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Polyketide synthases in *Cannabis sativa* L

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Polyketide synthases in *Cannabis sativa* L.



Isvett Josefina Flores Sanchez

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Cover photographs: *Cannabis sativa*, “Skunk” pistillate floral clusters (1, 4, 10, 14); “Skunk” leaf (2, 7); “Skunk” young leaves (9); “Skunk” seed and calyx (3, 18); “Kompolti” flowers (6, 11, 13, 16); “Skunk” seeded calyxes (8); “Kompolti” leaves (5, 12, 15); “Kompolti” staminate floral clusters (19); “Skunk” seeds (17); “Kompolti” seeds (21); “Skunk” and “Kompolti” seeds (20); “Kompolti” pistillate floral clusters (22).

Photograph: Isvett J. Flores-Sanchez

Polyketide Synthases in *Cannabis sativa* L.

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Contents

Chapter I

Introduction to secondary metabolism in cannabis	1
--	---

Chapter II

Plant Polyketide Synthases	29
----------------------------	----

Chapter III

Polyketide synthase activities and biosynthesis of cannabinoids and flavonoids in <i>Cannabis sativa</i> L. plants	43
--	----

Chapter IV

<i>In silicio</i> expression analysis of a <i>PKS</i> gene isolated from <i>Cannabis sativa</i> L.	73
--	----

Chapter V

Elicitation studies in cell suspension cultures of <i>Cannabis sativa</i> L.	93
--	----

Concluding remarks and perspectives	121
-------------------------------------	-----

Summary	123
---------	-----

Samenvatting	125
--------------	-----

References	127
------------	-----

Acknowledgements	167
------------------	-----

Curriculum vitae	168
------------------	-----

List of publications	169
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Chapter I

Introduction to secondary metabolism in cannabis

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Cannabis sativa L. is an annual dioecious plant from Central Asia. Cannabinoids, flavonoids, stilbenoids, terpenoids, alkaloids and lignans are some of the secondary metabolites present in *C. sativa*. Earlier reviews focused on isolation and identification of more than 480 chemical compounds; this review deals with the biosynthesis of the secondary metabolites present in this plant. Cannabinoid biosynthesis and some closely related pathways that involve the same precursors are discussed.

I.1 Cannabis plant

Cannabis is an annual plant, which belongs to the family Cannabaceae. There are only 2 genera in this family: *Cannabis* and *Humulus*. While in *Humulus* only one species is recognized, namely *lupulus*, in *Cannabis* different opinions support the concepts for a mono or poly species genus.

Linnaeus (1753) considered only one species, *sativa*, however, McPartland *et al.* (2002) described 4 species, *sativa*, *indica*, *ruderalis* and *afghanica*; and Hillig (2005) proposed 7 putative taxa, *ruderalis*, *sativa* ssp. *sativa*, *sativa* ssp. *spontanea*, *indica* ssp. *kafiristanica*, *indica* ssp. *indica*, *indica* ssp. *afghanica* and *indica* ssp. *chinensis*. Nevertheless, the tendency in literature is to refer to all types of cannabis as *Cannabis sativa* L. with a variety name indicating the characteristics of the plant.

The cultivation of this plant, native from Central Asia, and its use has been spread all over the world by man since thousands of years as a source of food, energy, fiber and medicinal or narcotic preparations (Jiang *et al.*, 2006; Russo, 2004; Wills, 1998).

Cannabis is a dioecious plant, i.e. it bears male and female flowers on separate plants. The male plant bears staminate flowers and the female plant pistillate flowers which eventually develop into the fruit and achenes (seeds). The sole function of male plants is to pollinate the females. Generally, the male plants commence flowering slightly before the females. During a few weeks the males produce abundant anthers that split open, enabling passing air currents to transfer the released pollen to the pistillate flowers. Soon after pollination, male plants wither and die, leaving the females maximum space, nutrients and water to produce a healthy crop of viable seeds. As result of special breeding, monoecious plants bearing both male and female flowers arose frequently in varieties developed for fiber production. The pistillate flowers consist of an ovary surrounded by a calyx with 2 pistils which trap passing pollen (Clarke, 1981; Raman, 1998). Each calyx is covered with glandular hairs (glandular trichomes), a highly specialized secretory tissue (Werker, 2000). In cannabis, these glandular trichomes are also present on bracts, leaves and on the underside of the anther lobes from male flowers (Mahlberg *et al.*, 1984).

I.2 Secondary metabolites of Cannabis

The phytochemistry in cannabis is very complex; more than 480 compounds have been identified (ElSohly and Slade, 2005) representing different chemical classes. Some belong to primary metabolism, e.g. amino acids, fatty acids and steroids, while cannabinoids, flavonoids, stilbenoids, terpenoids, lignans and alkaloids represent secondary metabolites. The concentrations of these compounds depend on tissue type, age, variety, growth conditions (nutrition, humidity and light levels), harvest time and storage conditions (Keller *et al.*, 2001; Kushima *et al.*, 1980; Roos *et al.*, 1996). The production of cannabinoids increases in plants under stress (Pate, 1999). Ecological interactions have also been reported (McPartland *et al.*, 2000). Feeding studies in grasshoppers indicated that minimum amounts of cannabinoids are stored in their exoskeletons, being excreted in their frass (Rothschild *et al.*, 1977); although a neurotoxic activity was reported in midge larvae using cannabis leaf extracts (Roy and Dutta, 2003).

I.2.1 Cannabinoids

This group represents the most studied compounds from cannabis. The term cannabinoid is given to the terpenophenolic compounds with 22 carbons (or 21 carbons for neutral form) of which 70 cannabinoids have been found so far and which can be divided into 10 main structural types (Figure 1). All other compounds that do not fit into the main types are grouped as miscellaneous (Figure 2). The neutral compounds are formed by decarboxylation of the unstable corresponding acids. Although decarboxylation occurs in the living plant, it increases during storage after harvesting, especially at elevated temperatures (Mechoulam and Ben-Shabat, 1999). Both forms are also further degraded into secondary products by the effects of temperature, light (Lewis and Turner, 1978) and auto-oxidation (Razdan *et al.*, 1972).

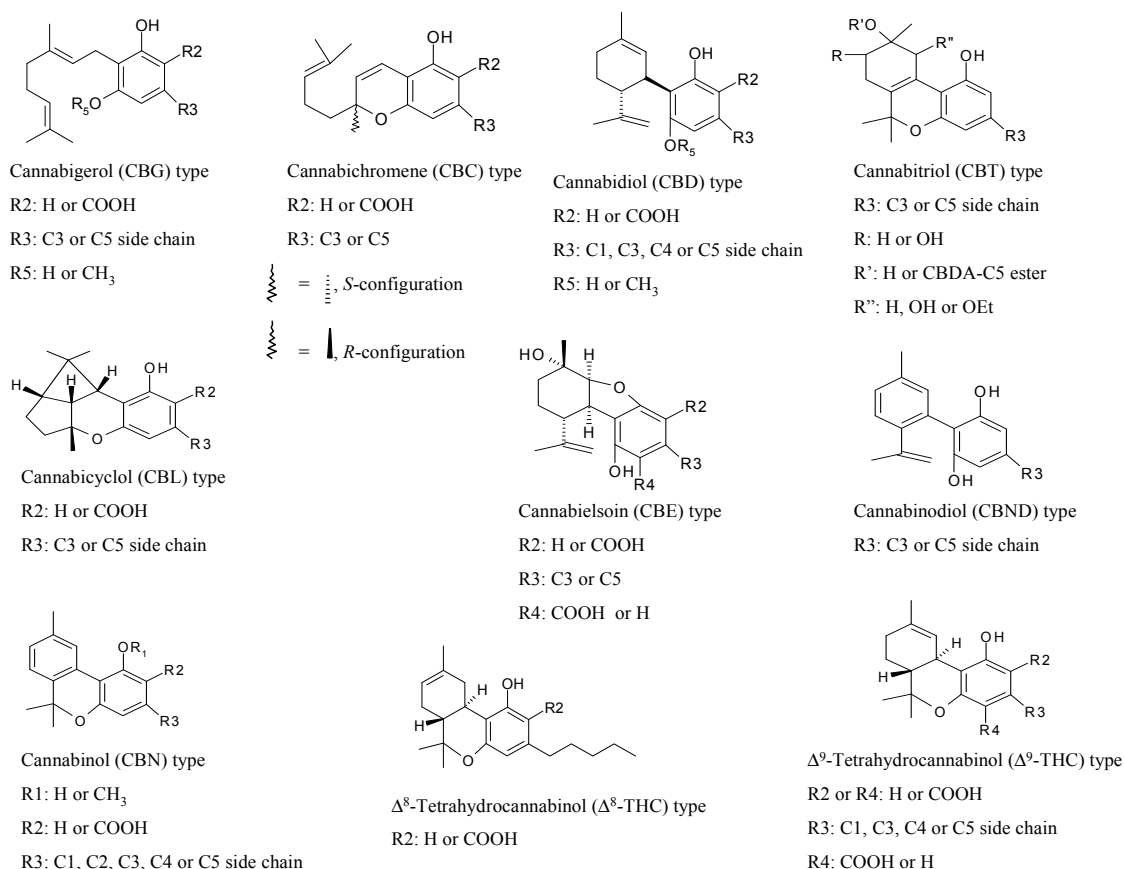


Figure 1. Cannabinoid structural types.

In cannabis, the most prevalent compounds are Δ⁹-THC acid, CBD acid and CBN acid, followed by CBG acid, CBC acid and CBND acid, while the others are minor compounds. Based on the absolute concentration of Δ⁹-THC (Δ⁹-THC + Δ⁹-THC acid) and CBD (CBD + CBD acid) obtained via HPLC or GC analyses, the plants are classified as follows: Drug type (chemotype I), the concentration of Δ⁹-THC is more than 2% and CBD concentration is less 0.5%; Fiber type (chemotype III), the Δ⁹-THC concentration is less than 0.3% and the concentration of CBD is more than 0.5%; Intermediate type (chemotype II), the concentrations of both are similar, usually more than 0.5% for each; and Propyl isomer/C3 type (chemotype IV), which can be differentiated by the dominant key cannabinoids Δ⁹-tetrahydrocannabivarinic acid (Δ⁹-THCVA) and Δ⁹-tetrahydrocannabivarin (Δ⁹-THCV), while also containing considerable amounts of Δ⁹-THC (Brenneisen and ElSohly, 1988; Fournier *et al.*, 1987; Lehmann and Brenneisen, 1995).

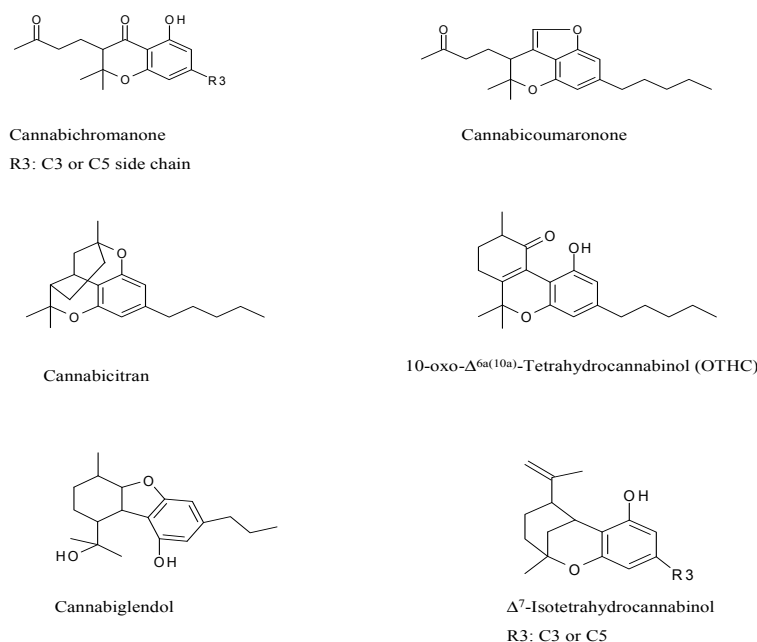


Figure 2. Miscellaneous cannabinoids.

The psychotropic activities of cannabinoids are well known (Paton and Pertwee, 1973; Ranganathan and D'Souza, 2006); however, in clinical studies, *in vitro* and *in vivo*, some other pharmacological effects of cannabinoids are observed such as antinociceptive, antiepileptic, cardiovascular, immunosuppressive (Ameri, 1999), antiemetic, appetite stimulation (Mechoulam and Ben Shabat, 1999), antineoplastic (Carchman *et al.*, 1976; Massi *et al.*, 2004), antimicrobial (ElSohly *et al.*, 1982), anti-inflammatory (Formukong *et al.*, 1988), neuroprotective antioxidants (Hampson *et al.*, 1988) and positive effects in psychiatric syndromes, such as depression, anxiety and sleep disorders (Grotenhermen, 2002; Musty, 2004). These effects could be due to agonistic nature of these compounds with respect to the cannabinoid CB₁- and CB₂ receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993) which compete with endocannabinoids (Mechoulam *et al.*, 1998), a family of cannabinoid receptor ligands participating in modulation of neurohumoral activity (Di Marzo *et al.*, 2007; Giuffrida *et al.*, 1999; Velasco *et al.*, 2005). Some therapeutic applications from cannabis, cannabinoids, cannabinoid analogs and CB receptor agonist/antagonist are shown in table 1.

Table 1. Some pharmacological applications of medicinal cannabis, THC, analogs and others.

Product	Components/ active ingredient	Prescription/ clinical effects	Administering	Country	Reference/ Company
Cannabis variety Bedrocan®	flos Dry flowers, 18% Δ^9 -THC and 0.2% CBD	Spasticity with pain in MS or spinal cord injury; nausea and vomiting by radiotherapy, chemotherapy and HIV-medication; chronic neuralgic pain and Gilles de la Tourette Syndrome; palliative treatment of cancer and HIV/AIDS	Smoking	NL	Office of Medicinal Cannabis (OMC)
Cannabis variety Bedrobinol®	flos Dry flowers, 13% Δ^9 -THC and 0.2% CBD	Spasticity with pain in MS or spinal cord injury; nausea and vomiting by radiotherapy, chemotherapy and HIV-medication; chronic neuralgic pain and Gilles de la Tourette Syndrome; palliative treatment of cancer and HIV/AIDS	Smoking	NL	Office of Medicinal Cannabis (OMC)
Marinol®	synthetic THC (capsules)	Nausea and vomiting by chemotherapy; appetite loss associated with weight loss by HIV/AIDS	Oral	USA	Solvay Pharmaceuticals, Inc.
Sativex®	Cannabis extract, 27 mg/ml Δ^9 -THC and 25 mg/ml CBD	Neuropathic pain in MS	Oromucosal	Canada	GW Pharm Ltd.
Cesamet™	THC analog (capsules)	Nausea and vomiting by cancer chemotherapy	Oral	USA	Valeant Pharmaceuticals International Karst <i>et al.</i> , 2003
Ajulemic acid (CT-3)	Δ^8 -THC-11-oic acid** analog, CB ₁ and CB ₂ agonist	Analgesic effect in chronic neuropathic pain	Oral	-	
Dexanabinol (HU-211)	11-OH- Δ^8 -THC* analog, <i>N</i> -methyl-D-aspartate antagonist	Neuroprotection	Intravenous	-	Knoller <i>et al.</i> , 2002/ Pharmos Ltd.
Rimonabant/ Acomplia® (SR141716A)	NPCDMPCH, CB ₁ selective antagonist	Adjunct to diet and exercise in the treatment of obese or overweight patients with associated risk factors such as type II diabetes or dyslipidaemia	Oral	Europe	Van Gall <i>et al.</i> , 2005; Aronne, 2007; Henness <i>et al.</i> , 2006 / Sanofi-Aventis

MS, Multiple Sclerosis; AIDS, acquired immunodeficiency syndrome; NL, The Netherlands

NPCDMPCH, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride* 11-OH- Δ^8 -THC is primary metabolite from Δ^8 -THC, which is further metabolized to ** Δ^8 -THC-11-oic acid by hepatic cytochrome P450s in humans

Table 2. Identified enzymes from cannabinoid pathway.

Enzyme	Source	MW (kDa)	K_m (μ M)	pH opt.	pI	V_{max} (nkat/mg)	K_{cat} (s^{-1})	(Sp activity, pKat/mg)	nce
Olivetol synthase	Flower, Leaf	-	Mal-CoA Hex-CoA	6.8				partially	Raharjo <i>et al.</i> 2004a
Geranyl diphosphate :olivetolate geranyltransferase (GOT)	Leaf		2000 GPP Olivetolic acid	7.0				partially	Fellermeier and Zenk 1998
			- NPP	7.0				partially	Fellermeier and Zenk 1998
CBGA synthase	Leaf	71	Olivetolic acid 23 CBGA	6.5	7.3	0.67	0.04	homogeneity (607)	Morimoto <i>et al.</i> 1998
CBDA synthase	Leaf	74	137 CBGA	5.0	6.1	2.57	0.19	homogeneity (1510)	Taura <i>et al.</i> 1996
			206 <i>trans</i> -CBGA	5.0		0.39	0.03	homogeneity	Taura <i>et al.</i> 1996
Δ^9 -THCA synthase	Leaf	75	134 CBGA	6.0	6.4	2.68	0.2	homogeneity	Taura <i>et al.</i> 1995a
Δ^9 -THCA synthase	Leaf (recombinant tobacco hairy roots)	58.6	- CBGA	5.0				homogeneity	Sirikantaramas <i>et al.</i> 2004
	Leaf (recombinant insect cells)	60	540 CBGA	5.0				homogeneity	Sirikantaramas <i>et al.</i> 2004

CBGA, cannabichromenic acid; CBDA, cannabidiolic acid; Δ^9 -THCA, Δ^9 -tetrahydrocannabinolic acid; Mal-CoA, malonyl-CoA; Hex-CoA, hexanoyl-CoA; GPP, geranyl diphosphate

I.2.1.1 Cannabinoid biosynthesis

Histochemical (André and Vercruysse, 1976; Petri *et al.*, 1988), immunochemical (Kim and Mahlberg, 1997) and chemical (Lanyon *et al.*, 1981) studies have confirmed that glandular hairs are the main site of cannabinoid production, although they have also been detected in stem, pollen, seeds and roots by immunoassays (Tanaka and Shoyama, 1999) and chemical analysis (Potter, 2004; Ross *et al.*, 2000).

The precursors of cannabinoids are synthesized from 2 pathways, the polyketide pathway (Shoyama *et al.*, 1975) and the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fellermeier *et al.*, 2001) (Figure 3). From the polyketide pathway, olivetolic acid is derived and from the DOXP/MEP pathway, geranyl diphosphate (GPP) is derived. Both are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (GOT) (Fellermeier and Zenk, 1998) to form cannabigerolic acid (CBGA), which is a common substrate for three oxydocyclases: Cannabidiolic acid synthase (Taura *et al.*, 1996), Δ^9 -Tetrahydrocannabinolic acid synthase (Taura *et al.*, 1995a) and Cannabichromenic acid synthase (Morimoto *et al.*, 1998), forming cannabidiolic acid (CBDA), Δ^9 -tetrahydrocannabinolic acid (Δ^9 -THCA) and cannabichromenic acid (CBCA), respectively (Morimoto *et al.*, 1999).

It is known that prenyltransferases condense an acceptor isoprenoid or non-isoprenoid molecule to an allylic diphosphate and depending on their specificities these prenyltransferases yield linear *trans*- or *cis*- prenyl diphosphates (Bouvier *et al.*, 2005). From *in vitro* assays it was observed that GOT could accept neryl diphosphate (NPP), the isomer of GPP which is formed by an isomerase (Shine and Loomis, 1974), as a substrate forming cannabinerolic acid (*trans*-CBGA) (Fellermeier and Zenk, 1998); this isomer of CBGA could be transformed to CBDA by a CBDA synthase (Taura *et al.*, 1996). The presence of *trans*-CBGA in cannabis has been shown (Taura *et al.*, 1995b). Probably, more than one enzymatic isoform coexist. It is known that depending on its degree of connectivity within the metabolic network, multiple isoforms of the same enzyme could preserve the integrity of the metabolic network; e.g. in the face of mutation. It has also been suggested that different organizations or associations from isoforms of the key biosynthetic enzymes into a metabolon, a

complex of sequential metabolic enzymes, could be differentially regulated (Jorgensen *et al.*, 2005; Sweetlove and Fernie, 2005).

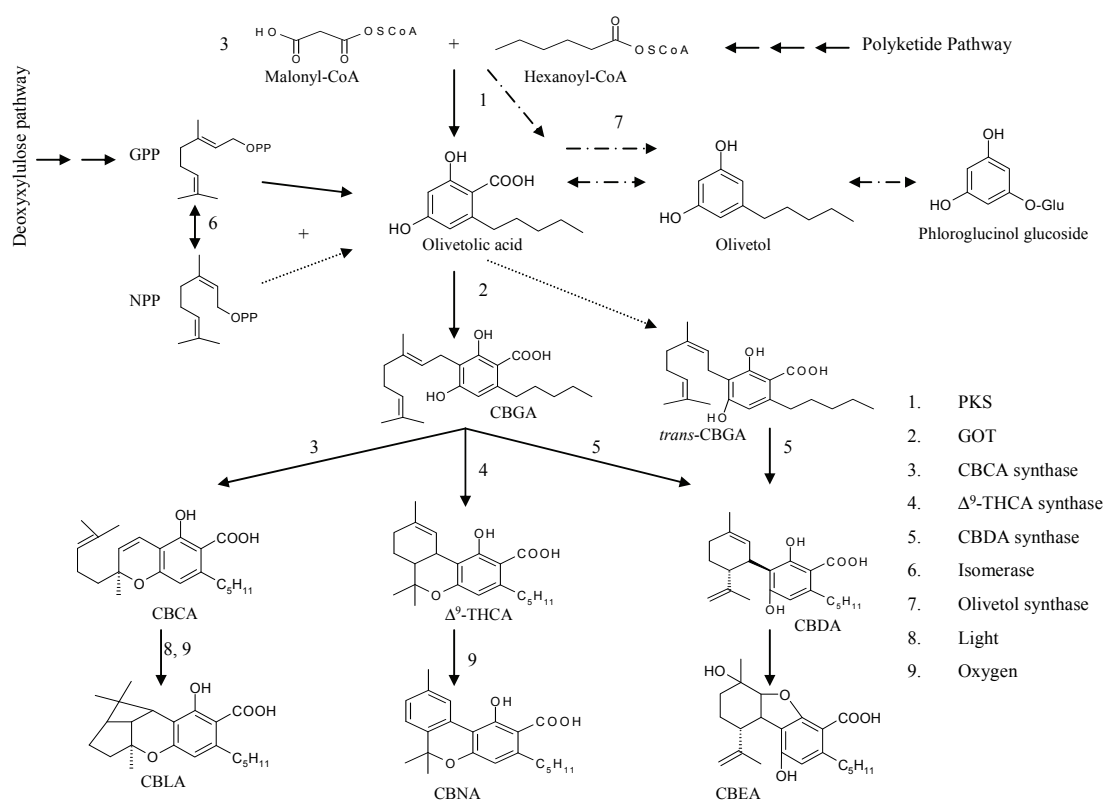


Figure 3. General overview of biosynthesis of cannabinoids and putative routes.

In table 2, some characteristics of the studied enzymes from the cannabinoid route are shown. The gene that encodes the enzyme THCA synthase has been cloned (Sirikantaramas *et al.*, 2004) and consists of a 1635-bp open reading frame, which encodes a polypeptide of 545 amino acids. The expressed protein revealed that the reaction is FAD-dependent and the binding of a FAD molecule to the histidine-114 residue is crucial for its activity. From the deduced amino acid sequence a cleavable signal peptide and glycosylation sites were found; suggesting post-translational regulation of the protein (Huber and Hardin, 2004; Uy and Wold, 1977). In addition, it was shown that THCA synthase is expressed exclusively in the glandular hairs and is also a secreted biosynthetic enzyme, which was localized to and functioned in the storage cavity of the glandular hairs; indicating that the storage cavity is not only the site for the accumulation of cannabinoids but also for the biosynthesis of THCA (Sirikantaramas *et al.*, 2005). This enzyme also has been crystallized (Shoyama *et al.*, 2005). The CBDA synthase gene has been cloned and expressed (Taura *et al.*, 2007b); the open reading frame encodes a 544 amino acid polypeptide, showing 83.9% of homology with THCA synthase. Furthermore, the expressed protein revealed a FAD-dependent reaction similar to THCA synthase and glycosylation sites were also found. In addition, it was suggested that a difference between the two reaction mechanisms from THCA and CBDA synthases is seen in the proton transfer step; while CBDA synthase removes a proton from the terminal methyl group of CBGA, THCA synthase takes it from the hydroxyl group of CBGA.

The transformation from CBD to CBE by cannabis suspension (Hartsel *et al.*, 1983), callus cultures (Braemer *et al.*, 1985) and *Saccharum officinarum* L. cultures (Hartsel *et al.*, 1983) have been reported, as well as the transformation of Δ^9 -THC to cannabicycoumarone (Braemer and Paris, 1987) by cannabis cell suspension cultures. From these studies, an epoxidation by epoxidases or cytochromes P-450 enzymes was proposed or a free radical-mediated oxidation mechanism (reactive oxygen species, ROS). It should be noted that the mentioned bioconversions all concern the decarboxylated compounds, i.e. not the normal biosynthetic products in the plant. Studies on the corresponding acids are required to reveal any relationship between the bioconversion experiments and the cannabinoid biosynthesis.

