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Elicitation studies in cell suspension cultures of *Cannabis sativa* L.

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ABSTRACT

Cannabis sativa L. plants produce a diverse array of secondary metabolites. Cannabis cell cultures were treated with biotic and abiotic elicitors to evaluate their effect on secondary metabolism. Metabolic profiles analysed by ^{1}H NMR spectroscopy and principal component analysis (PCA) showed variations in some of the metabolite pools. However, no cannabinoids were found in either control or elicited cannabis cell cultures. *Tetrahydrocannabinolic acid* (*THCA*) *synthase* gene expression was monitored during a time course. Results suggest that other components in the signaling pathway can be controlling the cannabinoid pathway.

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1. Introduction

Cannabis sativa L. is an annual dioecious plant from Central Asia. Two hundred and forty-seven secondary metabolites have been identified in this plant. Cannabinoids are a well-known group of natural products and more than 70 different cannabinoids have been found so far (ElSohly and Slade, 2005; Radwan et al., 2008). Several therapeutic effects of cannabinoids have been described (Williamson and Evans, 2000) and the discovery of an endocannabinoid system in mammals marks a renewed interest in these compounds (Di Marzo et al., 2007; Mackie, 2008; Moreira and Lutz, 2008). *C. sativa* cell cultures have been used for breeding (Jekkel et al., 1989; Mandolino and Ranalli, 1999), for studying secondary metabolite biosynthesis (Itokawa et al., 1977; Loh et al., 1983; Hartsel et al., 1983) and for secondary metabolite production (Veliky and Genest, 1972; Heitrich and Binder, 1982). However, cannabinoids have not been detected in cell suspension or callus cultures so far. Some of the strategies used to stimulate cannabinoid production from cell cultures involved media modifications and a variety of explants. Although, elicitation has been employed for inducing and/or improving secondary metabolite production in the cell cultures (Bourgaud et al., 2001) it would be interesting to observe elicitation effect on secondary metabolite production in *C. sativa* cell cultures.

Metabolomics has facilitated an improved understanding of cellular responses to environmental changes and analytical platforms have been proposed and applied (Sanchez-Sampedro et al., 2007; Hagel and Facchini, 2008; Zulak et al., 2008). ^{1}H NMR spectroscopy is one of these platforms which is currently being explored together with principal component analysis (PCA), the most common method to analyse the variability in a group of samples.

In this study biotic and abiotic elicitors were employed to evaluate their effect on secondary metabolism in *C. sativa* cell cultures. Metabolic profiles were analysed by ^{1}H NMR spectroscopy. Expression of the *THCA synthase* gene was also monitored by reverse transcription-polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Chemicals

$CDCl_3$ (99.80%) and CD_3OD (99.80%) were obtained from Eurisotop (Paris, France). D_2O (99%) was acquired from Spectra Stable Isotopes (Columbia, MD, USA). NaOD was purchased from Cortec (Paris, France). The cannabinoids Δ^9-THCA, CBGA, Δ^9-THC, CBG and CBN were isolated from plant material previously in our laboratory (Hazekamp et al., 2004). All chemical products and mineral salts were of analytical grade.

2.2. Plant material and cell culture methods

Seeds of *C. sativa*, drug type variety Skunk (The Sensi Seed Bank, Amsterdam, The Netherlands) were germinated and maintained

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under a light intensity of 1930 lx, at 26 °C and 60% relative humidity (RH) for continued growth until flowering. To initiate flowering, 2-month-old plants were transferred to a photoperiod chamber (12 h light, 27 °C and 40% RH). Leaves, female flowers, roots and bracts were harvested. Glandular trichome isolation was carried out according to the method reported by Zhang and Oppenheimer (2004). As negative control, cones of *Humulus lupulus* were collected in September 2004 from the Pharmacognosy gardens (Leiden University) and stored at −80 °C.

C. sativa cell cultures initiated from leaf explants were maintained in MS basal medium (Murashige and Skoog, 1962) supplied with B5 vitamins (Gamborg et al., 1968), 1 mg/l 2,4-D, 1 mg/l kinetin and 30 g/l sucrose. Cells were subcultured with a 3-fold dilution every two weeks. Cultures were grown on an orbital shaker at 110 rmp and 25 °C under a light intensity of 1000–1700 lx. Somatic embryogenesis was initiated from cell cultures maintained in hormone free medium. Cellular viability measurement was according to Widholm (1972).

2.3. Elicitation

Two fungal strains, *Pythium aphanidermatum* (Edson) Fitzp. and *Botrytis cinerea* Pers. (isolated from cannabis plants), were grown in MS basal medium containing B5 vitamins and 30 g/l sucrose. Cultures were incubated at 37 °C in the dark with gentle shaking for one week and subsequently after which they were autoclaved. The mycelium was separated by filtration and freeze-dried. *P. aphanidermatum* (313.33) was purchased from Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and *B. cinerea* was generously donated by Mr. J. Burton (Stichting Institute of Medical Marijuana, The Netherlands). For elicitation, dry mycelium suspensions were used. Cannabis pectin was obtained by extraction and hydrolysis according to the methods reported by Dornenburg and Knorr (1994) and Kurosaki et al. (1987). Yeast extract (Bacto™ Brunschwig Chemie, Amsterdam, The Netherlands), salicylic acid (Sigma, St. Louis, MO, USA), sodium alginate (Fluka, Buchs, Switzerland), silver nitrate, $CoCl_2{\cdot}6H_2O$ (Acros Organic, Geel, Belgium) and $NiSO_4{\cdot}6H_2O$ (Merck, Darmstadt, Germany), were dissolved in deionized water and sterilized by filtration (0.22 μm filter). Methyl jasmonate and jasmonic acid (Sigma) were dissolved in a 30% ethanol solution. Pectin suspensions from *Citrus fruits* (galacturonic acid 87% and methoxy groups 8.7%, Sigma) were prepared according to the method of Flores-Sanchez et al. (2002). For ultraviolet irradiation cannabis cell cultures were irradiated under UV 302 or 366 nm lamps (Vilber Lourmat, France).

Erlenmeyer flasks (250 ml) containing 50 ml fresh medium were inoculated with 5 g fresh cells. Five days after inoculation the suspensions were incubated in the presence of elicitors or exposed to UV-irradiation for different periods of time (Table 1).

Table 1
Elicitors, concentrations applied to cannabis cell cultures and harvest time.

