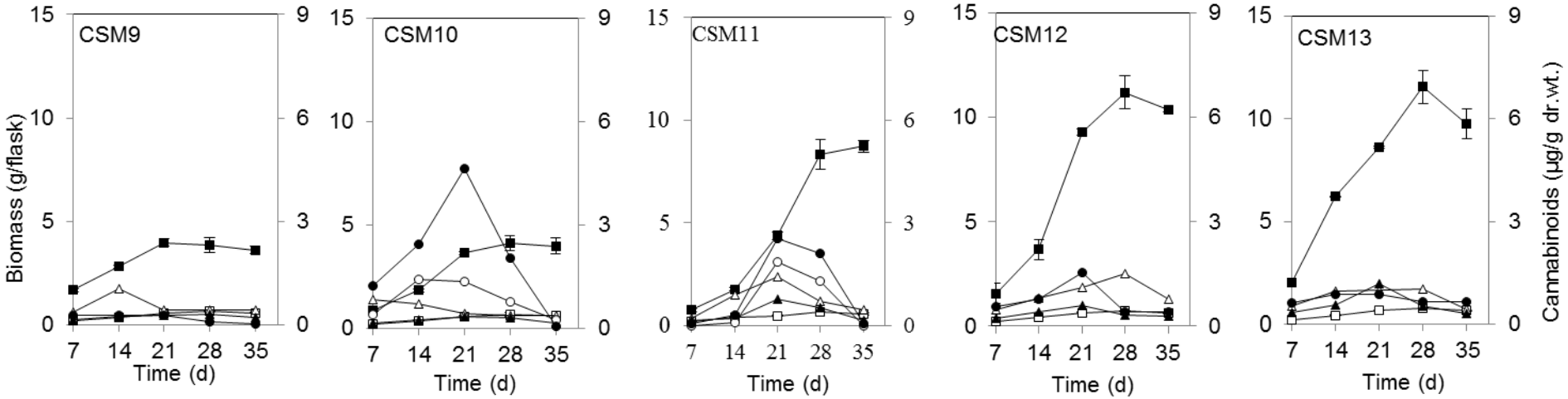


Figure 20: Cell growth and cannabinoid formation in suspension cultures of *C. sativa* under various cultivation conditions (sub-section 2.2.2.; Table 6); (■), fresh biomass; (□), dry biomass; (▲), CBGA; (∆), CBDA; (●), THCA; (○), THC. (fr. wt. and dr. wt. are the mean 3 replicate values ± SE).

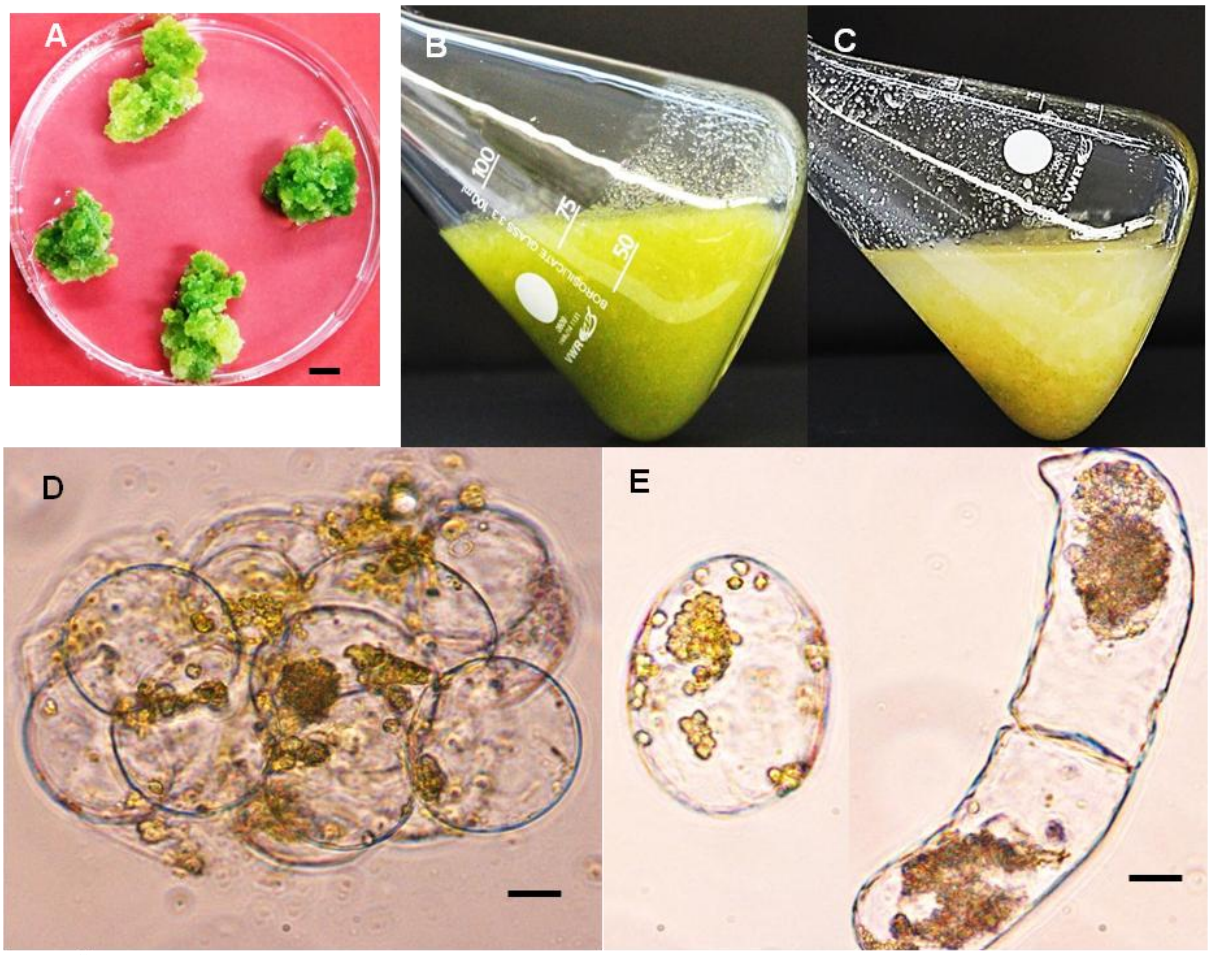


Figure 21: A: Friable callus of *C. sativa* on modified B5 medium; B: Shake flask cell suspension culture in CSM8 (0.5 mg/l BA and 1.5 mg/l GA_3) on day 14 of the growth cycle; C: Shake flask cell suspension culture in CSM8 after 35 days of cultivation; D-E: Morphological characteristics of the cultured cells on day 14 of the growth cycle. Scale bars equal: 1 cm (A) and 18 µm (D-E).

3.2.1.2.2. Characterization of low yielding cell cultures

In the cell cultures grown in media CSM1 (1 mg/l BA), CSM6 (1.5 mg/l TDZ) and CSM7 (1.5 mg/l GA_3) only low levels (< 0.38 µg/g dr. wt.) of THCA, THC, CBGA and CBDA were detected on day 28 of the culture cycle (Figure 21).

3.2.1.2.3. Cell culture morphology and aging

Morphological characteristics of the cells in suspension cultures of *C. sativa* are shown in Figure 22; individual and aggregated cells can be observed. On day 14 of the growth cycle, the cultures were green and most of the cells were isodiametric (~ 18 µm in diameter) and elliptic in shape. After 35 days of cultivation, however, the suspensions turned yellowish in colour.

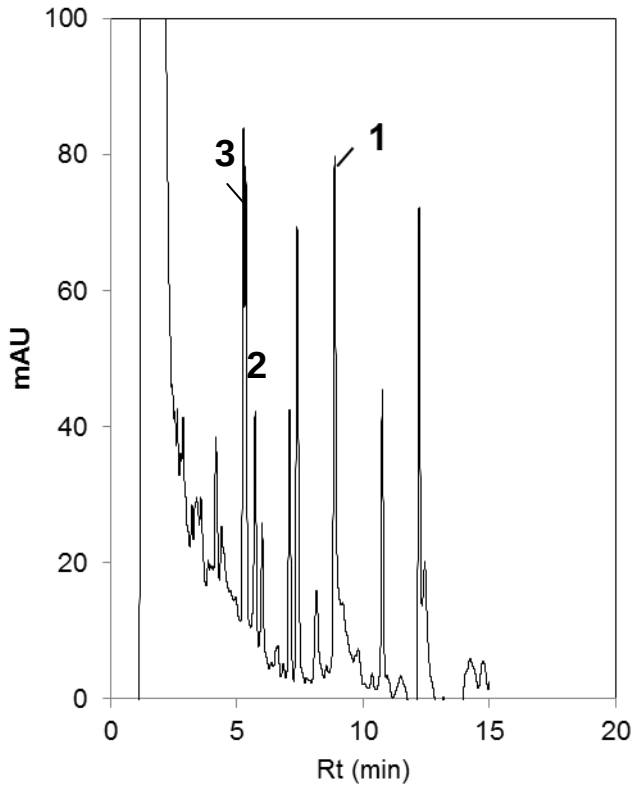


Figure 22: HPLC chromatogram of the extract derived from *C. sativa* cells cultivated in CSM13 (1 mg/l TDZ and 1.5 mg/l GA_3) on day 21 of the growth cycle (sub-section 2.2.6.1.2.). 1: THCA, t_R 9.03 min; 2: CBGA, t_R 5.7 min; 3: CBDA, t_R 5.3 min.

3.2.1.3. Fingerprinting of cannabinoids

3.2.1.3.1. LC-MS/MS analysis

Identification of cannabinoids in the samples extracted from cells cultured in CSM4 (0.5 mg/l TDZ) at different time points (14^{th}, 28^{th} and 35^{th} day of the growth cycle; sub-section 2.2.6.2.1.) was carried out by LC-MS (Figure 23; Table 15) and LC-MS/MS in positive ion mode. Table 16 and Figure 23 summarize the obtained results, while the detailed MS/MS spectra of selected cannabinoids found in the investigated crude extracts are illustrated in Figure 24. The high resolution mass spectra corroborated the presence of ions characteristic of the following cannabinoids: THCA, m/z 359.22; THC, m/z 315.23; CBN, m/z 311.20; CBGA, m/z 361.24; CBG, m/z 318.32 and CBD, m/z 316.24. THCA content was especially pronounced, with the heighest peak intensity recorded in the samples processed after seven days of cultivation. The retention time values and LC-MS/MS spectra of the detected metabolites were identical to those obtained for the standards.

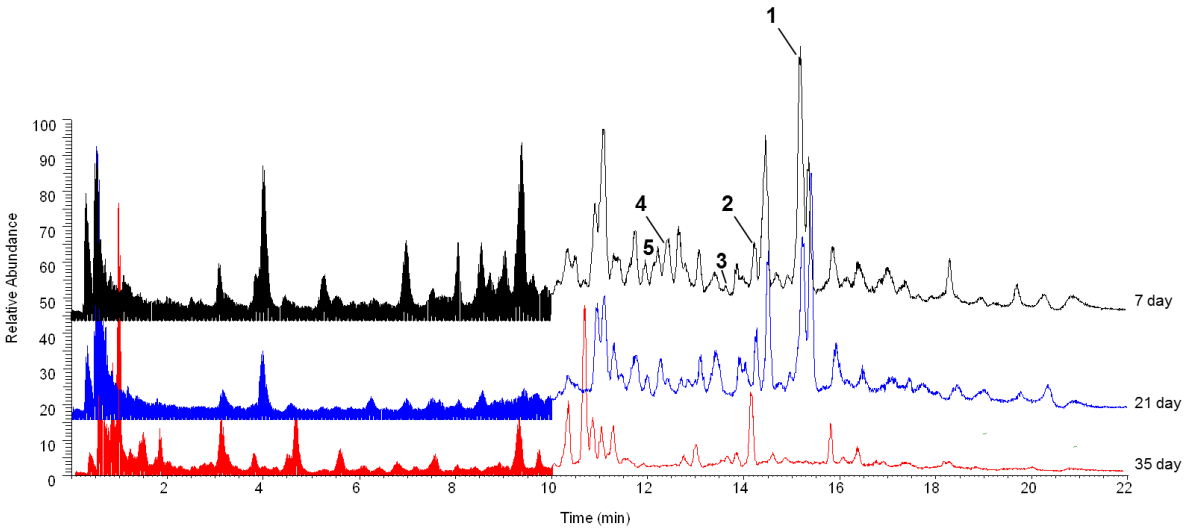


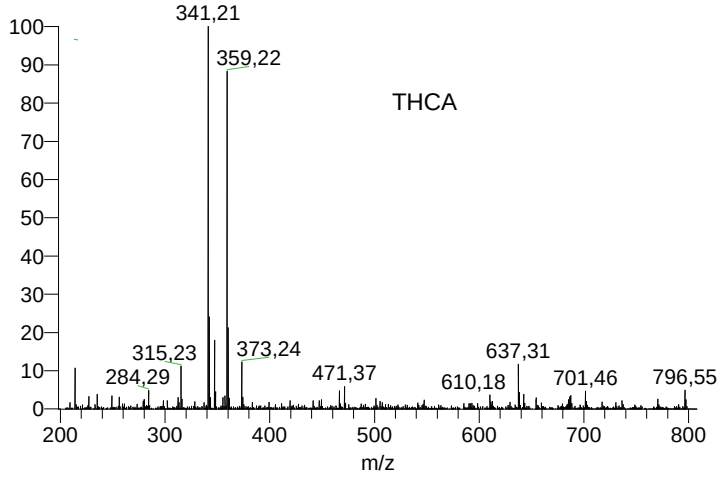
Figure 23: LC-MS total ion chromatograms of CSM4-grown (0.5 mg/l TDZ) cell suspension samples extraced on days 7, 21 and 35 of the cultivation cycle. 1: THCA, t_R 15.11 min; 2: THC; t_R 14.22 min; 3: CBN, t_R 13.62 min; 4: CBD, t_R 12.37 min; 5: CBDA, t_R 11.92 min.