Oxidative stress in plants can be induced by several factors such as anoxia or hypoxia (by excess of rainfall, winter ice encasement, spring floods, seed imbibition, etc.), pathogen invasion, UV stress, herbicide action and programmed cell death or senescence (Blokhina *et al.*, 2003; Jabs, 1999; Pastori and del Rio, 1997). The proposed mechanisms of oxidation from the neutral and acid forms of Δ^9 -THC to the neutral and acid forms of CBN or Δ^8 -THC by free radicals or hydroxylated intermediates (Miller, *et al.*, 1982; Turner and ElSohly, 1979) could originate from a production of ROS. Antioxidants and antioxidant enzymes such as tocopherols, phenolic compounds (flavonoids), superoxide dismutase, ascorbate peroxidase and catalase have been proposed as components of an antioxidant defense mechanism to control the level of ROS and protect cells under stress conditions (Blokhina *et al.*, 2003). Cannabinoids could fit in this antioxidant system, however, their specific accumulation in specialized glandular cells point to another function for these compounds, e.g. antimicrobial agent. Sirikantaramas *et al.* (2005) found that cannabinoids are cytotoxic compounds for cell suspension cultures from *C. sativa*, tobacco BY-2 and insects; suggesting that the cannabinoids act as plant defense compounds and would protect the plant from predators such as insects. The THCA synthase reaction produces hydrogen peroxide as well as THCA during the oxidation of CBGA (Sirikantaramas *et al.*, 2004), a toxic amount of hydrogen peroxide could be accumulated together with the cannabinoids which must be secreted into the storage cavity from the glandular hairs to avoid cellular damage itself. Additionally, Morimoto *et al.* (2007) have shown that cannabinoids have the ability to induce cell death through mitochondrial permeability transition in cannabis leaf cells, suggesting a regulatory role in cell death as well as in the defense systems of cannabis leaves. On the other hand, although CBN type cannabinoids have been isolated from cannabis extracts, they are probably artifacts (ElSohly and Slade, 2005).

Feeding studies using cannabigerovarinic acid (CBGVA) as precursor, showed that the biosynthesis of propyl cannabinoids (Shoyama *et al.*, 1984) probably follows a similar pathway (Figure 4) yielding cannabidivarinic acid (CBDVA), cannabichromevarinic acid (CBCVA), Δ^9 -tetrahydrocannabivarinic acid (Δ^9 -THCVA), cannabielsovarinic acid B (CBEVA-B) and cannabivarin (CBV).

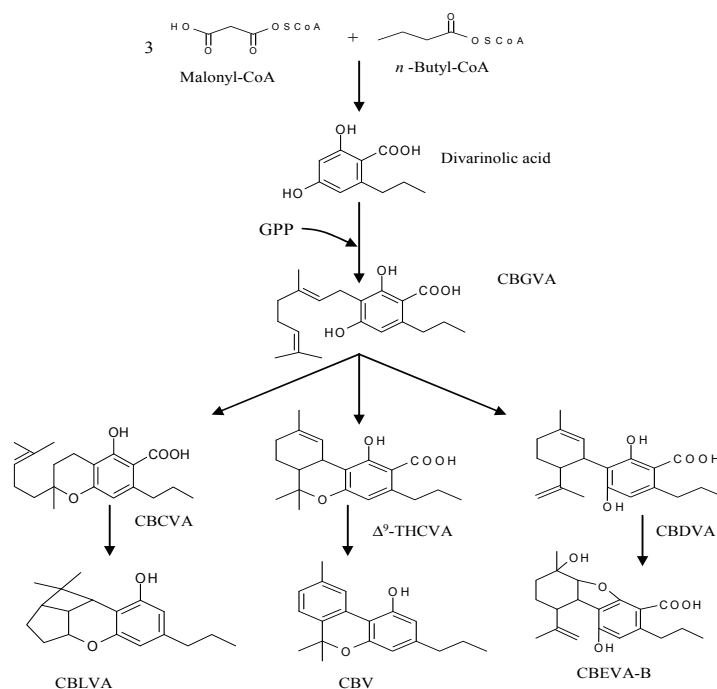


Figure 4. Proposed biogenetic pathway for cannabinoids with C3 side-chain.

Based on the structure of olivetolic acid (Figure 3), a polyketide synthase (PKS) could be involved in its biosynthesis. Raharjo *et al.* (2004a) found *in vitro* enzymatic activity for a PKS, though yielding the olivetol and not the olivetolic acid as the reaction product. It is known that olivetolic acid is the active form for the next biosynthetic reaction steps of the cannabinoids. Feeding studies (Kajima and Piraux, 1982), however, showed a low incorporation in cannabinoids using radioactive olivetol as precursor. Studies on the isoprenoid pathway suggest that the flux of active precursors (prenyl diphosphates) can be stopped by enzymatic hydrolysis by phosphatases, activated by kinases or even redirected to other biosynthetic processes (Goldstein and Brown, 1990; Meigs and Simoni, 1997). Furthermore, the presence of phloroglucinol glucoside in cannabis (Hammond and Mahlberg, 1994) suggests a regulatory role for olivetolic acid in the biosynthesis of cannabinoids (Figure 3), although, the presence of olivetolic acid and olivetol in ants from genus *Crematogaster* has been reported (Jones *et al.*, 2005); both olivetolic acid and olivetol are classified as resorcinolic lipids (alkylresorcinol, resorcinolic acid); these last ones have

been detected in several plants and microorganisms (Roos *et al.*, 2003; Jin and Zjawiony, 2006).

Kozubek and Tyman (1999) suggested that alkylresorcinols, such as olivetol, are formed from biosynthesized alkylresorcinolic acids by enzymatic decarboxylation or via modified fatty acid-synthesizing enzymes, where the alkylresorcinolic acid carboxylic group would be expected to be also attached either to ACP (acyl carrier protein) or to CoA. Thus, in the release of the molecule from the protein compartment in which it was attached or elongated, simultaneous decarboxylation of the alkylresorcinol may occur, otherwise the alkylresorcinolic acid would be the final product. Recently, it was shown that the fatty acid unit acts as a direct precursor and forms the side-chain moiety of alkylresorcinols (Suzuki *et al.*, 2003). The identification of methyl- (Vree *et al.*, 1972), butyl- (Smith, 1997), propyl- and pentyl-cannabinoids suggests the biosynthesis of alkylresorcinolic acids with different side-chain moieties, originating from different lengths of an activated short chain fatty acid unit (fatty acid-CoA). This side chain is important for the affinity, selectivity and pharmacological potency for the cannabinoids receptors (Thakur *et al.*, 2005).

Biotransformation of cannabinoids to glucosylated forms by plant tissues (Tanaka *et al.*, 1993; Tanaka *et al.*, 1996; Tanaka *et al.*, 1997) and various oxidized derivatives by microorganisms (Binder and Popp, 1980; Robertson *et al.*, 1978) have been reported; as well as biotransformations for olivetol (McClanahan and Robertson, 1984). However, the best studied biotransformations are in animals and humans (Mechoulam, 1970; Watanabe *et al.*, 2007)

1.2.2 Flavonoids

Flavonoids are ubiquitous and have many functions in the biochemistry, physiology and ecology of plants (Shirley, 1996; Gould and Lister, 2006), and they are important in both human and animal nutrition and health (Manthey and Buslig, 1998; Ferguson, 2001). In cannabis, more than 20 flavonoids have been reported (Clark and Bohm, 1979, Vanhoenacker *et al.*, 2002; ElSohly and Slade, 2005) representing 7 chemical structures which can be glycosylated, prenylated or methylated (Figure 5) Cannflavin A and cannflavin B are methylated isoprenoid flavones (Barron and Ibrahim, 1996). Some pharmacological effects from cannabis flavonoids have been detected such as inhibition of

prostaglandin E₂ production by cannaflavin A and B (Barrett *et al.*, 1986), inhibition of the activity of rat lens aldose reductase by *C*-diglycosylflavones, orientin and quercetin (Segelman *et al.*, 1976); other studies only suggest a possible modulation with the cannabinoids (McPartland and Mediavilla, 2002).

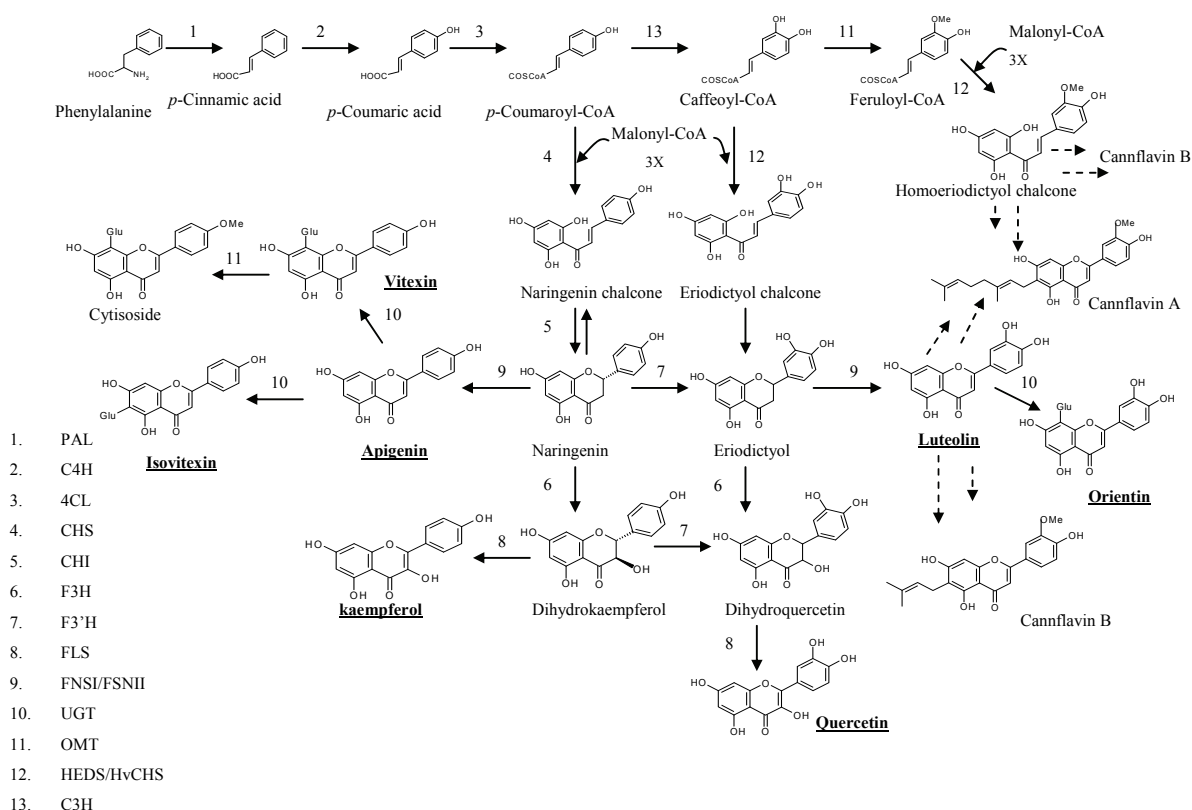


Figure 5. Proposed general phenylpropanoid and flavonoid biosynthetic pathways in *Cannabis sativa*. C3H, *p*-coumaroyl-CoA 3-hydroxylase; main structures of flavones and flavonols are in bold and underlined.

I.2.2.1 Flavonoid biosynthesis

Cannabis flavonoids have been isolated and detected from flowers, leaves, twigs and pollen (Segelman *et al.*, 1978; Vanhoenacker *et al.*, 2002; Ross *et al.*, 2005). There is no evidence indicating the presence of flavonoids in glandular trichomes, however, it is known that in *Betulaceae* family and in the genera *Populus* and *Aesculus* flavonoids are secreted by glandular trichomes or by a secretory epithelium (Wollenweber, 1980). Acylated kaempferol glycosides have

also been detected in leaf glandular trichomes from *Quercus ilex* (Skaltsa *et al.*, 1994), and flavone aglycones from *Origanum x intercedens* (Bosabalidis *et al.*, 1998) and from *Mentha x piperita* (Voirin *et al.*, 1993).

Although the flavonoid pathway has been extensively studied in several plants (Davies and Schwinn, 2006), there is no data on the biosynthesis of flavonoids in cannabis. The general pathway for flavone and flavonol biosynthesis as it is expected to occur in cannabis is shown in figure 5. The precursors are phenylalanine from the shikimate pathway and malonyl-CoA, which is synthesized by carboxylation of acetyl-CoA, a central intermediate in the Krebs tricarboxylic acid cycle (TCA cycle). Phenylalanine is converted into *p*-cinnamic acid by a Phenylalanine ammonia lyase (PAL), EC 4.3.1.5; this *p*-cinnamic acid is hydroxylated by a Cinnamate 4-hydroxylase (C4H), EC 1.14.13.11, to *p*-coumaric acid and a CoA thiol ester is added by a 4-Coumarate:CoA ligase (4CL), EC 6.2.1.12. One molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA are condensed by a Chalcone synthase (CHS), EC 2.3.1.74, a PKS, yielding naringenin chalcone. The naringenin chalcone is subsequently isomerized by the enzyme Chalcone isomerase (CHI), EC 5.5.1.6, to naringenin, a flavanone. This naringenin is the common substrate for the biosynthesis of flavones and flavonols. Hydroxy substitution to ring C at position 3 by a Flavanone 3-hydroxylase (F3H), EC 1.14.11.9; and to ring B at position 3' by a Flavonoid 3'-hydroxylase (F3'H), EC 1.14.13.21, occurs in naringenin. F3H is a 2-oxoglutarate-dependent dioxygenase (2ODG) and F3'H is a cytochrome P450. Subsequently, in the ring C at positions 2 and 3 a double bond is formed by a Flavonol synthase (FLS), EC 1.14.11.-, or Flavone synthase (FNS). FLS is a 2ODG and for FNS two distinct activities have been characterized that convert flavanones to flavones. In most plants FNS is a P450 enzyme (FNSII, EC 1.14.13.-), but in species from Apiaceae family FNS is a 2ODG (FNSI, EC 1.14.11.-). Modification reactions as glycosylation by UDP-glycosyltransferase (UGT, EC 2.4.1.-), methylation by a SAM-methyltransferase (OMT, EC 2.1.1.-) and prenylation by prenyltransferases are added to the flavone and flavonol. Alternative routes for luteolin, and cannflavin A / B biosynthesis starting from feruloyl-CoA or caffeoyl-CoA with malonyl-CoA are also proposed. Conversion of these substrates to homoeriodictyol or eriodictyol by Homoeriodictyol/eriodictyol synthase (HEDS or HvCHS), a PKS, has been shown (Christensen *et al.*, 1998). Feruloyl-CoA and caffeoyl-CoA are phenylpropanoids

which are derivatives from *p*-coumaric acid and are precursors for lignin biosynthesis (Douglas, 1996). HvCHS leads the production of the methylated flavanone homoeriodictyol and eliminate the need of the F3'H and the OMT. It has been shown that the flavonoid pathway is tightly regulated and several transcription factors have been identified (Davies and Schwinn, 2003; Davies and Schwinn, 2006), as well as formation of metabolons (Winkel-Shirley, 1999).

From biotransformation studies using *C. sativa* cell cultures, the transformation from apigenin to vitexin was shown, as well as glycosylations from apigenin to apigenin 7-*O*-glucoside and from quercetin to quercetin-*O*-glucoside (Braemer *et al.*, 1986). Regarding to PKS in cannabis, CHS activity was detected from flower protein extracts (Raharjo *et al.*, 2004a) and one PKS gene from leaf was identified (Raharjo *et al.*, 2004b), which expressed activity for CHS, Phlorisovalerophenone synthase (VPS) and Isobutyrophenone synthase (BUS). VPS, isolated from *H. lupulus* L. cones (Paniego *et al.*, 1999), and BUS, isolated from *Hypericum calycinum* cell cultures (Klingauf *et al.*, 2005), are PKSs that condense malonyl-CoA with isovaleryl-CoA or isobutyryl-CoA, respectively.

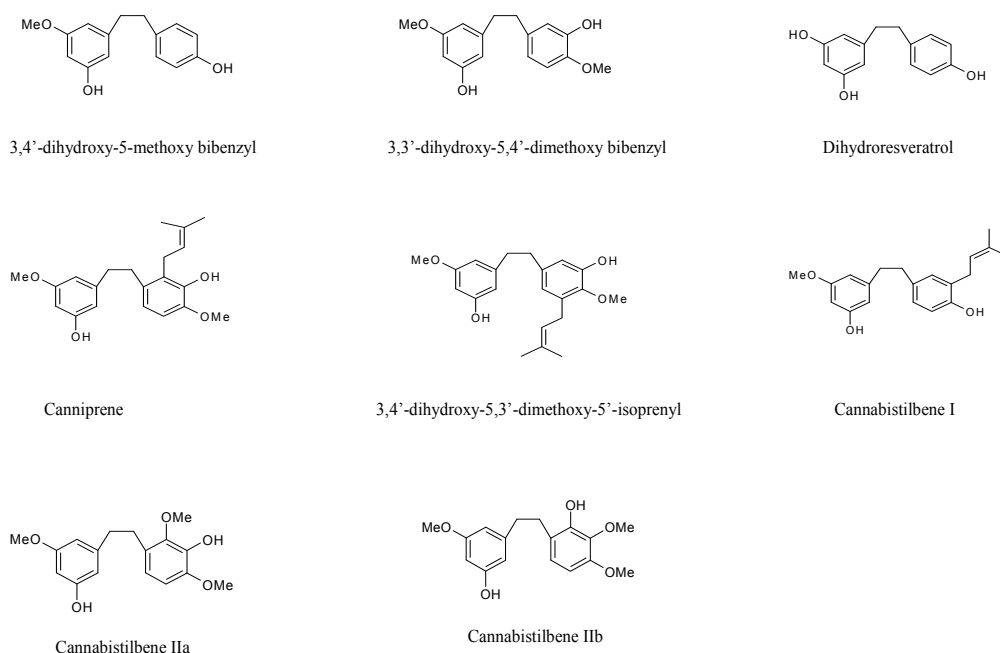


Figure 6. Bibenzyls compounds in *C. sativa*. The configuration of the structures is not given for simplicity reasons.

I.2.3 Stilbenoids

The stilbenoids are phenolic compounds distributed throughout the plant kingdom (Gorham *et al.*, 1995). Their functions in plants include constitutive and inducible defense mechanisms (Chiron *et al.*, 2001; Jeandet *et al.*, 2002), plant growth inhibitors and dormancy factors (Gorham, 1980). Frequently, the stilbenoids are constituents of heartwood or roots, and have antifungal and antibacterial activities (Kostecki *et al.*, 2004; Vastano *et al.*, 2000) or they are repellent towards insects (Hillis and Inoue, 1968). Nineteen stilbenoids have been identified in cannabis (Ross and ElSohly, 1995; Turner *et al.*, 1980) (Figures 6–8).



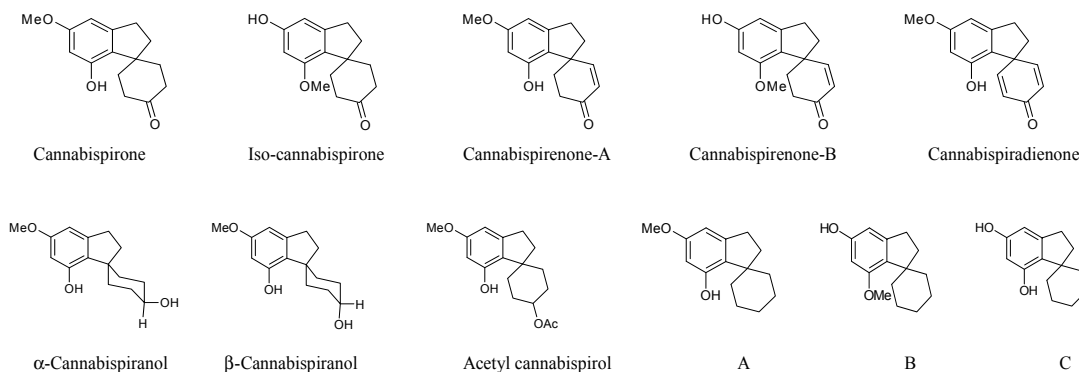


Figure 8. Spirans from *C. sativa*. A, 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; B, 5-hydroxy-7-methoxyindan-1-spiro cyclohexane; C, 5,7-dihydroxyindan-1-spiro-cyclohexane.

It has been observed that the stilbenoids show activities such as anti-inflammatory (Adams *et al.*, 2005; Djoko *et al.*, 2007; Leiro *et al.*, 2004), antineoplastic (Iliya *et al.*, 2006; Oliver *et al.*, 1994; Yamada *et al.*, 2006), neuroprotective (Lee *et al.*, 2006), cardiovascular protective (Leiro *et al.*, 2005; Estrada-Soto *et al.*, 2006), antioxidant (Stivala *et al.*, 2001) antimicrobial (Lee *et al.*, 2005), and longevity agents (Kaeberlein *et al.*, 2005; Valenzano *et al.*, 2006).

1.2.3.1 Stilbenoid biosynthesis

Cannabis stilbenoids have been detected and isolated from stem (Crombie and Crombie, 1982), leaves (Kettenes-van den Bosch and Saleminck, 1978) and resin (El-Feraly *et al.*, 1986).

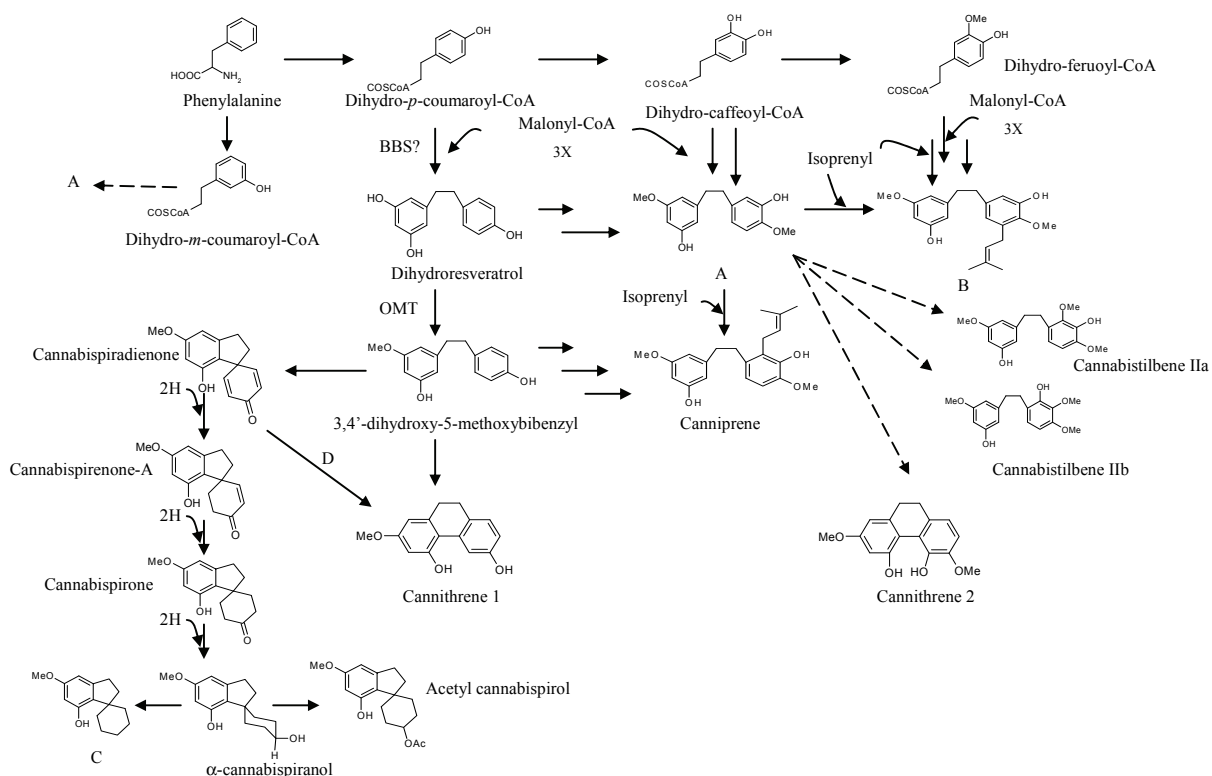


Figure 9. Proposed pathway for the biosynthesis of stilbenoids in *C. sativa*. A) 3,3'-dihydroxy-5,4'-dimethoxybibenzyl; B) 3,4'-dihydroxy-5,3'-dimethoxy-5'-isoprenylbibenzyl; C) 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; D) Dienone-phenol *in vitro* rearrangement (heat, acidic pH).