Elicitor	Final concentration	Harvest time after elicitation (days)
Biotic		
Microorganism and their cell wall fragments		
Yeast extract	10 mg/ml	2, 4 and 7
Pythium aphanidermatum	4 and 8 g/ml	2, 4 and 7
Botrytis cinerea	4 and 8 g/ml	1, 2 and 4
Signaling compounds in plant defense		
Salicylic acid	0.3, 0.5 and 1 mM	2, 4 and 7
Methyl jasmonate	0.3 mM	0, 6, 12, 24, 48 and 72 h
Jasmonic acid	100 μM	Every 2 days
Cell wall fragments		
Cannabis pectin extract	84 μg/ml	2 and 4
Cannabis pectin hydrolyzed	2 ml-aliquot	2 and 4
Pectin	0.1 mg/ml	Every 2 days
Sodium alginate	150 μg/ml	2 and 4
Abiotic		
$AgNO_3$	50 and 100 μM	2 and 4
$CoCl_2{\cdot}6H_2O$	50 and 100 μM	2 and 4
$NiSO_4{\cdot}6H_2O$	50 and 100 μM	2 and 4
UV 302 nm	30 s	2 and 4
UV 366 nm	30 min	2 and 4

2.4. Extraction of compounds for the metabolic profiling

Metabolite extraction was carried out as described by Choi et al. (2004a) with slight modifications. To 0.1 g of lyophilized plant material was added 4 ml MeOH:H_2O (1:1) and 4 ml CH_3Cl, vortexed for 30 s and sonicated for 10 min. The mixtures were centrifuged at 4 °C and 3000 rpm for 20 min. The MeOH:H_2O and CH_3Cl fractions were separated and evaporated. The extraction was performed twice. Alternatively, direct extraction with deuterated NMR solvents was performed in order to avoid possible loss or degradation of metabolites. Extracts were stored at 4 °C. For metabolite isolation and structure elucidation Sephadex LH-20 column chromatography eluted with MeOH:H_2O (1:1) and 2D NMR (HMBC, HMQC, J-Resolved and ^1H-^1H-COSY) was used. Ten fractions were collected and the profiles were analysed by TLC with silica gel $60F_{254}$ thin-layer plates developed in ethyl acetate–formic acid–acetic acid–water (100:11:11:26) and revealed with anisaldehyde–sulfuric acid reagent. Fraction 6 was subject to semi-preparative HPLC using a system formed by a Waters 626 pump, a Waters 600S controller, a Waters 2996 photodiode array detector and a Waters 717 plus autosampler (Waters, Milford, MA, USA), equipped with a reversed-phase C18 column (150 mm × 2.1 mm, 3.5 μm, ODS) and eluted with acetonitrile–water (10:90) at 1.0 ml/min and 254 nm. For LC–MS analyses, 5 μl of samples resuspended in MeOH were analysed in an Agilent 110 Series LC/MS system (Agilent Technologies, Palo Alto, CA, USA) with positive/negative atmospheric pressure chemical ionization (APCI), using an elution system MeOH:water with a flow rate of 1 ml/min. The gradient was 60–100% MeOH in 28 min followed by 100% MeOH for 2 min and a gradient step from 100 to 60% MeOH for 1 min. The optimum APCI conditions included a N_2 nebulizer pressure of 35 psi, a vaporizer temperature of 400 °C, a N_2 drying gas temperature of 350 °C at 10 l/min, a capillary voltage of 4000 V and a corona current of 4 μA. A reversed-phase C18 column (150 mm × 4.6 mm, 5 μm, Zorbax Eclipse XDB-C18, Agilent) was used.

2.5. NMR measurements, data analyses and quantitative analyses

The dried fractions were dissolved in $CDCl_3$ and MeOD:D_2O (1:1, v/v, pH 6), respectively. KH_2PO_4 was used as a buffering agent for MeOD:D_2O. Hexamethyldisilane (HMDS) and sodium trimethylsilyl propionate (TSP) were used as internal standards for $CDCl_3$ and MeOD:D_2O, respectively. Measurements were carried out using a Bruker AV-400 NMR. NMR parameters and data analyses were the same as previously reported by Choi et al. (2004a). Compounds were quantified by the relative ratio of the intensities of their peak-integrals and the ones of internal standard according to Choi et al. (2003, 2004b).

2.6. RNA and genomic DNA isolation

Trizol reagent (Invitrogen, Carlsband, CA, USA) was used for RNA isolation and Genomic DNA purification kit (Fermentas, St. Leon-Rot, Germany) for genomic DNA isolation following manufacturer's instructions.

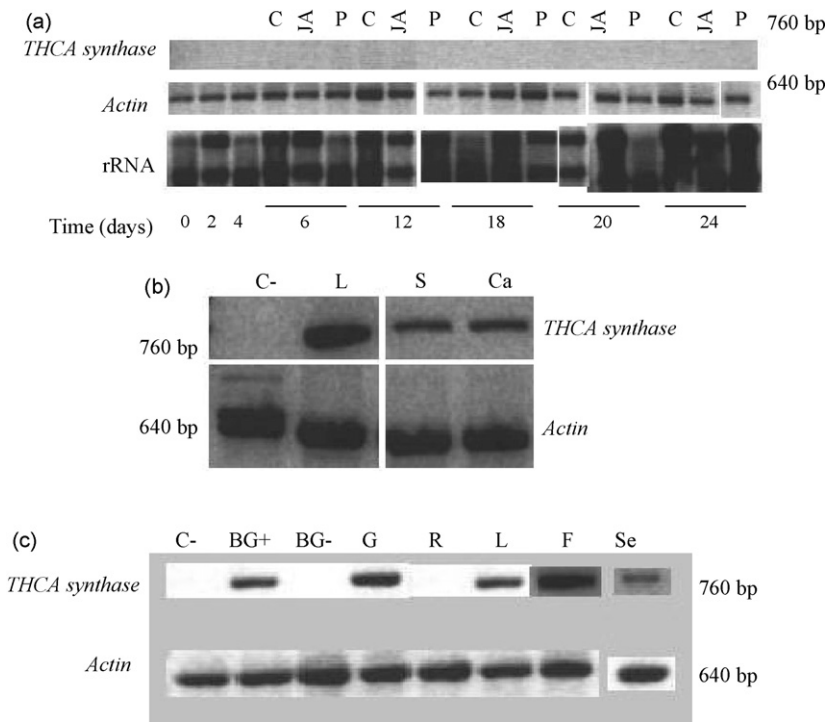


Fig. 1. Expression of *THCA synthase*. In panel (a) *THCA synthase* and *Actin* mRNAs in cannabis cell suspension cultures; C, control; JA, JA-treated cell suspension cultures; P, pectin-treated cell suspension cultures. In panel (b) the *THCA synthase* and *Actin* genes; C−, negative control (*Humulus lupulus*); L, cannabis leaf; S, cannabis cell suspension culture; Ca, cannabis calli. In panel (c) *THCA synthase* mRNAs in various tissues from cannabis plants; C−, negative control (*H. lupulus*); BG+, cannabis bracts covered with glandular trichomes; BG−, cannabis bracts without glandular trichomes; G, cannabis glandular trichomes; R, cannabis roots; L, cannabis leaf; F, cannabis flowers; Se, cannabis seedlings. Actin expression was used as a positive control.

2.7. RT-PCR and PCR conditions

The degenerated primers ActF (5′-TGGGATGAIATGGAGA-AGATCTGGCATCAIAC-3′) and ActR (5′-TCCTTYCTIATITCCACR-TCACACTTCAT-3′) (Biolegio BV, Malden, The Netherlands) were made based on conserved regions of *actin* gene or mRNA sequences from *Nicotiana tabacum* (accession number X63603), *Malva pusilla* (AF112538), *Picea rubens* (AF172094), *Brassica oleracea* (AF044573), *Pisum sativum* (U81047) and *Oryza sativa* (AC120533). The specific primers THCF (5′-GATACAACCCCAAAACCACTCGTTATTGTC-3′) and THCR (5′-TTCATCAAGTCGACTAGACTATCCACTCCA-3′) were made based on regions of the *THCA synthase* mRNA sequence (AB057805). RT-PCR was performed with total RNA as template. Reverse transcription was performed at 50 °C for 1 h followed by deactivation of the ThermoScript Reverse Transcriptase (Invitrogen) at 85 °C for 5 min. The PCR conditions for *actin* cDNA amplification were: five cycles of denaturation for 45 s at 94 °C, 1 min annealing at 48 °C, 1 min DNA synthesis at 72 °C; following five cycles with annealing at 50 °C and five cycles with annealing at 55 °C, and ending with 30 cycles with annealing at 56 °C. A PerkinElmer DNA Thermal Cycler 480 and a Taq PCR Core kit (QIAGEN, Hilden, Germany) were used. The PCR conditions for *THCA synthase* cDNA amplification were: denaturation for 40 s at 94 °C, 1 min annealing at 50 °C and 1 min DNA synthesis at 72 °C for 25 cycles. A final extension step for 10 min at 72 °C was included. The PCR products were separated on 1.5% agarose gel and visualized under UV light. DNA-PCR amplifications were performed with genomic DNA as template.