Table 15: Summary of detected peaks by LC-MS/MS

Compound and formula	Theoretical mass	t_R [min]	Major product ions $[m/z]^{+\cdot}$	Cannabinoids/ day		
				14	28	35
1 THCA- ($C_{22}H_{30}O_4$)	358.47	14.10	359.22 $[M+H]^+$,341.21, 285.15, 261.15, 219.10	+	+	+
2 THC- ($C_{21}H_{30}O_2$)	314.45	13.21	315.23 $[M+H]^+$, 259.17, 193, 135.12,	+	+	-
3 CBN- ($C_{21}H_{26}O_2$)	310.43	12.58	311.20 $[M+H]^+$, 293.19, 241.12, 223.11, 195.12	+	+	+
4 CBGA- ($C_{22}H_{31}O_4$)	360.5	11.55	361.24 $[M+H]^+$, 343.23, 271.17, 219.10, 193.12	-	-	+
6 CBD-($C_{21}H_{30}O_2$)	314.46	11.34	315.24 $[M+H]^+$, 259, 193.12, 135.12	+	+	-
8 CBDA- ($C_{21}H_{30}O_4$)	358.47	10.89	359.22 $[M+H]^+$,341.21, 298.35, 235.17, 214.10	+	+	+

+ = present; - = absent

c1 #2433-2512 RT: 14,09-14,44 AV: 80 NL: 2,30E7
T: FTMS + p ESI Full lock ms [200,0



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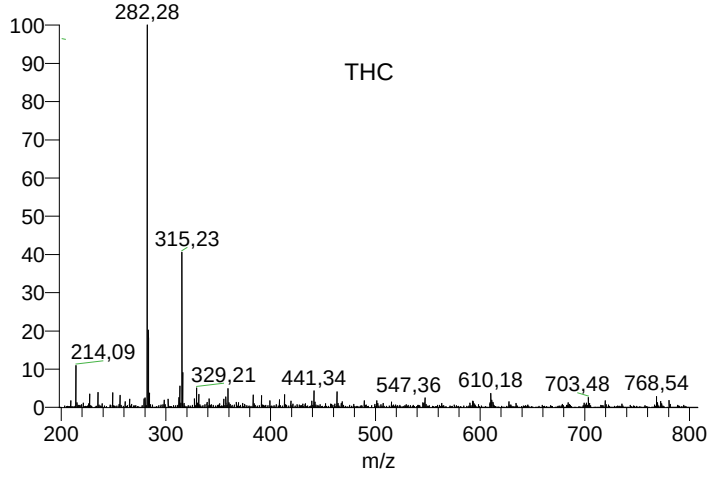

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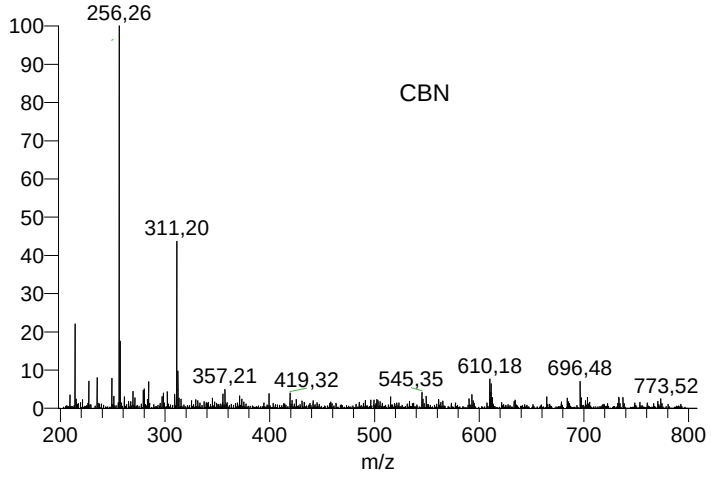

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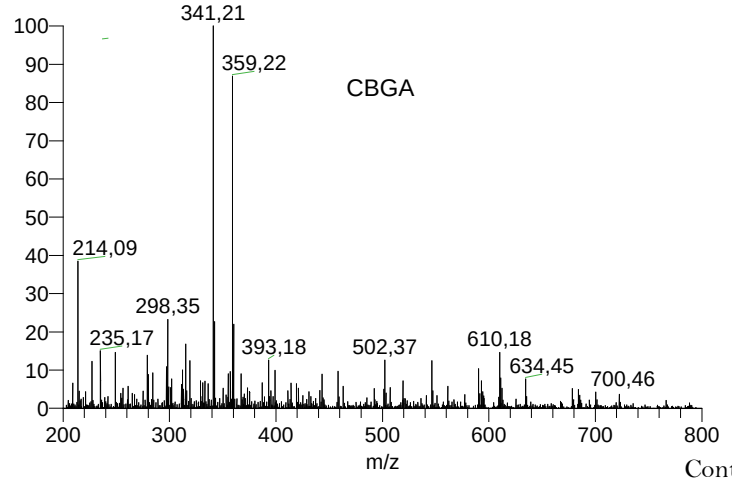

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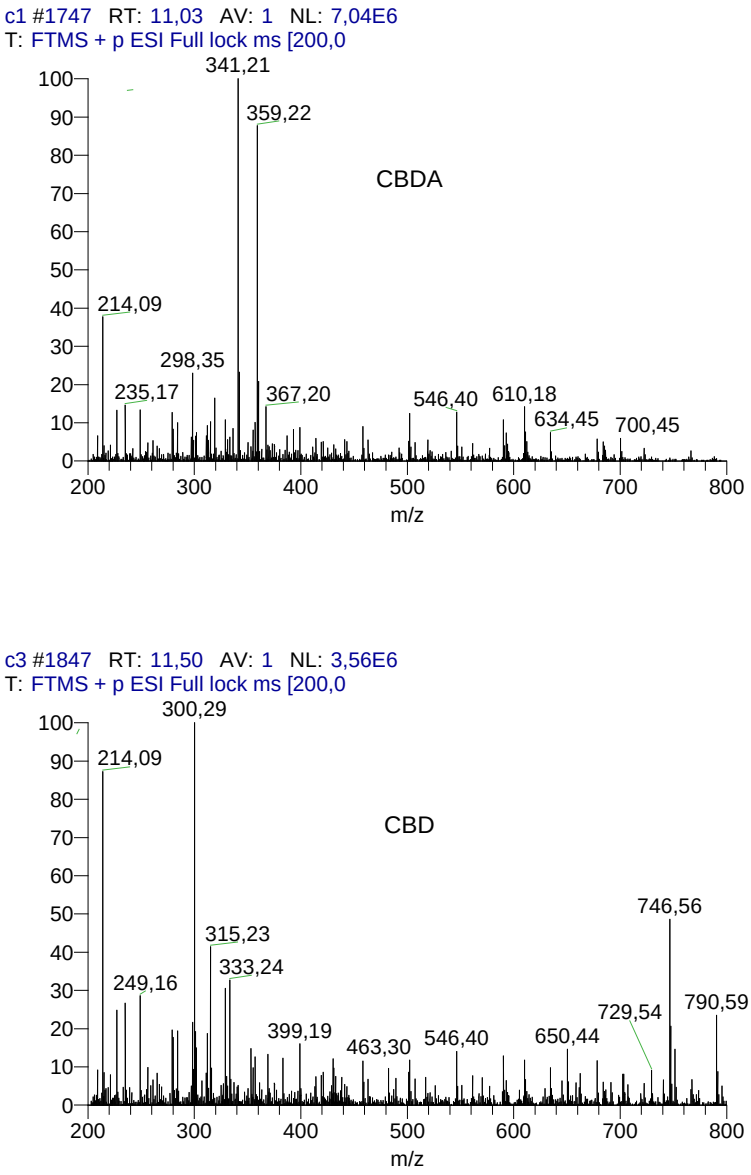

Figure 24: LC-MS/MS $[M+H]^+$ spectra of cannabinoids detected in the crude extracts of *C. sativa* cells cultured in CSM4 (0.5 mg/l TDZ).

3.2.1.3. 2. ^{1}H NMR

^{1}H NMR analysis of the investigated extracts (sub-section 2.2.6.2.4.) resulted in detection of minor THCA-specific signals in the sample processed after 21 days of cultivation in CSM8. The signals: δ 1.45 (s), δ 1.69 (s), δ 6.25 (s) and δ 6.40 (s) were assigned as corresponding to H-12, H-11, H-4 and H-10 of THCA, respectively. The results were further confirmed through comparison of the registered signals with the chemical shifts of the reference THCA (Figure 26).

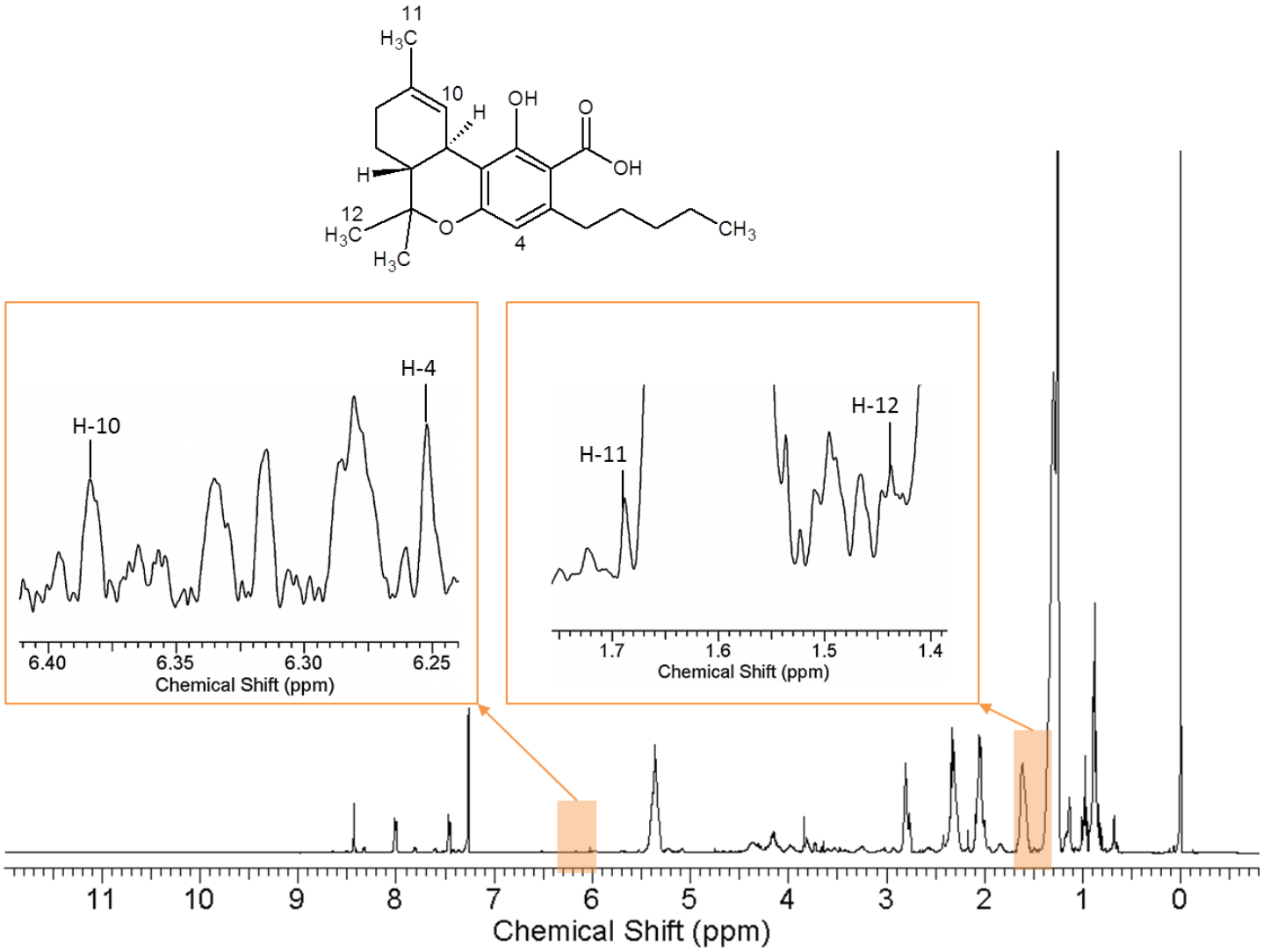

Figure 25: ^{1}H NMR spectrum of –the chloroform extract of the CSM8-grown (0.5 mg/l BA and 1.5 mg/l GA_3) cell suspension sample collected on day 21 of cultivation; the THCA-corresponding chemical shifts are highlighted.

3.2.2. Shake flask cultures of hairy roots

In the present investigation, high frequency-promotion of rhizogenesis in *C. sativa*-derived callus was obtained in IHR0 medium (B5 supplemented with 4 mg/l NAA;sub-section 2.2.3.). The flowchart in Figure 26 shows the consecutive steps of the procedure:

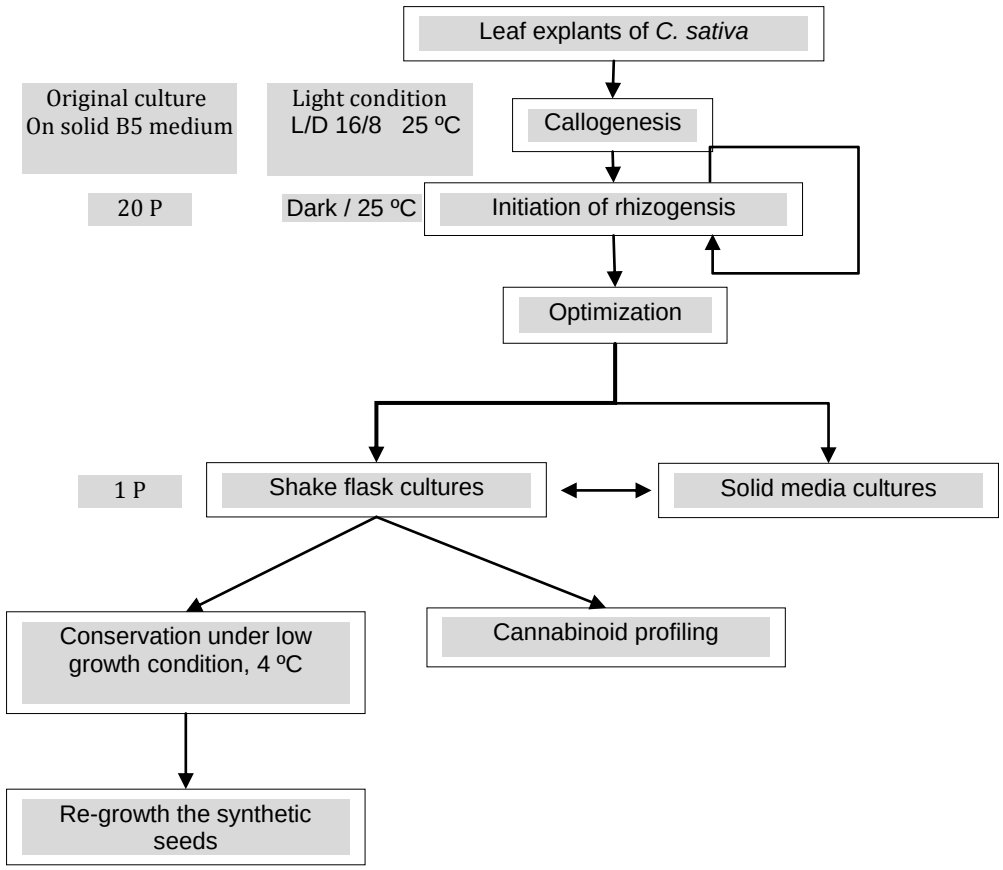

Figure 26: Schematic representation of the hairy root induction procedure from *C.sativa* calli. Passage numbers (P) are indicated on the left. The bold line denotes the most efficient hairy root shake flask culture. Subculturing to the new medium was performed, at the earliest, after 20 days of cultivation.

3.2.2.1. Initiation of adventitious hairy roots from calli

Of all the tested auxins (Table 16, Figure 27) only NAA, added to the full-strength B5 medium at the concentration of 4 mg/l, stimulated root differentiation. After 16-week incubation on the IHR0 medium in darkness at 25 °C, numerous hairy roots began to emerge vigorously from the surface of calli, with the final root formation quotient of 83.3 %. Eight weeks later, the formed roots showed the characteristics of typical adventitious roots, having numerous branches covered with root hairs.