It has been suggested (Crombie and Crombie, 1982; Shoyama and Nishioka, 1978) that their biosynthesis could have a common origin (Figure 9). The first step could be the formation of bibenzyl compounds from the condensation of one molecule of dihydro-*p*-coumaroyl-CoA and 3 molecules of malonyl-CoA to dihydroresveratrol. It was shown that in cannabis both dihydroresveratrol and canniprene are synthesized from dihydro-*p*-coumaric acid (Kindl, 1985). In orchids, the induced synthesis by fungal infection of bibenzyl compounds by a PKS, called Bibenzyl synthase (BBS), was shown to condense dihydro-*m*-coumaroyl-CoA and malonyl-CoA to 3,3',5-trihydroxybibenzyl (Reinecke and Kindl, 1994a). It was also found that this enzyme can accept dihydro-*p*-coumaroyl-CoA and dihydrocinnamoyl-CoA as substrates, although to a lesser degree. Dihydropinosylvin synthase is an enzyme from *Pinus sylvestris* (Fliegmann *et al.*, 1992) that accepts dihydrocinnamoyl-CoA as substrate to form bibenzyl dihydropinosylvin. Gehlert and Kindl (1991) found a relationship

between induced formation by wounding of 3,3'-dihydroxy-5,4'-dimethoxybibenzyl and the enzyme BBS in orchids. This result also suggests that in cannabis the 3,3'-dihydroxy-5,4'-dimethoxybibenzyl compound could have the 3,3',5-trihydroxybibenzyl formed from dihydro-*m*-coumaroyl-CoA or dihydro-caffeoyl-CoA as intermediate. In orchids, however, the incorporation of phenylalanine into dihydro-*m*-coumaric acid, dihydrostilbene and dihydrophenanthrenes was shown (Fritzemeier and Kindl, 1983); indicating an origin from the phenylpropanoid pathway. Similar to flavonoid biosynthesis, modification reactions such as methylation and prenylation could form the rest of the bibenzyl compounds in cannabis. A second step could involve the synthesis of 9,10-dihydrophenanthrenes from bibenzyls. It is known that *O*-methylation is a prerequisite for the cyclization of bibenzyls to dihydrophenanthrenes in orchids (Reinecke and Kindl, 1994b) and a transient accumulation of the mRNAs from *S*-adenosyl-homocysteine hydrolase and BBS was also detected upon fungal infection (Preisig-Müller *et al.*, 1995). The cyclization mechanism in plants is unknown. An intermediate step between bibenzyls and 9,10-dihydrophenanthrenes could be involved in the biosynthesis of spirans. It has been proposed that spirans could be derived from *o-p*, *o-o* or *p-p* coupling of dihydrostilbenes followed by reduction (Crombie, 1986; Crombie *et al.*, 1982) and that 9,10-dihydrophenanthrenes could be derived by a dienone-phenol rearrangement from the spirans. No reports about the biosynthesis of spirans or about the regulation of the stilbenoid pathway in cannabis exist.

1.2.4 Terpenoids

The terpenoids or isoprenoids are another of the major plant metabolite groups. The isoprenoid pathway generates both primary and secondary metabolites (McGarvey and Croteau, 1995). In primary metabolism the isoprenoids have functions as phytohormones (gibberellic acid, abscisic acid and cytokinins) and membrane stabilizers (sterols), and they can be involved in respiration (ubiquinones) and photosynthesis (chlorophylls and plastoquinones); while in secondary metabolism they participate in the communication and plant defense mechanisms (phytoalexins). In cannabis 120 terpenes have been identified (ElSohly and Slade, 2005): 61 monoterpenes, 52 sesquiterpenoids, 2 triterpenes, one diterpene and 4 terpenoid derivatives

(Figure 10). The terpenes are responsible for the flavor of the different varieties of cannabis and determine the preference of the cannabis users. The sesquiterpene caryophyllene oxide is the primary volatile detected by narcotic dogs (Stahl and Kunde, 1973). It has been observed that terpene yield and floral aroma vary with the degree of maturity of female flowers (Mediavilla and Steinemann, 1997) and it has been suggested that terpene composition of the essential oil could be useful for the chemotaxonomic analysis of cannabis plants (Hillig, 2004). Pharmacological effects have been detected for some cannabis terpenes and they may synergize the effects of the cannabinoids (Burstein *et al.*, 1975; McPartland and Mediavilla, 2002). Terpenes have been detected and isolated from the essential oil from flowers (Ross and ElSohly, 1996), roots (Slatkin *et al.*, 1971) and leaves (Bercht *et al.*, 1976; Hendriks *et al.*, 1978); however, the glandular hairs are the main site of localization (Malingre *et al.*, 1975).

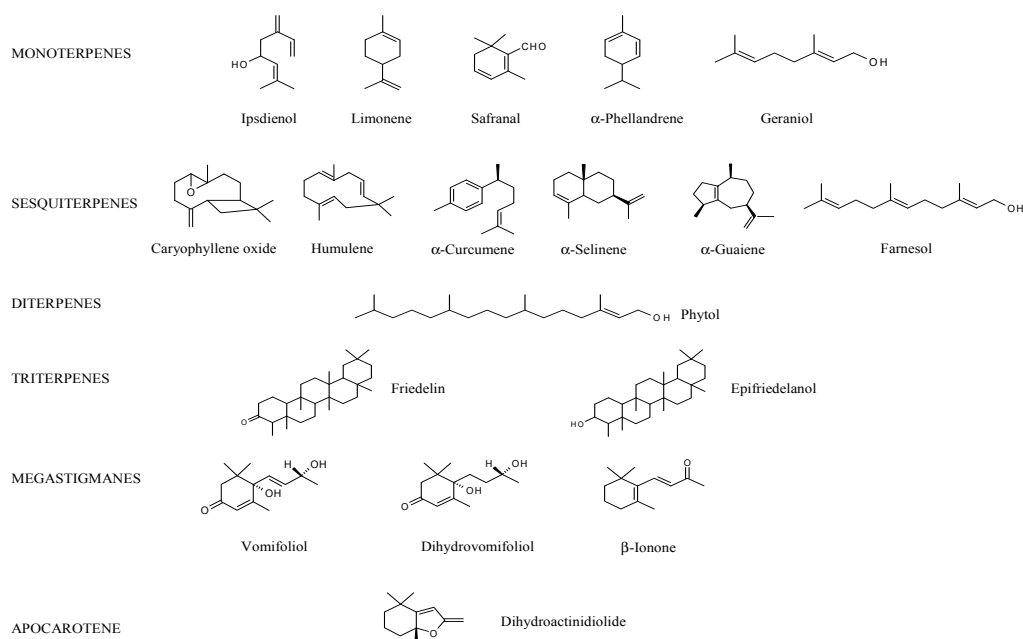


Figure 10. Some examples of isolated terpenoids from *C. sativa*.

I.2.4.1 Terpenoid biosynthesis

The isoprenoid pathway has been extensively studied in plants (Bouvier *et al.*, 2005). The terpenoids are derived from the mevalonate (MVA) pathway, which is active in the cytosol, or from the plastidial deoxyxylulose phosphate/methyl-

erythritol phosphate (DOXP/MEP) pathway (Figure 11). Both pathways form isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Condensation reactions by prenyl transferases produce a series of prenyl diphosphates. Generally, it is considered that the MVA pathway provides precursors for the synthesis of sesquiterpenoids, triterpenoids, steroids and others; while the DOXP/MEP pathway supplies precursors for monoterpenoids, diterpenoids, carotenoids and others. In cannabis both pathways could be present, DOXP/MEP pathway for monoterpenes and diterpenes, and MVA pathway for sesquiterpenes and triterpenes. As it was previously mentioned the DOXP/MEP pathway supplies the GPP precursor for the biosynthesis of cannabinoids. There is little knowledge about the regulation of both pathways in the plant cells and which transcriptional factors control them.

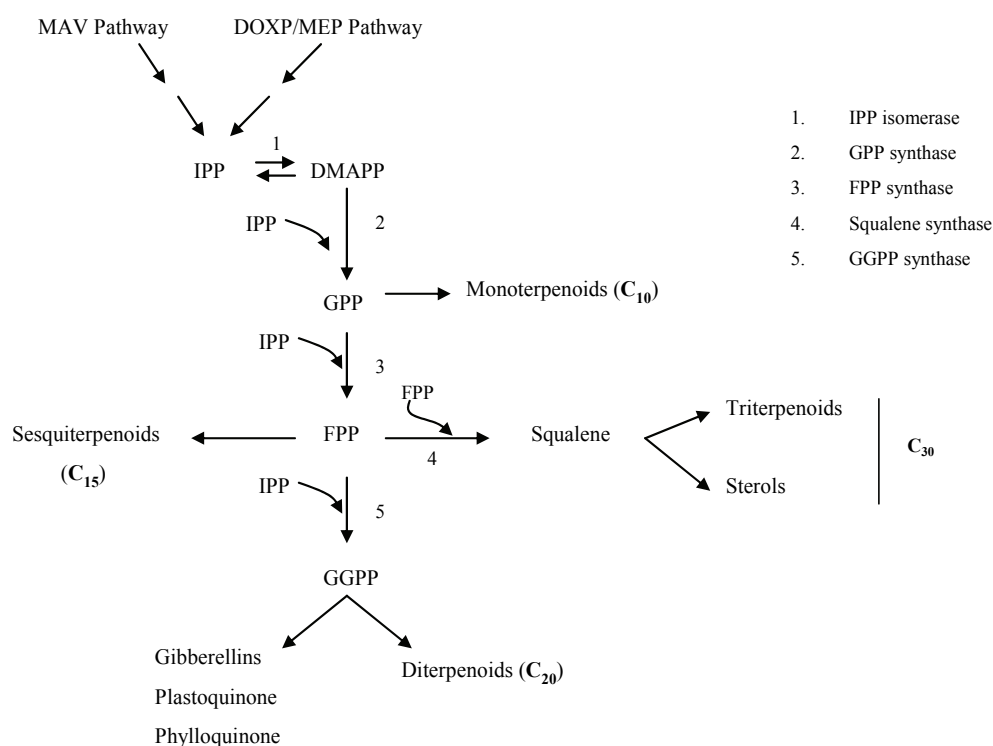


Figure 11. General pathway for the biosynthesis of terpenoids.

1.2.5 Alkaloids

The alkaloids are another major group of secondary metabolites in plants. Alkaloids are basic, nitrogenous compounds usually with a biological activity in

low doses and they can be derived from amino acids. In cannabis 10 alkaloids have been identified (Ross and ElSohly, 1995; Turner *et al.*, 1980). Choline, neurine, L-(+)-isoleucine-betaine and muscarine are protoalkaloids; hordenine is a phenethylamine and trigonelline is a pyridine (Figure 12). Cannabisisativine and anhydrocannabisisativine are polyamines derived from spermidine and are subclassified as dihydroperiphylline type (Bienz *et al.*, 2002). They are 13-membered cyclic compounds where the polyamine spermidine is attached *via* its terminal *N*-atoms to the β -position and to the carboxyl carbon of a C_{14} -fatty acid (Figure 13). Piperidine and pyrrolidine were also identified in cannabis. These alkaloids have been isolated and identified from roots, leaves, stems, pollen and seeds (El-Feraly and Turner, 1975; ElSohly *et al.*, 1978; Paris *et al.*, 1975). The presence of muscarine in cannabis plants has been questioned (Mechoulam, 1988; ElSohly, 1985).

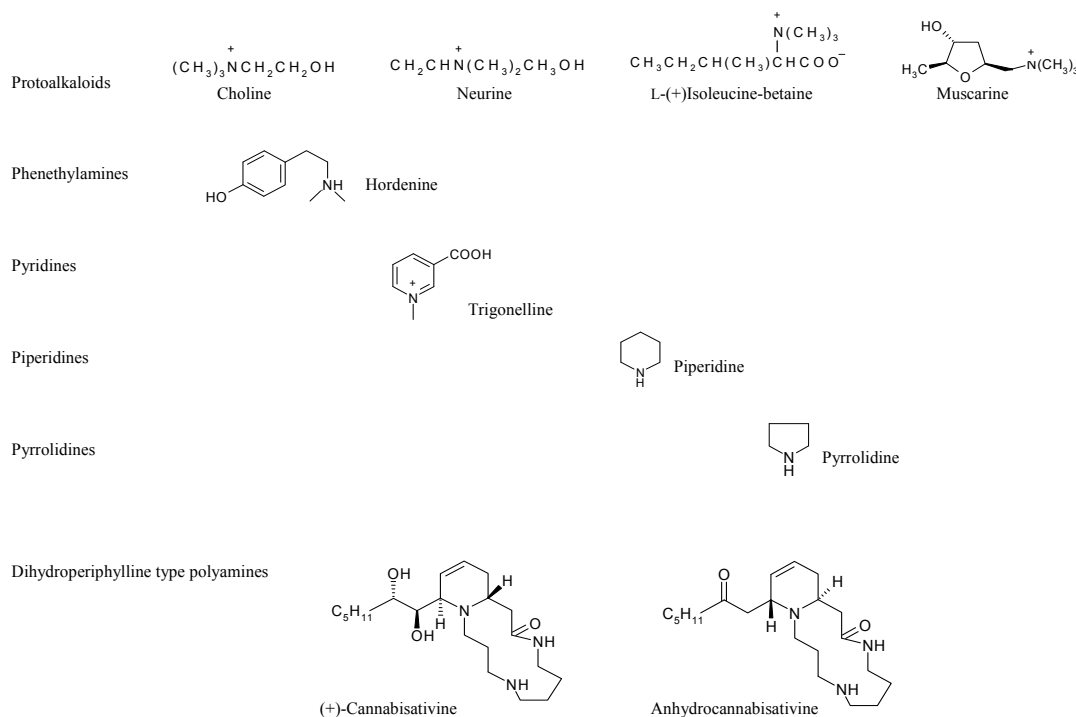


Figure 12. Alkaloids isolated from *C. sativa*.

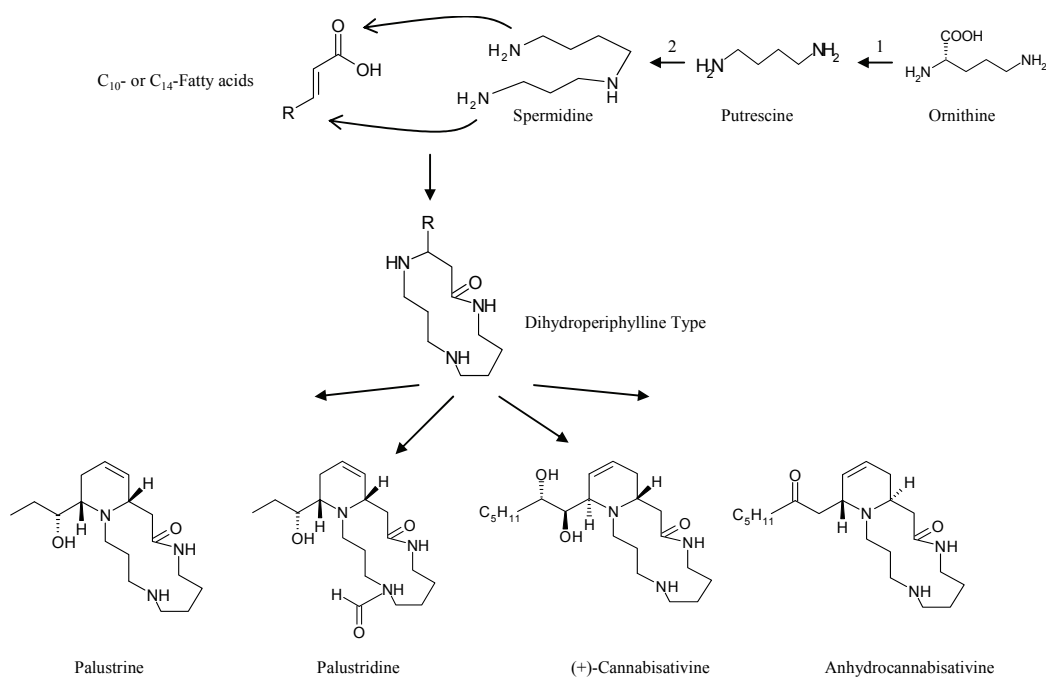


Figure 13. Spermidine alkaloids of the dihydroperiphylline type. 1) Ornithine decarboxylase, 2) Spermidine synthase.

I.2.5.1 Alkaloid biosynthesis

Kabarity *et al.* (1980) reported induction of C-tumors (tumor induced by colchicine) and polyploidy on roots of bulbs from *Allium cepa* by polar fractions from cannabis. It is known that hordenine is a feeding repellent for grasshoppers (Southon and Backingham, 1989) and its presence in cannabis plants could suggest a similar role. The decarboxylation of tyrosine gives tyramine, which on di-*N*-methylation yields hordenine (Brady and Tyler, 1958; Dewick, 2002). Trigonelline is found widely in plants and it has been suggested that it participates in the pyridine nucleotide cycle which supplies the cofactor NAD. Trigonelline is synthesized from the nicotinic acid formed in the pyridine nucleotide cycle (Zheng *et al.*, 2004). Choline is an important metabolite in plants because it is the precursor of the membrane phospholipid phosphatidylcholine (Rhodes and Hanson, 1993) and is biosynthesized from ethanolamine, for which the precursor is the amino acid serine (McNeil *et al.*, 2000). Piperidine originates from lysine and pyrrolidine from ornithine (Dewick, 2002). The structures of cannabisativine and anhydrocannabisativine are similar

to the alkaloids palustrine and palustridine from several *Equisetum* species (Figure 13). A common initial step in biosynthesis of the ring has been proposed starting with an enantioselective addition of the amine from the spermidine to an α,β -unsaturated fatty acid (Schultz *et al.*, 1997). However, there are no studies about the biosynthesis and biological functions of cannabissativine and anhydrocannabissativine. It is known that spermidine is biosynthesized from putrescine, which comes from ornithine (Tabor *et al.*, 1958; Dewick, 2002). In the therapeutic field, Bercht *et al.* (1973) did not find analgesic, hypothermal, rotating rod and toxicity effects on mice by isoleucine betaine. Some other studies suggest pharmacological activities of smoke condensate and aqueous or crude extracts containing cannabis alkaloids (Johnson *et al.*, 1984; Klein and Rapoport, 1971). Due to the low alkaloid concentration in cannabis [the concentration of choline and neurine from dried roots is 0.01% (Turner and Mole, 1973), while THCA from bracts is 4.77% (Kimura and Okamoto, 1970)] chemical synthesis or biosynthesis could be options to have sufficient quantities of pure alkaloids for biological activity testing. New methods for synthesis for cannabissativine (Hamada, 2005; Kuethe and Comins, 2004) as well as the biosynthesis of choline and atropine by hairy root cultures of *C. sativa* (Wahby *et al.*, 2006) have been reported.

1.2.6 Lignanamides and phenolic amides

Cannabis fruits and roots (Sakakibara *et al.*, 1995) have yielded 11 compounds identified as phenolic amides and lignanamides. *N-trans*-coumaroyltyramine, *N-trans*-feruloyltyramine and *N-trans*-caffeoyltyramine are phenolic amides; while cannabisin-A, -B, -C, -D, -E, -F, -G and grossamide are lignanamides (Figure 14). The lignanamides belong to the lignan group (Bruneton, 1999b) and the cannabis lignanamides are classified as lignans of the Arylnaphthalene derivative type (Lewis and Davin, 1999; Ward, 1999).

The phenolic amides have cytotoxic (Chen *et al.*, 2006), anti-inflammatory (Kim *et al.*, 2003), antineoplastic (Ma *et al.*, 2004), cardiovascular (Yusuf *et al.*, 1992) and mild analgesic activity (Slatkin *et al.*, 1971). For the lignanamides grossamide, cannabisin-D and -G a cytotoxic activity was reported (Ma *et al.*, 2002). The presence and accumulation of phenolic amides in response to wounding and UV light suggests a chemical defense against predation in plants (Back *et al.*, 2001; Majak *et al.*, 2003). Furthermore, it has been suggested that

they have a role in the flowering process and the sexual organogenesis, in virus resistance (Martin-Tanguy, 1985; Ponchet *et al.*, 1982), as well as in healing and suberization process (Bernards, 2002; King and Calhoun, 2005). For the lignanamides cannabisin-B and -D a potent feeding deterrent activity was reported (Lajide *et al.*, 1995). It is known that lignans have insecticidal effects (Garcia and Azambuja, 2004).

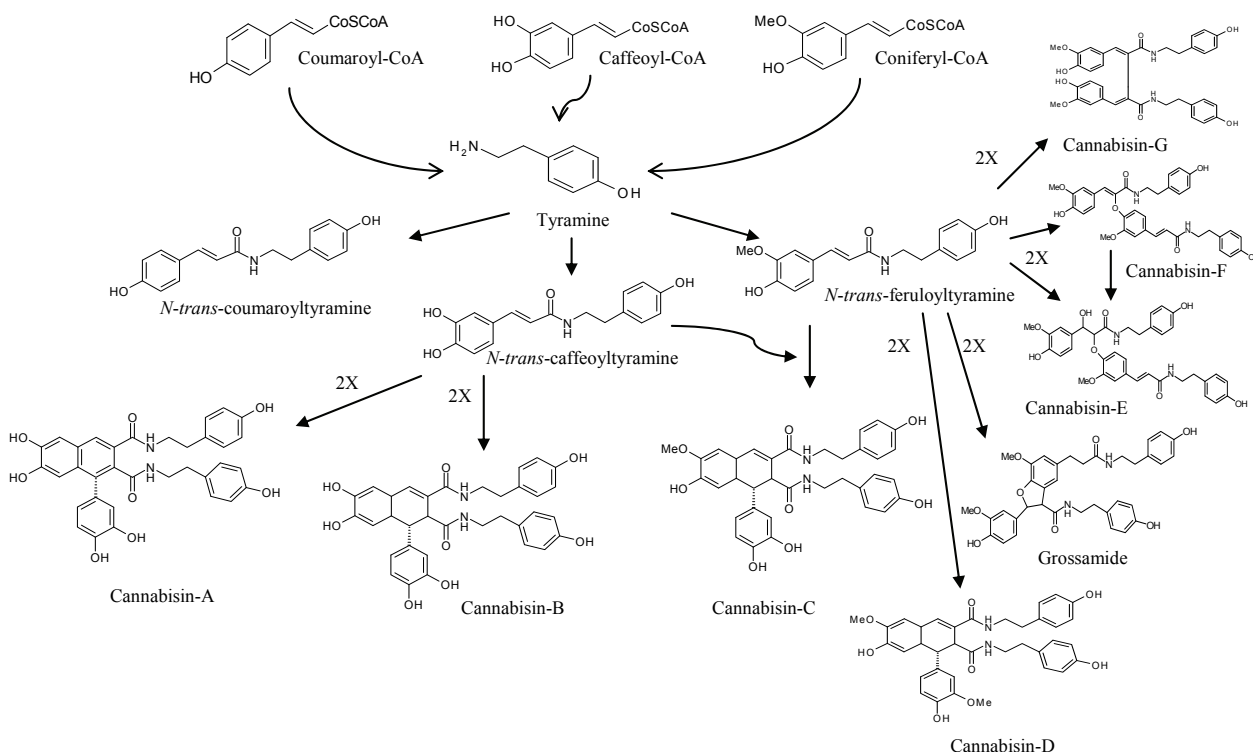


Figure 14. Proposed route for the biosynthesis of phenolic amides and lignanamides in cannabis plants.

I.2.6.1 Lignanamide and phenolic amide biosynthesis

The structures of the lignanamides and phenolic amides from cannabis suggest condensation and polymerization reactions in their biosynthesis starting from the precursors tyramine and CoA-esters of coumaric, caffeic and coniferic acid (Figure 14). It is known that the enzyme Hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase, E.C. 2.3.1.110 (THT) condenses hydroxycinnamoyl-CoA esters with tyramine (Hohlfeld *et al.*, 1996; Yu and Facchini, 1999). As it was mentioned previously, tyramine comes from tyrosine and the phenylpropanoids from phenylalanine. The amides *N-trans*-

feruloyltyramine and *N-trans*-caffeoyltyramine could be the monomeric intermediates in the biosynthesis of these lignanamides. It has been suggested that these lignanamides could be formed by a random coupling mechanism *in vivo* or they are just isolation artifacts (Ayres and Loike, 1999; Lewis and Davin, 1999); however, biosynthesis studies are necessary to elucidate their origin.

1.3 Conclusion

Cannabis sativa L. not only produces cannabinoids, but also other kinds of secondary metabolites which can be grouped into 5 classes. Little attention has been given to the pharmacology of these compounds. The isolation and identification of the cannabinoids, the identification of the endocannabinoids and their receptors, as well as their metabolism in humans have been extensively studied. However, the biosynthetic pathway of the cannabinoids and its regulation is not completely elucidated in the plant, the same applies for other secondary metabolite groups from cannabis. In three of the mentioned secondary metabolite groups (cannabinoids, flavonoids and stilbenoids), enzymes belonging to the polyketide synthase group could be involved in the biosynthesis of their initial precursors. Only one gene of CHS has so far been identified and more PKS genes are thought to be present for the flavonoid pathway as well as the stilbenoid and cannabinoid pathway. Cannabinoids are unique compounds only found in the cannabis. However, in *Helichrysum umbraculigerum* Less., a species from the family Compositae, the presence of CBGA, CBG and analogous to CBG was reported (Bohlmann and Hoffmann, 1979). Moreover, in liverworts from *Radula* species the isolation of geranylated bibenzyls analogous to CBG was reported (Asakawa *et al.*, 1982), suggesting homology of PKS and prenylase genes from the cannabinoid pathway in other species. Crombie *et al.* (1988) reported the chemical synthesis of bibenzyl cannabinoids.

Plants, including *C. sativa*, have developed intricate control mechanisms to be able to induce defense pathways when are required and to regulate secondary metabolite levels in the various tissues at specific stages of their life cycle. Figure 15 shows the currently known various secondary metabolite pathways in cannabis. Research on the secondary metabolism of *C. sativa* as well as its regulation will allow us to control or manipulate the production of the

important metabolites, as well as the biosynthesis of new compounds with potential therapeutic value.