2.8. Statistics

Data were analysed by SIMCA-P 11.0 software (Umetrics Umeå, Sweden) and MultiExperiment Viewer MEV 4.0 software (Saeed et al., 2003). For analyses involving two and three or more groups paired *t*-test, ANOVA and PCA were used, respectively with $\alpha = 0.05$ for significance. Values are expressed as means of three replicates with standard deviations.

3. Results and discussion

3.1. Effect of elicitors on cannabinoid biosynthesis from C. sativa cell suspension cultures

For cannabinoid identification, $CHCl_3$ extracts were investigated. Characteristic signals for cannabinoids in ^1H NMR spectrum of the $CHCl_3$ extracts from cannabis female flowers (Choi et al., 2004a) were absent both on control and elicitor-treated cell cultures. Increased cannabinoid production in plants under stress has been observed (Pate, 1999). Although, environmental stress or elicitation appear to be a direct stimulus for enhanced secondary metabolite production by plants or cell cultures it seems that in cannabis cell suspension cultures the biotic or abiotic stress did not have any activating or stimulating effect on cannabinoid production. Stimulation of the biosynthesis of constitutive secondary

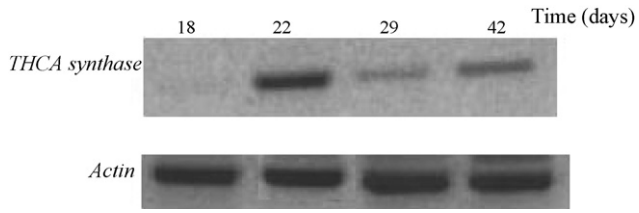


Fig. 2. Expression of *THCA synthase* during the development of glandular trichomes on flowers from cannabis plants.

metabolites during the exponential or stationary stages of cellular growth from cell tissues or upon induction by elicitation has been reported. The accumulation of the constitutive triterpene acids ursolic and oleanolic acid in *Uncaria tomentosa* cell cultures increased by elicitation during the stationary stage (Flores-Sanchez et al., 2002), while in *Rubus idaeus* cell cultures increasing amounts of raspberry ketone (*p*-hydroxyphenyl-2-butanone) and benzalacetone were observed during the exponential stage (Pedapudi et al., 2000). Also, secondary metabolite biosynthesis induction by elicitation such as the stilbene resveratrol in *Arachis hypogaea* (Rolfs et al., 1981) and *Vitis vinifera* (Liswidowati et al., 1991) cell cultures or the alkaloid sanguinarine in *Papaver somniferum* cell cultures (Facchini et al., 1996; Eilert and Constabel, 1986) has been reported. As cannabinoids are constitutive secondary metabolites in *C. sativa* (reviewed in Flores-Sanchez and Verpoorte, 2008a) a time course was made after induction with jasmonate and pectin. Both are known to induce the plant defense system (Zhao et al., 2005). These elicitors were used to induce the metabolism of the cell cultures during the exponential and stationary phases of cellular growth. Cellular growth was not significantly affected by the treatments (Supplementary Fig. 1, $n = 3$ per group, Anova, $P < 0.05$). However, no signals for cannabinoids in ^{1}H NMR spectrum of the $CHCl_3$ extracts were detected during the time course of the elicitation cell cultures with methyl jasmonate (MeJA), jasmonic acid (JA) and pectin. Analyses by LC–MS of the chloroform fractions revealed similar results.

An analysis of the expression of the *THCA synthase* gene from elicited cell cultures was performed by RT-PCR. No expression of the gene was detected in control and elicitor-treated cell cultures (Fig. 1a). DNA amplification of *THCA synthase* in cannabis leaf and cannabis cell cultures confirms that conditions and primer concentration were optimal (Fig. 1b). The results suggest that in cell cultures cannabinoid biosynthesis was absent and could not be induced as a plant defense response. Although, MeJA, JA and salicylic acid (SA) are transducers of elicitor signals it seems that in cell suspension cultures cannabinoid accumulation or biosynthesis was not related to JA or SA signaling pathways. Moreover, cannabinoid biosynthesis was neither induced as a response to pathogen-derived signals (pectin, cannabis pectin, alginate or components from fungal elicitors or yeast extract). Elicitor recognition by plants is assumed to be mediated by high-affinity receptors at the plant cell surface or occurring intracellularly which subsequently initiates an intracellular signal transduction cascade leading to stimulation of a characteristic set of plant defense responses (Nurnberger, 1999).

On the other hand, in the plant itself, secondary metabolites mostly accumulate in specific or specialized cells, tissues or organs. Although, cell cultures are derived, mostly, from parenchyma cells present in the explant prepared to initiate the cultures, sometimes a state of differentiation in the cultures is required for biosynthesis and accumulation of the secondary metabolites (Ramawat and Mathur, 2007). The accumulation of hypericin in cell cultures of *Hypericum perforatum* is dependent on cellular and tissue differentiation. Callus and cell suspension lines never accumulate hypericin, but hypericin accumulation has been shown in shoot cultures of this species and has been related with the formation of secretory structures (black globules) in the regenerated vegetative buds (Dias, 2003; Pasqua et al., 2003). Similar results have been observed in *P. somniferum* cell cultures, where differentiated tissues (roots or somatic embryos) are required for morphinan alkaloid biosynthesis (Laurain-Mattar et al., 1999). Furthermore, tissue specificity of the gene expression of secondary metabolite biosynthetic pathways has been shown. In *Citrus* cell cultures the production of flavonoids was closely related to embryogenesis together with the expression of the chalcone synthase, *CitCHS2*, gene (Moriguchi et al., 1999). In *P. somniferum*, tyrosine/dopa decarboxylase (*TYDC*) gene expression is associated with the developmental stage of the plant. TYDC catalyses the formation of the precursors tyramine and dopamine in the biosynthesis of alkaloids (Facchini and De Luca, 1995). Developmental, spatial and temporal control of gene expression is also known. Anthocyanin biosynthesis in flowers from *Gerbera hybrida* (Helariutta et al., 1995), *Ipomoea purpurea* (Durbin et al., 2000), Asiatic hybrid lily (Nakatsuka et al., 2003) and *Daucus carota* (Hirner and Seitz, 2000), as well as aroma and color of raspberry fruits (Kumar and Ellis, 2003) are some examples of a developmental, spatial, temporal and tissue-specific regulation. Cannabinoid accumulation and their biosynthesis have been shown to occur in glandular trichomes (Turner et al., 1978; Lanyon et al., 1981; Sirikantaramas et al., 2005) and a physiological function of the cannabinoid production in these trichomes has been suggested