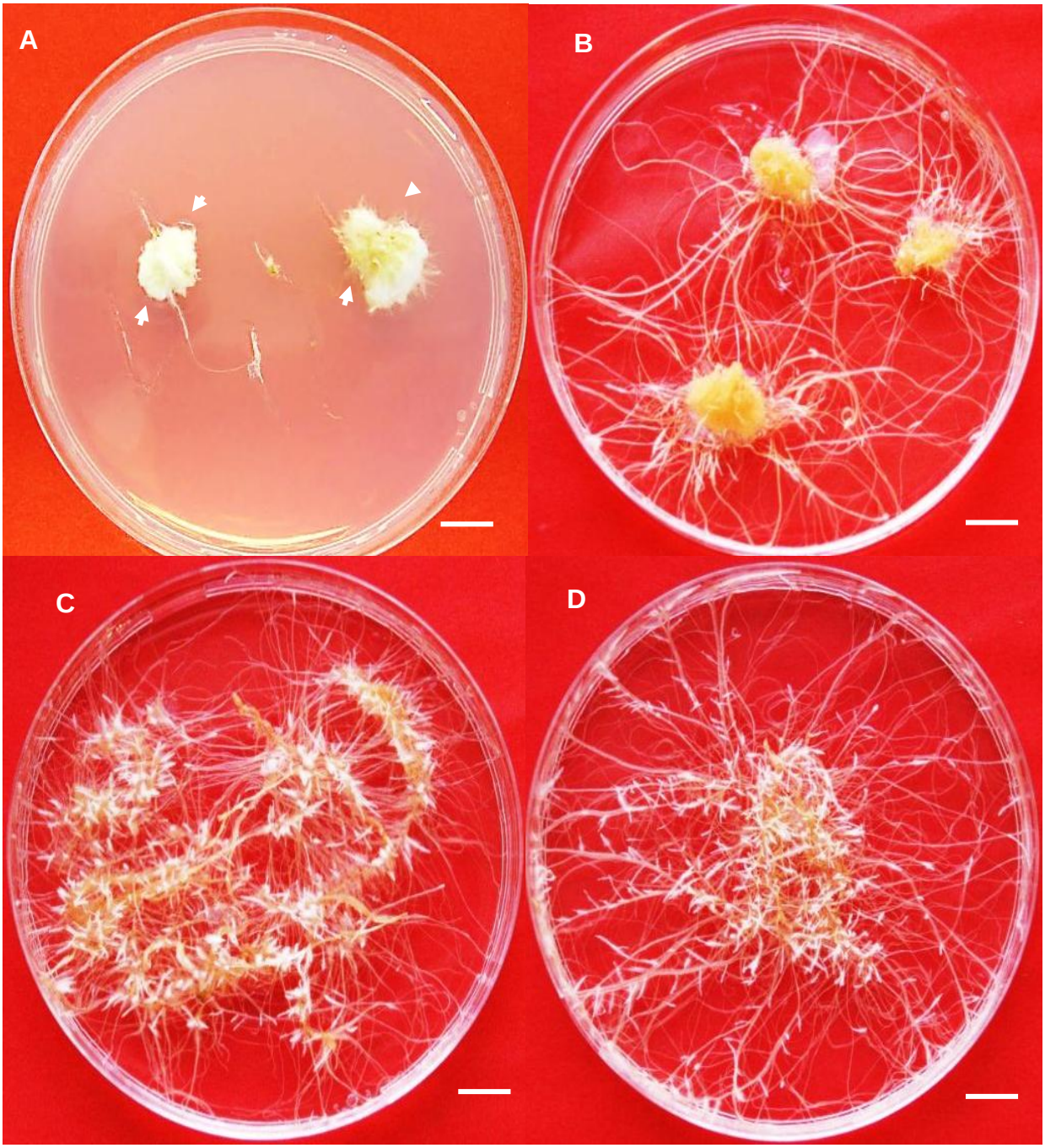

Figure 27: Morphology of *C. sativa* hairy root cultures. A: Hairy root initiation from leaf-derived calli cultured on IHR0 (after 16 weeks); B-D: Proliferation of hairy roots on the IHR0 medium. Scale bars equal 1 cm.

Table 16: Induction media for hairy root growth stimulation.

Medium	Phytohromone (mg/l)			No. of roots/callus ($\overline{\times}$ ± SE) †	Root length (cm) /callus ($\overline{\times}$ ± SE) †	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
SHR7[b]			2.5	0.0	0.0	0.0
SHR8[b]			1.5	0.0	0.0	0.0

PGR-free, full-strength B5 medium without PGRs serving as control.
[a] full-strength B5 with 4 mg/l NAA serving as a reference medium
[b] various treatments on half-strength B5
† all data are presented as mean values of 3 replicates with 2 callus pieces per treatment ± SE;
0.0, no response
Induction of hairy roots from callus cultures was observed after 4 subculturing cycles.

3.2.2.2. *Optimization of hairy root cultures*

The applied auxin type influenced root growth and multiplication (section 2.2.3. ;Tables 16, Figure 28A). Calli cultured on solid media SHR5 (½ B5, 0.5 mg/l IBA) and SHR8 (½ B5, 0.5 mg/l IAA) gave rise to high numbers of hairy roots, with an average of 37 ± 4.7 and 27.25 ± 4.9 roots per culture, respectively. On the other hand, utilization of media SHR2 (½ B5, 0.5 mg/l NAA) and SHR8 (½ B5, 1.5 mg/l IAA) resulted in pronounced lateral hairy root formation with an average length of 6.3 ± 0.14 and 5.2 ± 0.28 cm per culture, respectively. Further, auxin concentration affected root phenotype: root hairs formed on SHR0 (B5, 4 mg/l NAA), SHR2 and SHR5 were rather different than those induced on SHR8. From morphological perspective, and as depicted in Figure 28G, three distict zones can be differenciated within the hairy root structure (Bhadra and Shanks, 1997; Datta *et al*., 2011): the meristematic zone (MZ), where cell division and formation of lateral branches takes place, the elongation zone (EZ), where cells expand along the longitudinal axis and the differentiation zone (DZ), where root hairs develop.

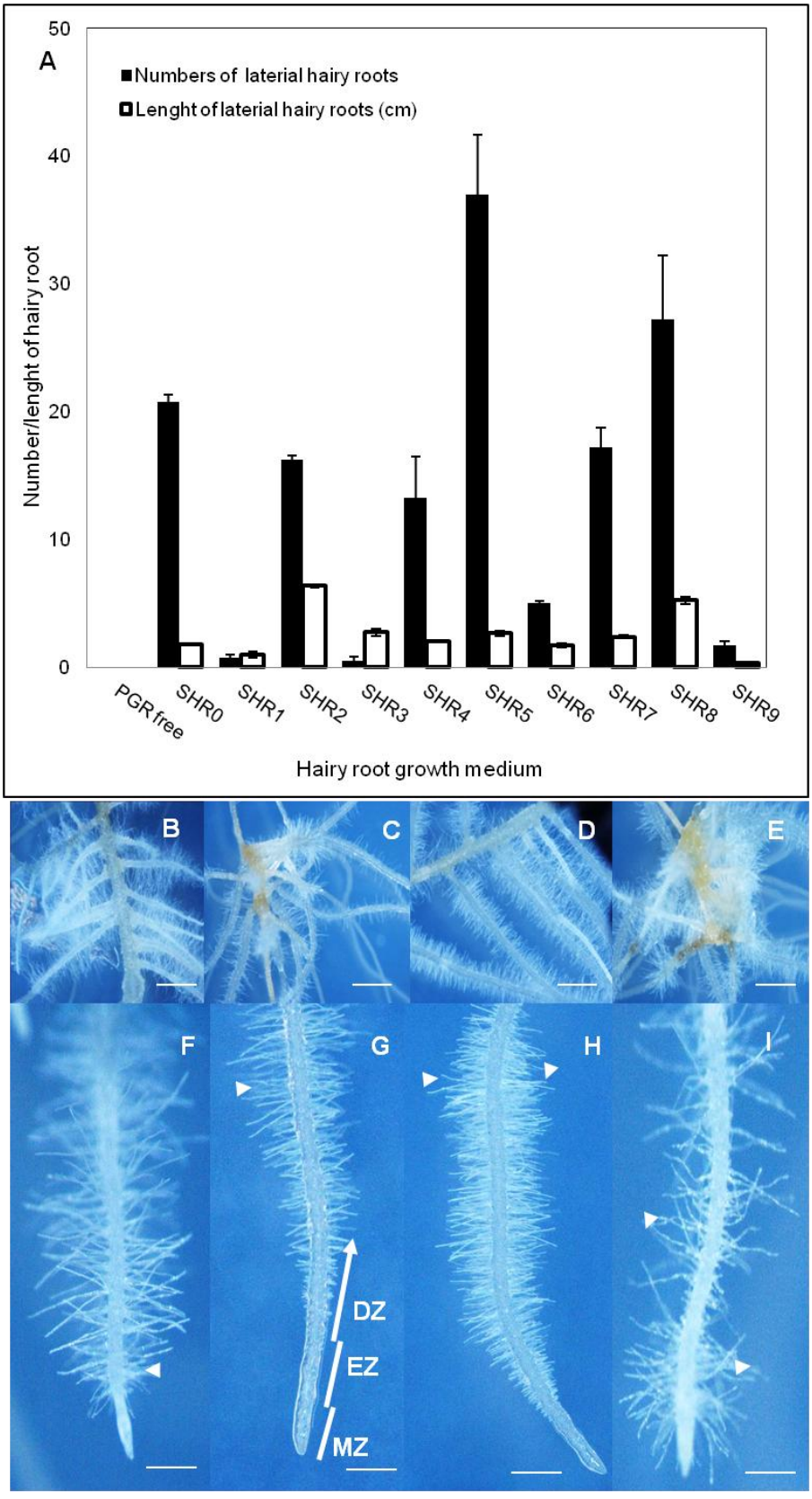

Figure 28: A: Effect of different auxins (NAA, IBA and IAA) on growth of *C. sativa* hairy roots after 4 weeks of cultivation (mean values of five replicates with four root tips per treatment, repeated twice ± SE). B-E: Venation of lateral hairy roots of the oldest rootlets after 4 weeks of culturing on SHR0, SHR2, SHR5 and SHR8; scale bars equal 50 µm. F-I: Phenotypes of root hairs (arrowheads) after 4 weeks of culturing on SHR0, SHR2, SHR5 and SHR8; scale bars equal 200 µm). MZ, meristematic zone; EZ, elongation zone; DZ, differentiation zone

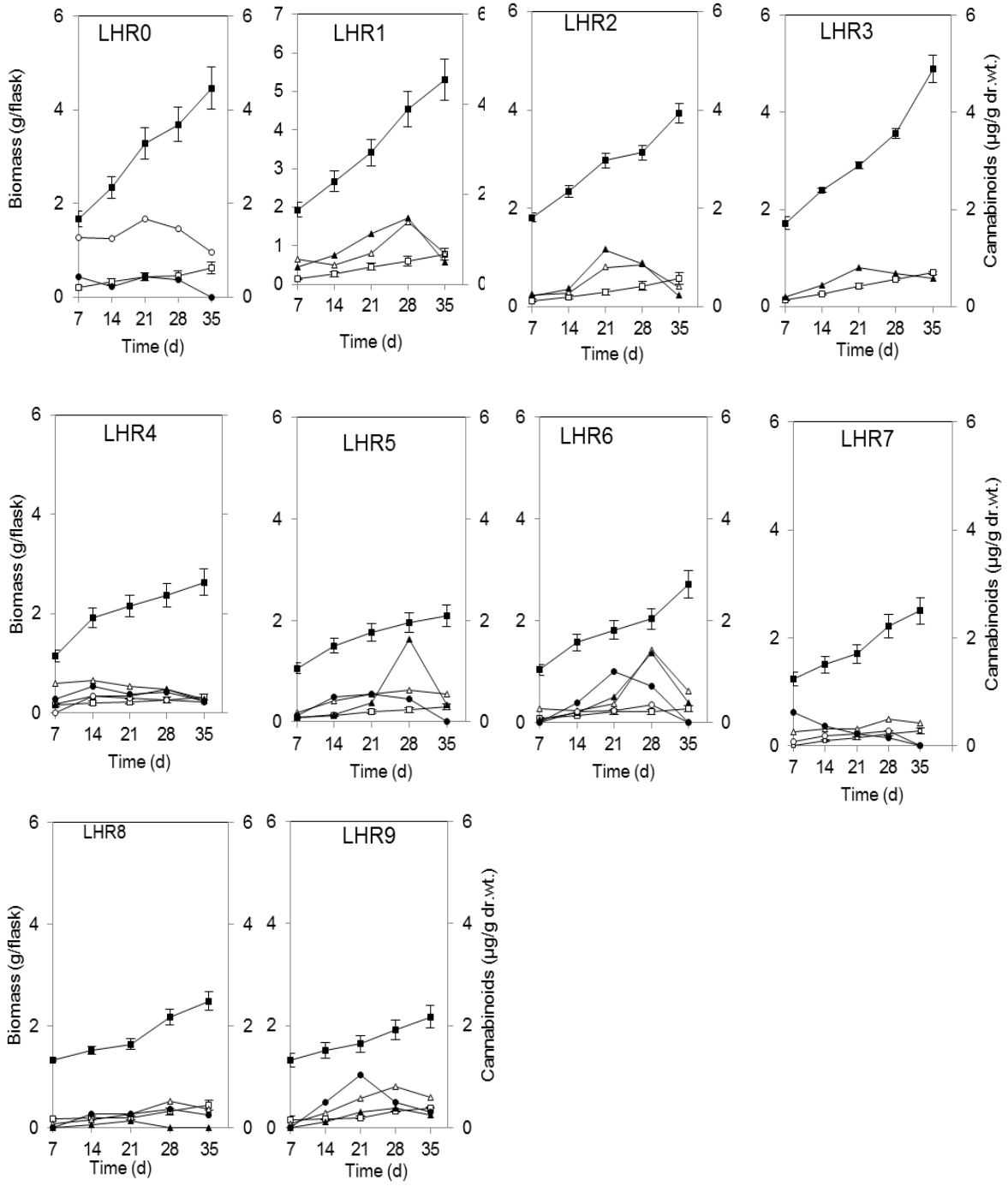

Figure 29: Growth and cannabinoid production profiles in hairy roots of *C. sativa* under various cultivation conditions (sub-section 2.2.3.2.; Table 8); (■), fresh biomass; (□), dry biomass; (▲), CBGA; (Δ), CBDA; (●), THCA; (○), THC (fr. wt. and dr. wt. are the mean 3 replicates± SE).

3.2.2.3. Characterization of cannabinoids

Effects of exogenous auxins on hairy root growth kinetics and cannabinoid formation were determined and illustrated in Figure 29.