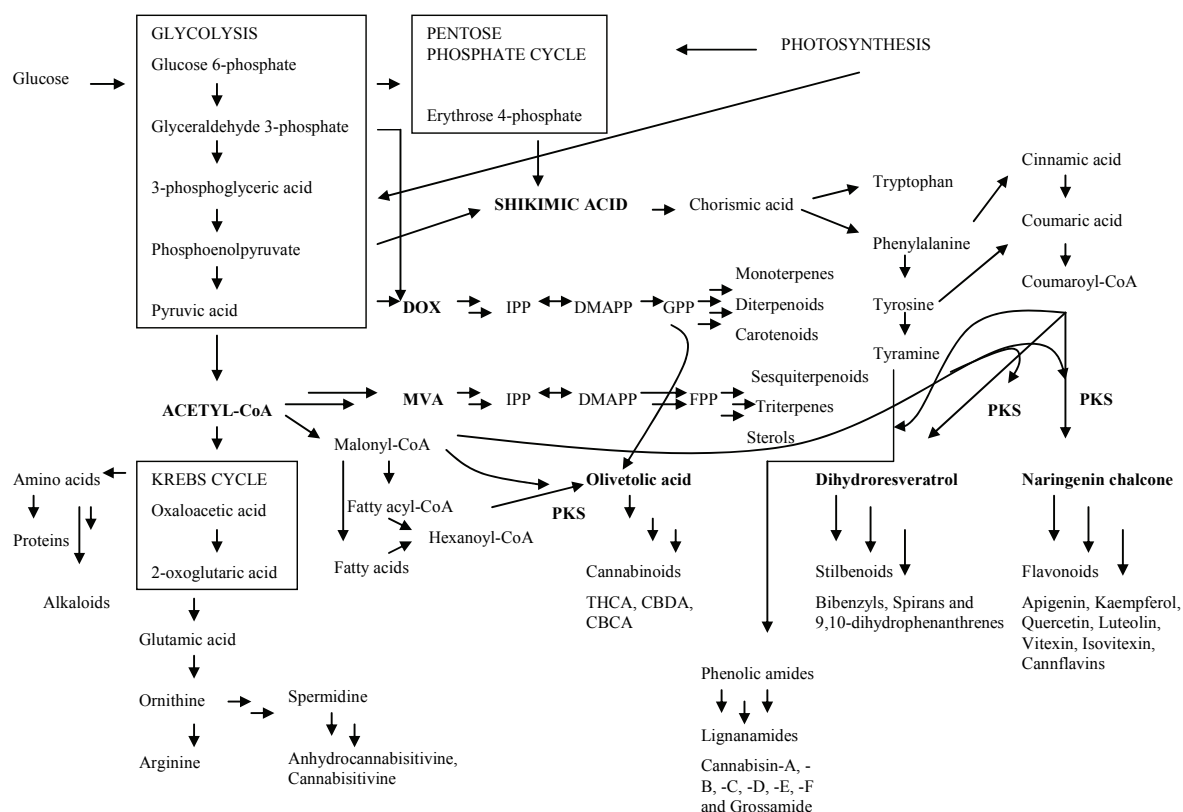


Figure 15. A general scheme of the primary and secondary metabolism in *C. sativa*. For a complete detail of proposed pathways of secondary metabolism see previous figures.

1.4 Outline of the thesis

The studies described in this thesis are focused on biochemical and molecular aspects of PKSs involved in the biosynthesis of precursors from cannabinoid, flavonoid or stilbenoid pathways. A review about general aspects of plant PKS is given in **Chapter 2**. Enzymatic activities of PKSs in plant cannabis tissues and a correlation with the content of cannabinoids and flavonoids is described in **Chapter 3**. Isolation of *PKS* mRNAs and an expression *in silico* are presented in **Chapter 4**. Finally, as cell cultures can be used as model systems to study secondary metabolite biosynthesis, cannabis cell suspension cultures were treated with biotic and abiotic elicitors to evaluate their effect on the cannabinoid biosynthesis (**Chapter 5**).

Chapter II

Plant Polyketide Synthases

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Abstract:

The Polyketide Synthases (PKSs) are condensing enzymes which form a myriad of polyketide compounds. In plants several PKSs have been identified and studied. This mini-review summarizes what is known about plant PKSs, and some aspects such as specificity, reaction mechanisms, structure, as well as their possible evolution are highlighted.

II.1 Introduction

The polyketide natural products are one of the largest and most diverse groups of secondary metabolites. They are formed by a myriad of different organisms from prokaryotes to eukaryotes. Antibiotics and mycotoxins produced by fungi and actinomycetes, and stilbenoids and flavonoids produced by plants are examples of polyketide compounds. They have an important role in medicine, due to their activities such as antimicrobial, antiparasitic, antineoplastic and immunosuppressive (Rawlings, 1999; Sankawa, 1999; Whiting, 2001).

II.2 Polyketide Synthases

The Polyketide Synthases (PKSs) are a group of enzymes that catalyzes the condensation of CoA-esters of acetic acid and other acids to give polyketide compounds. They are classified according to their architectural configurations as type I, II and III (Hopwood and Herman, 1990; Staunton and Weissman, 2001; Fischbach and Walsh, 2006). The type I describes a system of one or more multifunctional proteins that contain a different active site for each enzyme-catalyzed reaction in polyketide carbon chain assembly and modification. They are organized into modules, containing at least acyltransferase (AT), acyl carrier protein (ACP) and β -keto acyl synthase (β -KS) activities. Type I PKSs are sub-grouped as iterative or modular; usually present in fungal or bacterial systems, respectively (Moore and Hopke, 2001; Moss *et al.*, 2004). The type II is a system of individual enzymes that carry a single set of iteratively acting activities and a minimal set consists of two ketosynthase units (α - and β -KS) and an ACP, which serves as an anchor for the growing polyketide chain. Additional PKS subunits such as ketoreductases, cyclases or aromatases define the folding pattern of the polyketo intermediate and further post-PKS modifications, such as oxidations, reductions or glycosylations are added to the polyketide (Rix *et al.*, 2002; Hertweck *et al.*, 2007). The only known group of organism that employs type II PKS systems for polyketide biosynthesis is soil-borne and marine Gram-positive actinomycetes. The type III is present in bacteria, plants and fungi (Austin and Noel, 2003; Seshime *et al.*, 2005; Funa *et al.*, 2007); they are essentially condensing enzymes that lack ACP and act directly on acyl-CoA substrates.

II.3 Plant Polyketide Synthases

In plants several type III PKSs have been found and all of them participate in the biosynthesis of secondary metabolites (Table 1 and Figure 1); chalcone synthase (CHS), 2-pyrone synthase (2-PS), stilbene synthase (STS), bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS), acridone synthase (ACS), benzophenone synthase (BPS), phlorisovalerophenone synthase (VPS), isobutyrophenone synthase (BUS), coumaroyl triacetic acid synthase (CTAS), benzalacetone synthase (BAS), *C*-methyl chalcone synthase (PstrCHS2), anther-specific chalcone synthase-like (ASCL) and stilbene carboxylate synthase (STCS) are some examples from this group (Atanassov *et al.*, 1998; Austin and Noel, 2003; Eckermann *et al.*, 2003; Klingauf *et al.*, 2005; Wu *et al.*, 2008). As CHS and STS are the most studied enzymes, this group is often called the family of the CHS/STS type. It is known that plant PKSs share 44–95% amino acid sequence identity and utilize a variety of different substrates ranging from aliphatic-CoA to aromatic-CoA substrates, from small (acetyl-CoA) to bulky (*p*-coumaroyl-CoA) substrates or from polar (malonyl-CoA) to nonpolar (isovaleroyl-CoA) substrates giving to the plants an extraordinary functional diversification.

Table I. Examples of type III polyketide synthases, preferred substrates and reaction products.

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
Plant:				
<i>None cyclization reaction</i>				
Benzalacetone synthase (BAS), EC 2.3.1.-	<i>p</i> -coumaroyl-CoA, Malonyl-CoA (1X)		Benzalacetone (1)	Borejsza-Wysocki and Hrazdina, 1996; Abe <i>et al.</i> , 2001; Zheng and Hrazdina, 2008
	Feruloyl-CoA, Malonyl-CoA (1X)		Methoxy-benzalacetone (12)	
<i>One cyclization reaction</i>				
Benzalacetone synthase (BAS), EC 2.3.1.-	<i>N</i> -methylanthraniloyl-CoA (or anthraniloyl-CoA), Malonyl-CoA (or methyl-malonyl-CoA) (1X)	-, heterocyclic	4-hydroxy-2(1 <i>H</i>)quinolones (3)	Abe <i>et al.</i> , 2006a
<u>CTAS type</u>		Lactonization, heterocyclic		
C-methylchalcone synthase (PstrCHS2)	Diketide-CoA, Methyl-malonyl-CoA (1X)		Methyl-pyrone (4)	Schröder <i>et al.</i> , 1998
Styrylpyrone synthase (SPS) or Bismoryangonin synthase (BNS)	<i>p</i> -coumaroyl-CoA, Malonyl-CoA (2X) Caffeoyl-CoA, Malonyl-CoA (2X)		Bismoryangonin (5) Hispidin (6)	Beckert <i>et al.</i> , 1997; Herderich <i>et al.</i> , 1997; Schröder Group
2-pyrone synthase (2-PS)	Acetyl-CoA, Malonyl-CoA (2X)		Triacetic acid lactone (TAL) (7)	Eckermann <i>et al.</i> , 1998
<i>p</i> -Coumaroyltriatic acid synthase (CTAS)	<i>p</i> -coumaroyl-CoA, Malonyl-CoA (3X)		<i>p</i> -coumaroyltriatic acid lactone (8)	Akiyama <i>et al.</i> , 1999
<u>CHS type</u>		Claisen, aromatic		
Chalcone synthase (CHS), EC 2.3.1.74	<i>p</i> -coumaroyl-CoA, Malonyl-CoA (3X)		Naringenin chalcone (9)	Whitehead and Dixon, 1983; Ferrer <i>et al.</i> , 1999
Phlorisovalerophenone synthase (VPS), EC 2.3.1.156	Isovaleroyl-CoA, Malonyl-CoA (3)		Phlorisovalerophenone (10)	Paniego <i>et al.</i> , 1999; Okada and Ito, 2001

Table 1. *Continued.*

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
Isobutyrophenone synthase (BUS)	Isobutyryl-CoA, Malonyl-CoA (3X)		Phlorisobutyrophenone (11)	Klingauf <i>et al.</i> , 2005
Benzophenone synthase (BPS), EC 2.3.1.151	<i>m</i> -hydroxybenzoyl-CoA, Malonyl-CoA (3X)		2,3',4,6-tetrahydroxybenzophenone (12)	Beerhues, 1996
	Benzoyl-CoA, Malonyl-CoA (3X)		2,4,6-trihydroxybenzophenone (13)	Liu <i>et al.</i> , 2003
Acridone synthase, EC 2.3.1.159 (ACS)	<i>N</i> -methylantraniloyl-CoA, Malonyl-CoA (3X)		1,3-dihydroxy- <i>N</i> -methylacridone (14)	Junghanns <i>et al.</i> , 1998; Springo <i>et al.</i> , 2000
Homoeiodictyol/eriodictyol synthase (HEDS or HvCHS)	Feruloyl-CoA, Malonyl-CoA (3X) Caffeoyl-CoA, Malonyl-CoA (3X)		Homoeiodictyol (15) Eriodictyol (16)	Christensen <i>et al.</i> , 1998
STS type				
Stilbene synthase (STS), EC 2.3.1.95	<i>p</i> -coumaroyl-CoA, Malonyl-CoA (3X)	Aldol, aromatic	Resveratrol (17)	Schöppner and Kindl, 1984; Austin <i>et al.</i> , 2004a
Pinosylvin synthase, EC 2.3.1.146	Cinnamoyl-CoA, Malonyl-CoA (3X)		Pinosylvin (18)	Raiber <i>et al.</i> , 1995; Schanz <i>et al.</i> , 1992; Fliegmann <i>et al.</i> , 1992
Bibenzyl synthase (BBS)	Dihydro- <i>m</i> -coumaroyl-CoA, Malonyl-CoA (3X)		3,3',5-trihydroxybibenzyl (19)	Reinecke and Kindl, 1994; Preisig-Müller <i>et al.</i> , 1995
Biphenyl synthase (BIS)	Benzoyl-CoA, Malonyl-CoA (3X)		3,5-dihydroxybiphenyl (20)	Liu <i>et al.</i> , 2007
Stilbenecarboxylate synthase (STCS)	Dihydro- <i>p</i> -coumaroyl-CoA, Malonyl-CoA (3X)	Aldol without decarboxylation, aromatic	5-hydroxylunularic acid (21)	Eckermann <i>et al.</i> , 2003; Schröder Group

Table 1. Continued.

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
<i>More than 2 cyclization reactions</i>				
<u>Miscellaneous type</u>				
Pentaketide chromone synthase (PCS)	Acetyl-CoA, Malonyl-CoA (4X)	- , heterocyclic or aromatic	5,7-dihydroxy-2-methylchromone (22)	Abe <i>et al.</i> , 2005a
Hexaketide synthase (HKS)	Acetyl-CoA, Malonyl-CoA (5 X)		6-(2',4'-dihydroxy-6'-methyl-phenyl)-4-hydroxy-2-pyrone (23)	Springob <i>et al.</i> , 2007; Jindaprasert <i>et al.</i> , 2008
Aloesone synthase (ALS)	Acetyl-CoA, Malonyl-CoA (6X)		Aloesone (24)	Abe <i>et al.</i> , 2004a
Octaketide synthase (OKS)	Acetyl-CoA, Malonyl-CoA (7X)		SEK4 (25) and SEK4b (26) (octaketides)	Abe <i>et al.</i> , 2005b
<u>Bacteria</u>				
PKS18	Lauroyl-CoA, Malonyl-CoA (1X)	Pyrone type ring-folding	Lauroyl triketide pyrone (27), Lauroyl tetraketide pyrone (28)	Saxena <i>et al.</i> , 2003; Sankaranarayanan <i>et al.</i> , 2004
Monoacetylphloroglucinol synthase (PhID)	Malonyl-CoA (3X)	CHS type ring-folding	phloroglucinol (29)	Achkar <i>et al.</i> , 2005; Zha <i>et al.</i> , 2006
3,5-dihydroxyphenylacetate synthase (DHPAS), (DpgA)	Malonyl-CoA (4X)	STS type ring-folding	3,5-dihydroxyphenylacetic acid (30)	Li <i>et al.</i> , 2001; Pfeifer <i>et al.</i> , 2001
1,3,6,8-tetrahydroxynaphthalene synthase (THNS, RppA)	Malonyl-CoA (5X)	- , two cyclization reactions	1,3,6,8-tetrahydroxynaphthalene (31), THN	Funa <i>et al.</i> , 1999; Funa <i>et al.</i> , 2002
Fungi 2'-oxoalkylresorcylic acid synthase (ORAS)	Stearoyl-CoA, Malonyl-CoA (4X)	STS type ring-folding without decarboxylation	2,4-dihydroxy-6-(2'-oxononadecyl)-benzoic acid (32)	Funa <i>et al.</i> , 2007
-, undefined				

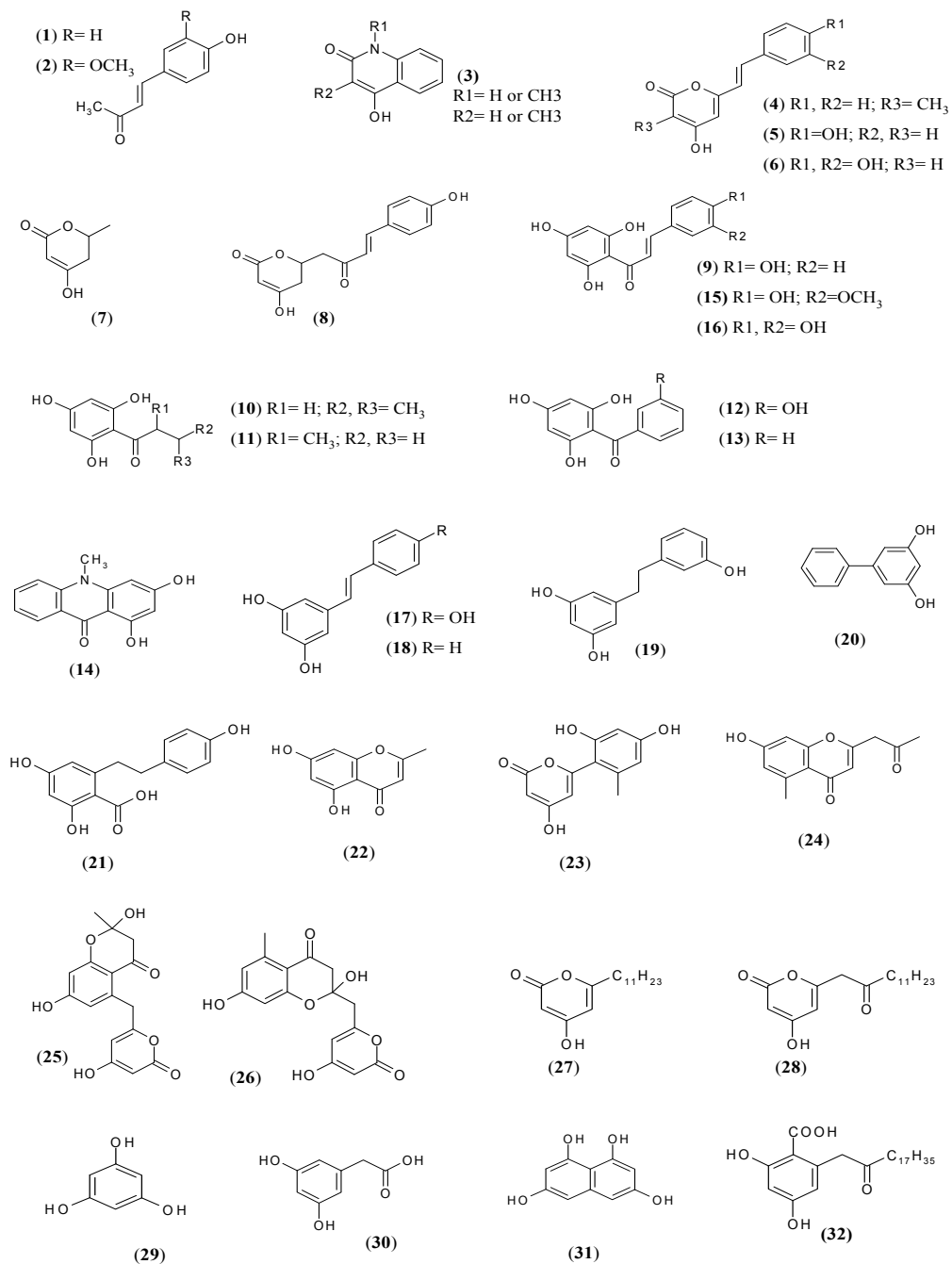


Figure 1. Some compounds biosynthesized by type III PKS.

II.3.1 Type of cyclization reaction

Divergences by the number of condensation reactions (polyketide chain elongation), the type of the cyclization reaction and the starter substrate are characteristic of the type III PKSs (Schröder, 2000). Based on the mechanism of the cyclization they are classified as CHS-, STS- and CTAS-type (Figure 2).

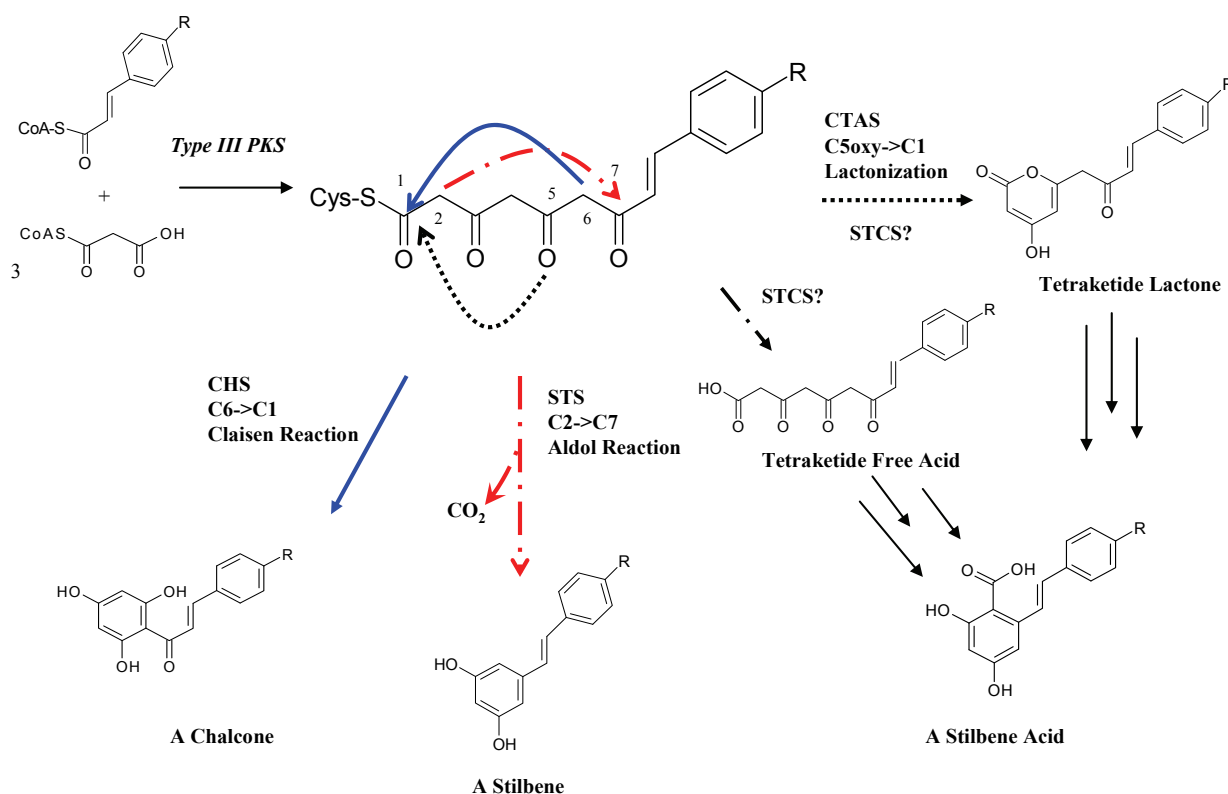


Figure 2. Type of cyclization by plant PKS. R, OH, H. Modified from Austin *et al.*, 2004a.

In the CHS-type the intramolecular cyclization from C6 to C1 is called Claisen condensation; this mechanism for the carbon-carbon bond formation is not only used for the biosynthesis of polyketides, but also for fatty acids (Heath and Rock, 2002). In the STS -type the cyclization is from C2 to C7, with an additional decarboxylative loss of the C1 as CO₂, this reaction is an Aldol type of condensation. In the CTAS-type there is a heterocyclic lactone formation

between oxygen from C5 to C1, called lactonization. Regarding the biosynthesis of stilbene carboxylic acids, Eckermann *et al.* (2003) reported the expression of a PKS with STCS activity from *Hydrangea macrophylla* L. and it was proposed to be an Aldol condensation without decarboxylation of the C1. The same group reported expression of STCSs in *Marchantia polymorpha* (Schröder Group). Although, the formation of the stilbenecarboxylate represented 40–45% of the product mixture pyrone formation was predominant. It has been suggested that the formation of a tetraketide free acid or lactone is the product of the STCS and undergoes spontaneous cyclization to yield the stilbenecarboxylate. Aromatization and reduction could be additional steps to stilbenecarboxylic acid formation (Akiyama *et al.*, 1999; Schröder Group). Some examples of metabolites which could be formed by a STCS-type PKS in *Cannabis sativa* (Fellermeier and Zenk, 1998; Fellermeier *et al.*, 2001), *Ginkgo biloba* (Adawadkar and ElSohly, 1981), liverworts species (Valio and Schwabe, 1970; Pryce, 1971), *Amorpha fruticosa* (Mitscher *et al.*, 1981), *Gaylussacia baccata* (Askari *et al.*, 1972), *Helichrysum umbraculigerum* (Bohlmann and Hoffmann, 1979), *Syzygium aromaticum* (Charles *et al.*, 1998) and *H. macrophylla* (Asahina and Asano, 1930; Gorham., 1977) are shown in figure 3. Together with the different types of cyclization mentioned above some PKSs only catalyze condensation reactions without a cyclization reaction. BAS, which has been isolated from raspberries and *Rheum palmatum* (Borejsza-Wysocki and Hrazdina, 1996; Abe *et al.*, 2001), catalyzes a single condensation of malonyl-CoA to *p*-coumaroyl-CoA starter to form *p*-hydroxybenzalacetone. In *Oryza sativa* curcuminoid synthase (CUS) condenses two *p*-coumaroyl-CoAs and one malonyl-CoA to form bisdemethoxycurcumin (Katsuyama *et al.*, 2007) and for the initial step in diarylheptanoid biosynthesis from *Wachendorfia thyrsiflora* a PKS was identified (Brand *et al.*, 2006).