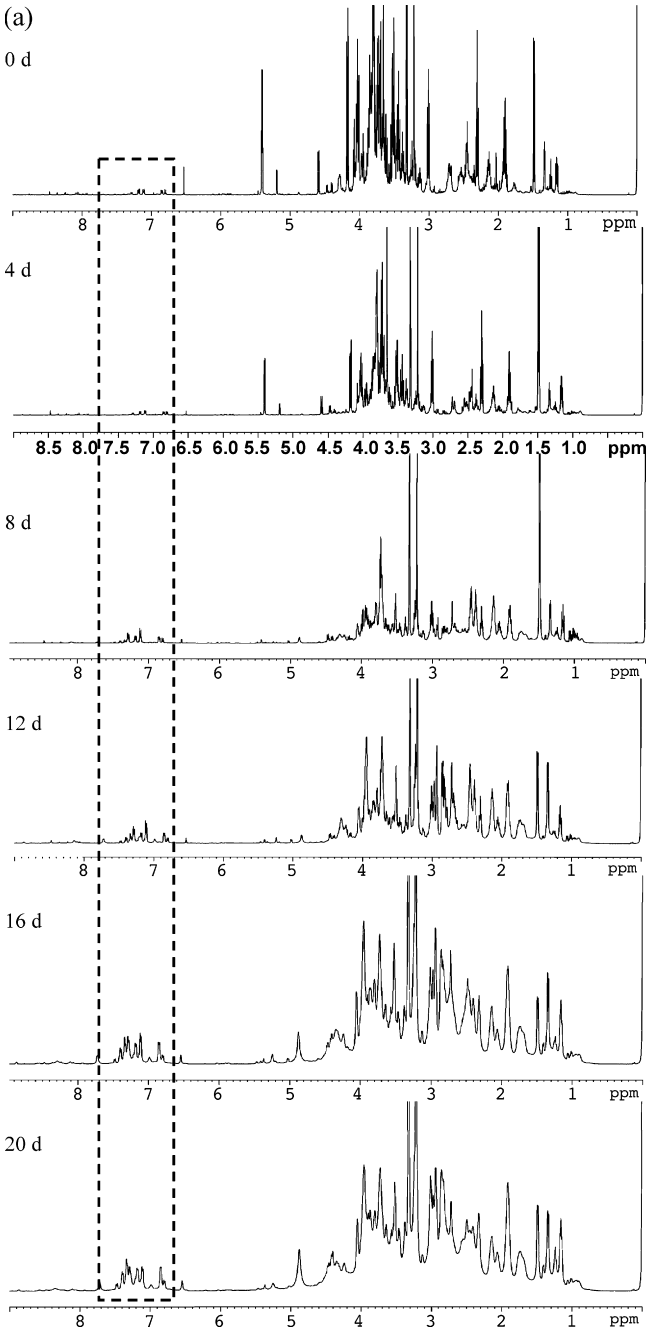


Fig. 3. ^{1}H NMR spectra of MeOH:water extracts from control (a), JA- (b) and pectin-treated (c) cannabis cell suspension cultures.

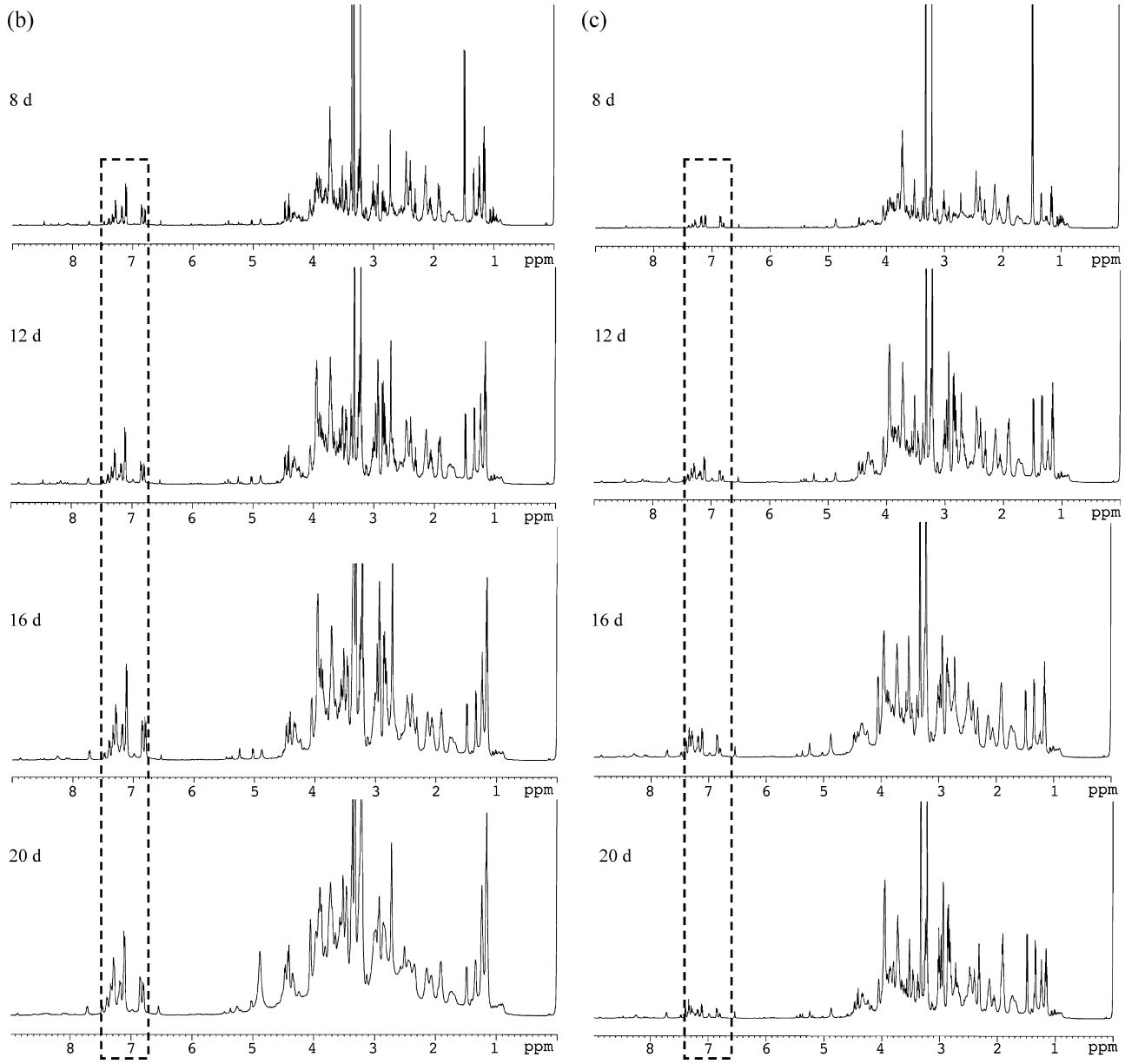


Fig. 3. (*Continued*).

(Taura et al., 2007a). Glandular trichomes, which secrete lipophilic substances, can serve in chemical protection against herbivores and pathogens by deterring or poisoning them. Moreover, trichomes can be both production and storage sites of phytotoxic materials (Werker, 2000). In *H. perforatum* plants the phototoxin hypericin accumulats in secretory glands on leaves and flowers (Fields et al., 1990; Zobayed et al., 2006). It has been confirmed that cannabinoids are cytotoxic compounds and thus they should be biosynthesized and accumulated in highly specialized cells such as glandular trichomes (Morimoto et al., 2007).