3.2.2.3.1. Growth rates

Growth curves representative of in the investigated shake flask hairy root cultures (Table 8) are shown in Figure 29. Auxins had a positive influence on biomass accumulation (nearly linear increase within the 35-day cultivation cycle). Hairy roots grown in LHR1 (½ B5, 0.25 mg/l NAA) amassed most efficiently (fr. wt. 5.31 ± 0.15 g/flask and dr. wt. 0.77 ± 0.008 g/flask on day 35 of the growth cycle), as compared to those cultured in the control (wild type), LHR0 (B5, 4 mg/l NAA; fr. wt. 4.46 ± 0.05 g/flask and dr. wt. 0.62 ± 0.03 g/flask on day 35 of the growth cycle).

3.2.2.3.2. Time course of cannabinoid production

Regarding cannabinoid production (Figures 29 and 32), the highest levels of THCA, as determined by HPLC (1.04 µg/g dr. wt.; 0.32 ± 0.01 g/flask) were noted in samples derived from the cultures grown in LHR3 (½ B5, 1.0 mg/l IAA) on day 28 of the growth cycle (sub-section 2.2.2.1.). Interestingly, the highest quantities of CBGA (1.63 µg/g dr. wt.; 0.23 ± 0.02 g/flask) were detected in extracts of LHR9-grown roots (½ B5, 0.5 mg/l IBA), while the most pronounced CBDA levels 1.67 µg/g dr. wt.; 0.42 ± 0.01 g/flask) were recorded in those derived from cultures grown in the reference medium, LRH0 (B5, 4 mg/l NAA); both on day 28 of the growth cycle. It was further noted that the formation of cannabinoids decreased after 28 days of cultivation. On the other hand ,The efficient de novo biosynthesis and formation of cannabinoids in dark-grown hairy root cultures observed herein are not surprising, and could be indicate many the up-regulation of pathway enzymes in the absence of light in pathway up-regulated in dark. Thus, the obtained results suggest from these results we conclude that cannabinoids biosynthesis is not dependent on light-independent, as in our case the hairy root cultures were maintained in darkness.

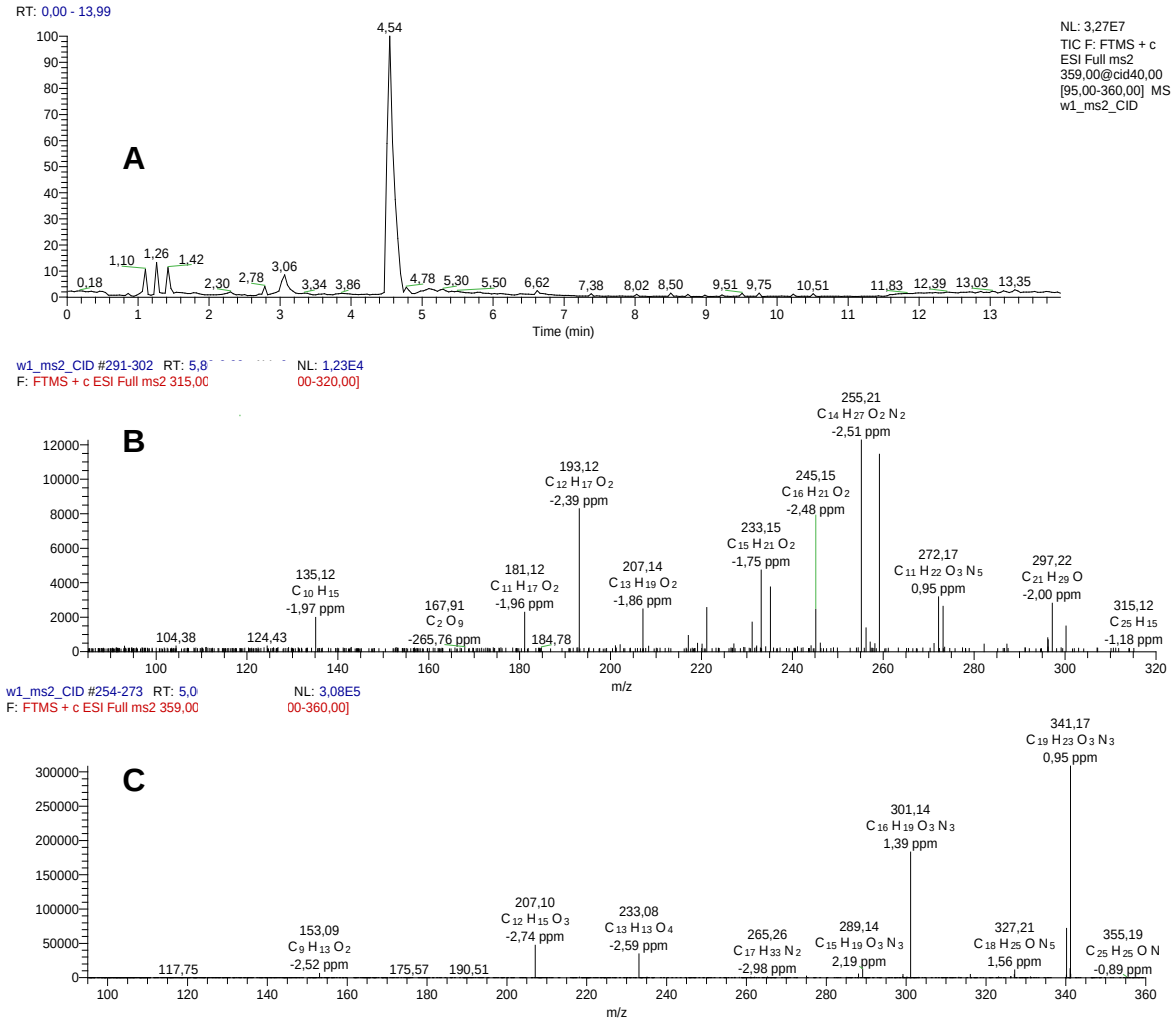

Figure 30: LC-MS/MS analysis of the extract derived from LHR0-grown hairy roots. A: Total ion current (TIC) chromatogram; B: MS2 spectrum of THC; C: MS2 spectrum of CBDA.

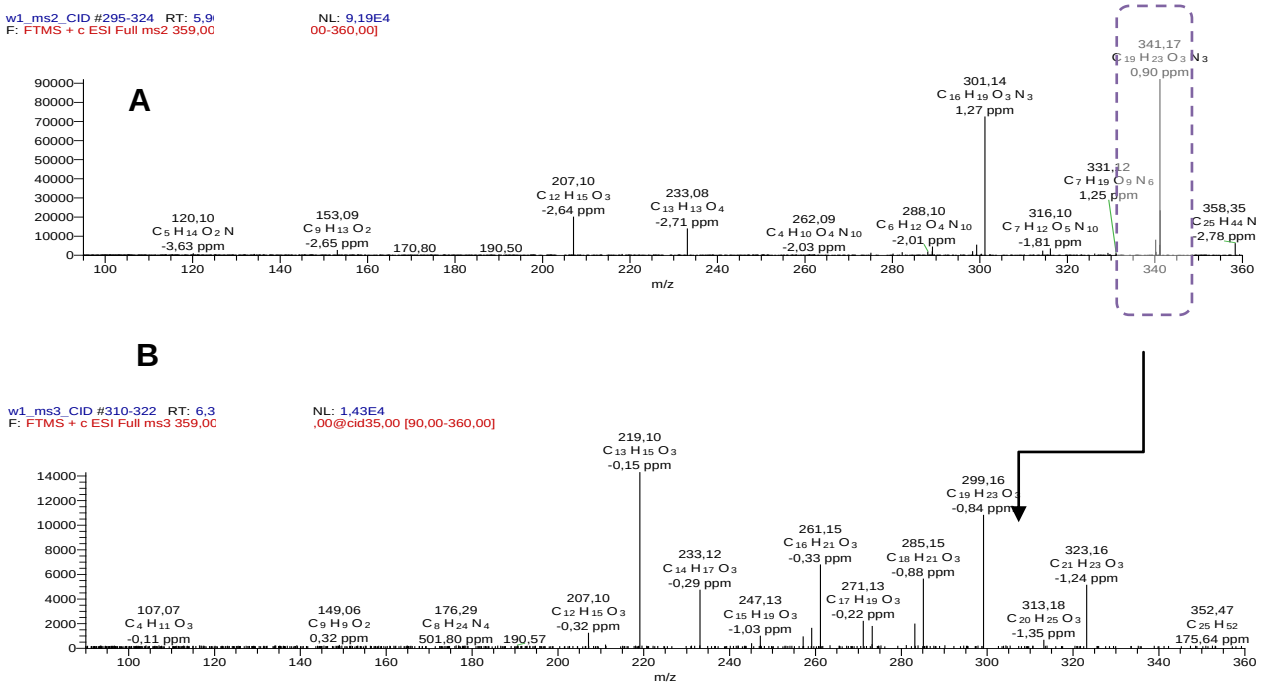

Figure 31: THCA spectra. A: MS2;B: MS3.

Table 17: Summary of LC-MS/MS results of hairy root cultures.

Compound and formula	Theoretical mass	t_R [min]	Major product ions [m/z]+	Cannabinoids/day				
				7	14	21	28	35
THCA ($C_{22}H_{30}O_4$)	358.47	5.90	358.35 $[M+H]^+$, 341.17, 331.12, 301.14, 207.10	+	+	+	-	-
THC ($C_{21}H_{30}O_2$)	314.45	5.80	315.12 $[M+H]^+$, 297.22, 272.17, 255.21, 207.14	+	+	+	+	-
CBDA ($C_{21}H_{30}O_4$)	358.47	5.06	355.19 $[M+H]^+$,341.17, 327.21, 301.14, 207.10	+	+	+	-	-

+ = present; - = absent

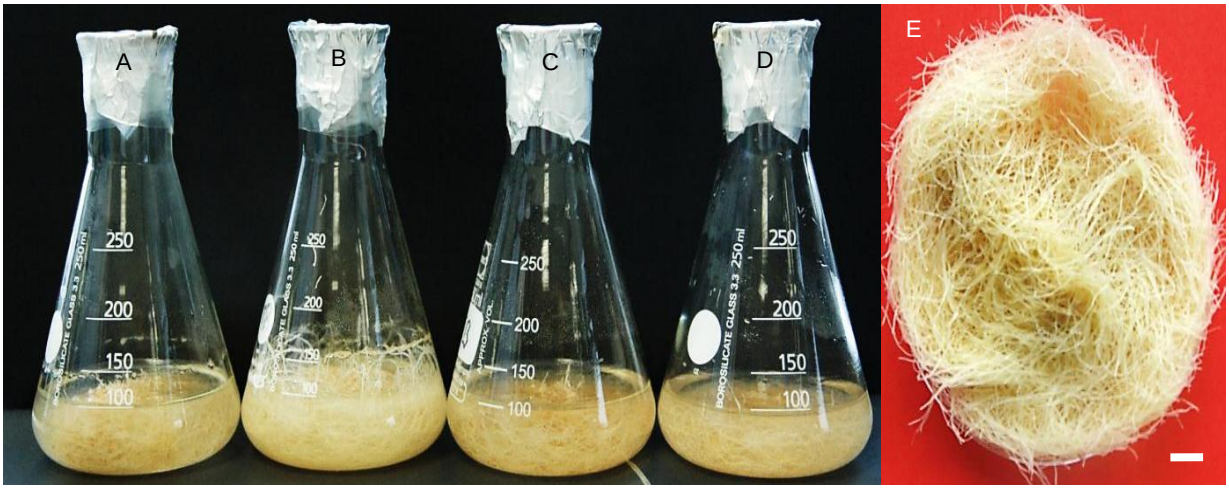

Figure 32: Suspension cultures of *C. sativa* adventitious roots (growth in flasks recorded after 14 days of cultivation); A: LHR0; B: LHR2; C: LHR5; D: LHR8; E: Harvested fresh roots after 35 days of growth. Scale bar equals 1 cm.

3.2.2.3.3. Fingerprinting of cannabinoids

3.2.2.3.3.1. LC-MS/MS analysis

Identification of cannabinoids in the extracted samples of LHR0-grown hairy root cultures at different time points (7th, 14th, 21st, 28th and 35th day of the cultivation cycle) was carried out by means of LC--ESI-MS/MS (sub-section 2.2.6.2.2., Figures 30 and 31). Table 17 summarizes the obtained results, while the detailed MS/MS spectra of selected cannabinoids found in the extracts of LHR0-grown (B5, 4 mg/l NAA) root sampes are illustrated in Figure 30. The high resolution mass spectra corroborated the presence of ions characteristics of the following cannabinoids:

THCA, m/z 358.35; THC, m/z 315.12 and CBDA, m/z 355.19. Highest THCA-, THC-, and CBDA-specific peak intensity values were recorded after seven days of cultivation. After 21 days, however, the compounds were not detectable. The retention time values and LC-MS/MS spectra of the detected compounds were identical to those obtained for the standards.

The fragmentation pattern of THCA was further confirmed through comparison of MS^2 and MS^3 spectra of the standard sample with those of the investigated extract. While the MS^2 spectrum of the THCA ion $[M + H]^+$showed an intense signal at m/z 341, its further analysis by MS^3 (Figure 31) revealed a dominant fragmentation pattern: m/z 323 → m/z 285 → m/z 261 → m/z 233 → m/z 219. The enumerated product ions were identical to those of the standard.

3.2.2.4. Conservation of hairy root cultures

Hairy roots were conserved through encapsulation-dehydration in the form of synthetic seeds (beads). In all experiments, the preservation efficiency was monitored, starting with the observation of the seeds for ruptures caused by the emerging root tips followed by root elongation (Figure 33). Newly formed lateral roots were developed for 10 to 12 days after treatment.

3.2.2.4.1. Effect of the encapsulation-dehydration procedure

According to previous reports, the intracellular moisture content of synthetic seeds should be reduced by laminar airflow desiccation prior to conservation (Lata *et al*., 2012). The investigated *Cannabis* hairy root beads were dehydrated for different time periods to determine the optimal desiccation time (they were weighed every hour within the timespan of 0-4 h and the percentage of moisture content before and after desiccation was determined). The moisture content in the desiccated seeds decreased rapidly until the 2nd hour of treatment; from then on, the values remained approximately constant, reaching a minimum after 4 h with 13 %. After 7, 14, 30 and 180 days of storage at 4 °C, the encapsulated hairy roots desiccated for one h showed 100 % recovery, whereas the non-dessicated beads – about 75 %. On the other hand, the bead-encapsulated root tips desiccated for up to three-four h lost their viability (Figure 34).

3.2.2.4.2. Post-conservation characteristics of hairy root cultures

3.2.2.4.2.1. Growth profiling: solid medium

Solid IHR0 medium (B5, 4 mg/l NAA) was used for recovery of cold-stored seeds. The highest number of lateral roots, 17 ± 7.0 was regenerated from non-desiccated seeds (storage, 180 days), whereas one h of dessication decreased that number to 10 ± 3.0. After two subcultures with 30-day intervals the recovered roots could be further used for *in vitro* applications.