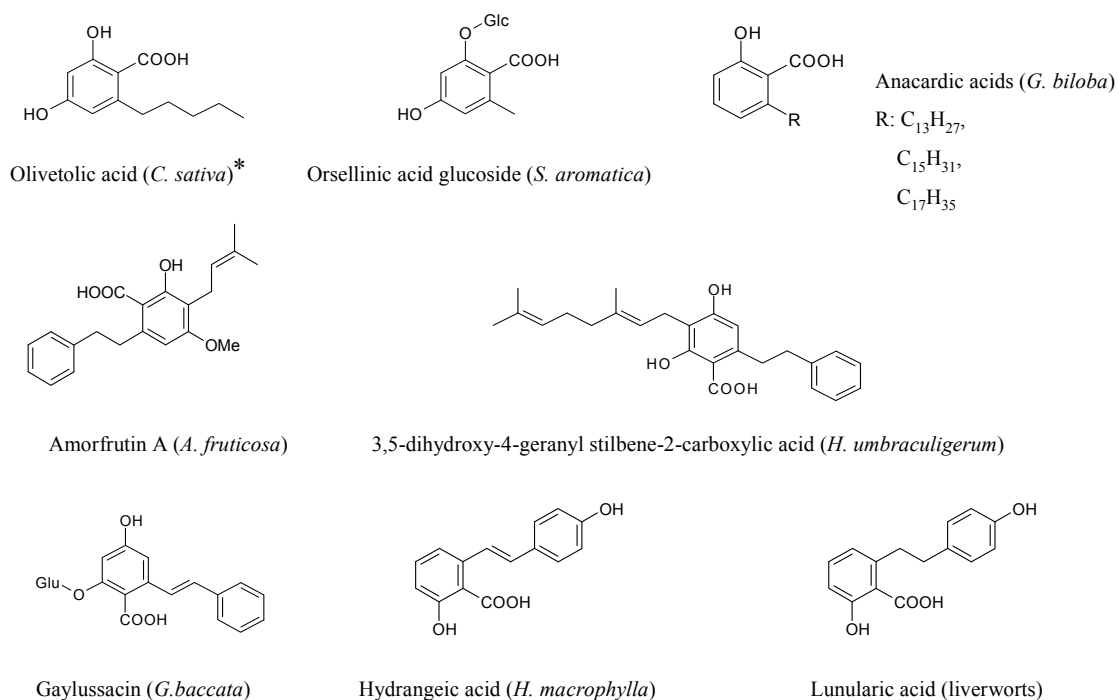


Figure 3. Some examples of alkyl-resorcinolic acids and stilbene carboxylic acids isolated from plants. * Putative intermediate of cannabinoid biosynthesis.

II.3.2 Structure and reaction mechanism

From data bases (NCBI) more than 859 nucleotide sequences have been reported from plant PKSs and several PKS crystalline structures have been characterized (Ferrer *et al.*, 1999; Austin *et al.*, 2004a; Shomura *et al.*, 2005; Jez *et al.*, 2000a; Schröder Group, PDB: 2p0u, MMDB: 45327; Morita *et al.*, 2007; Morita *et al.*, 2008), as well as bacterial type III PKSs (Austin *et al.*, 2004b; Sankaranarayanan *et al.*, 2004). There are no significant differences on the conformation of these crystalline structures, PKSs form a symmetric dimer displaying a $\alpha\beta\alpha\beta\alpha$ five-layered core and in each monomer an independent active site is present. Besides, that dimerization is required for activity and an allosteric cooperation type between the two active sites from the monomers was suggested (Tropf *et al.*, 1995). Furthermore, it was found that the Met 137 (numbering in *M. sativa* CHS) in each monomer helps to shape the active site cavity of the adjoining subunit (Ferrer *et al.*, 1999).

The basic principle of the reaction mechanism consists of the use of a starter CoA-ester to perform sequential condensation reactions with two Carbon units,

from a decarboxylated extender, usually malonyl-CoA. A linear polyketide intermediate is formed which is folded to form an aromatic ring system (Schröder, 1999). In particular, the active site is composed of a CoA-binding tunnel, a starter substrate-binding pocket and a cyclization pocket, and three residues conserved in all the known PKSs define this active site: Cys 164, His 303 and Asn 336. Each active site is buried within the monomer and the substrates enter *via* a long CoA-binding tunnel. The Cys 164 is the nucleophile that initiates the reaction and attacks the thioester carbonyl of the starter resulting in transfer of the starter moiety to the cysteine side chain. Asn336 orients the thioester carbonyl of malonyl-CoA near His303 with Phe215, providing a nonpolar environment for the terminal carboxylate that facilitates decarboxylation and a resonance of the enolate ion to the keto form allows for condensation of the acetyl carbanion with the enzyme-bound polyketide intermediate. Phe215 and Phe265 perform as gatekeepers (Austin and Noel, 2003). The recapture of the elongated starter-acetyl-diketide-CoA by Cys164 and the release of CoA set the stage for additional rounds of elongation, resulting in the formation of a final polyketide reaction intermediate. Later an intramolecular cyclization of the polyketide intermediate takes place (Abe, *et al.*, 2003a; Jez *et al.*, 2000b; Jez *et al.*, 2001a; Lanz *et al.*, 1991; Suh *et al.*, 2000). The GFGPG loop is a conserved region on plant PKSs that provides a scaffold for cyclization reactions (Austin and Noel, 2003; Suh *et al.*, 2000).

The remarkable functional diversity of the PKSs derives from small modifications in the active site, which greatly influence the selection of the substrate, number of polyketide chain extensions and the mechanism of cyclization reactions. The volume of the active site cavity influences the starter molecule selectivity and limits polyketide length. The 2-PS cavity is one third the size of the CHS cavity. The combination of three amino acids substitutions on Thr197Leu, Gly256Leu and Ser338Ile on CHS sequence changes the starter molecule preference from *p*-coumaroyl-CoA to acetyl-CoA and results in formation of a triketide instead of a tetraketide product (Jez *et al.*, 2000a). From homology modeling studies, it was found that the cavity volume of octaketide synthase (OKS) (Abe *et al.*, 2005b) and aloesone synthase (ALS) (Abe *et al.*, 2004a) is slightly larger than that of CHS; while that of pentaketide chromone synthase (PCS) is almost as large as of ALS (Abe *et al.*, 2005a). The replacing of the residues Ser132Thr, Ala133Ser and Val265Phe fully transformed the ACS to

a functional CHS (Lukacin *et al.*, 2001). The change from His166–Gln167 to Gln166–Gln167 converts the STS from *A. hypogaea* to a dihydropinosilvin synthase (Schröder and Schröder, 1992). It was shown that Gly256, which resides on the surface of the active site, is involved in the chain-length determination from CHS (Jez *et al.*, 2001b); while in ALS Gly256 determines starter substrate selectivity, Thr197 located at the entrance of the buried pocket controls polyketide chain length and Ser338 in proximity of the catalytic Cys164 guides the linear polyketide intermediate to extend into the pocket, leading to the formation of a heptaketide (Abe *et al.*, 2006b).

The cyclization specificities in the active site of CHS and STS are given by electronic effects of a water molecule rather than by steric factors (Austin *et al.*, 2004a). In BAS, the residue Ser338 is important in the steric guidance of the diketide formation reaction and probably BAS has an alternative pocket to lock the coumaroyl moiety for the diketide formation reaction (Abe *et al.*, 2007). Dana *et al.* (2006) analyzed mutant alleles of the *Arabidopsis thaliana* CHS locus by molecular modeling and found that changes in the amino acid sequence on regions not located at or near residues that are of known functional significance can affect the architecture, the dynamic movement of the enzyme, the interactions with others proteins, as well as have dramatic effects on enzyme function.

II.3.2.1 Specificity and byproducts

Probably in *vivo* PKSs are highly substrate-specific and product-specific, as they are confined to specific organelles, tissues or present in organized enzymatic complexes (metabolons). However, *in vitro* PKSs are not very substrate-specific and enzymatic reactions yield derailment byproducts together with the final product in a highly variable proportion. Benzalacetone, bisnoryangonin and *p*-coumaroyltriatic acid lactone are reaction byproducts from CHS, STS and STCS using *p*-coumaroyl-CoA as starter (Schröder Group). It is known that CHS (Morita *et al.*, 2000; Novak *et al.*, 2006; Raharjo *et al.*, 2004b; Schüz *et al.*, 1983; Springob *et al.*, 2000), STS (Samappito *et al.*, 2003; Zuurbier *et al.*, 1998) and VPS (Okada *et al.*, 2001; Paniego *et al.*, 1999) can use efficiently acetyl-CoA, cinnamoyl-CoA, caffeoyl-CoA, butyryl-CoA, isovaleryl-CoA, hexanoyl-CoA, benzoyl-CoA and phenylacetyl-CoA as starter substrates; moreover, it has been found that CHS (Abe *et al.*, 2003b), OKS (Abe

et al., 2006c), STS and BAS (Abe *et al.*, 2002) could use methylmalonyl-CoA as extender substrate. Morita *et al.* (2001) reported the biosynthesis of novel polyketides by a STS using halogenated starter substrates of cinnamoyl-CoA and *p*-coumaroyl-CoA, as well as analogs in which the coumaroyl moiety was replaced by furan or thiophene. The formation of long-chain polyketide pyrones by CHS and STS using CoA esters of C₆-, C₈-, C₁₀-, C₁₂-, C₁₄-, C₁₆-, C₁₈-, and C₂₀- fatty acids has been demonstrated (Abe *et al.*, 2005c; Abe *et al.*, 2004b). Recently, a type III PKS from *Huperzia serrata* with a versatile enzymatic activity was reported (Wanibuchi *et al.*, 2007). This PKS can accept from aromatic to aliphatic CoA as starter substrates, including the bulky starter substrates *p*-methoxycinnamoyl-CoA and *N*-methylantraniloyl-CoA to produce chalcones, benzophenones, phloroglucinols, pyrones and acridones. It was suggested that this enzyme possesses a larger starter substrate-binding pocket at the active site, giving a substrate multiple capacity. The crystallization of this PKS was also reported (Morita *et al.*, 2007).

II.3.2.2 Homology and Evolution

Type III PKSs have around 400 amino acid long polypeptide chains (41–44 kDa) and share from 44 to 95% sequence identity. The PKS reactions share many similarities with the condensing activities in the biosynthesis of fatty acids in plants and microorganisms as well as of microbial polyketides. It has been recognized that all three types of PKSs likely evolved from fatty acid synthases (FASs) of primary metabolism (Austin and Noel, 2003; Schröder, 1999). All PKSs, like their FASs ancestors, possess a β -KS activity that catalyzes the sequential head-to-tail incorporation of two-carbon acetate units into a growing polyketide chain; while FAS performs reduction and dehydration reactions on each resulting β -keto carbon to produce an inert hydrocarbon, PKS omits or modifies some of these latter reactions, thus preserving varying degrees of polar chemical reactivity along portions of the growing linear polyketide chain. The use of CoA-ester rather than of ACP-ester is a long line of evolution that separates type III PKSs from the other PKSs. It has been suggested that STS, 2-PS and CHS isoforms have evolved from CHS by duplication and mutation (Durbin *et al.*, 2000; Eckermann *et al.*, 1998; Helariutta *et al.*, 1996; Lukacin *et al.*, 2001; Tropf *et al.*, 1994). Several phylogenetic analyses (Abe *et al.*, 2001; Abe *et al.*, 2005c; Liu *et al.*, 2003;

Springob *et al.*, 2007; Wanibuchi *et al.*, 2007) have revealed that the CHS/STS type family is grouped into subfamilies according to their enzymatic function. Hypothesis about evolution of the plant PKSs and its ecological role in the biosynthesis of secondary metabolites have been suggested (Moore and Hopke, 2001; Seshime *et al.*, 2005; Jenke-Kodama *et al.*, 2008).

II.4. Concluding remarks

The type III PKSs appears widespread in fungi and bacteria, as well as in plants. Enormous progress has been made in understanding the reaction mechanism of type III PKSs, several crystalline structures have been identified and some reaction mechanisms, e.g. CHS and STS, have been deciphered; however, from others, like STCS, it is still unclear. Systems, such as microorganism (Beekwilder *et al.*, 2006; Katsuyama *et al.*, 2007; Watts *et al.*, 2004; Watts *et al.*, 2006; Xie *et al.*, 2006), mammal cells (Zhang *et al.*, 2006) and plants (Schijlen *et al.*, 2006), for the production of plant polyketides have been developed. Improvement of plant microbial resistance (Hipskind and Paiva, 2000; Hui *et al.*, 2000; Serazetdinova *et al.*, 2005; Stark-Lorenzen *et al.*, 1997; Szankowski *et al.*, 2003), quality of crops (Husken *et al.*, 2005; Kobayashi *et al.*, 2000; Morelli *et al.*, 2006; Ruhmann *et al.*, 2006) or sometimes to give plant specific traits such as color (Aida *et al.*, 2000; Courtney-Gutterson *et al.*, 1994; Deroles *et al.*, 1998; Elomma *et al.*, 1993; van der Krol *et al.*, 1988) or sterility (Fischer *et al.*, 1997; Höfig *et al.*, 2006; Taylor and Jorgensen, 1992) are also reported by expression or antisense expression from plant PKSs. Further (novel) polyketides will be produced in the future as well as more PKSs and polyketides will be discovered in nature (Wilkinson and Micklefield, 2007).

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Chapter III

Polyketide synthase activities and biosynthesis of cannabinoids and flavonoids in *Cannabis sativa* L. plants.

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Abstract

Polyketide synthase (PKS) enzymatic activities were analyzed in crude protein extracts from cannabis plant tissues. Chalcone synthase (CHS, EC 2.3.1.74), stilbene synthase (STS, EC 2.3.1.95), phlorisovalerophenone synthase (VPS, EC 2.3.1.156), isobutyrophenone synthase (BUS) and olivetol synthase activities were detected during the development and growth of glandular trichomes on bracts. Cannabinoid biosynthesis and accumulation take place in these glandular trichomes. In the biosynthesis of the first precursor of cannabinoids, olivetolic acid, a PKS could be involved; however, no activity for an olivetolic acid-forming PKS was detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant, from plant tissues revealed differences in their distribution, suggesting a diverse regulatory control on these biosynthetic fluxes in the plant.

III.1 Introduction

Cannabis sativa L. is an annual dioecious plant from Central Asia. Cannabinoids are the best known group of natural products in *C. sativa* and 70 of these have been found so far (ElSohly and Slade, 2005). Several therapeutic effects of cannabinoids have been reported (reviewed in Williamson and Evans, 2000) and the discovery of an endocannabinoid system in mammals marks a renewed interest in these compounds (Di Marzo and De Petrocellis, 2006; Di Marzo *et al.*, 2007). The cannabinoid biosynthetic pathway has been partially elucidated (Figure 1). It is known that the geranyl diphosphate (GPP) and the olivetolic acid are initial precursors, which are derived from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fellermeier *et al.*, 2001) and from the polyketide pathway (Shoyama *et al.*, 1975), respectively. These precursors are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (Fellermeier and Zenk, 1998) to yield CBGA; which is further oxido-cyclized into CBDA, Δ^9 -THCA and CBCA (Morimoto *et al.*, 1999) by the enzymes cannabidiolic acid synthase (Taura *et al.*, 2007b), Δ^9 -tetrahydrocannabinolic acid synthase (Sirikantaramas *et al.*, 2004) and cannabichromenic acid synthase (Morimoto *et al.*, 1998), respectively. On the other hand, the first step leading to olivetolic acid, an alkylresorcinolic acid, is less known and it has been proposed that a polyketide synthase (PKS) could be involved in its biosynthesis. Raharjo *et al.* (2004a) found *in vitro* enzymatic activity for a PKS from leaves and flowers, though yielding olivetol and not the olivetolic acid as the reaction product. Olivetolic acid is the active form for the next biosynthetic reaction step of the cannabinoids. Later, a *PKS* mRNA was detected from leaves, which expressed activity for the PKSs chalcone synthase (CHS), phlorisovalerophenone synthase (VPS) and isobutyrophenone synthase (BUS), but not for the formation of olivetolic acid (Raharjo *et al.*, 2004b).

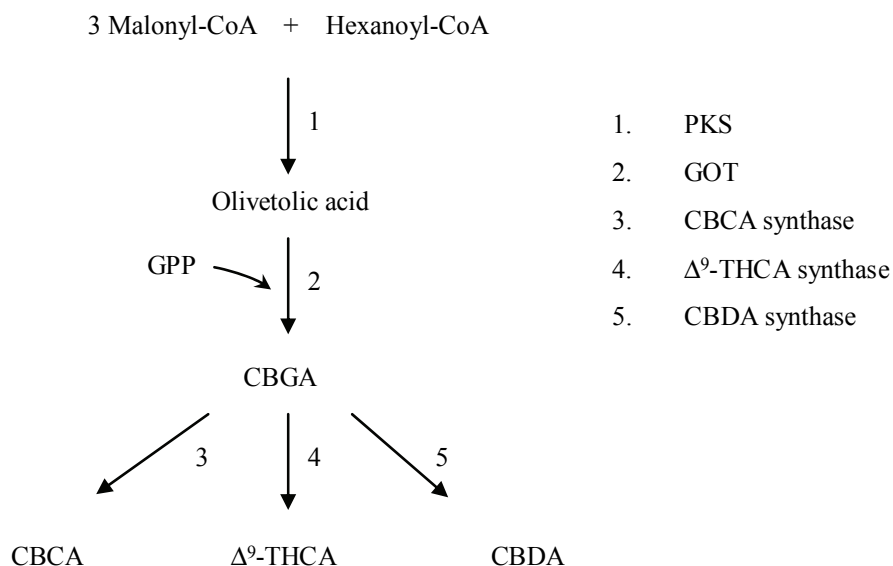


Figure 1. General pathway for biosynthesis of cannabinoids. PKS, polyketide synthase; GPP, geranyl diphosphate; GOT, geranyl diphosphate:olivetolate geranyltransferase; CBGA, cannabigerolic acid; Δ⁹-THCA, Δ⁹-Tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; CBCA, cannabichromenic acid.

PKSs are a group of condensing enzymes that catalyzes the initial key reactions in the biosynthesis of a myriad of secondary metabolites (Schröder, 1997). In plants several PKSs have been found, which participate in the biosynthesis of compounds from the secondary metabolism. CHS, STS, VPS, BUS, bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS) and stilbene carboxylate synthase (STSC) are some examples from type III PKSs as they have been classified (Austin and Noel, 2003; Eckermann *et al.*, 2003; Klingauf *et al.*, 2005; Chapter II). Type III PKSs use a variety of thioesters of coenzyme A as substrates from aliphatic-CoA to aromatic-CoA, from small (acetyl-CoA) to bulky (*p*-coumaroyl-CoA) or from polar (malonyl-CoA) to nonpolar (isovaleryl-CoA). For example, CHS (Kreuzaler and Hahlbrock, 1972) and STS (Rupprich and Kindl, 1978) condense one molecule of *p*-coumaroyl-CoA with 3 molecules of malonyl-CoA forming naringenin-chalcone and resveratrol, respectively. VPS (Paniego *et al.*, 1999) and biphenyl synthase (Liu *et al.*, 2007) uses isovaleryl-CoA and benzoyl-CoA, respectively, as starter substrates instead of *p*-coumaroyl-CoA.

Here, we report the PKS enzymatic activities found in different tissues of cannabis plants and show a correlation between the production of polyketide derived secondary metabolites and the activity of these PKSs in the plant.

III.2 Materials and methods

III.2.1 Plant material

Seeds of *Cannabis sativa*, variety Skunk (The Sensi Seed Bank, Amsterdam, The Netherlands), were germinated and 9 day-old seedlings were planted in 11 LC pots with soil (substrate 45 L, Holland Potgrond, Van der Knaap Group, Kwintsheul, The Netherlands) and maintained under a light intensity of 1930 lux, at 26 °C and 60% relative humidity (RH). After 3 weeks the small plants were transplanted into 10 L pots 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). Young leaves from 13 week-old plants, female flowers in different stages of development and male flowers from 4 month-old plants were harvested. Three month-old male plants were used for pollination of female plants. The fruits were harvested 18 days after pollination. Roots from 4 month-old female plants were harvested and washed with cold water to remove residual soil. All vegetal material was weighed and stored at -80 °C.

III.2.2 Chemicals

Benzoyl-CoA, hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, malonyl-CoA, resveratrol, naringenin and 2,4-dihydroxy-benzoic acid were obtained from Sigma (St. Louis, MO, USA). Olivetol was acquired from Aldrich Chem (Milwaukee, WI, USA) and 4-hydroxybenzylidenacetone (PHBA) from Alfa Aesar (Karlsruhe, Germany). Orcinolic acid (orsellinic acid) was from AApin Chemicals Ltd (Abingdon, UK) and resorcinol (1,3-dihydroxy-benzene) from Merck Schuchardt OHG (München, Germany). *p*-Coumaroyl-CoA was synthesized according to Stöckigt and Zenk (1975), and phlorisovalerophenone (PiVP) and phlorisobutyrophenone (PiBP) were previously synthesized in our laboratory (Fung *et al.*, 1994). Olivetolic acid was obtained from hydrolysis of methyl olivetolate (Horper and Marner, 1996) and methyl olivetolate was a gift from Prof. Dr. J. Tappey (Virginia Military Institute, USA). The cannabinoids Δ^9 -THCA,

CBGA, Δ^9 -THC, Δ^8 -THC, CBG, CBD and CBN were isolated from plant materials previously in our laboratory (Hazekamp *et al.*, 2004). Δ^9 -THVA was identified based on its relative retention time and UV spectra (Hazekamp *et al.*, 2005) and its quantification was relative to Δ^9 -THCA. The flavonoids kaempferol, orientin and luteolin were purchased from Extrasynthese (Genay, France), and vitexin, isovitexin and apigenin from Sigma-Aldrich (Buchs, Switzerland). Quercetin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were from our standard collection. All chemical products and mineral salts were of analytical grade.

III.2.3 Protein extracts

Frozen plant material was homogenized in a mortar with nitrogen liquid, the powder was thawed in polyvinylpolypyrrolidone (PVPP) and extraction buffer (0.1 M potassium phosphate buffer, pH 7, 0.5 M sucrose, 3 mM EDTA, 10 mM DTT and 0.1 mM leupeptin), squeezed through Miracloth and centrifuged at 14,000 rpm for 20 min. Per each gram of fresh weight, 0.1 g of PVPP and 2 ml of extraction buffer were used. The crude protein extracts were desalted using Sephadex G-25 M (PD-10) columns, eluted with same extraction buffer without addition of leupeptin. All steps were performed at 4 °C.

III.2.4 Polyketide synthase assays

Polyketide synthase activity was measured by the conversion of starter CoA esters and malonyl-CoA into reaction products.

The standard reaction mixture, in a final volume of 500 μ l, contained 50 mM K-Pi buffer (pH 7), 20 μ M starter-CoA, 40 μ M malonyl-CoA 0.5 M sucrose and 1 mM DTT. The reaction was initiated by addition of 250 μ l of desalted crude protein extracts (100–440 μ g of protein) and was incubated for 90 min at 30 °C. Reactions were stopped by addition of 20 μ l of 4N HCl then extracted twice with 800 μ l of ethyl acetate and centrifuged for 2 min. The combined organic phases were evaporated in vacuum centrifuge and the residue was kept at 4 °C. Samples were resuspended in 100 μ l and in 40 μ l MeOH for analysis by HPLC and LC/MS, respectively.

VPS was isolated previously in our laboratory (Paniego *et al.*, 1999), and CHS and STS were a gift from Prof. Dr. J. Schröder (Freiburg University, Germany).

III.2.5 Protein determination

Protein concentration was measured as described by Peterson (1977) with bovine serum albumin as standard.

III.2.6 HPLC analysis

The system consisted of 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 (250 x 4.6 mm, Inertsil ODS-3, GL Sciences, Tokyo, Japan). 80 µl of sample was injected, the gradient solvent system consisted of MeOH and Water, both containing 0.1% TFA: Method 1) 0–40 min, 20–80% MeOH; 40–43 min, 80% MeOH; 43–48 min, 80–20% MeOH; 40–50 min, 20% MeOH. Method 2) 0–30 min, 40–60% MeOH; 30–33 min, 60% MeOH; 35–38 min, 60–40% MeOH; 38–40 min 40% MeOH. Method 3) 0–40 min, 40–60% MeOH; 40–43 min, 60% MeOH; 43–44 min, 40–60% MeOH; 44–45 min 40% MeOH. Method 4) 0–40 min, 50–100% MeOH; 40–43 min, 100% MeOH; 43–44 min, 100–50% MeOH; 44–45 min, 50% MeOH. Method 5) 0–20min, 50–80% MeOH; 20–30min, 80% MeOH; 30–35 min, 80–50% MeOH; 35–40 min, 50% MeOH. Flow rate was 1 ml/min at 25 °C; olivetol, methyl olivetolate, olivetolic acid, PiVP, PiBP, naringenin and resveratrol were detected at 280 nm, orcinolic acid at 260 nm, orcinol at 273 nm and 2,4-dihydroxy-benzoic acid at 256 nm. PHBA was detected at 320 nm. Calibration curves with the respective standards were made.

III.2.7 LC–MS analysis

For the confirmation of the identity of enzymatic products, 20 µl of samples were analyzed in an Agilent 1100 Series LC/MS system (Agilent Technologies, Palo Alto, CA, USA) with positive/negative atmospheric pressure chemical ionization (APCI), using elution system method 5 with a flow rate of 0.5 ml/min. The optimum APCI conditions included a N₂ nebulizer pressure of 45 psi, a vaporizer temperature of 400 °C, a N₂ drying gas temperature of 350 °C at 10 L/min, a capillary voltage of 4000 V, a corona current of 4 µA, and a fragmentor voltage of 100 V. A reversed-phase C18 column (150 x4.6 mm, 5 µm, Zorbax Eclipse XDB-C18, Agilent) was used.

III.2.8 Extraction of compounds

Extraction was carried out as described by Choi *et al.* (2004) with slight modifications. To 0.1 g of lyophilized and ground plant material was added 4 ml MeOH:H₂O (1:1, v/v) and 4 ml CHCl₃, vortexed for 30 s and sonicated for 10 min. The mixtures were centrifuged in cold at 3000 rpm for 20 min. The MeOH:H₂O and CHCl₃ fractions were separated and evaporated. The extraction was performed twice. The extracts were resuspended on 1 ml of MeOH:H₂O (1:1) and CHCl₃, respectively; for the subsequent cannabinoid and flavonoid analyses.