We did not detect cannabinoids in cell suspension cultures of *C. sativa* or in somatic embryos induced from cell suspension cultures. Expression analyses of the *THCA synthase* gene revealed that only in cannabis plant tissues containing glandular trichomes such as leaves and flowers, there was *THCA synthase* mRNA (Fig. 1c). No *THCA synthase* gene expression was found in glandular trichome-free bracts or in roots. Sirikantaramas et al. (2005) found *THCA synthase* gene expression in glandular trichomes as well. Although, seedlings did not accumulate cannabinoids (Flores-Sanchez and

Table 2
^{1}H NMR and ^{13}C NMR assignments for tryptophan measured in deuteromethanol. Chemical shifts (ppm) were determined with reference to TSP.

Position	^{1}H NMR	^{13}C NMR	HMBC
1		175.8	
2	3.86 (*dd*, 8.0, 4.0 Hz)	56.5	C-1,3,4
3	3.51 (*dd*, 15.9, 4.0 Hz)	28.0	C-2,4,5,11
	3.14 (*dd*, 15.9, 8.9 Hz)		C-2,4,5,11
4		109.0	
5		128.5	
6	7.68 (*d*, 8.0 Hz)	118.1	C-4,8,10
7	7.03 (*t*, 8.0 Hz)	120.0	C-5,9
8	7.10 (*t*, 8.0 Hz)	122.5	C-6,10
9	7.35 (*d*, 8.0 Hz)	112.0	C-5,7
10		138.9	
11	7.18 (*s*)	125.1	C-3,4,5,10

Table 3
^{1}H NMR and ^{13}C NMR assignments for tyramine and glutamyl-tyramine measured in deuteromethanol. Chemical shifts (ppm) were determined with reference to TSP.

Position	Tyramine			Glutamyl-tyramine		
	^{1}H NMR	^{13}C NMR	HMBC	^{1}H NMR	^{13}C NMR	HMBC
1		127.0			129.6	
2	7.07 (*d*, 8.0 Hz)	129.4	C-4,6,1′	7.01 (*d*, 8.0 Hz)	129.3	C-4,6,1′
6			C-4,2,1′			C-4,2,1′
3	6.75 (*d*, 8.0 Hz)	115.5	C-1,5	6.69 (*d*, 8.0 Hz)	115.0	C-1,5
5			C-1,3			C-1,3
4		156.5			155.5	
1′	2.84 (*t*, 8.8 Hz)	32.2	C-1,2(6),2′	2.68 (*t*, 8.0 Hz)	34.2	C-1,2(6),2′
2′	3.10 (*t*, 8.8 Hz)	41.0	C-1,1′	3.34 (*t*, 8.0 Hz)	41.2	C-1,1′,5′
Glutamic acid moiety	–	–	–			
1″	–	–	–		172.5	
2″	–	–	–	3.56 (*dd*, 15.0, 7.2 Hz)	54.0	C-1″,3″,4″
3″	–	–	–	2.05 (*m*)	26.5	C-1″,2″,4″,5″
4″	–	–	–	2.38 (*t*, 7.2 Hz)	31.0	C-2″,3″,5″
5″	–	–	–		173.5	

Table 4
^{1}H NMR and ^{13}C NMR assignments for phenylalanine measured in deuteromethanol. Chemical shifts (ppm) were determined with reference to TSP.

Position	^{1}H NMR	^{13}C NMR	HMBC
1		174.8	
2	3.91 (*dd*, 8.0, 4.0 Hz)	57.0	C-1,3,4
3	3.07 (*dd*, 15.3, 8.0 Hz)	36.5	C-1,2,4,5 (9)
	3.29 (*dd*, 15.3, 4.0 Hz)	36.5	C-1,2,4,5 (9)
4		135.4	
5	7.31 (*dd*, 8.4, 1.6 Hz)	129.2	C-7,9
9			C-7,5
6	7.39 (*t*, 8.4 Hz)	129.1	C-3,4,8
8			C-3,4,6
7	7.33 (*t*, 8.4)	126.8	C-9

Verpoorte, 2008b), low expression of the *THCA synthase* gene was observed by RT-PCR (Fig. 1c). On the other hand, it was found that expression of the *THCA synthase* gene is linked to the development and growth of glandular trichomes on flowers. After 18 days the development of gland trichomes on flowers became visible, after which the *THCA synthase* mRNA was expressed (Fig. 2). This suggests that cannabinoid biosynthesis is under tissue-specific and/or developmental control. The genes that encode the enzymes THCA synthase and cannabidiolic acid (CBDA) synthase have been characterized (Sirikantaramas et al., 2004; Taura et al., 2007b) and analyses of their promoters should be one of the subsequent steps to figure out the metabolic regulation of this pathway.

3.2. Effect of elicitors on metabolism in C. sativa cell suspension cultures

Analyses on the ^{1}H NMR spectra of methanol–water extracts from elicitor-treated cell cultures showed differences with the control (Supplementary Fig. 2).

In the MeJA-treated cell cultures, tryptophan (Supplementary Fig. 1) was identified in fraction 9 (Table 2). From fraction 7 tyramine (Supplementary Fig. 2) and glutamyl-tyramine (Supplementary Fig. 3) were identified (Table 3). Phenylalanine (Supplementary Fig. 4) was identified from subfraction 3 (Table 4).

Table 5
Chemical shifts (δ) of metabolites detected in CH_3OH-d_4-KH_2PO_4 in H_2O-d_2 (pH 6.0) from ^{1}H NMR, J-resolved 2D and COSY 2D spectra. TSP was used as reference.

Metabolite	δ (ppm) and coupling constants (Hz)
Alanine	1.48 (H-β, *d*, 7.2), 3.73 (H-α, *q*, 7.2)
Aspartic acid	2.83 (H-β, *dd*, 17, 7.9), 2.94 (H-β′, *dd*, 17, 4.0), 3.95 (H-α, *dd*, 8.1, 4.0)
GABA	1.90 (H-3, *m*, 7.5), 2.31 (H-2, *t*, 7.5), 3.00 (H-4, *t*, 7.5)
Fumaric acid	6.54 (H-2, H-3, *s*)
Threonine	1.33 (H-γ, *d*, 6.5), 3.52 (H-α, *d*, 4.9), 4.24 (H-β, *m*)
Valine	1.00 (H-γ, *d*, 7.0), 1.05 (H-γ′, *d*, 7.0)
Tryptophan	3.27 (H-3), 3.50 (H-3′), 3.98 (H-2), 7.14 (H-8, *t*, 7.7), 7.22 (H-7, *t*, 7.7), 7.29 (H-11, *s*), 7.47 (H-9, *dt*, 8.0, 1.3), 7.72 (H-6, *dt*, 8.0, 1.3)
Tyrosine	3.01 (H-β), 3.20 (H-β′), 3.86 (H-α), 6.85 (H-3, H-5, *d*, 8.4), 7.18 (H-2, H-6, *d*, 8.4)
Phenylalanine	3.09 (H-3, *dd*, 14.4, 8.4), 3.30 (H-3′, *dd*, 14.4, 9.6), 3.94 (H-2), 7.36 (H-5, H-6, H-7, H-8, H-9, *m*)
Glutamic acid	2.05 (H-β, *m*), 2.36 (H-γ, *m*)
Glutamine	2.13 (H-β, *m*), 2.49 (H-γ, *m*)
Sucrose	4.19 (H-1′, *d*, 8.5), 5.40 (H-1, *d*, 3.8)
α-Glucose	5.19 (H-1, *d*, 3.8)
β-Glucose	4.58 (H-1, *d*, 7.9)
Gentisic acid[a]	6.61 (H-3, *d*, 8.2), 6.99 (H-4, *dd*, 8.2, 2.5), 7.21 (H-6, *d*, 2.5)
Isoleucine	0.95 (H-5, *t*, 7.5), 1.02 (H-6, *d*, 6.8)
Leucine	0.97 (H-5, *d*, 6.7), 0.98 (H-6, *d*, 6.7)
Ethanol glucoside	1.24 (H-2, *t*, 6.9)
Adenosine	6.04 (H-1′, *d*, 6.6), 8.23 (H-8, *s*), 8.35 (H-2, *s*)
Cytidine	5.86 (H-5, *d*, 8.0), 5.91 (H-1′, *d*, 4.3), 7.93 (H-6, *d*, 8.0)
Choline	3.21 (H-1′, H-2′, H-3′, *s*)
Trigonelline	8.86 (H-4, H-6, *m*), 9.15 (H-2, *s*)

[a] In CH_3OH-d_4.