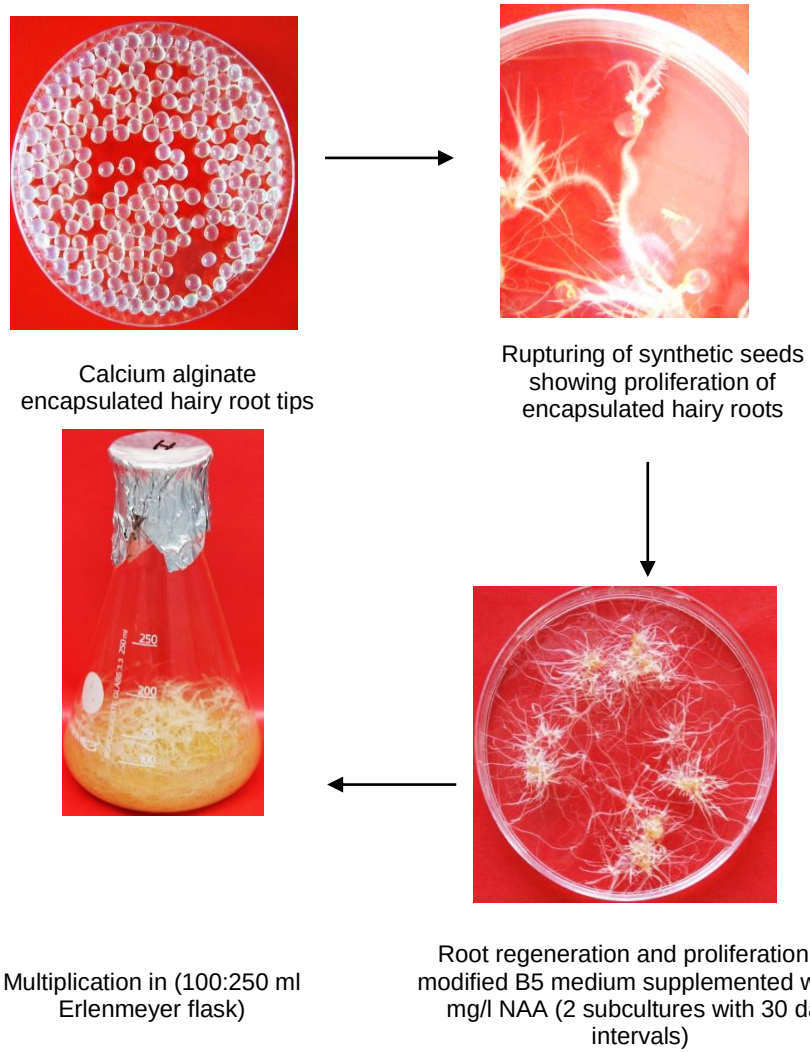

Figure 33: Re-establishment and propagation of *C. sativa* root cultures from synthetic seeds stored at 4 °C. All cultures were incubated on modified B5 medium supplemented with 4 mg/l NAA at 25 °C in darkness.

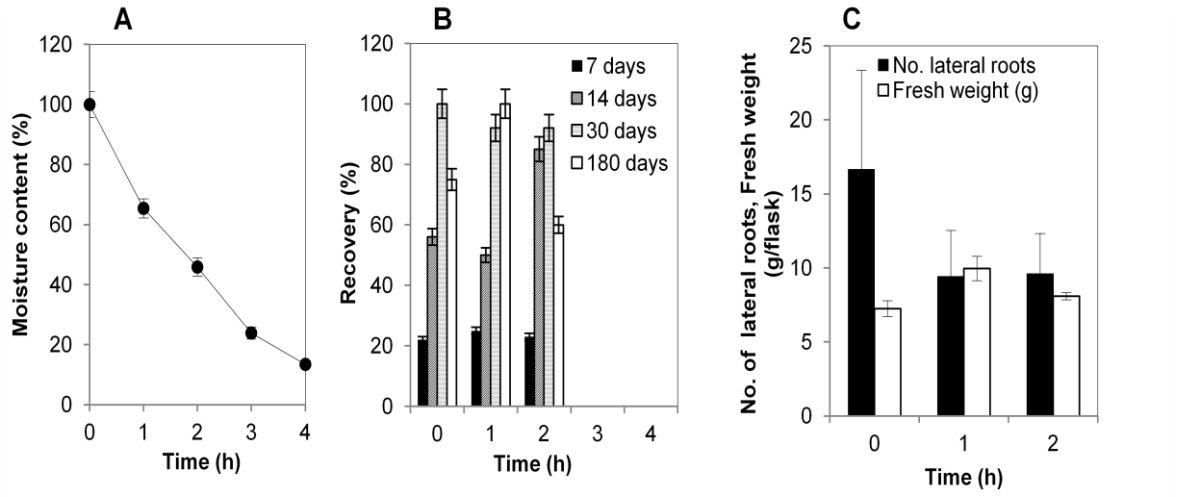

Figure 34: Hairy root conservation and recovery. A: Desiccation of encapsulated hairy roots (mean values of 10 bead/time point replicates ± SE); B: Recovery (%) of the desiccated hairy roots (mean values of 10 bead/time point replicates ± SE); C: Characteristics of shake flask cultures of hairy roots recovered after 180 days of cold-storage, grown in modified B5 medium with 4 mg/l NAA for 21 days (mean values of 3 shake flask/time point replicates ± SE). Axes x: measurement time points during desiccation process.

3.2.2.4.2.2. Growth profiling: shake flask cultures

Liquid LHR0 medium was used for multiplication of the recovered roots (dessication, 0-3 h; storage, 180 days) and their growth profiles were investigated. The highest fresh biomass of 9.96 ± 0.8 g/flask on the 21st day of growth was recorded in case of roots regenerated from desiccated seeds (dessication, 1 h; storage, 180 days), whereas for non-dessicated beads, the value reached 7.47 ± 0.13 g/flask (Figures 33 and 34). The amount and viability of newly regenerated roots were sufficient for re-establishment of flask cultures.

3.2.3. Initiation of trichome formation

3.2.3.1. Morphotypes of trichomes

Experimental procedure applied in the present work resulted in successful induction of trichome-like structures on calli of *C. sativa* (section 2.2.5.,Table 18). The induced trichomes were very similar in shape to those characteristic of the intact adult *Cannabis* plant (Happyana *et al*., 2013), with varying sizes of 9-25 µm. Microscopic examination (light and scanning electron microscope, SEM) revealed that the investigated callus samples (Table 18) encompassed unicellular non-glandular (hairy) trichomes and glandular trichomes of two different types: capitate-sessile and capitate-stalked trichomes (Figures 35-36).

Figure 35: Microscopic images of trichome morphotypes induced *in vitro* from *C. sativa* callus. A and B: Capitate-sessile trichomes (B5= 0.5 mg/l TDZ); C: Capitate-stalked trichomes (B5= 1.0 mg/l TDZ); D-G: Hairy trichomes (B5= 1.0 mg/l TDZ + 3 mg/l GA_3). Scale bars equal: 25 µm (A-C and G) and 10 µm (D-F).

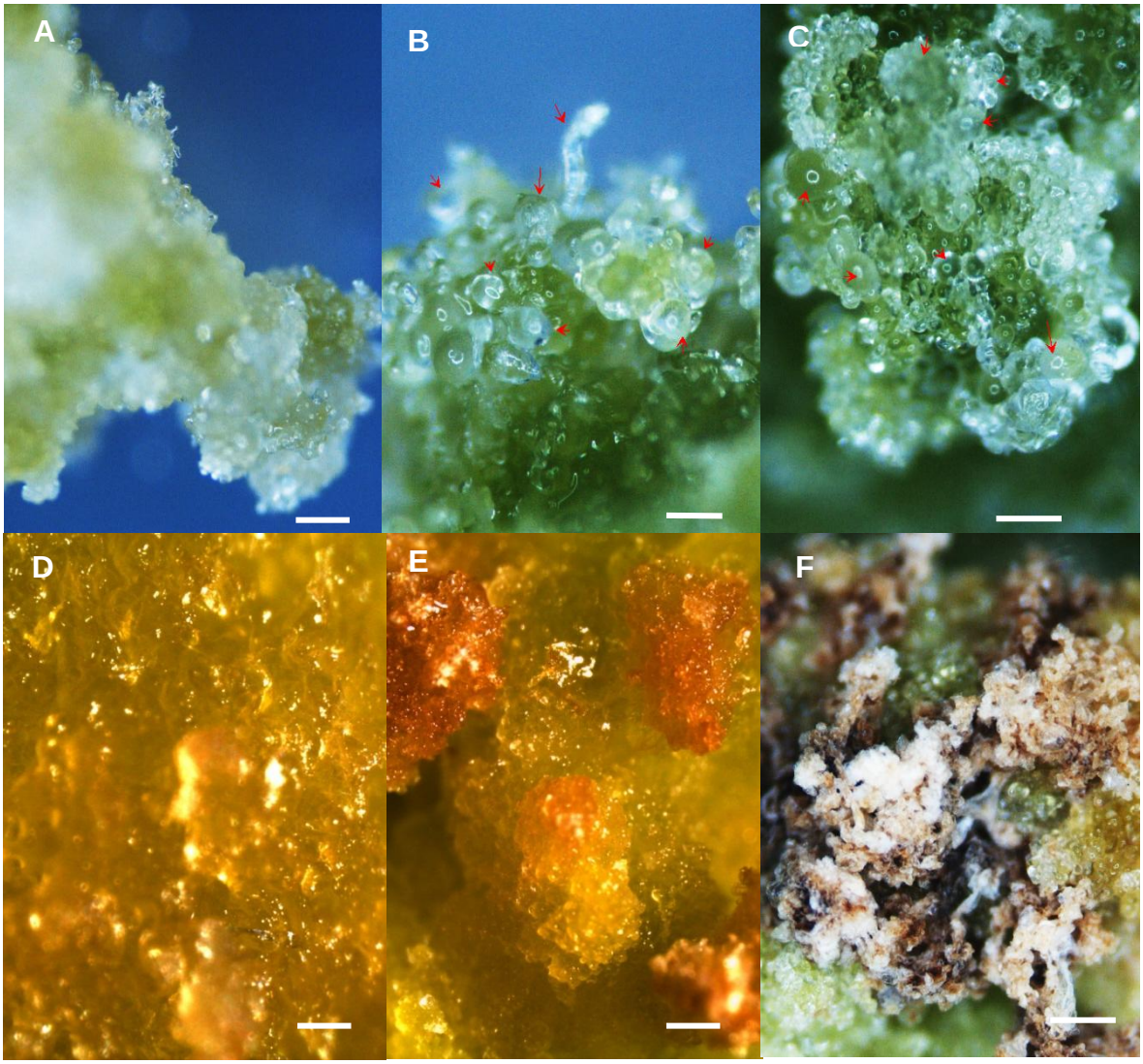

Figure 36: *In vitro*-generated trichomes at different stages of development on B5 medium with 0.5 mg/l TDZ. A: After 7 days; B: After 14 days; C: After 21 days; D: After 28 days; E-F: After 35 days. Scale bars equal 100 µm.

Hairy and capitate-stalked trichomes were less abundant than the capitate-sessile ones, induced on modified B5 medium supplemented with TDZ and GA_3 (Table 18). Trichome senescence (Figure 36D-F) and initiation of their functionality coincided with changes in colour (clear → yellow → amber) after 20 days of cultivation.

Table 18 : Effects of different concentrations of TDZ and GA_3 on trichomes inducion

Medium†	Phytohromone (mg/l)		Trichome density (score)††		
	TDZ	GA_3	CSE	CST	HA
T0 (PGR-free)	0	0	-	-	-
T1	0.1	-	++	+	+
T2	0.3	-	++	+	+
T3	0.5	-	+++	+	+
T4	1.0	-	++	+	+
T5	-	3	++	+	+
T6	0.1	3	+++	-	+
T7	0.3	3	+++	-	+
T8	0.5	3	+++	++	++
T9	1.0	3	+++	++	+

† B5 basal medium was applied; T0 (free of PGRs) served as a control medium
††Trichome density after 21 days; - = no response; ++ = moderate; +++ = exuberant.
CSE: capitate-sessile trichomes; CST: capitate-stalke trichomes; HA: hairy trichomes

3.2.3.2. Profling of cannabinoids

3.2.3.2.1. LC-MS analysis

Identification of cannabinoids in the trichome-callus biomass was achieved through comparison with authentic reference standards using LC-MS/MS, while quantitative determination (LC-ESI-MS) of cannabinoid content in trichomes induced in calli grown on the T8 medium for 14 days indicated THCA yield of 7.4 ± 2.05 µg/g fr. wt. . The retention time values and mass fragmentation patterns of THCA in the test samples were identical to those of the authentic standard (Figure 37).

3.2.3.2.2. MALDI imaging MS

For determination of spatial localization of cannabinoids in *in vitro*-generated trichomes, MALDI imaging MS was applied. The analysis focused on glandular and capitate-stalked trichomes as centres of synthesis and storage of cannabinoids (Turner *et al.*, 1978, 1981). Only one ion (trace mass $[M + H]^+$, m/z 359.9; extracted ion chromatogram ± 2 ppm) localized to the trichomes, as shown in Figure 38 (raster points indicated in red). The obtained results confirmed that THCA was the dominant compound in trichome-generating callus cultures.

Due to low THCA concentrations and small sizes of the investigated trichomes (preventing efficient sample preparation), the applied MALDI imagining MS procedure did not prove reproducible.

3.2.3.2.2.1. MALDI imaging MS profiling of cannabinoids trichomes

Our goal was to determine if MALDI-imagining MS could be accomplished on *in vitro* trichomes for determining the spatial localization of cannabinoids trichomes. In this context, glandular trichome and capitate stalked was used, because of their capability of synthesis and storage of cannabinoids (Turner *et al.* 1978, 1981). To accomplish the imaging on trichome, a 15 *μ*m thick section was prepared using a cryo-microtome and mounted onto a MALDI plate, the sample was then covered with a uniform crystalline matrix and subjected to MALDI-imagining MS. Using this approach, only one ion localized to trichome with trace mass $[M + H]^+$ m/z 359.9 (extracted ion chromatogram with ± 2 ppm) from the indicated raster points (red region) as shown in Figure 38. The data in clearly provides supported the result of LC-MS/MS that THCA is the dominant compound in callus cultures. However, MALDI- imagining MS procedure was not reproducible for detection of THCA due to its low concentration and fine scale (size) of trichome which prevents obtaining good slices. In the available literature no comparable data were reported, since trichomes were not investigated with this technique.