III.2.9 Cannabinoid analysis by HPLC

The column used was a Grace Vydac (WR Grace, Columbia, MD, USA) C₁₈ (250x4.6 mm MASS SPEC 218MS54, 5 µm) with a Waters Bondapak C₁₈ guard column (2x20 mm, 50 µm). The solvent system and the operational conditions were the same as previously reported by Hazekamp *et al.* (2004). For preparation of samples, 100 µl of the CHCl₃ fraction from extraction was evaporated using N₂ gas. The samples were dissolved in 1 ml of EtOH and 20 µl was injected in the HPLC system. Cannabinoids were detected at 228 nm. Calibration curves with their respective standards were made.

III.2.11 Flavonoid analysis by HPLC

A reversed-phase C₁₈ column (250 x4.6 mm, Inertsil ODS-3) was used. The solvent system and the operational conditions were as described by Justesen *et al.* (1998) with slight modifications. The mobile phase consisted of MeOH:Water (30:70, v/v) with 0.1% TFA (A) and MeOH with 0.1% TFA (B). The gradient was 25–86% B in 40 min followed by 86% B for 5 min and a gradient step from 86–25% B for 5 min at a flow-rate of 1 ml/min and at 25 °C. Twenty µl of resuspended hydrolyzed samples was injected. Retention times for aglycones were as follows: apigenin 23.02 min, kaempferol 21.95 min, luteolin 18.37 min, quercetin 16.37 min, isovitexin 5.32 min, vitexin 4.71 min and orientin 3.64 min; and for apigenin-7-*O*-Glc 10.7 min and luteolin-7-*O*-Glc 7.42 min. Flavones and flavonols were detected at their maximal UV absorbance (quercetin, 255 nm; kaempferol, 265.8 nm; apigenin, isovitexin and apigenin-7-*O*-Glc, 270 nm; and orientin, luteolin and luteolin-7-*O*-Glc, 350 nm). Flow rate was 1 ml/min at 25 °C. Calibration curves with their respective standards

were made. The standards apigenin and vitexin were dissolved in MeOH:DMSO (7:3), orientin in MeOH:DMSO (8:2, v/v), apigenin-7-*O*-Glc and luteolin-7-*O*-Glc in MeOH:DMSO (9:1, v/v); the rest of them only in MeOH.

The optimum APCI conditions for LC-MS analyses were as described above.

III.2.12 Acid hydrolysis for flavonoids

Five hundred microliters of the MeOH:H₂O fraction from extraction were hydrolyzed at 90 °C for 60 min with 500 µl of 4N HCl to which 2 mg of antioxidant *tert*-butylhydroquinone (TBHQ) was added. Hydrolysates were extracted with EtOAc three times. The organic phase was dried over anhydrous NaSO₄ and evaporated with N₂ gas.

III.2.13 Statistics

All data were analyzed by MultiExperiment Viewer MEV 4.0 software (Saeed *et al.*, 2003; Dana-Faber Cancer Institute, MA, USA). For analyses involving two and three or more groups paired *t*-test and ANOVA were used, respectively with $\alpha = 0.05$ for significance.

III.3 Results and discussion

III.3.1 Activities of PKSs present in plant tissues from *Cannabis sativa*

For positive control of PKS activity, CHS from *Pinus sylvestris*, STS from *Arachis hypogaea* and VPS from *Humulus lupulus* were used (Table 1). The activities of these enzymes were similar to the ones previously reported of STS (58.6 pKat/mg protein) from peanut cell cultures (Schoppner and Kindl., 1984), CHS (30 pKat/mg protein) from *Phaseolus vulgaris* cell cultures (Whitehead and Dixon., 1983) and VPS (35.76 pKat/mg protein) from hop (Okada *et al.*, 2000), respectively. Negative control assays consisted on standard reaction mixture adding 50 µl water as starter and extender substrate. The final pH for CHS and benzalacetone synthase (BAS) assays was 8, which is optimum for the naringenin (Schröder *et al.*, 1979; Whitehead and Dixon, 1983) and benzalacetone (Abe *et al.*, 2001; Abe *et al.*, 2007) formation, while for the rest of PKS assays was maintained at 7. Due to limited availability of substrates and standards, for detection of STS type activity in cannabis protein extracts we decided to perform the assay using the starter substrate *p*-coumaroyl-CoA for

resveratrol formation as general indicator from STS activities. For detection of CHS type activities, the assay was carried out with *p*-coumaroyl-CoA as starter substrate and naringenin-chalcone formation was an indicator of CHS type activity. For detection of VPS and BUS activities, the assays were achieved with the starter substrates isovaleryl-CoA and isobutyryl-CoA, respectively.

Table 1. PKSs activities used as positive control. The enzymatic assays were made in a final reaction volume of 400 μ l with 100 μ l of purified enzyme (35-66 μ g of protein).

PKS	Sp Act (pKat/mg protein)	Product
CHS (<i>Pinus sylvestris</i>)	33.30 $\pm$ 3.45	Naringenin
STS (<i>A. hypogaea</i>)	70.50 $\pm$ 5.02	Resveratrol
VPS (<i>H. lupulus</i>)	31.97 $\pm$ 6.86	Forming PiVP
VPS (<i>H. lupulus</i>)	27.66 $\pm$ 14.83	Forming PiBP

For the analysis of the assays of PKS activities by HPLC, we started with the eluent system reported by Robert *et al.* (2001), which was slightly modified as is described in material and methods (method 1). Narigenin (Rt 33.55 min) and resveratrol (Rt 26.36 min) had a good separation in this solvent system; however, the retention times of olivetol, PiVP and PiBP (Table 2) were longer than naringenin. Four elution gradients were tested in order to reduce the retention times of these standards and the method 5 was used subsequently for the analysis by HPLC and LC-MS.

Table 2. Retention times (min) of standards employed for analyses using a elution system of MeOH:H₂O in different gradient profiles.

Standard	Naringenin	Resveratrol	PiVP	PiBP	PHBA	Olivetol	Olivetolic acid	Methyl olivetolate	Orcinolic acid	Orcinol	2,4-dBZ acid	Resorcinol
Solvent system* 1	33.55	26.36	37.85	33.71	24.71	37.97	-	-	-	-	-	-
Solvent system* 2	24.69	13.65	33.95	26.09	10.88	33.22	-	-	-	-	-	-
Solvent system* 3	30.21	15.96	39.80	31.68	12.75	40.10	-	-	-	-	-	-
Solvent system* 4	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	-	-	-	-	-	-
Solvent system* 5	14.50	9.01	18.50	15.32	8.54	18.37	23.45	26.83	9.07	5.53	7.15	4.36

*see material and methods

2,4-dihydroxy-benzoic acid, 2,4-dBZ acid

n.r., no resolution

-, not measured

CHS activity was detected in the plant tissues analyzed (Figure 2) and maximum activities were observed in roots (24.86 ± 4.38 pKat/mg protein). No significant differences were found in the CHS activity from the rest of the tissues analyzed ($P < 0.05$) which were until 16 times lesser than that one in roots. STS type activities were also detected in the same plant tissues. The STS activities from fruits and male leaves were no significant different (0.96 ± 0.07 pKat/mg protein and 1.05 ± 0.04 pKat/mg protein, respectively) as well as those ones from female leaves and male flowers (2.11 ± 0.12 pKat/mg protein and 1.76 ± 0.12 pKat/mg protein, respectively). The STS activities from bracts, seedlings and roots were 5 times higher than that one in fruits and they were not significant different. No VPS activities were detected in fruits and roots. The VPS activity in seedlings was until 15 times lesser than those in bracts and male flowers, which were not significantly different. The VPS activities detected in leaves (female and male) were until 7 times bigger than that one in seedlings (0.39 ± 0.06 pKat/mg protein) and they were not significant different by gender. Significant differences were observed in BUS activities from bracts, seedlings and leaves. The BUS activities from female leaves (7.98 ± 2.98 pKat/mg protein) and male leaves (5.76 ± 2.5 pKat/mg protein) were highly significant; no BUS activity was found in fruits, roots and male flowers.

PKS activities expressed during the development of the glandular trichomes on the bracts were significantly different ($P < 0.05$), except at day 31 (Figure 3). CHS activity was increased at day 23 during the growth and development of glandular hairs. The CHS activities at days 17 and 35 were not significantly different to the BUS and VPS activities at the same days. No significant differences were found in STS-type activity during the time course, except at day 35 when it had increased three fold. VPS and BUS activities increased during the growth and development of the glandular trichomes on female flowers with a maximum activity at day 23 (7.07 ± 1.05 pKat/mg protein) and 29 (15.99 ± 4.5 pKat/mg protein), respectively. During the accumulation of resin the VPS activities were not significantly different (from days 31 to 35), but BUS activities were significantly different during the time course. The activities from days 17, 29 and 31 were significantly different between BUS and VPS. No activity for an olivetolic acid-forming PKS was detected during the time course of the growth and development of glandular trichomes on female flowers. However, HPLC and LC-MS analyses confirmed formation of olivetol (retention time 18.21 ± 0.24

min and m/z 181 $[M+H]^+$) using hexanoyl-CoA as starter substrate. This PKS activity forming olivetol was not detected in seedlings, fruits and roots; but significant differences were found in bracts, male flowers and between the leaves of the two genders (Figure 2). The activity for this olivetol-forming PKS was seven times higher in bracts than that in male leaves (5.35 ± 1.07 pKat/mg protein). A time-course of the growth and development of glandular trichomes on female flowers showed that the activity of the olivetol-forming PKS increased at day 29 and decreased later until no activity was detected anymore in female flowers from 35 days-old (Figure 3). Raharjo *et al.* (2004a) suggested that olivetol was formed by a PKS and Kozubek and Tyman (1999) proposed that alkylresorcinols, such as olivetol, are formed from biosynthesized alkylresorcinolic acids by enzymatic decarboxylation or via modified fatty acid-synthesizing enzymes, where the olivetolic acid carboxylic group would be expected to be also attached either to ACP (acyl carrier protein) or to CoA. Thus, in the release of the molecule from the protein compartment in which it was attached or elongated, simultaneous decarboxylation of the olivetol may occur, otherwise the olivetolic acid would be the final product. PKS isolation and gene identification forming alkylresorcinolic acids (Gaucher and Shepherd, 1968; Gaisser *et al.*, 1997; Funa *et al.*, 2007) and stilbene carboxylic acids (Eckermann *et al.*, 2003; Schröder Group) has been reported. Conversion of tetraketides (free acids or lactones) synthesized *in vivo* by stilbene carboxylic acid synthases (Schröder Group) or by chemical synthesis (Money *et al.*, 1967) into the carboxylic acids at a suitable pH (mildly acidic or basic conditions) has been suggested too.

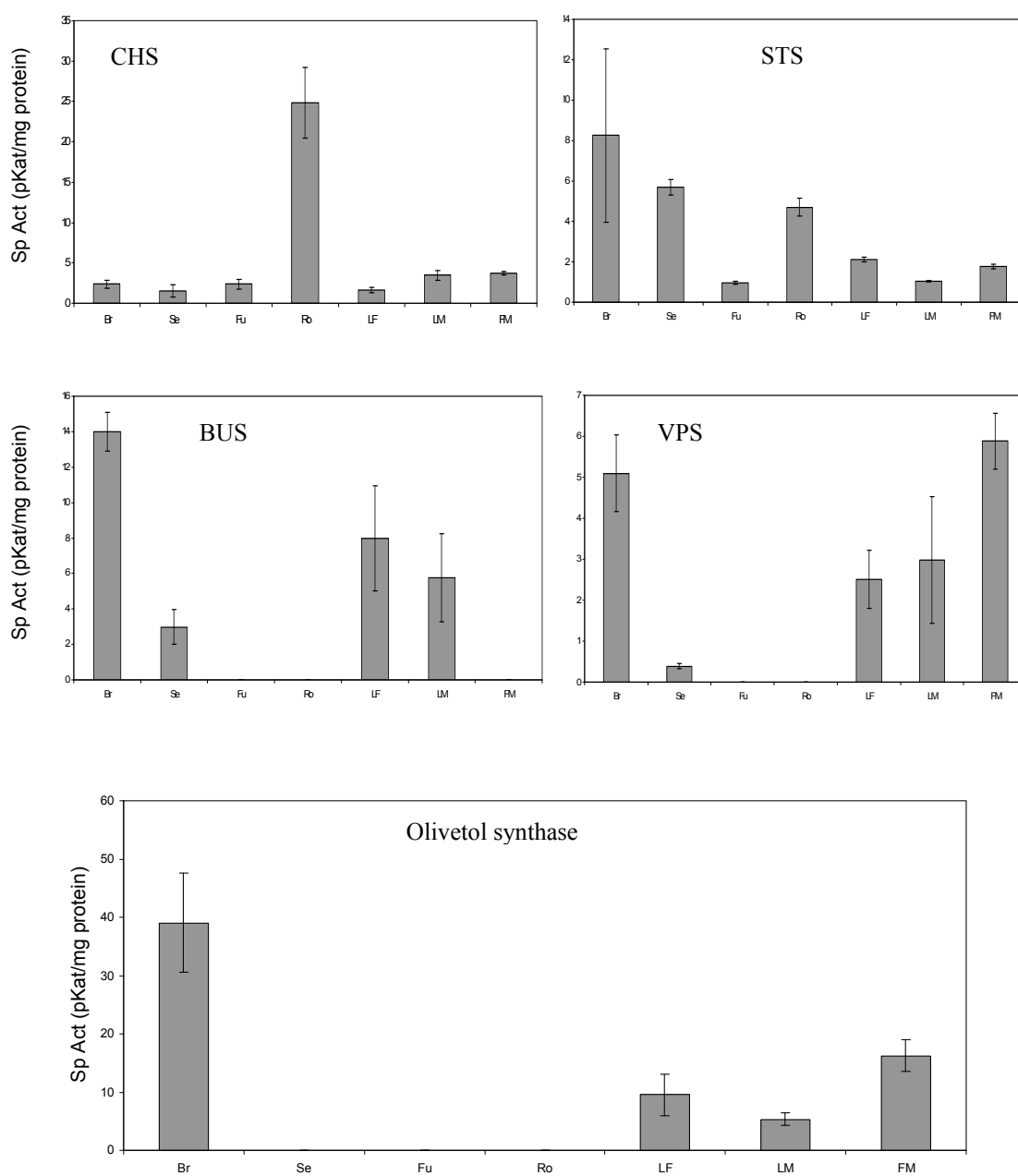


Figure 2. PKS activities in several crude extracts from different cannabis tissues. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower. Bracts of flowers from 29 day-old. Values are expressed as means of three replicates with standard deviations.

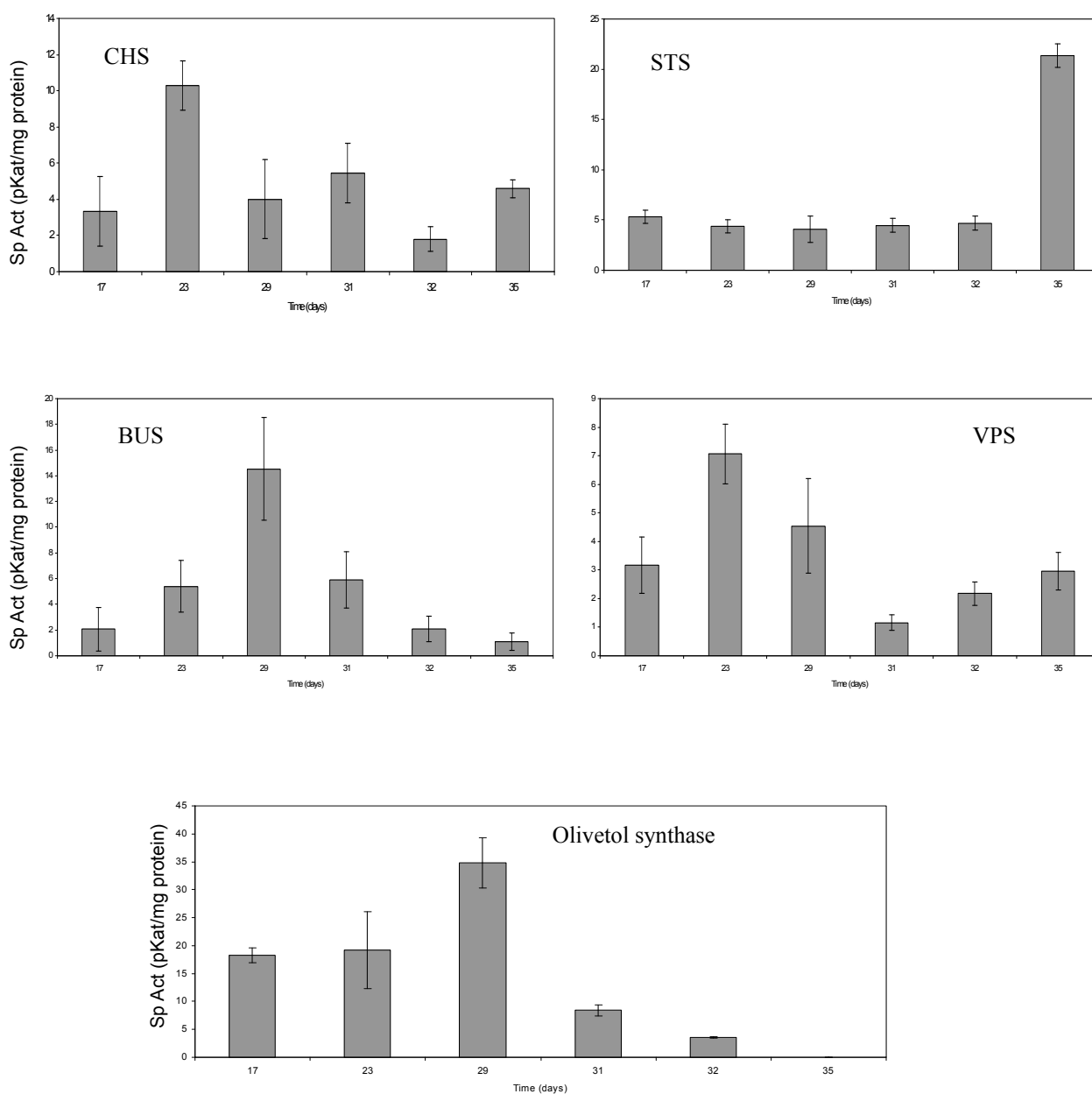


Figure 3. PKS activities during the development of glandular trichomes on female flowers. Values are expressed as means of three replicates with standard deviations.

Table 3. Recovery of olivetolic acid, orcinolic acid, 2,4-dihydroxy-benzoic acid (2,4-dBZ acid) and methyl-olivetolate from cannabis protein crude extracts.

Tissue	Standard	Addition	Added concentration	Calculated concentration	Recovery (%)
Bracts:					
(C-)	Olivetolic acid	After Rx	0.5 mg	0.48 ± 0.02	96.00
			60 μM	57.52 ± 2.18	95.87
		Before Rx	120 μM	116.31 ± 4.72	96.92
			120 μM	117.81 ± 0.54	98.18
(C-)	Orcinolic acid	After Rx	60 μM	58.76 ± 0.94	97.93
			60 μM	56.67 ± 1.66	94.45
		Before Rx	0.06 mg	0.058 ± 0.004	96.67
			60 μM	57.94 ± 1.51	96.57
(C-)	2,4-dBz acid	After Rx	120 μM	117.31 ± 2.13	97.76
			120 μM	118.00 ± 1.41	98.33
		Before Rx	60 μM	59.42 ± 0.64	99.03
			60 μM	58.53 ± 2.05	97.55
(C-)	Methyl-olivetolate	After Rx	0.01 mg	0.0098 ± 0.0002	98.00
			60 μM	57.26 ± 1.03	95.43
		Before Rx	120 μM	118.19 ± 1.86	98.50
			120 μM	118.87 ± 0.13	99.06
(C-)		After Rx	60 μM	57.69 ± 3.09	96.15
			60 μM	57.31 ± 1.07	95.52
		Before Rx	0.5 mg	0.49 ± 0.017	98.00
			60 μM	58.87 ± 1.23	98.12
(C-)		After Rx	120 μM	118.43 ± 1.89	98.69
			120 μM	119.47 ± 0.68	99.56
		Before Rx	60 μM	56.59 ± 2.59	94.32
			60 μM	58.41 ± 0.53	97.35
Leaves:					
	Olivetolic acid	Before Rx	20 μM	19.15 ± 0.01	95.75
	Orcinolic acid	Before Rx	20 μM	19.27 ± 0.92	96.35
	2,4-dBz acid	Before Rx	20 μM	19.24 ± 0.31	96.20

(C-), negative controls without addition of protein extract

Raharjo *et al.* (2004a) did not observe any effect on the formation of the olivetol by neither the incubation time of the PKS assays nor the mildly acidic conditions used. Enzymatic decarboxylation *in vitro* and *in vivo*, and purification of carboxylic acid decarboxylases has been reported from liverworts (Pryce, 1972; Pryce and Linton, 1974), lichens (Mosbach and Ehrensvar, 1966) and microorganism (Pettersson, 1965; Huang *et al.*, 1994; Dhar *et al.*, 2007; Stratford *et al.*, 2007). We did not observe formation of olivetol by an enzymatic or chemical decarboxylation from olivetolic acid (Table 3). Although, the

recovery for the standards orcinolic acid and 2,4-dihydroxy-benzoic acid was more than 95% no orcinol or resorcinol (1,3-dihydroxy-benzene) was detected; methyl-olivetolate was used as negative control of decarboxylation. Purification of this olivetol-forming PKS is required in order to characterize it and analyze the mechanism of the reaction. In addition, no activity was detected with benzoyl-CoA at pH 7.0, 7.5 or 8.0 and no BAS activity was found. Slightly small amounts of derailment byproducts were detected from the PKS assays.

III.3.2 Cannabinoid profiling by HPLC

Figure 4 shows the variations in the cannabinoid content with respect to tissues analyzed. Eight times higher concentration of Δ^9 -THCA was detected in female flowers than in male flowers. No significant differences were found in the contents in male flowers, fruits and male or female leaves ($P < 0.05$). Previous studies confirm that there is no significant difference in the cannabinoid content in leaves of the two genders from the same variety (Holley *et al.*, 1975; Kushima *et al.*, 1980). Δ^9 -THVA was only identified in male and female flowers, and fruits. The concentration of this cannabinoid in flowers was more than seven times higher than the content in fruits but the Δ^9 -THVA content from male flowers was not significantly different from fruits. The CBGA contents from female flowers and, male and female leaves were not significantly different. The content of this cannabinoid in fruits was six times lesser than in female flowers. The CBGA concentration detected from male flowers was not significantly different from fruits. CBDA was identified in flowers and leaves; the CBDA content from female flowers was 2.6 times higher than in male flowers. The CBDA contents from leaves were not significantly different from male flowers. The increment of the concentration of cannabinoids corresponds with the development and growth of the glandular trichomes on the bracts (Table 4 and Figure 5). No significant differences were found in the CBGA and CBDA contents. Although cannabinoid content in the individual glandular trichomes can vary with age, type and location (Turner *et al.*, 1977; Turner *et al.*, 1978), a correlation exists between glandular density and cannabinoid content at each stage of bract development (Turner *et al.*, 1981). As CBGA is the precursor of Δ^9 -THCA and CBDA, its concentration slightly decreased (from 0.18 ± 0.087 mg/100 mg dry weight to 0.12 ± 0.099 mg/100 mg dry weight). Δ^9 -THCA content increased 1.6 times at day 31 (7.82 ± 2 mg/100 mg dry weight). On the

other hand, Δ^9 -THVA accumulation started only after day 24. Natural (plant decarboxylation) or artificial degradation (oxidation, isomerization, UV-light) of cannabinoids occurred on lesser extent in our plant material (Table 4). No cannabinoids and neutral forms were found in seedlings and roots.

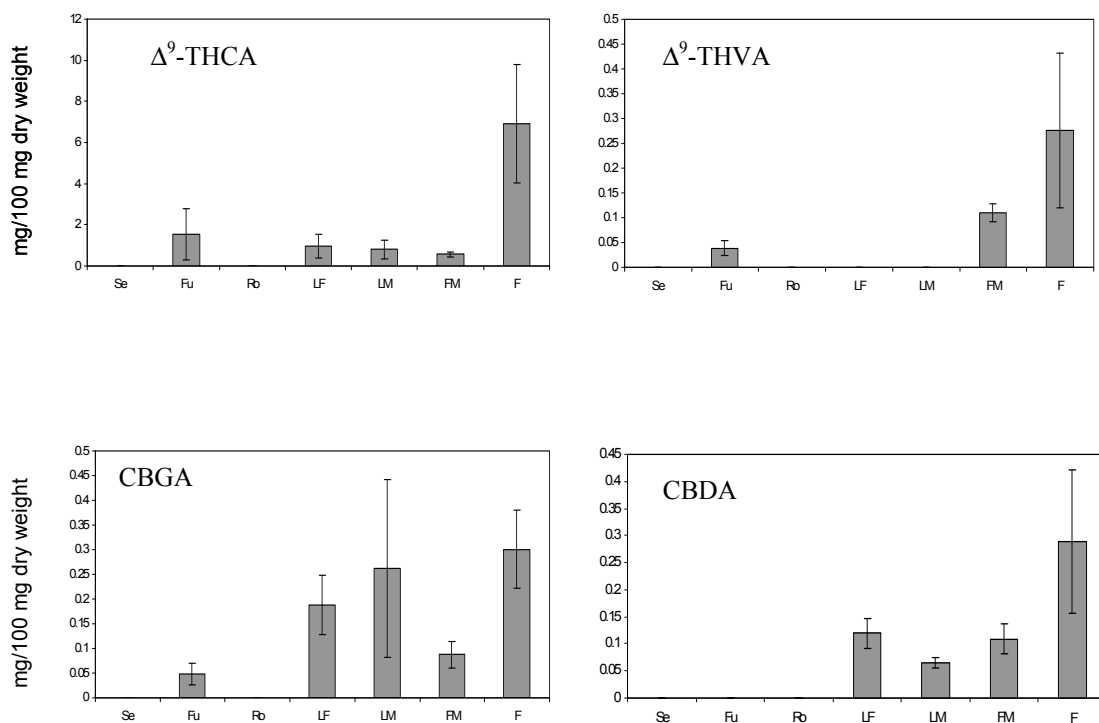


Figure 4. Cannabinoid content in different cannabis plant tissues. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower; F, female flower. Female flowers from 35 day-old. Values are expressed as means of three replicates with standard deviations.