In the others treatments with biotic and abiotic elicitors, except with UV exposure, the signal at δ7.34 was increased and corresponded to phenylalanine. An overview of ^{1}H NMR spectra of methanol–water fractions of a time course from elicited cell cultures with JA and pectin is shown in Fig. 3.

Principal component analysis (PCA) showed that the separations (Fig. 4) are based on the elicitor-treated cells (PC4) and on culture age or harvest-time (PC3). During the logarithmic growth phase alanine (δ1.48 and δ3.72; Table 5) is the predominant compound, glutamic acid and glutamine (δ2.12, δ2.16, δ2.40 and δ2.44), and valine (δ0.96, δ1.00 and δ3.56) were predominant compounds in JA-treated cells, while aspartic acid (δ2.80, δ2.84 and δ3.96) and γ-aminobutyric acid (GABA, δ1.92, δ2.32 and δ3.0) are the predominant compounds in pectin-treated and control cells. In the stationary phase of cellular growth tyrosine (δ3.88 and δ3.24), phenylalanine (δ3.92) and tryptophan (δ3.48) increased. These results are similar to those from MeJA-treated cells, where alanine (δ1.49) and tyramine (δ7.12) were predominant from 0 to 12 h after treatment; phenylalanine (δ7.34) reached a maximum concentration at 24 h (Fig. 5) and tryptophan content was also induced after 12 h by elicitation with MeJA (Supplementary Fig. 3). Ethanol glucoside (δ1.24) was a predominant compound after 48–72 h in MeJA-treated cells and was also present in cells treated with JA during the stationary phase. The presence of ethanol glucoside in MeJA-treated plant cell cultures has been reported (Kraemer et al., 1999; Sanchez-Sampedro et al., 2007) and it was suggested that glucosylation is a detoxification process of the ethanol used to dissolve MeJA and JA.

The content of some amino acids, organic acids and sugars in the cell suspension cultures during the time course after elicitation with JA and pectin were analysed (Fig. 6). No significant differences were found in the pools of sucrose and glucose in elicited

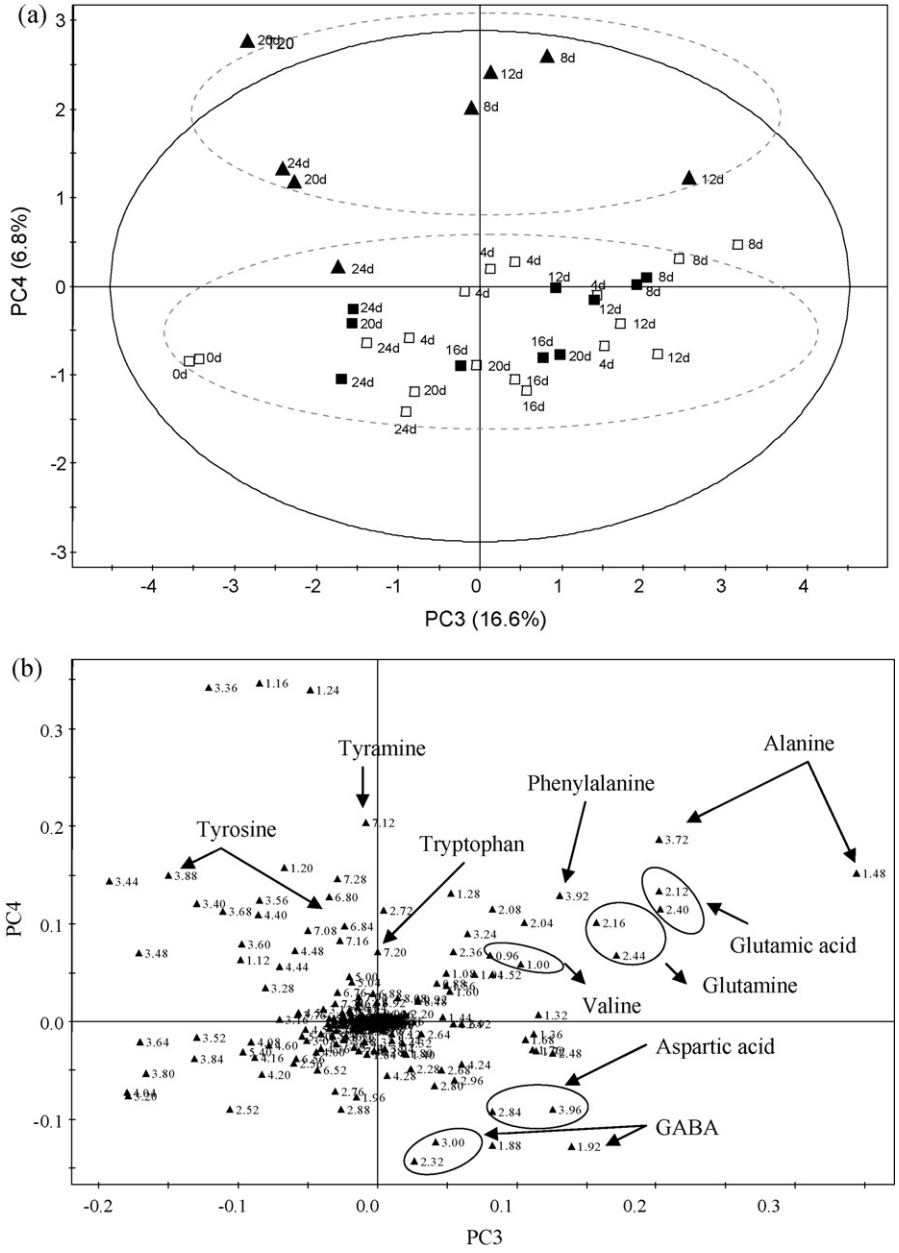


Fig. 4. Principal component analysis (PCA) of ^{1}H NMR data of MeOH:water fractions from cannabis cell. (a) Score and (b) loading plot of PCA; open squares, control cells; closed squares, pectin-treated cell; closed triangles, JA-treated cells; d, day. The ellipse represents the Hotelling T2 with 95% confidence in score plots.