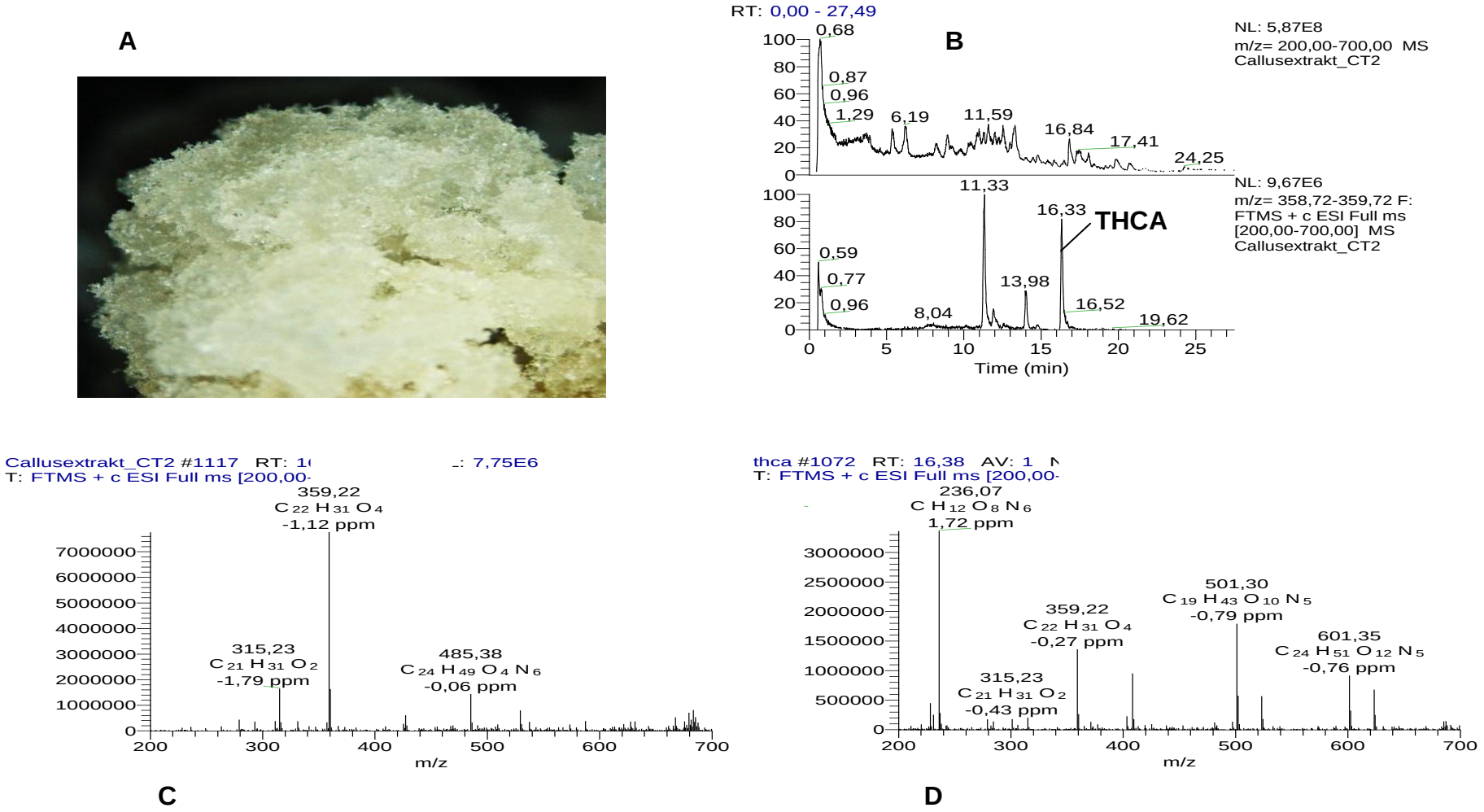

Figure 37: A: Trichome-callus culture; B: LC-MS/MS total ion chromatograms of the trichome-callus extract collected on day 14 of the growth cycle (sub-section 2.2.6.2.1.); C THCA molecular ion, m/z 359.22 ; D: THC molecular ion, m/z 236.07.

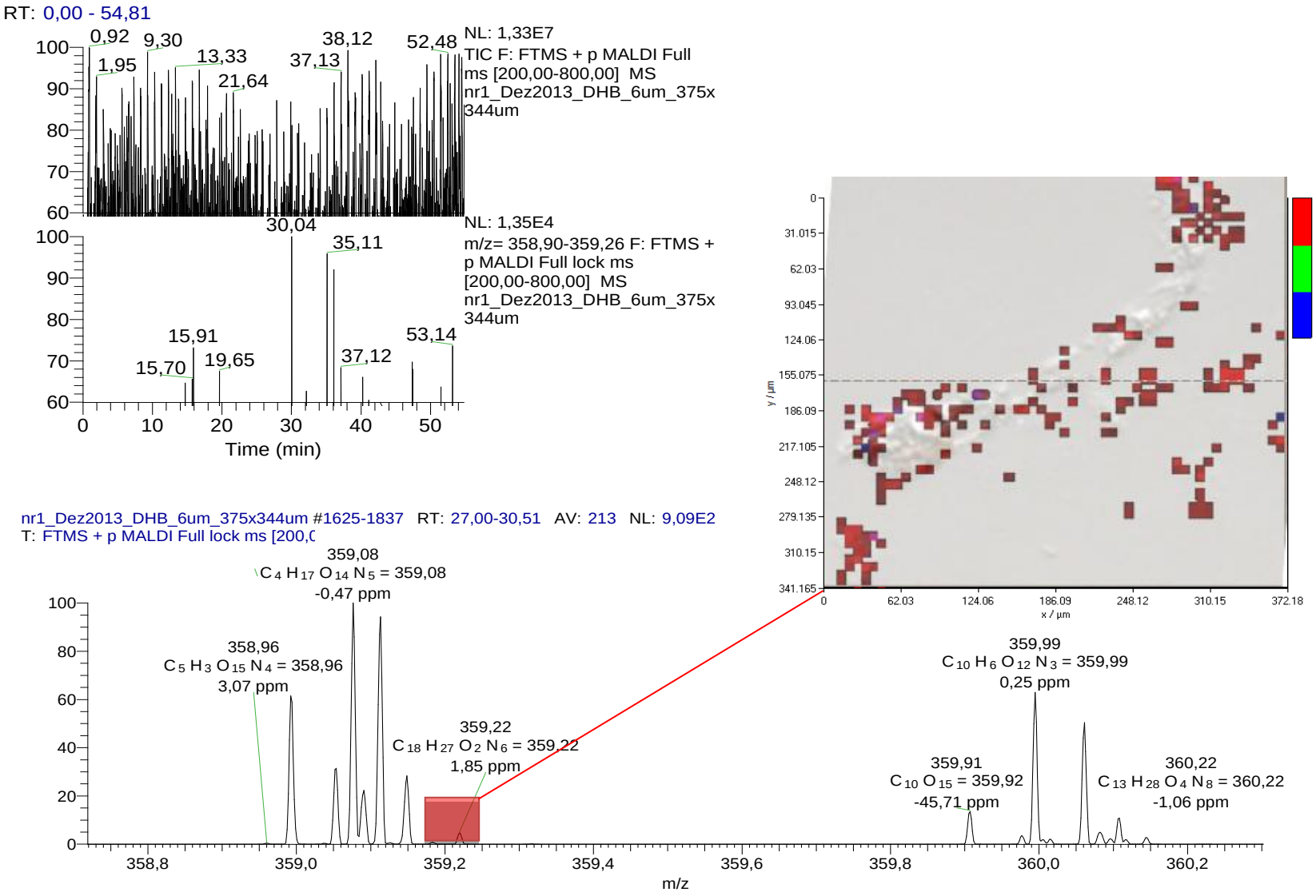

Figure 38. MALDI-imaging- MS of *in vitro* trichomes

4. Discussion

The aim of the present investigation was to produce cannabinoids, valuable secondary metabolites of *C. sativa*, using state-of-the-art tissue culture techniques.

4.1. Establishment of leaf-derived callus cultures

In this work, juvenile leaves, as the most favourable *C. sativa* organ (Appendino *et al*., 2011), were selected for callus initiation. Optimal callus generation and growth was obtained using CNm7 medium (Table 9 and Figure 18A), comprising modified B5 medium with addition of 50 mg/l myo-inositol, 30 g/l sucrose, 1 g/l casein hydrolysate, vitamins (1 mg/l nicotinic acid,1 mg/l pyridoxine-HCl and 10 mg/l thiamine-HCl) as well as 1 mg/l NAA and 1 mg/l BA as phytohormones. Other investigated media were disqualified for either inhibiting callus response (supplementation with 2,4-D and KIN or generation of calli of low biomass or brownish colour – yellow and brown colouration indicate accumulation of phenolic and quinone compounds that may be enzyme inhibitors (Harborne *et al*., 1975). Efficiency of the Gamborg B5 medium in callus initiation has been well-documented (Ikenaga *et al*., 1995; Jacob and Malpathak, 2005). While augmentation with myo-inositol has been said to improve callus growth (Nigra *et al.,* 1985, 1987, 1989 and 1990), the supplemented vitamins have been reported to enhance healthy development of cell and tissue cultures (Narayanaswamy, 1994). In addition, the obtained results are in agreement with the findings of Deka *et al*. (2005), who noted similar effects of NAA and BA on callogenesis of *Pogostemon cablin.*

4.2. Micropropagation *via* leaf-derived calli

To date, only one report on *in vitro* plant regeneration of *C. sativa via* organogenesis has been published (Lata *et al*., 2010). The protocol implemented herein comprised four steps: meristemoid initiation, shootlet induction, rooting of shootlets and *ex vitro* acclimatization.

4.2.1. Meristemoid initiation

In the present study, regeneration trials of *C. sativa* from calli were performed using four different media (MNm1, MNm2, MNm3 and MNm4; Table 10). Among these, MNm2 medium, containing NAA, BA and AS at respective concentrations of 0.5, 5

and 40 mg/l proved most favourable. Small organized clusters of cells, the so-called meristemoids, could be observed on the calli after 20 weeks of cultivation (Figure 18B).

In this context, many observations on meristemoid formation were reported for *Papaver somniferum* and *Nicotiana tabacum* (Furuya *et al*., 1972; Ross *et al*., 1973; Maeda and Thorpe, 1979; Yoshikawa and Furuya, 1983). Moreover, it is generally accepted that by altering the auxin-cytokinin ratio, shootlet and/or root formation can be obtained (Skoog and Miller, 1957). Furthermore, addition of AS proved important for initiating *de novo* organogenesis from calli of *Camellia sinensis*, *Leguminous liana* and *Phaseolus vulgaris* (Das *et al*., 1996; Dhar and Upreti, 1999; Arias *et al*., 2010). Nevertheless, further investigation is necessary to assess whether the observed increased meristemoid formation rate was due to the applied AS concentration or the interaction between all supplemented phytohormones.

4.2.2. Shootlet induction and multiplication

Kut *et al*. (1984) stated that shootlet initiation from calli or explants depends on: 1) type and concentration of growth regulators in culture media, 2) growth conditions of the donor plant, 3) age of the donor plant, 4) original explant selection and 5) genotype of the donor plant. In this study, adventitious shootlets were induced from the newly formed meristemoids on B5 medium fortified with various combinations of cytokinins and GA_3 (Table 11). The regenerated shootlets were much healthier when grown on full-strength medium. The average number of regenerated shootlets per callus was strongly influenced by the PGR composition and varied from 1 to 8. Media SNm18, containing 0.5 mg/l GA_3, and SNm25, supplemented with 0.25 mg/l TDZ and 3 mg/l GA_3, proved most advantageous, generating the average of 8.5 ± 1.73 and 7.25 ± 1.03 shootlets per callus, respectively. The regenerated shootlets showed healthy growth without any significant morphological variation (Figure 18C-I). A corresponding effect of GA_3 supplementation on shootlet induction was reported in cultures of *Atriplex canescens* (Wochok and Sluis, 1980) and *Lotus corniculatus* (Nikolic *et al*., 2010). Further, the obtained resultas are in agreement with the research of Li and Qu (2000), postulating advantageous impact of exogenous GA_3 augmentation on breaking the dormancy of somatic embryos and acceleration of micropropagation. Similarly, the beneficial influence of TDZ/GA_3 combination on shootlet formation was shown in *Rosa hybrid* and *Rosa chinensis* (Hsia and Korban,

1996), while according to other reports, growth of shootlets was initiated from calli cultured on media supplemented with GA_3 and alternative cytokinins, Z or BA (Lata *et al*., 2010). In conclusion, it is possible to produce clones of *C. sativa* and thus propagate the valuable plant for commercial uses.

4.2.3. Rooting of shootlets

Roots were generated from the bases of shootlets of *C. sativa* using B5 medium supplemented with different auxins (NAA, IBA or IAA) at concentrations ranging from 0.5 to 1.5 mg/l (Table 12). In accord with previous reports on stimulation of rhizogenesis in *Malus domestica* (Zimmerman, 1984) and *Acacia mangium* (Monteuuis and Bon, 2000) through exposure of shootlets to a period of darkness, *Canabis* roots were formed on 100 % of the investigated shootlets, reaching the mean length of 8.7 cm, after one week of cultivation in the dark followed by a four week culture period with a light/dark cycle of 16/8 h (Figure18J-N). The inducing effect of IAA on *in vitro* rooting of shootlets was documented previously in *C. sativa*, *M. domestica* cv. Jork 9 and *Adesmia latifolia* (Lata *et al*., 2009 ab; DeKlerk *et al*., 1997; Muniz *et al*., 2013).

4.2.4. Ex vitro acclimatization (indoor cultivation)

The success of any micropropagation protocol depends chiefly on rooting efficiency and indoor environmental conditions applied in the *ex vitro* acclimatization procedure (Zimmerman, 1988). The latter constitutes a critical experimental step, as the *in vitro*-grown plantlets are forced to attune their system to autotrophy and, in most cases, mortality of plantlets proves high under *ex vitro* conditions (Hazarika, 2003 & 2006). Wang *et al*. (2007) reported that *ex vitro* cultivated *Camptotheca acuminata* required high humidity and protection from light stress to survive under greenhouse conditions. Of the propagated plantlets, 80 % developed into healthy whole plants after eight weeks of growth. The following 16 weeks of cultivation resulted in specimens reaching the average height of 53.16 ± 16.34 cm, with 6.2 ± 20.78 true leaves and 9.25 ± 0.47 nodes per plant (Table 13). In some cases, abnormal leaf morphology was observed (single or three leaflets instead of the usual seven), as well as abnormal plant appearancecharacterized by a shrub-like growth with multiple stems (Figure 19B-C). In this context, Pontaroli and Camadro (2005) suggested the term “somaclonal variation”. The noted morphological disparities may have been

brought about by environmental factors, like high concentration of salts in the hydroponic solution, and possibly other determinants, not yet identified.