Table 4. Cannabinoid content from different cannabis tissues.

Tissue	cannabinoids* (acid forms)	Δ^9 -THC	CBG	CBD	CBN	Total
Bracts:						
24 d	5.19	0.42 ± 0.03	-	-	-	5.61
31 d	8.40	0.12 ± 0.03	-	-	0.08 ± 0.002	8.60
Fruits	1.63	0.04 ± 0.02	-	-	-	1.67
Leaves:						
Female	1.27	0.43 ± 0.31	-	-	0.06 ± 0.010	1.76
Male	1.13	-	-	-	-	1.13
Flowers:						
Female	7.78	0.16 ± 0.01	-	-	0.09 ± 0.005	8.03
Male	0.86	-	-	-	-	0.86

* concentration expressed in mg/100 mg dry weight; (Δ^9 -THCA, Δ^9 -THVA, CBDA and CBGA)
d, day

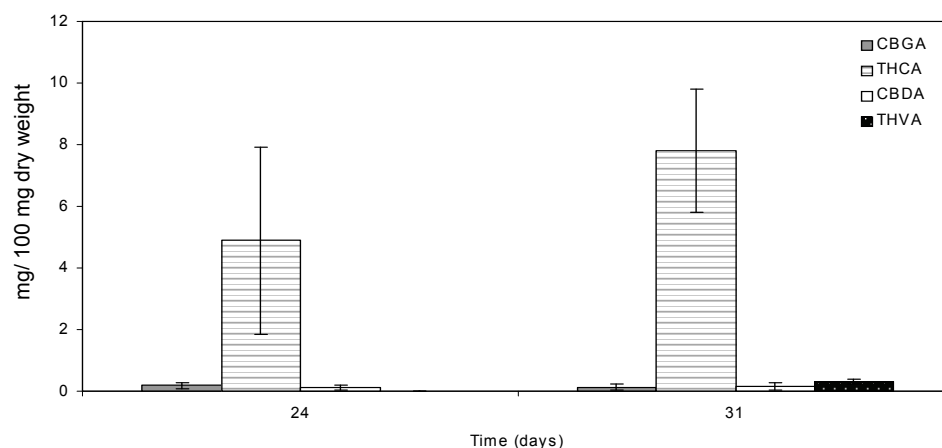


Figure 5. Cannabinoid content in bracts during the growth and development of glandular trichomes on female flowers.

III.3.4 Flavonoid profiling by HPLC

As standards for most flavonoid glycosides are not commercially available, we proceeded to hydrolyze the samples in order to analyze the aglycones. Apigenin, luteolin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were used as internal standards. Percentage of recovery of aglycones from standards was more than 90% (Table 5). Typical profiles corresponding to a standard mixture of the selected flavones and flavonols with our samples are shown in figure 6 and analyses by LC-MS confirmed the identity of the aglycones (Figure 7).

Table 5. Recovery percentage of aglycones from standard acid hydrolysis.

Name	Concentration (mg)	Calculated concentration (mg)	% Recovery
Apigenin-7- <i>O</i> -Glc	0.3	0.283 ± 0.011	94
Apigenin	0.3	0.244 ± 0.012	81
Luteolin-7- <i>O</i> -Glc	0.3	0.277 ± 0.021	92
Luteolin	0.3	0.246 ± 0.019	82

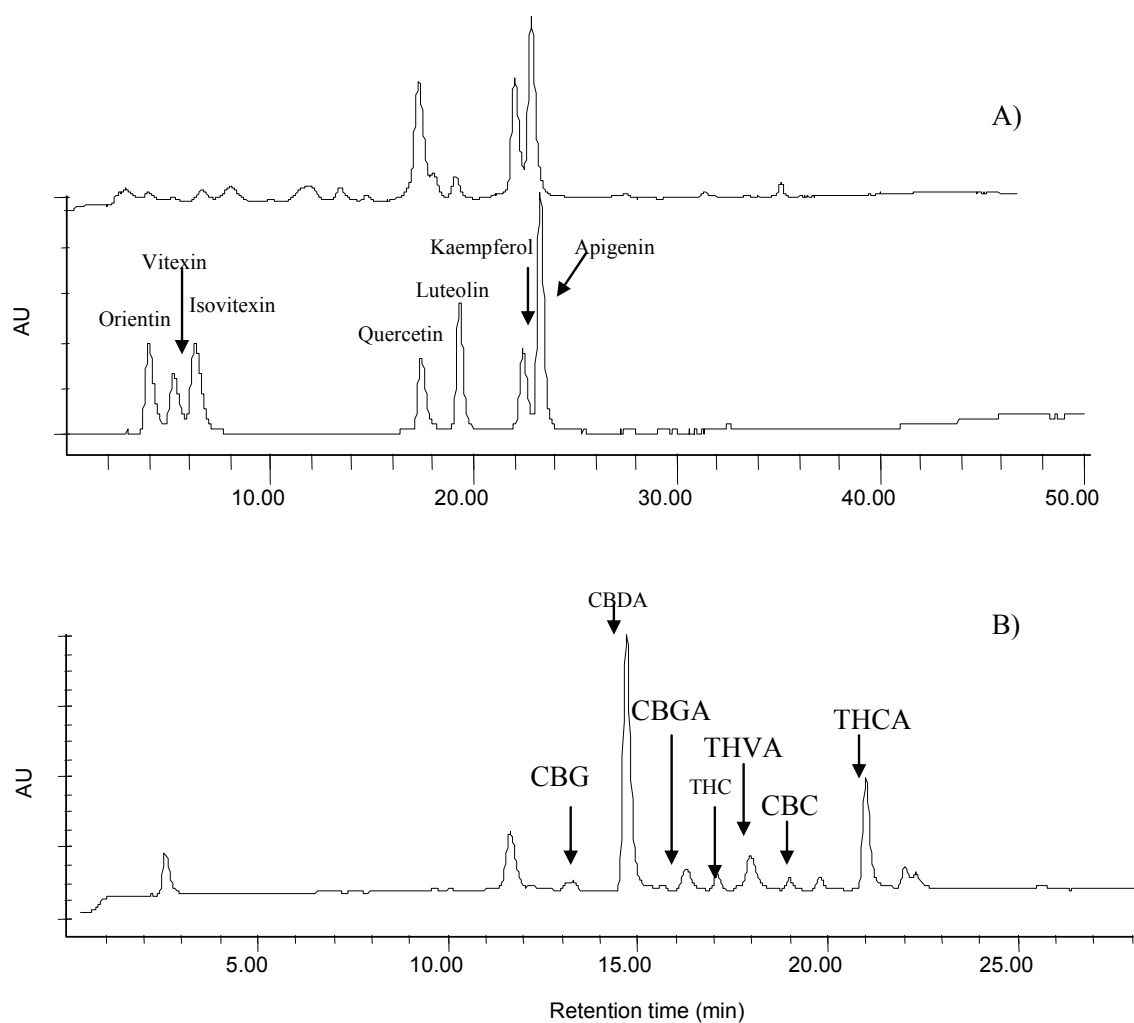
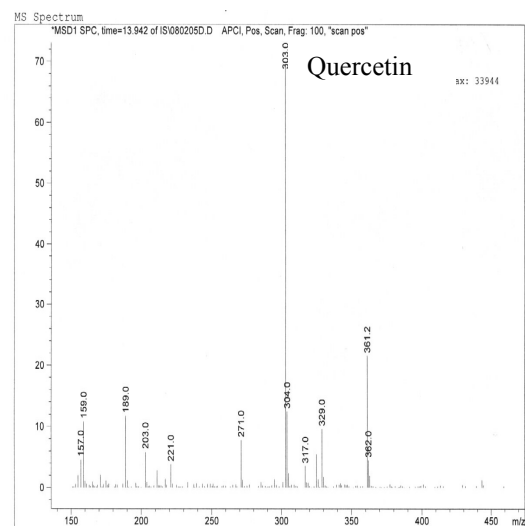
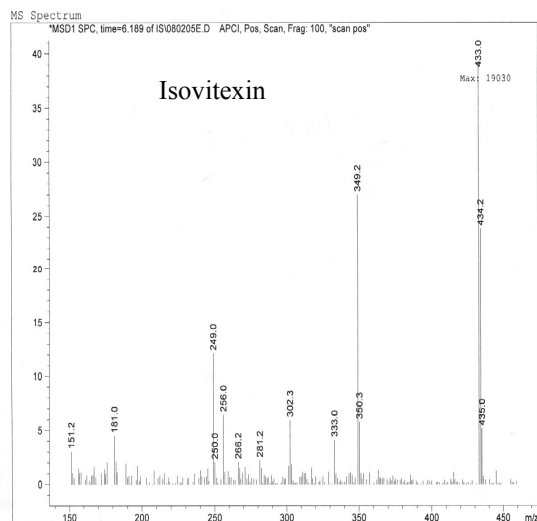
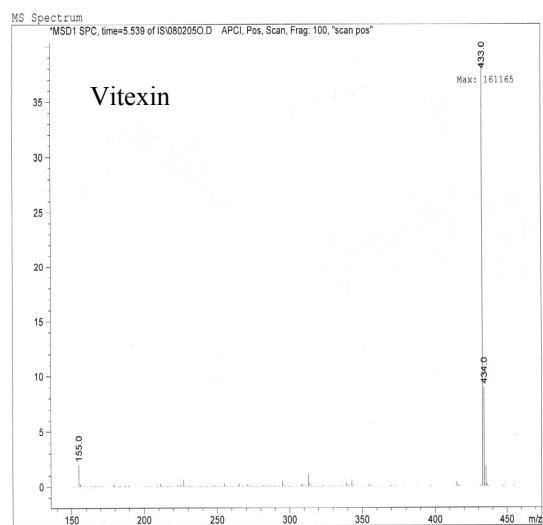
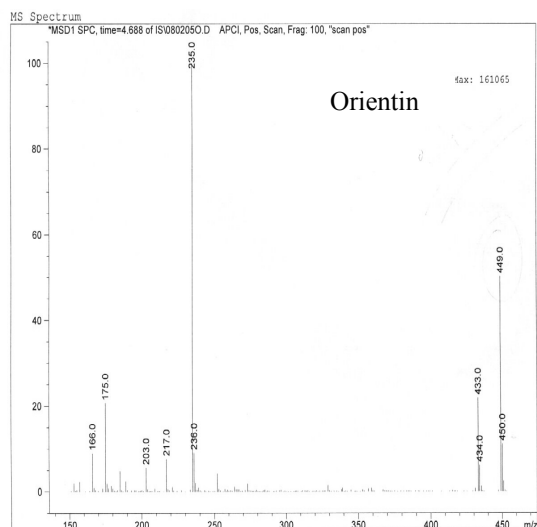


Figure 6. A) Comparison of HPLC chromatograms of the standard mixture of aglycones and a hydrolyzed MeOH:Water fraction (350 nm) and B) HPLC chromatogram of the chloroform fraction from bracts variety "Kompolti" (280 nm).



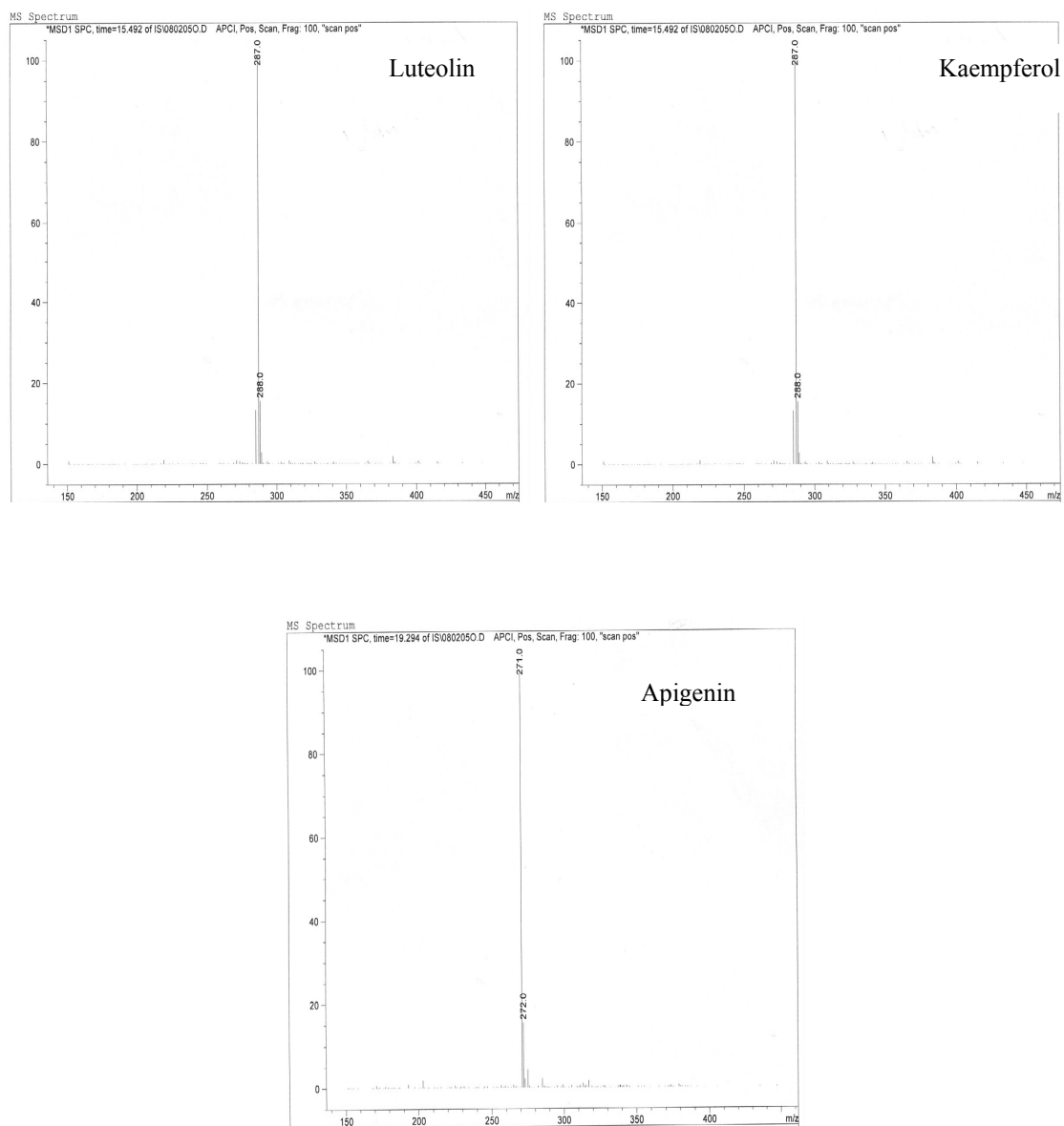


Figure 7. Mass-spectra of hydrolyzed flavonoids from MeOH:Water fraction in the range of m/z 150-450 obtained by LC-MS. Peak values correspond to [M+H]. *MW*: orientin, 448.4; vitexin, 432.4, isovitexin, 432.4; quercetin, 302.25; luteolin, 286.25; kaempferol, 286.25 and apigenin, 270.25.

Flavonoid content varied from a plant tissue to another (Figure 8). No flavonoids were detected in roots. Orientin content in flowers and leaves was not significant different by gender, but a significant difference was found between the contents from seedlings (0.040 ± 0.025 mg/100 mg dry weight) and fruits 0.026 ± 0.019 mg/100 mg dry weight). Vitexin content in fruits was the lowest and the contents in leaves and flowers were not significantly different. Isovitexin contents from female and male leaves were not significantly different, as well as the contents in seedlings and female flowers, and fruits and male flowers. Lowest amounts of quercetin were detected in fruits and highest amounts in male flowers. No significant differences were found in the contents in leaves and seedlings. The contents of luteolin in leaves (female and male), male flowers and seedlings were not significantly different and lowest contents were detected in fruits, which were not significantly different from the contents in male flowers. The kaempferol contents of leaves (female and male) and male flowers were not significantly different. Lowest contents were detected in fruits (0.0025 ± 0.0013 mg/100 mg dry weight) and the contents in seedlings and female flowers were seventeen times higher than in fruits. Apigenin contents from leaves were not significantly different for gender, but the contents in flowers were significantly different for gender. Lowest contents were detected in fruits (0.0048 ± 0.0028 mg/100 mg dry weight). Luteolin and vitexin contents are similar to results reported by Vanhoenacker *et al.*, (2002) but apigenin and orientin contents are higher in our samples. Though Raharjo (2004) only reported apigenin and luteolin in leaves and flowers of *C. sativa* Fourway plants the contents were different from our results, probably because of differences in plant tissue age and the variety. Contrary to the cannabinoid accumulation during the growth and development of glandular trichomes the flavonoid content decreased (Figure 9 and Table 6).

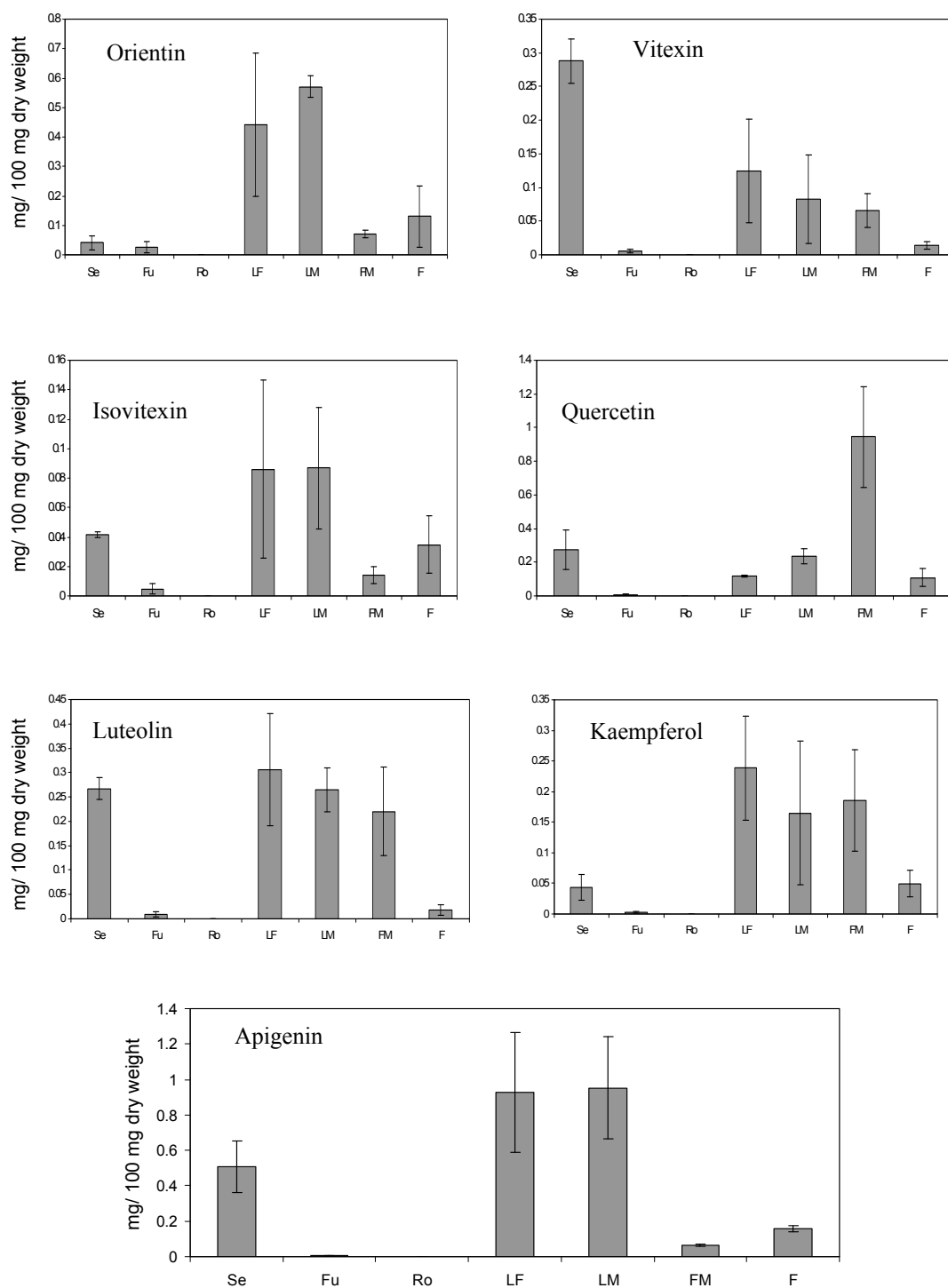


Figure 8. Flavonoid content in different cannabis plant tissues. Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; F, female flower; LM, male leaf; FM, male flower. Female flowers from 35 days-old. Values are expressed as means of three replicates with standard deviations.

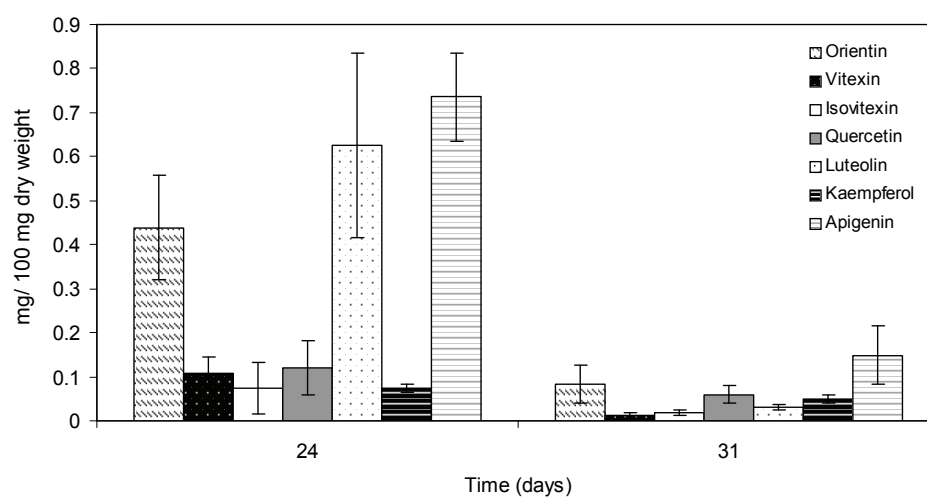


Figure 9. Flavonoid content in bracts during the growth and development of glandular trichomes on female flowers.

Table 6. Flavonoid content in different plant tissues from *C. sativa*.

Tissue	Flavonoid total content (mg/100 mg dry weight)
Bracts:	
24 d	2.18
31 d	0.40
Fruits	0.06
Seedlings	1.46
Leaves:	
Female	2.24
Male	2.36
Flowers:	
Female	1.56
Male	0.51

d, day

III.3.5 PKS activities and secondary metabolites in *C. sativa*

In plant tissues from *C. sativa*, *in vitro* PKS activities of CHS, STS, BUS and VPS, as well as activity for an olivetol-forming PKS were detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant (Chapter 1), revealed differences in their distribution, suggesting a diverse regulatory control on the biosynthetic fluxes in the plant. Apigenin, luteolin, kaempferol are widespread compounds in plants (Valant-Vetschera and Wollenweber, 2006). Quercetin and kaempferol have a role in fertility of male flowers (Vogt *et al.*, 1995; Napoli *et al.*, 1999) and higher levels of these two flavonols in cannabis male flowers than in female flowers (Figure 8) support this role. UV-B (280–315 nm) protection by flavone or flavonol glycosides has been reported (Lois and Buchanan, 1994; Rozema *et al.*, 2002) and their occurrence in aerial tissues from cannabis should be vital. Furthermore, roles as growth regulators have been suggested (Ylstra *et al.*, 1994; Gould and Lister, 2006). Quercetin, apigenin and kaempferol can modulate auxin-mediated processes (Jacobs and Rubery, 1988) and this role should not be excluded in cannabis. It has been reported that luteolin and apigenin derivatives acted as feeding deterrents of Lepidoptera larvae (Erhard *et al.*, 2007). On the other hand, it is known that cannabinoids are cytotoxic compounds (Rothschild *et al.*, 1977; Roy and Dutta, 2003; Sirikantaramas *et al.*, 2005) and they can act as plant defense compounds against predators such as insects. Moreover, a regulatory role in cell death has been suggested as cannabinoids have the ability to induce cell death through mitochondrial permeability transition (Morimoto *et al.*, 2007).