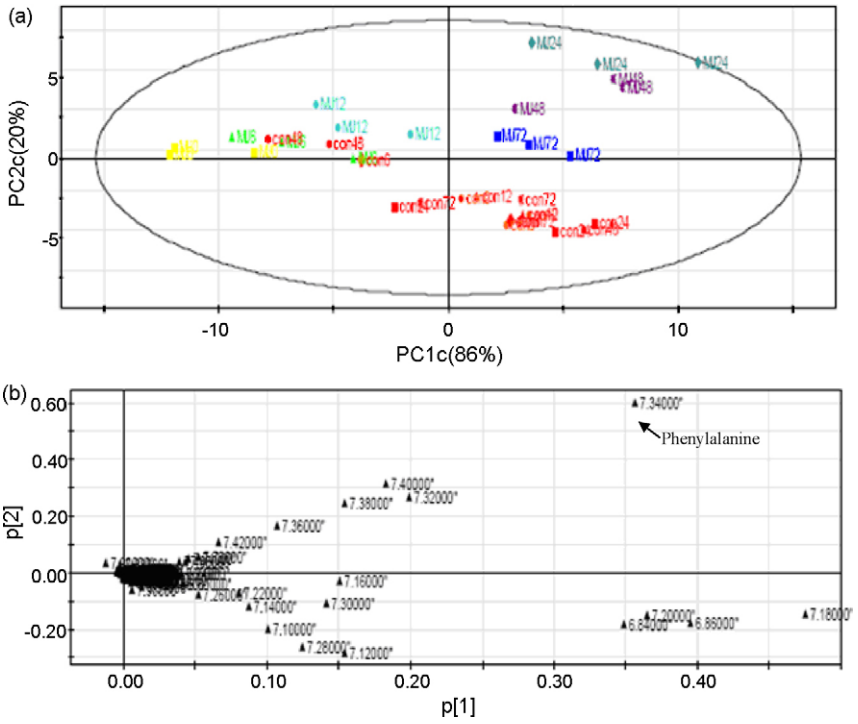


Fig. 5. Principal component analysis based on ^{1}H NMR data corresponding to aromatic region of MeOH:water fractions from cannabis cell. (a) Score and (b) loading plot of PCA; Con, control cells (hours) in red spots; MJ, MeJA-treated cells (hours after treatment). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and control cultures ($n = 3$ per group, Anova, $P < 0.05$). Fumaric acid content from pectin- and JA-treated cell suspensions increased at the end of the time course to levels of 9- and 14-fold, respectively; while the content in the control was 0 μmol/100 mg DW. Threonine content from control cell suspensions reached a maximum during the stationary phase and decreased at the end of the time course. Although, the threonine content was 1.5 times less in the JA-treated and pectin-treated cell suspensions during the first part of the growth cycle an accumulation of 10 and 12 times was found at day 24, respectively. No significant differences were observed between JA and pectin treatments ($n = 3$ per group, t-test, $P < 0.05$). Alanine content was not affected by the treatments, except at day 12 the alanine content from JA-treated cell suspensions was twice higher than those from controls and pectin-treated cell suspensions ($n = 3$ per group, Anova, $P < 0.05$). Maximum accumulation of aspartic acid was observed during the stationary phase. In controls this content decreased after day 16, but an increase of 35 and 37 times was found in the elicited cell cultures at the end of the time course. There were no significant differences between the two treatments ($n = 3$ per group, t-test, $P < 0.05$).

Maximum accumulation of tryptophan was also found in the stationary phase but significant differences in the accumulation levels during the time course were observed among controls and, pectin and JA elicitation ($n = 3$ per group, Anova, $P < 0.05$). It seems that JA increased twice the tryptophan level in the logarithmic growth phase reaching a maximum in the stationary phase of 1.4 times more than control and pectin elicitation. But whereas the tryptophan pool in controls returned to basal levels at day 24, in pectin and JA elicited cells the pools were still 26 and 14 times higher. The plant defense requires a coordinated regulation of primary and secondary metabolism (Henstrand et al., 1992; Batz et al., 1998; Zulak et al., 2007, 2008), the differences in pools of some of the metabolites analysed were observed after elicitation treatments before day 20 (Fig. 6) when the cellular viability started to decrease (Supplementary Fig. 4).

After day 20, larger differences were found in cultures with more than 95% of dead cells (no significant differences, $n = 3$ per group, Anova, $P < 0.05$). Gentisic acid (2,5-dihydroxybenzoic acid, δ6.61, δ6.99 and δ7.21; Supplementary Fig. 5) was identified in culture medium and was not affected by the pectin- and JA-treatment. Although, the metabolites isoleucine, leucine, choline, trigonelline, adenosine and cytidine were identified in the cell suspension cultures (Table 5) their pools were also not affected by the elicitor-treatments.

Fig. 7 shows the most likely metabolic interconnections of the compounds identified in this study. Although, glutamyl-tyramine has been detected in the horseshoe crab *Limulus polyphemus* (Battelle et al., 1988) and in the snail *Helix aspersa* (Zhou et al., 1993), presence of glutamyl-tyramine has not been reported in plants so far. γ-Glutamyl conjugates and tyramine conjugates have been identified as neurotransmitters in insects (Maxwell et al., 1980; Sloley et al., 1990), crustaceans (Battelle and Hart, 2002), mollusks (MacCaman et al., 1985; Karhunen et al., 1993) and mammals (Macfarlane et al., 1989). In plants such as soybean (Garcez et al., 2000), tomato (Zacares et al., 2007), rice (Jang et al., 2004), *Lycium chinense* (Han et al., 2002; Lee et al., 2004), *Chenopodium album* (Cutillo et al., 2003), *Solanum melongena* (Whitaker and Stommel, 2003), *Citrus aurantium* (Pellati and Benvenuti, 2007), *Piper caninum* (Ma et al., 2004) and *Cyathobasis fructiculosa* (Bunge) Aallen (Bahceevli et al., 2005), hydroxycinnamic acid conjugates such as the *N*-hydroxycinnamic acid amides and amine conjugates such as the phenethylamine alkaloids have been identified as constitutive, induced or overexpressed metabolites of plant defense. Alkaloids, *N*-hydroxycinnamic acid amides (phenolic amides) and lignans have been identified in cannabis plants (Flores-Sanchez and Verpoorte, 2008a). These secondary metabolites were not identified in the NMR spectra and further analyses using more sensitive