4.2.5. Cannabinoids in the acclimatizated plants

Cannabinoid analysis was performed on leaf-extracts of IV plantlets and *ex vitro* plants IVH. Mother plant leaves were extracted to provide a control sample. Investigation of cannabinoids of IV origin revealed that their concentrations varied within the entire plantlet. While the recorded amounts of THCA and CBGA reached 0.3 %and 0.45 % (relative to the control), respectively, THC and CBG were not detected. Concurrently, the noted CBDA accumulation was 157 %. Still higher CBDA content, exceeding that of the control eleven-fold, was recorded in flower tops of IVH plants (at the flowering stage of their development), eight weeks after transfer to the hydroponic system (Table 14). The observed changes in cannabinoid concentration were probably the result of somaclonal variation, reported for many *in vitro* propagated plants (Larkin and Scowcroft, 1981; Meins, 1983; Mangolin *et al*., 1994; Mohanty *et al*., 2008). Moreover, while cannabinoids are enzymatically biosynthesized in the plant as their corresponding carboxylic acid forms (Taura *et al*., 2007), they are thermally unstable and can be readily converted, through exposure to light, heat or as a result of prolonged storage, to their corresponding neutral forms *via* decarboxylation (Thakur *et al*., 2005). Further oxidative degradation or isomerization of the metabolites of interest was reported to occur as a result of exposure to various influences, such as those of UV-light or oxidative agents (Turner and Elsohly, 1979; Razdan *et al*., 1972; Trofin *et al*., 2012). Composition of the acclimatizated plant extracts described in this work was comparable, but not identical, to the data presented by Lata *et al*. (2010). The observed disparities may be the result of different plant growth regulators applied in each step of culture maintenence.

4.3. Shake flask cell suspension cultures

Growth kinetics parameters, such as changes in fresh (fr. wt.) and dry cell weight (dr. wt.) as well as the corresponding cannabinoid content values were recorded in course of a 35-day cultivation cycle of the established *C. sativa* cell suspensions.

4.3.1. Characterization of cannabinoids

4.3.1.1. Growth rates

Maximum values of fresh and dry cell weight were variable, especially within the period between the 21st and 28th day of the cultivation period, depending on the type and concentration of the applied plant growth regulators. Growth curves (Figure 21) were delineated to determine growth characteristics and of the established cultures. The obtained and illustrated development patterns comprised three phases:

– The lag phase, noted within the first week of cultivation, during which only little growth was observed. Such cell behaviour was expected, since their suspension cultures had to adjust and establish themselves in the new medium.

– The log phase, in which a moderate, then dramatic increase in growth occurred, throughout the 2nd and 3rd weeks of the culture period. At the early stages of development, the cell cultures were actively proliferating through cell multiplication and enlargement. During this period, synthesis of proteins, nucleic acids and phospholipids, as well as multiplication of organelles and utilization of energy in the form of ATP occur (Kumar, 1999). The investigated shake flask cell cultures were green and most of the cells were isodiametric (~ 18 µm) and elliptic in shape (Figure 22).

– After the log phase (4-5 weeks of cultivation), the growth slightly or even sharply decreased, while the cultures turned yellowish in colour (Figures 21 and 22). De and Roy (1985) attributed reduction in fresh weight values of biomass to the degradation of compounds in synthetic processes and/or the production and release of extracellular material accumulating in the medium. Similar results were considered an outcome of cell culture aging due to chromosome variation (Deus-Neumann and Zenk, 1984; Sierra *et al*., 1992), or cell aging (Roychoudhury *et al*., 2006) and supported by free radical theory-based argumentation (Bremner *et al*., 1997). In addition, it was reported that the THCA synthase-catalyzed reaction resulted not only in THCA formation, but also in the release of hydrogen peroxide – an agent inducing cell death in *C. sativa* (Sirikantaramas *et al*., 2004; Sirikantaramas *et al*., 2005; Morimoto *et al*., 2007). Other reports suggested that the loss of productivity was related to nutrient depletion (Liu and Zhong, 1998; Abreu *et al*., 2007).

4.3.1.2. Time course of cannabinoid production in high yielding cell cultures

Several studies have shown that optimized application of plant growth regulators

results in enhancement of secondary metabolite yields (Suthar *et al*., 1980). Until now, however, only a few studies concerning cell suspension cultures of *C. sativa* as a potential cannabinoid production platform have been published (Pacifico *et al.,* 2008; Flores-Sanchez *et al*., 2009; Pec *et al*., 2010), while no specific reports regarding the effect of PGRs on the generation of the secondary metabolites of interest are available. In this investigation, cannabinoid content values were rather low in cell extracts derived from most of the tested cultures (Figure 21). Highest concentrations of THCA and THC (8.12 and 4.45 µg/g dr. wt., respectively) were recorded after 21 days of cultivation in CSM8 medium containing 0.5 mg/l BA and 1.5 mg/l GA_3. In addition, CBDA formation reached its maximum level of 1.5 µg/g dr. wt. in cell extracts derived from cultures grown in CSM12 medium, supplemented with 1 mg/l TDZ and 1.5 mg/l GA_3, on day 28 of the culture cycle. Concurrently, CBGA content reached its peak (1.18 µg/g dr. wt. after 21 days of cultivation) in cultures maintained in CSM13 medium containing 1.5 mg/l TDZ and 1.5 mg/l GA_3. The obtained results are in agreement with the available literature, reporting on modulation of secondary metabolite biosynthesis by addition of cytokinins (BA and TDZ) to the culture media (Nam *et al*., 2012; Bhargava *et al*., 2013). Similarly, the regulatory role of GA_3 in mevalonate (MVA) and methyl erythritol phosphate (MEP) pathways in *C. sativa* has been reported (Crozier *et al*., 2000; Mansouri *et al*., 2009).

4.3.2. Fingerprinting of cannabinoids

4.3.2.1. LC-MS/MS analysis

The liquid chromatography – tandem mass spectrometry analyses were carried out upon the standard cannabinoid samples and the extracts of cells cultured in CSM4 medium containing 0.5 mg/l TDZ, processed at different time points of the cultivation cycle (day 14, 28 and 35). The investigation was aimed at higher degree identity confirmation of the extracted analytes. On the one hand, the LC-peaks were matched with those of the standard compounds: THCA, t_R 14.10 min; THC, t_R 13.21 min; CBN, t_R 12.58 min; CBGA, t_R 11.55 min; CBD, t_R 11.34 min and CBDA, t_R 10.89 min. Further, the mass-to-charge ratio values of their protonated molecular ions $[M + H]^+$ ultimately confirmed detection of cannabinoids in the investigated cell samples: THCA, m/z 359.22; THC, m/z 315.23; CBN, m/z 311.20; CBGA, m/z 361.24; CBG, m/z 318.32 and CBD, m/z 316.24 (Table 15, Figures 24 and 25). Presence of the enumerated cannabinoids in *C. sativa* cell cultures has not been previously reported

(Pacifico *et al*., 2008; Flores-Sanchez *et al*., 2009; Pec *et al*., 2010). It is important to note that THCA, CBN, CBGA and CBDA were detected after 35 days of cultivation, while the presence of THC and CBD was recorded in the early stages of the growth cycle (the compounds were undetectable after 28 days of cultivation).

4.3.2.2. ^{1}H NMR measurements

Analysis of cannabinoid content of the investigated cell extracts by ^{1}H NMR spectroscopy resulted in detection of but minor THCA-specific signals in the sample processed after 21 days of cultivation in CSM8 medium supplemented with 0.5 mg/l TDZ and 1.5 mg/l BA (Figure 26). However, no signals corresponding to THCA, or any other cannabinoids, were detected in the samples extracted on days 7, 14, 28 and 35 of the culture cycle. Neither THCA nor other secondary metabolites of interest have been previously detected in *C. sativa* cell cultures (Pacifico *et al*., 2008; Flores-Sanchez *et al*., 2009; Pec *et al*., 2010).

4.4. Hairy root cultures

Detailed *in vitro C. sativa* hairy root induction procedure as well as analyses of the corresponding biomass accumulation and cannabinoid generation, in the presence of auxins, have not been reported before. The main objective of this study was twofold: to increase fundamental understanding of the effects of exogenously applied plant growth regulators on hairy root induction from leaf-derived calli of *C. sativa* and to develop an optimized shake flask root culture system for cannabinoid production.

4.4.1. Initiation of adventitious hairy roots from leaf-derived calli

Generally, the presence of auxins, accompanying the carbon source, in the culture medium has been known to stimulate rhizogensis (Blazich, 1988). The induced roots of *C. sativa* displayed similar growth patterns after 16 weeks of cultivation, with 83.3 % root formation when the initiating calli were incubated on full-strength B5 medium supplemented with 4 mg/l NAA (IHR0), in darkness at 25 °C (Table 16 and Figure 28). The obtained result is in accord with a previous report on rhizohgenesis stimulating effect of NAA in *C. sativa* callus cultures (Fisse *et al*., 1981). In contrast, all calli tested with various IAA concentrations (1.5, 2.5 and 4 mg/l) did not generate hairy roots, although the ability of callus cultures in other plant systems to proliferate hairy roots in response to PGRs, including IAA, was studied and documented

(Drozdowska and Rogozinska, 1984; Whitney, 1996). Perhaps the investigated phytohormone concentrations were insufficient to stimulate rhizogenesis.

4.4.2. Optimization of hairy root cultures on solid media

In this study, various auxins (NAA, IBA and IAA) in several concentrations (0.25, 0.5 and 1.0 mg/l) were applied to establish optimal conditions for growth and multiplication of roots initiated on the reference medium (IHR0). All root cultures grown on solid media SHR5 (0.5 mg/l IBA), and SHR8 (0.5 mg/l IAA) developed high numbers of hairy roots, with the average values of 37 ± 4.7 and 27.25 ± 4.9 roots per culture, respectively. In addition, media SHR2 (0.5 mg/l NAA) and SHR8 provided for significant development of lateral hairy roots, with average length values of 6.3 ± 0.14 and 5.2 ± 0.28 cm per culture, respectively (Table 16 and Figure 29A). Moreover, the applied auxin levels affected root phenotype (Figure 29B). The obtained results are in agreement with those reported by Liu *et al*. (2002), stating that auxins (NAA, IBA and IAA) are more efficient in initiation and stimulation of phenotypic variation of hairy roots than other phytohormones.

4.4.3. Characterization of cannabinoids in shake flask root cultures

4.4.3.1. Growth rates

Hairy root growth profiles in shake flask cultures increased periodically during the 35-day dark-cultivation cycle in all tested auxin-augmented media (Figures 30 and 31). The maximum biomass accumulation values were recorded in case of roots cultured in LHR1 medium (½ B5, 0.25 mg/l NAA); these were: fr. wt., 5.31 ± 0.15 g/flask and dr. wt., 0.77 ± 0.008 g/flask, on day 35 of the growth cycle (Figure 30).

4.4.3.2. Time course of cannabinoid production

In the present study, cannabinoid accumulation recorded in roots reached but minor levels, below 2.0 µg/g dry weight. While THCA content amounted to 1.04 µg/g dr. wt. in roots cultured in LHR3 medium (½ B5, 1.0 mg/l IAA), CBGA accumulation was most pronounced in LHR9 root cultures (½ B5, 0.5 mg/l IBA; 1.63 µg/g dr. wt.) and that of CBDA, in roots grown in LRH0 (B5, 4 mg/l NAA; 1.67 µg/g dr. wt.). All the aforementioned maximum values were recorded on day 28 of the cultivation cycle, after which cannabinoid formation rates decreased (Figure 30). No further relation between the maximum biomass and cannabinoid accumulation was observed.

Unfortunately, the biosynthetic capabilities of the investigated hairy root clones did not provide sufficient levels of cannabinoids to warrant scaled-up cultivation. The results indicate that, the efficient *de novo* biosynthesis and formation of cannabinoids in dark-grown hairy root cultures observed herein is light-independent.

4.4.4. Fingerprinting of cannabinoids in root suspensions

Liquid chromatography – tandem mass spectrometry was applied to analyze samples of standard cannabinoids and the hairy root extracts obtained from LHR0-grown cultures (B5, 4 mg/l NAA) at different time points of the cultivation cycle (day 14, 28 and 35). The investigation was aimed at higher degree identity confirmation of the extracted analytes. On the one hand, the LC-peaks were matched with those of the standard compounds: THCA, t_R 5.9 min; THC, t_R 5.8 min and CBDA, t_R 5.06 min. On the other, the mass-to-charge ratio values for their protonated molecular ions $[M + H]^+$ ultimately confirmed the presence of cannabinoids in the investigated root samples: THCA, m/z 358.22; THC, m/z 315.12 and CBDA, m/z 355.19 (Table 17, Figures 32 and 33). Mass fragmentation pattern of THCA was investigated further, corroborating its specific transitions, as compared to the standard: m/z 358 → m/z 341 and m/z 331 in the MS^2 spectrum and m/z 323 → m/z 285 and m/z 261 in the MS^3 spectrum.

4.5. Conservation of hairy root cultures

Well-established plant cell and tissue culture protocols require that a back-up stock of hairy roots be maintained in 500 ml shake flasks and subcultured manually (Grout, 1995). However, the postulated collection and subculturing procedure is time consuming, cost-ineffective and bears high risk of culture contamination and operator mistakes (Grout, 1995; Gangopadhyay *et al*., 2011). Consequently, conservation *via* synthetic seed (bead) generation emerges as the most reliable method for sustainable management of plant cell and tissue cultures, effectively avoiding the aforementioned problems (Grout, 1995). Despite the progress in plant material conservation that has been made in the last decade, only few reports on preservation of hairy root cultures are available (Lambert *et al*., 2009; Oh *et al*., 2009). *In vitro* conservation of *C. sativa* hairy root cultures was performed at low incubation temperature of 4 °C through application of encapsulation-dehydration procedure. Desiccation of the generated synthetic seeds was performed under laminar airflow

(0-4 h) and was followed by their cold storage (7, 14, 30 and 180 days). Synthetic seeds dessicated for one hour showed 100 % root recovery, whereas the restoration rate of non-dehydrated beads reached about 75 % (Figures 34 and 35). On the other hand, the bead-encapsulated root tips desiccated for up to three to four hours lost their viability. Morphology of the recovered hairy roots was identical to that characteristic of the *in vitro* stock cultures. Fresh biomass accumulation in shake flask cultures of roots restored from desiccated seeds after 180 days of cold storage was significantly more pronounced than that recorded for cultures developed from non-desiccated beads. In general, this preservation technique has been successfully used in different plant species (Takagi, 2000; Lambert *et al*., 2009; Lata *et al*., 2012), as it does not require dealing with cytotoxic compounds (Surenciski *et al*., 2012). The present study is the first report on synthetic seed generation by encapsulation-dehydration of hairy roots of *C. sativa*. It further confirms the applicability of the described technique in *in vitro* and *ex vitro* cultivation protocols, as well as germplasm banks *ex situ* conservation affording prevention of genetic shifts, reduction of operational costs and space requirements.

4.6. Initiation of trichome formation

4.6.1. Trichome morphotypes

In *C. sativa*, glandular trichomes constitute the main site of cannabinoid production (Appendino *et al.*, 2011; Happyana *et al.*, 2013). In the present study, trials for *in vitro* induction of different morphotypes of *C. sativa* trichomes resulted in generation of unicellular non-glandular (hairy) and glandular trichomes of two different types, capitate-sessile and capitate-stalked, especially pronounced on T8-maintained (B5, 0.5 mg/l TDZ and 3 mg/l GA_3) leaf-derived calli (Figure 32). SEM imaging was performed to confirm the initial, light microscopic examination (Figure 38). Hairy and capitate-stalked trichomes were observed less frequently than capitate-sessile ones on calli maintained on most of the tested PGR-augmented media (Table 18). Among the applied phytohormones, TDZ is considered superior to other cytokinins used for morphogensis stimulation in plant tissue culture protocols, due to its unique mode of action resulting in accumulation of endogenous auxins and cytokinins (Jones *et al*., 2007). Further reports indicated that GA_3 was favourable for *in vitro* promotion of growth and biomass production (Lata *et al*., 2009ab). Thus, the hereby recorded *in vitro* induction of trichomes does not contradict the hypothesis postulating that all

plant cells are totipotent (Guha and Maheshwari 1964). Further, trichome senescence was observed after 20 days of cultivation. This may have been due to cellular accumulation of cannabinoids resulting in cell death (Sirikantaramas *et al*., 2004; Sirikantaramas *et al*., 2005; Morimoto *et al*., 2007), or exhaustion of carbon sources and nutrients from the culture medium (Liu and Zhong, 1998; Abreu *et al*., 2007).