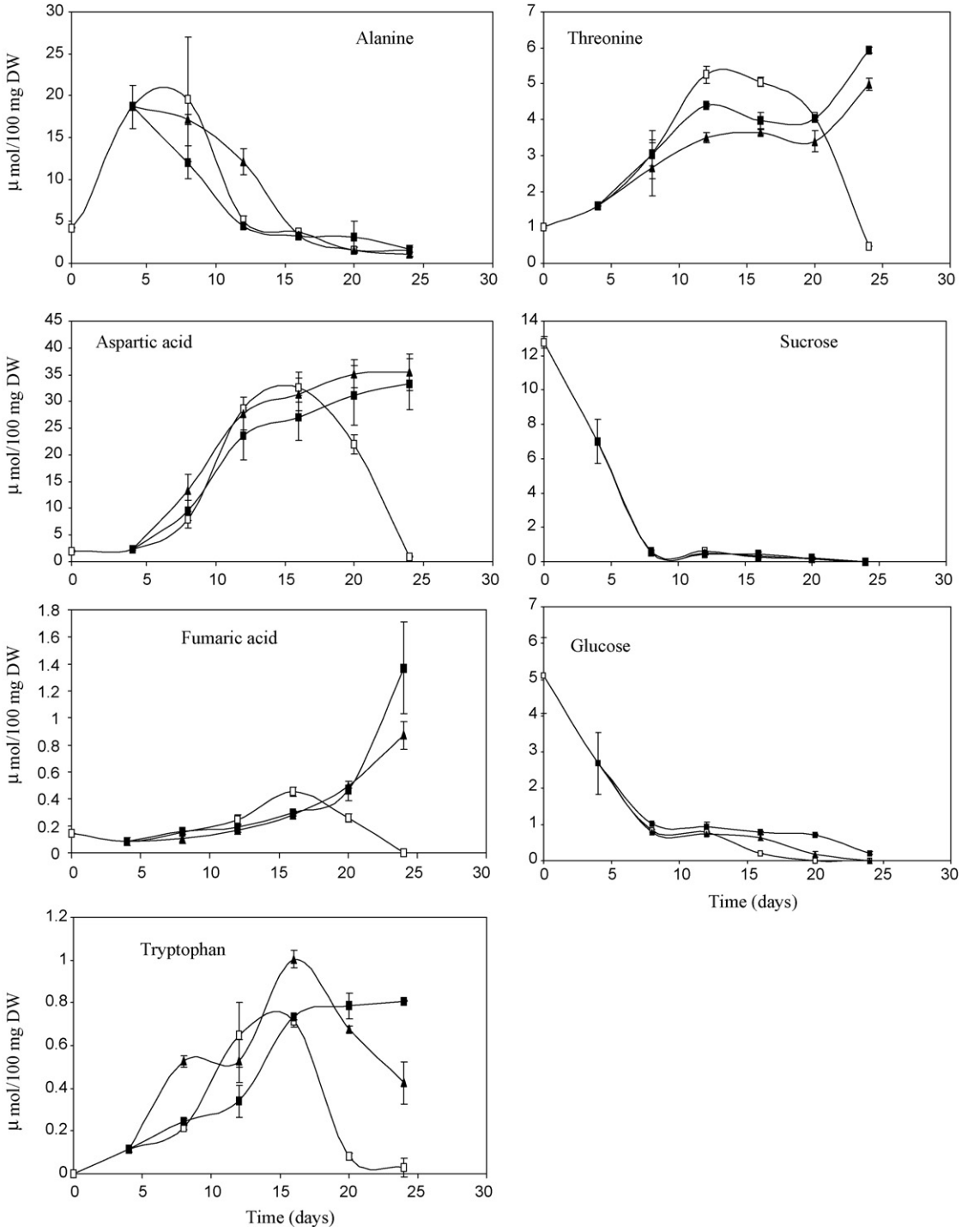


Fig. 6. Time course of identified metabolite content in control (open symbols) and elicited (closed symbols) cultures of *C. sativa*. Pectin-treated cell cultures (squares) and JA-treated cell cultures (triangles). TSP was used as internal standard (1.55 μmol). Values are expressed as means of three replicates with standard deviations.

methods or hyphenated methods (LC/GC–MS and HPLC–SPE–NMR, Jaroszewski, 2005) are necessary in order to prove their presence in the cannabis cell cultures. The results generated from NMR analyses and PCA are not conclusive, however, it seems that the main effect of the JA-, MeJA- and pectin-treatments was in the biosynthesis of primary precursors which could go into secondary biosynthetic pathways. It has been reported that *N*-hydroxycinnamic acid amide biosynthesis in *Theobroma cacao* (Alemanno et al., 2003) and maize (LeClere et al., 2007) is developmentally and spatially regulated. Similarly cannabinoid biosynthesis can be linked to development and spatial and temporal control, including other pathways of secondary metabolite biosynthesis. However, this control is probably not active in the cannabis undifferentiated/dedifferentiated and redifferentiated cultures such as cell suspensions, calli or embryo cultures. Biondi et al. (2002) reported that in *Hyoscyamus muticus* a relationship exists between the plant differentiation degree and the response to elicitors to form secondary metabolites.

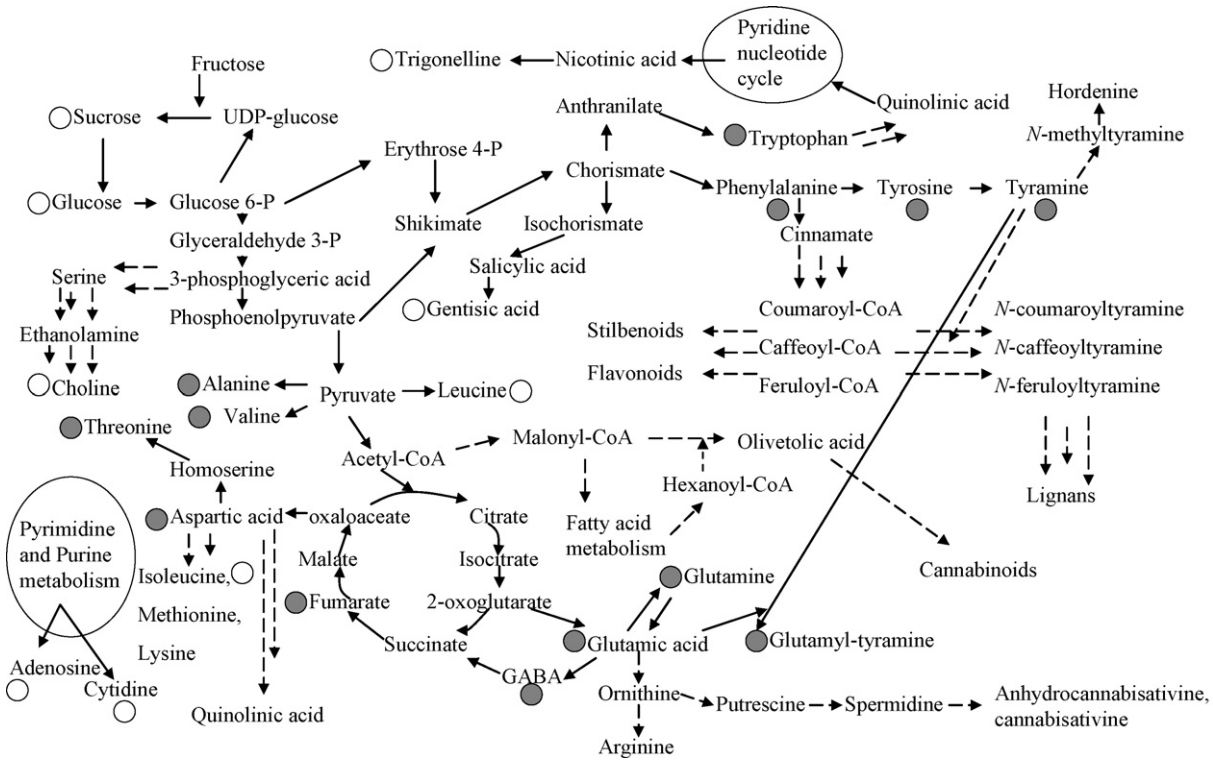


Fig. 7. Proposed metabolite linkage map between primary and secondary metabolism in cannabis cell suspension cultures. Metabolites identified in this study are associated with circles. Open circles, unaffected by elicitation; closed circles, metabolites affected by elicitation; dashed line, proposed pathways for biosynthesis of metabolites in cannabis plants according to Flores-Sanchez and Verpoorte (2008a).

4. Conclusions

In cannabis cell cultures, cannabinoid biosynthesis was not stimulated or induced by biotic and abiotic elicitors. A developmental, spatial, temporal or tissue-specific regulation could be controlling this pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.05.006.

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