4.6.2. Profling of cannabinoids

4.6.2.1. LC-MS/MS analysis

In the present study, yield of THCA amounting to 7.4 ± 2.05 µg/g fr. wt. was recorded in the trichome-callus biomass maintained on T8 medium by means of LC-ESI-MS. Detection of high levels of THCA in calli may be attributed to the role of glandular trichomes in regulation of metabolic pathways (Turner *et al.*, 1978). Retention time value as well as ESI-MS/MS fragmentation pattern of the detected THCA were identical to the corresponding data obtained for the standard compound (Figure 39), while the high resolution mass spectra ultimately confirmed its molecular identity.

4.6.2.2 MALDI imaging MS

Determination of spatial localization of secondary metabolites of interest focused on glandular and capitate-stalked trichomes as centres of synthesis and storage of cannabinoids (Turner *et al.*, 1979, 1981). The applied MALDI imaging MS investigation resulted in detection of but one ion signal (trace mass $[M + H]^+$, m/z 359.9) confined to the trichomes (Figure 40), thus confirming THCA as the dominant compound in trichome-generating callus cultures. Due to low THCA concentrations and small sizes of the investigated trichomes (preventing efficient sample preparation), the applied MALDI imagining MS procedure did not prove reproducible. No comparable reports are available, as trichome analysis with the aforementioned technique has not been previously attempted.

5. Concluding remarks and perspectives

The main objective of the hereby presented work was to apply plant tissue culture techniques to produce cannabinoids of *C. sativa*.

The most significant results are as follows:

– Establishment of callus cultures:

Juvenile *C. sativa* leaf explants provided for superior callogenesis (95 %), as compared to those originating from other plant organs, when cultured on modified B5 basal medium supplemented with 1 mg/l NAA and 1 mg/l BA.

– Plant regeneration from leaf-derived calli:

Meristemoid initiation was achieved by subculturing calli derived from leaf explants on modified B5 medium supplemented with NAA, BA and AS. The optimal combination of the aforementioned phytohormones was 0.5, 5 and 40 mg/l and promoted meristemoids with the efficiency of 50 %. Mature meristemoids were then transferred to regeneration media for induction, development and multiplication of shootlets. The optimal media for shootlet induction and differentiation were B5 supplemented with 0.5 mg/l GA_3 and B5 augmented with 0.25 mg/l TDZ and 3 mg/l GA_3, respectively. Shoots bearing true leaves were subsequently transferred to B5 medium with various concentrations of auxins (NAA, IBA and IAA) for rooting and, eventually, plantlet formation. Roots formed on 100 % of shoots when cultured on B5 medium supplemented with 1.5 mg/l IAA, after one week of cultivation in the dark followed by a four week culture period with a light/dark cycle of 16/8 h. For acclimatization, plantlets were transferred to a hydroponic system under controlled environmental conditions. Of the regenerated plantlets, 80.0 % survived the following 16week *ex vitro* cultivation period. Significant differences in metabolic profiles of *in vitro* micropropagated plantlets and hydroponically grown plants, as compared to the mother plant-derived control, were recorded. While in the former the respective relative amounts of THCA, CBGA and CBDA reached about 0.33, 0.45 and 157.1 mg/g fr. wt., in the latter, THCA, THC, CBGA, CBG and CBDA were detected, after eight weeks of hydroponic cultivation, in respective concentrations amounting to about 1.54, 28.30, 6.0, 0.125 and 1121.4 mg/g fr. wt., as compared to the control mother plant. The hereby observed variation in cannabinoid content could be correlated to the influence of the plant growth regulators applied at all stages of the

micropropagation procedure. Further optimization is necessary to reduce the total time period of plant regeneration.

– Cannabinoids in the established shake flask cell suspension cultures:
Modified B5 medium supplemented with different concentrations of phytohormones was used to stimulate and optimize cannabinoid production in the established cell suspensions. Highest concentration values of THCA and THC (8.12 and 4.45 µg/g dr. wt.) were recorded after 21 days of cultivation in medium containing 0.5 mg/l BA and 1.5 mg/l GA_3. Similarly, a 3-week culture period in medium supplemented with 1.5 mg/l TDZ and 1.5 mg/l GA_3 resulted in highest CBGA accumulation reaching 1.18 µg/g dr. wt. On the other hand, CBDA formation reached its maximum level of 1.5 µg/g dr. wt. on day 28 of the cultivation cycle in medium augmented with 1 mg/l TDZ and 1.5 mg/l GA_3. Moreover, gradual reduction in cannabinoid accumulation during the culture cycle was noted; further investigation is needed to fully elucidate this phenomenon. Improvement of cannabinoid production in cell cultures through elicitor augmentation and/or precursor feeding warrants additional experimental efforts. Similarly, manipulation of culture conditions, such as fermenter aeration rate and agitation speed, should be further studied.

– Cannabinoids in the established hairy root cultures; root culture conservation:
Hairy roots were induced by incubation of aseptically grown callus cultures on solid B5 medium supplemented with 4 mg/l NAA in darkness at 25 °C. Hairy root growth in shake flask cultures increased periodically during the 35-day cultivation cycle. Cannabinoids were produced in minor amoints and remained below 2.0 µg/g dr. wt.. Additionally, encapsulation-dehydration procedure was applied to conserve adventitious roots of *C. sativa*. Hairy root tips were successfully encapsulated in calcium alginate beads and stored at 4 °C for up to 6 months, with maximum viability of 100 % in case of beads dessicated for one hour and highest biomass accumulation of 9.96 ± 0.7 g/flask recorded after 21 days of cultivation in B5 medium supplemented with 4 mg/l NAA. Advantages of the applied encapsulation-dehydration technique include: high survival rates, rapid recovery, reduced labour, work-space and time input as well as prevention of mutations and contaminations.

Further investigations are being conducted to improve 1) cannabinoid production in root cultures to commercially interesting levels through implementation of various strategies and 2) viability of post-freeze root cultures.

– Induction of trichomes:

Trichome-like structures were induced from *C. sativa* calli. B5 medium supplemented with 0.5 mg/l TDZ and 3 mg/l GA_3 proved most favourable. Micromorphology of the induced trichomes was investigated by means of SEM, which led to their classification into three categories: unicellular non-glandular (hairy) trichomes and glandular trichomes of two different types: capitate-sessile and capitate-stalked. Quantitative determation of cannabinoid content in callus-trichomes (14 days of incubation on B5 medium with 0.5 mg/l TDZ) by LC-MS pointed to THCA accumulation (7.4 ± 2.05 µg/g fr. wt.). Spatial distribution of the valuable metabolite in the *in vitro*-induced capitate-stalked trichomes was further confirmed by MALDI imaging MS. However, the procedure was not reproducible due to low THCA concentrations and fine sacle (small size) of trichomes, preventing efficient sample preparation.

5. References

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Appendix I-IV

I. List of Abbreviations

δ	chemical shift (NMR)
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2iP	6-(γ,γ-dimethylallylamino)purine
B	boron
Cu	copper
Cl	chlorine
Fe	iron
N_2	nitrogen gas
Ni	nickel
Mn	manganese
Mo	molybdenum
N	nitrogen
Ni	nickel
NH_4^+	ammonium cation
NO_3^-	nitrate anion
Mo	molybdenum
Zn	zinc
P	phosphorus
S	sulfur
^{1}H-NMR	proton nuclear magnetic resonance
$CDCl_3$	deuterochloroform
ABA	abscisic acid
AS	adenine hemisulfate salt
B5	Gamborg B5 medium (Gamborg *et al*., 1968)
BA	6-benzylaminopurine
BCE	before the christian era
CEA	controlled environment agriculture
CBN	cannabinol
CBD	cannabidiol
CBL	cannabicyclol
CBC	cannabichromene
CBDA	cannabidiolic acid
CBCA	cannabichromenic acid
CBGA	cannabigerolic acid
CBLA	cannabicyclolic acid
CBNA	cannabinolic acid
CBG	cannabigerol
CBC	cannabicoumaronone
CBE	cannabielsoin
CID	collision-induced dissociation
cm	centimeter
CO_2	carbon dioxide
CDKs	cyclin dependent protein kinases
Cdc-2	cell division cycle 2
COSY	correlation spectroscopy
DHB	2,5-dihydroxybenzoic acid
DNA	desoxyribonucleic acid

T-DNA	transfer DNA
Dicamba	2-methoxy-3,6-dichlorobenzoic acid
dr. wt.	dry weight
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
FID	free induction decay
fr. wt.	fresh weight
ft	feet
GPP	geranylpyrophosphate
GRIN	germplasm resources information network
GAs	gibberellins
GA_3	gibberelic acid
GLC	gas-liquid chromatography
HID	high intensity discharge
HPS	high pressure sodium
HPLC	high performance liquid chromatography
HCE	hexane crude extract
HMBC	heteronuclear multiple bond correlation
h	hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IV plantlets	plantlets grown *in vitro*
IVH plants	*ex vitro* plants in a growth room
IMS	ion-mobility spectrometry
ISSR	inter simple sequence repeat
KIN	*N*-furfuryl-7-*H*-purin-6-amine (kinetin)
LS	Linsmaier and Skoog medium (Linsmaier and Skoog,1965)
LTQ	linear ion trap
lx	lux
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
L/D	light/dark
MEP	methyl erythritol phosphate
MH	metal halide
MCPA	2-methyl-4-chlorophenoxyacetic acid
MS	Murashige and Skoog medium (Murashige and Skoog, 1962)
MS	multiple sclerosis
m	meter
NAA	1-naphthaleneacetic acid
OA	olivetolic acid
PAR	photosynthetically active radiation
PGRs	plant growth regulators
Picloram	4-amino-3,5,6-trichloropicolinic acid
PCA	principal component analysis
PLS-DA	partial least square-discriminant analysis
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rH	relative humidity

ppm	parts per million
SE	standard error
SD	standard deviation
s	second
SEM	scanning electron microscope
TOF	time of flight
TDZ	thidiazouron
THC	tetrahydrocannabinol, Δ-9-tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
Ti	tumour-inducing
TMS	tetramethylsilane
t_R	retention time
UV	ultraviolet radiation
UV-B	ultraviolet radiation (medium wavelength, 315-280 nm)
v/v	volume to volume
vir	virulence
W	watt
Z	zeatin

II. Acknowledgements

I am most grateful to my supervisor **Prof. Dr. Oliver Kayser**, Head of the Technical Biochemistry Group at the Technical University of Dortmund, for accepting me as a Ph.D. student and giving me the opportunity to work on a fascinating topic. I also thank him for his continuous guidance and invaluable advice throughout my scientific work and for reviewing this manuscript.

I would like to thank the **Technical University of Dortmund** for financing my work during the four years of my Ph.D project.

I sincerely thank **Dr. Armin Quentmeier** for her support in the preparation of my thesis, for her critical review of this manuscript and helpful comments on my work. I also express my thanks to Dr. Felix Stehle for valuable tips, new ideas and stimulating discussions.

This work would not have been possible without the brilliant expertise in mass spectrometry of **Dr. Marc Lamshöft**, **Dr. Sebastian Zühlke** and **Selahaddin Sezgin** (Analytical and Environmental Chemistry, Institute of Environmental Research, Department of Chemistry and Chemical Biology, Technical University of Dortmund), to whom I am extremely grateful.

For the good atmosphere in the lab, many thanks go to my current and former colleagues from the TB chair: **Torsten**, **Nizar** (a good office colleague), **Marcello**, **Sara, Evamaria**, **Parija**t, **Bastian**, **Kathleen** (always ready to help), **Jörg**, **Fabian**, **Bettina** and **Friederike**.

Finally, I would like to thank my **parents** and my brothers for their love and moral support over the course of my research away from home. And special thanks to my wife **Rasha** for caring, understanding and supporting me during our stay in Germany. I highly appreciate your skills in tending to our loving son (**Omar**) and daughter (**Qamar**) and managing my schedules very efficiently.

Sayed

III. Curriculum Vitae

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2005-2008	Master studies in phytochemistry (M. Sc.) at the Department of Phytochemistry, National Research Centre (NRC), Cairo, Egipt, under the supervision of Prof. Dr. Medhat M. Seif-El-Nasr
2003-2005	Postgraduate studies, Faculty of Science, Al-Azhar University, Cairo, Egypt
1999-2003	Bachelor studies in Botany (B. Sc.) Faculty of Science, Al-Azhar University, Cairo, Egypt
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Professional experience

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IV. List of Publications

Peer Reviewed Articles

1. **Sayed Farag**, Sebastian Zühlke, Michael Spiteller, Oliver Kayser. *In vitro* micropropagation of leaf-derived calli from *Cannabis sativa* L.: Cannabinoid content of the regenerated plants, 2014 (submitted to *In vitro* cellular and developmental biology – plant).
2. **Sayed Farag**, Oliver Kayser. Cannabinoids production by hairy root cultures of *Cannabis sativa* L. 2014 (submitted to American journal of plant science).
3. **Sayed Farag**, Nizar Happyana, Marc Lamshöft, Michael Spiteller, Oliver Kayser "Characterization of cannabinoids biosynthesis in *Cannabis sativa* L. cell suspensioncultures". 2014 (submitted to Plant cell reports).
4. **Sayed Farag**, Oliver Kayser Encapsulation-dehydration of *in vitro* hairy root cultures of *Cannabis sativa* L.: Towards a practical conservation protocol".2014 (submitted to Journal of Horticultural Science).

Book Chapters

1. Book Chapter 10:**Sayed Farag**, Oliver Kayser (2015) Cultivation and Breeding of *Cannabis sativa* L. for Preparation of Standardized Extracts for Medicinal Purposes. In: Máthé Ákos (ed) Medicinal and Aromatic Plants of the World, Springer (inpress)
2. Book Chapter (II):**Sayed Farag**, Oliver Kayser (2015).The Cannabis Plant: Botanical Aspects. In: Victor Preedy (ed) The Comprehensive Handbook of Cannabis Related Pathology.Elsevier. (inpress).

Poster

Sayed Farag, Marc Lamshöft, Michael Spiteller, Oliver Kayser (2013) LCMS Spectral Evidence of the Occurrence of Cannabinoid in *Cannabis sativa* Cell Cultures. Planta Med 2013; 79 - PM2 DOI: 10.1055/s-0033-1352335. 61st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), 1st – 5th of September, 2013, Münster.