The accumulation of cannabinoids in bracts during the growth and development of glandular trichomes from flowers (Figure 5) could be related to floral protection and consequently during the seed maturation the cannabinoid content may decrease. Lower contents of cannabinoids were detected in fruits (seed and cup-like bracteole) than in female flowers (Table 3). It seems that cannabinoid accumulation is correlated with maximum activities for an olivetol-forming PKS (Figures 3 and 5) and the CHS activity preceded the accumulation of flavonoids at day 24 (Figures 3 and 9). A significant STS-type activity was detected at day 35 (Figure 3). Although, significant enzymatic activities for VPS and BUS were also detected in crude protein extracts no acylphloroglucinols have been identified in cannabis so far (Chapter I). Acylphloroglucinols and activities of VPS and BUS have been detected in *Humulus lupulus* (Paniego *et al.*, 1999) and *Hypericum perforatum* (Hoelzl and Petersen, 2003; Klingauf *et al.*, 2005). It is known that PKSs can use efficiently a broad range of substrates (Novak *et al.*, 2006; Springob *et al.*, 2000; Samappito *et al.*, 2003; Chapter II) and probably the cannabis PKSs have this notorious *in vitro* substrate promiscuity. Zuurbier *et al.* (1998) showed that CHS and STS enzymes can have VPS- and BUS-type activities and the VPS and BUS activities identified in this study could be from CHS or olivetol-forming PKS, even from STS. Although, a significant activity of CHS and STS activities were detected in crude protein extracts from roots (Figures 2) no flavonoids were identified in these tissues (Figure 8). There are no reports about isolation or detection of flavonoids and stilbenoids in roots (Chapter I) and contradict the CHS- and STS-type activities detected in roots. Low expression of the *CHS-type PKS* gene in roots and the absence of flavonoids in this plant tissue was previously reported (Raharjo *et al.*, 2004b; Raharjo 2004). Stilbenoids have been isolated from cannabis leaves and resin (Chapter I) but they could not be identified in the methanol:water fractions from leaves and bracts by LC-MS analysis, this could be due to the low STS-type activity (Figures 3). Gehlert and Kindl (1991) found a relationship between induced formation by wounding of stilbenes and the PKS BBS in orchids. Stilbenoid functions in plants include constitutive and inducible defense mechanisms (Chiron *et al.*, 2001; Jeandet *et al.*, 2002), plant growth inhibitors and dormancy factors (Gorham, 1980).

It is known that induction of enzymatic activity in early steps from a biosynthetic pathway precedes the accumulation of final products (Figure 10).

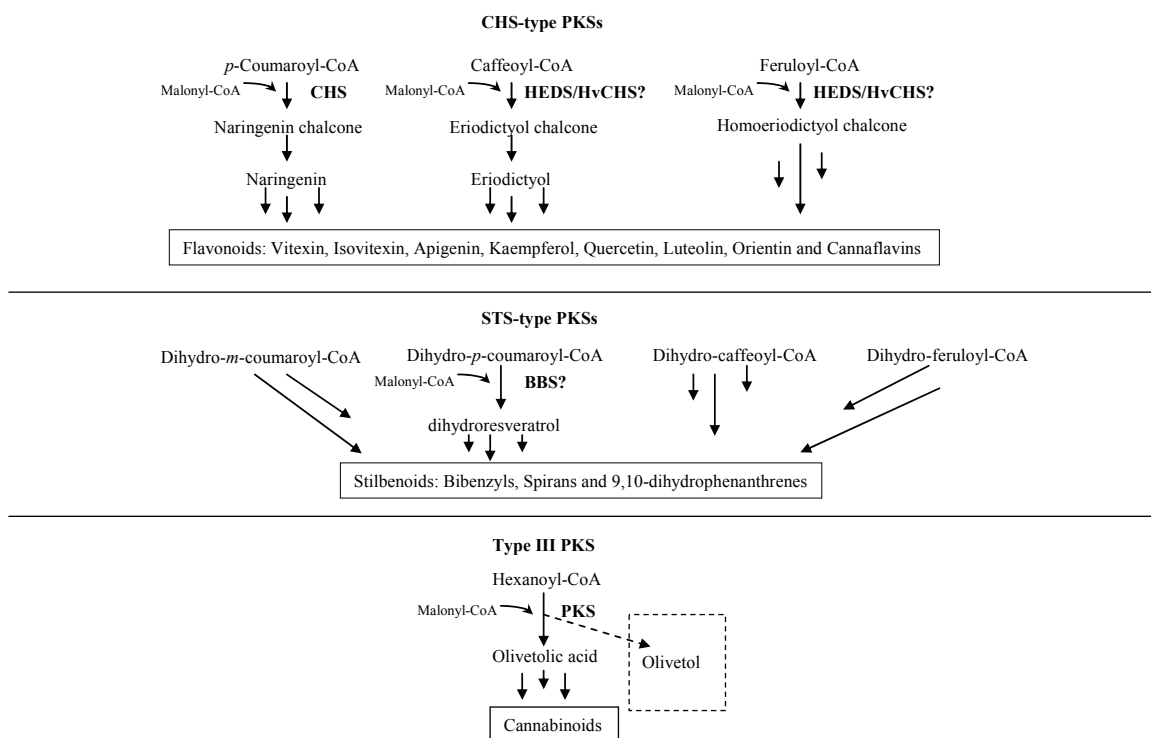


Figure 10. Proposed reactions for PKSs in the biosynthesis of precursors from flavonoid, stilbenoid and cannabinoid pathways in cannabis plants. Dashed square represent the compound found in crude extracts.

The cannabinoid content in female flowers was 5 times higher than the flavonoid content (Table 4) and during the development of the glandular trichomes on the flowers the activity of the olivetol-forming PKS at day 29 was 8 times higher than the CHS activity (Figure 3). Although, STS activity detected during the time course was low it increased at the end being 4 times and 21 times higher than the CHS and olivetol-forming PKS, respectively. This STS activity can be associated to the precursor formation in stilbenoid biosynthesis. The results shown here suggest the presence of three PKS activities, one CHS type, one STS type and another for the olivetol biosynthesis. However, further studies are required to identify the substrate specificities of these PKSs in cannabis plants. Purification and characterization of the PKS enzymes will be necessary to know their catalytic potential and their regulation, which may lead to the identification of their role in the plant.

Acknowledgements

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Chapter IV

***In silico* expression analysis of a *PKS* gene isolated from *Cannabis sativa* L.**

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Abstract:

In the annual dioecious plant *Cannabis sativa* L., the compounds cannabinoids, flavonoids and stilbenoids have been identified. Of these, the cannabinoids are the best known group of natural products. Polyketide synthases are responsible for biosynthesis of diverse secondary metabolites, including flavonoids and stilbenoids. Using a RT-PCR homology search, a PKS cDNA was isolated (PKSG2). The deduced amino acid sequence showed 51–72% identity to other CHS/STS type sequences of the PKS family. Further, phylogenetic analysis revealed that this PKS cDNA grouped with other non-chalcone-producing PKSs. Homology modeling analysis of this cannabis PKS predicts a 3D overall fold similar to alfalfa CHS2 with small steric differences on the residues that shape the active site of the cannabis PKS.

IV.1 Introduction

In plants, polyketide synthases (PKSs) play an important role in the biosynthesis of a myriad of secondary metabolites (Schröder, 1997, Chapter II). They are a group of homodimeric condensing enzymes that catalyze the initial key reactions in the biosynthesis of several compounds, such as flavonoids and stilbenoids. PKSs are classified into three types (Chapter II). Chalcone synthase (CHS, EC 2.3.1.74) and stilbene synthase (STS, EC 2.3.1.95) are the most studied enzymes from the group of type III PKSs (Austin and Noel, 2003; Schröder, 2000). Plant PKSs have 44–95% amino acid identity and are encoded by similarly structured genes. For example, CHSs from *Petunia hybrida*, *Petroselinum hortense*, *Zea mays*, *Antirrhinum majus* and *Hordeum vulgare*, and STS from *Arachis hypogaea* have 70–75% identity on the protein level and the *CHS* and *STS* genes contain an intron at the same conserved position (Schröder and Schröder, 1990; Schröder *et al.*, 1988). Families of PKS genes have been reported in many plants, such as alfalfa (Junghans *et al.*, 1993), bean (Ryder *et al.*, 1987), carrot (Hirner and Seitz, 2000), *Gerbera hybrida* (Helariutta *et al.*, 1996), vine (Goto-Yamamoto *et al.*, 2002; Wiese *et al.*, 1994), *Humulus lupulus* (Novak *et al.*, 2006), *Hypericum androsaemum* (Liu *et al.*, 2003), *Ipomoea purpurea* (Durbin *et al.*, 2000), pea (Harker *et al.*, 1990), petunia (Koes *et al.*, 1989), pine (Preisig-Müller *et al.*, 1999), *Psilotum nudum* (Yamazaki *et al.*, 2001), raspberry (Kumar and Ellis, 2003), rhubarb (Abe *et al.*, 2005), tomato (O'Neill *et al.*, 1990), *Ruta graveolens* (Springob *et al.*, 2000), *Sorghum bicolor* (Lo *et al.*, 2002), soybean (Shimizu *et al.*, 1999) and sugarcane (Contessotto *et al.*, 2001). Their expression is differently controlled and it has been suggested that PKSs have evolved by duplication and mutation, providing to plants an adaptative differentiation (Durbin *et al.*, 2000; Lukacin *et al.*, 2001; Tropf *et al.*, 1994). As PKSs are in vital branch points for biosynthesis of secondary metabolites, the presence of families of PKSs in one single species emphasizes the importance of their characterization to understand their functional divergence and their contribution to function(s) in different cell types of the plant.

Cannabis sativa L. is an annual dioecious plant from Central Asia. Several compounds have been identified in this plant. Cannabinoids are the best known group of natural products and 70 different cannabinoids have been found so far (ElSohly and Slade, 2005). Several therapeutic effects of cannabinoids have been

reported (reviewed in Williamson and Evans, 2000) and the discovery of an endocannabinoid system in mammals marks a renewed interest in these compounds (Di Marzo and De Petrocellis, 2006; Di Marzo *et al.*, 2007). However, other groups of secondary metabolites have been described also, such as flavonoids and stilbenoids (Flores-Sanchez and Verpoorte, 2008; Chapter I). It is known that the PKSs CHS and STS catalyze the first committed step of the flavonoid and stilbenoid biosynthesis pathways, respectively. Cannabinoid biosynthesis could be initiated by a PKS (Shoyama *et al.*, 1975). Previously, a PKS cDNA was generated from *C. sativa* leaves. It encodes an enzyme with CHS, phlorisovalerophenone synthase (VPS) and isobutyrophenone synthase (BUS) activities, but lacking olivetolic acid synthase activity (Raharjo *et al.*, 2004b). The co-existence of cannabinoids, flavonoids and stilbenoids in *C. sativa* could be correlated to different enzymes of the PKS family. This report deals with the generation and molecular analysis of one PKS cDNA obtained from tissues of cannabis plants.

IV.2 Materials and methods

IV.2.1 Plant material

Seeds of *Cannabis sativa*, drug type variety Skunk (The Sensi Seed Bank, Amsterdam, The Netherlands) were germinated and 9 day-old seedlings were planted into 11 LC pots with soil (substrate 45 L, Holland Potgrond, Van der Knaap Group, Kwintsheul, The Netherlands) and maintained under a light intensity of 1930 lux, at 26 °C and 60 % relative humidity (RH). After 3 weeks the small plants were transplanted into 10 L pots 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). Young leaves from 13 week-old plants, female flowers in different stages of development and male flowers from 4 month-old plants were harvested. Besides, cones of *Humulus lupulus* at different stages of development were collected in September 2004 from the Pharmacognosy gardens (Leiden University). All vegetal material was weighed and stored at -80 °C.

IV.2.2 Isolation of glandular hairs and lupulin glands

Six grams of frozen female flowers containing 17-, 23-, 35- and 47-day-old glandular trichomes from cannabis plants were removed by shaking frozen material through a tea leaf sieve and collected in a mortar containing liquid N₂ and immediately used for RNA extraction. For lupulin glands, frozen cones of hop were ground in liquid nitrogen using a mortar and pestle only to separate the bracteoles and were shaken using the same system as for cannabis glandular hairs.

IV.2.3 Total RNA and mRNA isolation

For total RNA isolation from flowers, leaves, glandular hairs, glandular lupulins and hop cones, frozen tissues (0.1–0.5 g) were ground to a fine powder in a liquid nitrogen-cooled mortar, resuspended and vortexed in 0.5 ml extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA and 4% SDS) and 0.5 ml water-saturated phenol. The suspension was centrifuged at 1400 rpm for 2 min to separate phenol and water phases. The RNA was precipitated from the water phase after addition of in 1/3 volume 8M LiCl at 4 °C overnight. The RNA was collected by centrifugation at 14000 rpm for 10 min, and resuspended in 0.1 ml H₂O. The suspension was heated at 60 °C for 20 min and centrifuged. Five µl 3M Na-acetate (pH 4.88) was added to the supernatant to initiate the precipitation with 0.25 ml 100% EtOH at –20 °C for 30 min and centrifuged at 14000 rpm for 7 min. The pellet was washed with 250 µl 70% EtOH, centrifuged for 2 min at 14000 rpm, dried at 60 °C for 15 min, dissolved in 50 µl H₂O and incubated at 50 °C for 10min.

Alternatively, Micro-fast track 2.0 kit and Trizol reagent (Invitrogen, Carlsband, CA, USA) were used for mRNA and total RNA isolation following manufacturer's instructions. Isolated RNA was stored at –80 °C.

IV.2.4 RT-PCR

Degenerated primers, HubF (5'–GAGTGGGGYCARCCCAART–3'), HubR (5'–CCACCIGGRTGWGYAATCCA–3'), STSF (5'–GGITGCIIGCIGGIGGMAC–3'), STSR (5'–CCIGGICCRAAICCRAA–3') (Biolegio BV, Malden, The Netherlands) were made, based on CHS, STS and stilbene carboxylate synthase (STCS) sequences from *H. lupulus*, peanut, *Rheum tataricum*, *Pinus strobus*, vine and *Hydrangea macrophylla*. For primers HubF and HubR the conserved regions were from CHS

and VPS (accession number AJ304877, AB061021, AB061022, AJ430353 and AB047593), while for STSF and STSR from STS and STCS (accession number AB027606, AF508150, Z46915, AY059639, AF456446). RT-PCR was performed with total RNA or mRNA as template using different combinations of primers. 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 were: 45s denaturation at 94 °C, 1 min annealing at 45 °C, 1 min DNA synthesis at 72 °C for 30 cycles using a Perkin Elmer DNA Thermal Cycler 480 and a Taq PCR Core kit (QIAGEN, Hilden, Germany). A final extension step of 10 min at 72 °C was included. The PCR products were separated on 1.5% agarose gel, visualized under UV light, and recovered using Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA, USA) or QIAquick PCR Purification kit (QIAGEN) according manufacturer's instructions.

IV.2.5 RACE-PCR

For generation of 5' and 3' end cDNAs, we used total RNA, gene specific primers and a SMART RACE kit (ClonTech, Palo Alto, CA, USA). The cycling parameters were: 94 °C for 1 min followed by 35 cycles at 94 °C for 35 s, annealing temperature for 35 s and 72 °C for 3 min. A final elongation step of 10 min at 72 °C was included. Gene-specific, amplification and sequencing primers, as well as annealing temperatures are shown in table 1. The PCR products were separated on 1.5% agarose gel and visualized under UV light. For generation of complete sequences, total RNA and amplification primers were used. Nested amplifications were made with gene-specific primers to select PKS sequences for sequencing. PKS full-length cDNAs were re-sequenced with sequencing primer in order to confirm that the ORF of the sequences were correct. The corresponding amplification products were ligated into pGEM-T vector and cloned into JM109 cells according to the manufacturer's instructions (Promega, Madison WI, USA). Plasmids containing the inserted fragment were sequenced (BaseClear, Leiden, The Netherlands).

IV.2.6 Homology modeling

The PKS 3D models were generated by the web server Geno3D (Combet *et al.*, 2002; <http://genoed-pbil.ibcp.fr>), using as template the X-ray crystal structures of *M. sativa* CHS2 (1BI5.pdb, 1CHW.pdb and 1CML.pdb). The models

were based on the sequence homology of residues Arg5–Ile383 of the PKS PKSG2. The VPS model was based on the sequence homology of the residues Val4–Val390. The corresponding Ramachandran plots confirm that the majority of residues grouped in the energetically allowed regions. All models were displayed and analyzed by the program DeepView–the Swiss–Pdbviewer (Guex and Peitsch, 1997; <http://www.expasy.org/spdbv/>).

IV.3 Results and discussion

IV.3.1 Glandular hair isolation

In a previous study (Raharjo *et al.*, 2004b) a *PKS* cDNA was isolated from young cannabis leaves, which expressed PKS activity but did not form the first precursor of cannabinoids, olivetolic acid. It is known that glandular hairs are the main site of cannabinoid production (Chapter I). Moreover, it was shown that the cannabinoid THCA is biosynthesized in the storage cavity of the glandular hairs and the expression of THCA synthase was also found in these trichomes (Sirikantaramas *et al.*, 2005; Taura *et al.*, 2007a). So it is imperative to isolate RNA from these glandular trichomes in order to be able to produce *PKS* cDNAs associated to the cannabinoid biosynthesis.

For glandular hair isolation from cannabis flowers, we followed the method reported by Hammond and Mahlberg (1994). However, we observed under the microscope (data not shown) that the glandular hairs remained attached to the tissue after 5 s of blending. Increasing the blending time to 12 s resulted in increased breakage of the tissues and glandular hair heads. Therefore we tested the method reported by Zhang and Oppenheimer (2004), which consisted of gentle rubbing using an artist's paintbrush. Using this method we had 100% of recovery of glandular hairs. However, this method was tedious and the handling of the tissue was difficult because it was very fragile. We made some modifications in order to improve the tissue handling to preserve the frozen tissues and avoid degradation of RNA. We found that shaking the tissue frozen with liquid nitrogen through a tea leaf sieve was easier and resulted on approximately 90% recovery of trichomes. The effectiveness of this method is comparable to the method reported by Yerger *et al.* (1992), which consists of vortexing the tissues with powdered dry ice and sieving.

Table 1. Oligonucleotide primers and annealing temperatures used in this study.

Primers	Sequence (5' → 3')	Annealing temperature (°C)
Gene-specific primers		
2F	CATGACGGCTTGCTTGTTTCGTGGGCCCTTCAGATTCTAACC	64
2R	GGTTAGAACTCTGAAGGCCACGAAACAAGCAAGCCGTCATG	
Amplification primers		
PKSF _w	ATGAATCATCTTCGTGCTGAGGGTCCGGCC	63
PKSR _v	TTAATAATTGATCGGAACACTACGCAGGACCAC	
Sequencing primer		
Sq	GTCCCTCAGTGAAGCGTGTGATGATGTATCAACTAGGCTGTTA	63

IV.3.2 Amplification of cannabis *PKS* cDNAs

RNA isolated from glandular hairs of cannabis flowers was used as a template for reverse transcription–polymerase chain reaction (RT–PCR) amplification of segments of *PKS* mRNAs using degenerate primers (Figure 1). RNA from hop tissues was used as a positive control. The degenerated primers corresponded to conserved regions surrounding Gln 119, the catalytic domain around Cyst 164, a region surrounding His 303 and the C-terminal region of the selected protein sequences from CHS, STS and STCS.

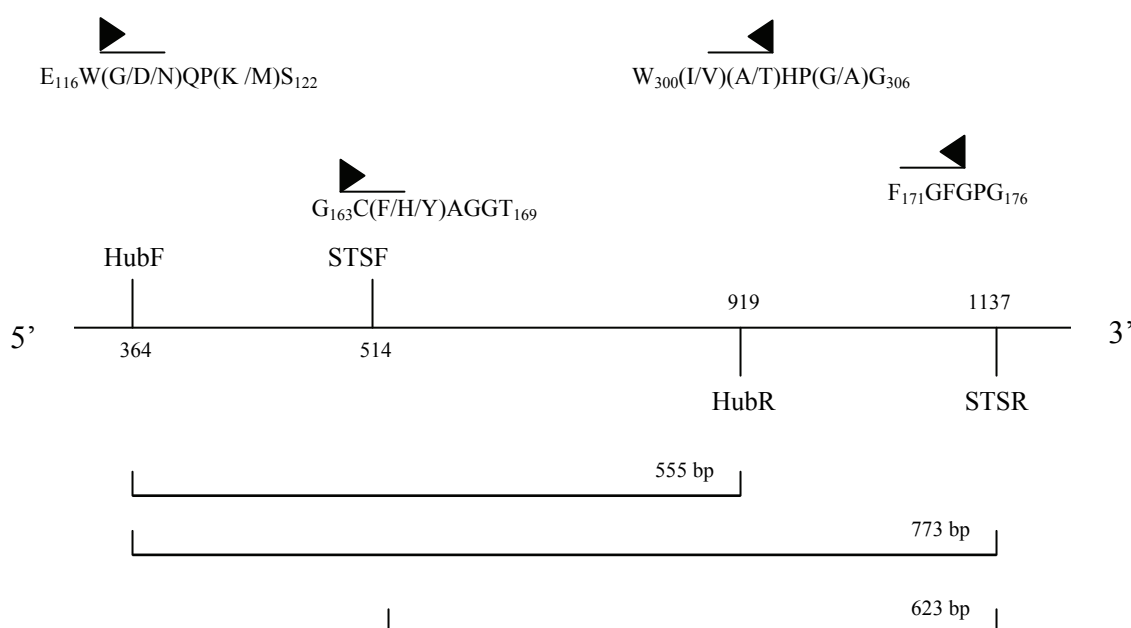


Figure 1. Positions of degenerate primers and of the amplified PCR products, and size of PCR products, relative to *CHS3* from *H. lupulus* (AB061022). Closed arrow heads indicate the sense and position of the degenerate primers relative to the amino acid sequences of the *PKS*s CHS, STS and STCS. These amino acid positions have been numbered relative to *M. sativa* CHS.

The various amplification products had nucleotide sequences encoding open reading frames (ORFs) for proteins with a size and amino acid sequence similar to *PKS*s from other plants (Table 2).

Table 2. Homology percentage of amino acid partial sequences with CHSs from *H. lupulus* (accession numbers CAC19808, BAB47195, BAB47196, CAD23044, BAA29039) and *C. sativa* (AAL92879); STSs from *R. tataricum* (AAP13782), *Pinus strobus* (CAA87013), peanut (BAA78617), grape (AAB19887), *P. densiflora* (BAA94593) and STCS from *H. macrophylla* (AAN76183).

Name sequence	Tissue	CHS1 <i>H. lupulus</i>	CHS2 <i>H. lupulus</i>	CHS3 <i>H. lupulus</i>	CHS4 <i>H. lupulus</i>	VPS <i>H. lupulus</i>	CHS type PKS <i>C.sativa</i>	STCS <i>H. macrophylla</i>	STS <i>P.strobus</i>	STS peanut	STS <i>R. tataricum</i>	STS grape	Pinosylvin synthase <i>P. densiflora</i>
Set 1													
PKS1	FF	92	66	72	70	72	99	71	73	72	69	76	73
	GH	95	68	76	75	77	100	75	75	73	70	78	73
	MF	95	68	76	75	77	100	75	75	73	70	78	75
Set 2													
PKS2	GH	67	70	77	75	73	68	63	63	62	64	66	62
	L	67	70	77	75	73	68	63	63	62	64	66	62
Control:													
HopPKS	LG	100				70							
	LG					100							
FF, female flower; GH, glandular hairs; MF, male flower; L, leaf; LG, lupulin glands													

Two sets of sequences were obtained. Set 1 consisted of sequences identified in female and male flowers, and glandular hairs that were a 99–100% identical to the PKS with CHS-type activity previously isolated from *C. sativa* (Raharjo *et al.*, 2004b). The second set (Set 2), was derived from mRNA of leaves and glandular hairs and showed 77% homology with CHS3 from *H. lupulus* and a 68% homology with the known cannabis CHS-type PKS. The homology among the various sequences within each set was more than 99%. Regarding the positive controls performed on hop mRNA, we obtained the partial sequences of VPS and CHS2 from the hop cone's secretory glands (also called lupulin glands). It is known that *VPS* and *CHS_1* are expressed in lupulin glands (Matousek *et al.*, 2002a, 2002b; Okada and Ito, 2001) and the presence of a gene family of *VPS* as well as one of *CHS* has been suggested. Figure 2 shows the strategy to obtain the full-length cDNAs of the likely PKS gene.

IV.3.3 Nucleotide and protein sequence analyses

A full-length PKS cDNA, *PKSG2*, of 1468bp containing an ORF of 1158 bp was obtained from mRNA of *C. sativa* glandular trichomes. The nucleotide sequence data was deposited at GenBank database with the accession number EU551164 (Figure 3). The *PKSG2* ORF encodes a protein of 385 amino acids with a calculated Mw of 42.61 kDa and a pI of 6.09. According to the percentage of identity at amino acid level (Table 3), *PKSG2* showed to have more homology with the CHSs 3, 4 and VPS from *H. lupulus* than other PKSs. Conserved amino acid residues present in type III PKSs are also preserved in the amino acid sequence from *PKSG2* (Figure 4). The catalytic triad (Cys157, His297 and Asn330), the “gatekeeper” phenylalanines (Phe208 and Phe259) and Met130, which ties one catalytic site up to the other one in the homodimeric complex, as well as Gly250, which determines the elongation cavity volume of the active site, are strictly preserved when compared to CHS2 from alfalfa (Ferrer *et al.*, 1999; Jez *et al.*, 2000b; Jez *et al.*, 2001b). The GFGPG loop, which is important for the cyclization reactions in CHS/STS type PKSs (Suh *et al.*, 2000), is also preserved in our *PKSG2*. In the starter substrate-binding pocket, the amino acid residues Ser126, Ser332 and Thr187 are preserved as on alfalfa CHS2, but Glu185 and Thr190 are replaced by an Asp and a Leu, respectively. In the PKS 2-pyrone synthase (2PS), the amino acid residue Thr190 is replaced by a Leu. All these amino acid residues are important for the selectivity of the

starter substrate. In alfalfa CHS2, the catalytic efficiency of the *p*-coumaroyl-CoA-binding pocket was affected by replacement of these residues (Jez *et al.*, 2000a).

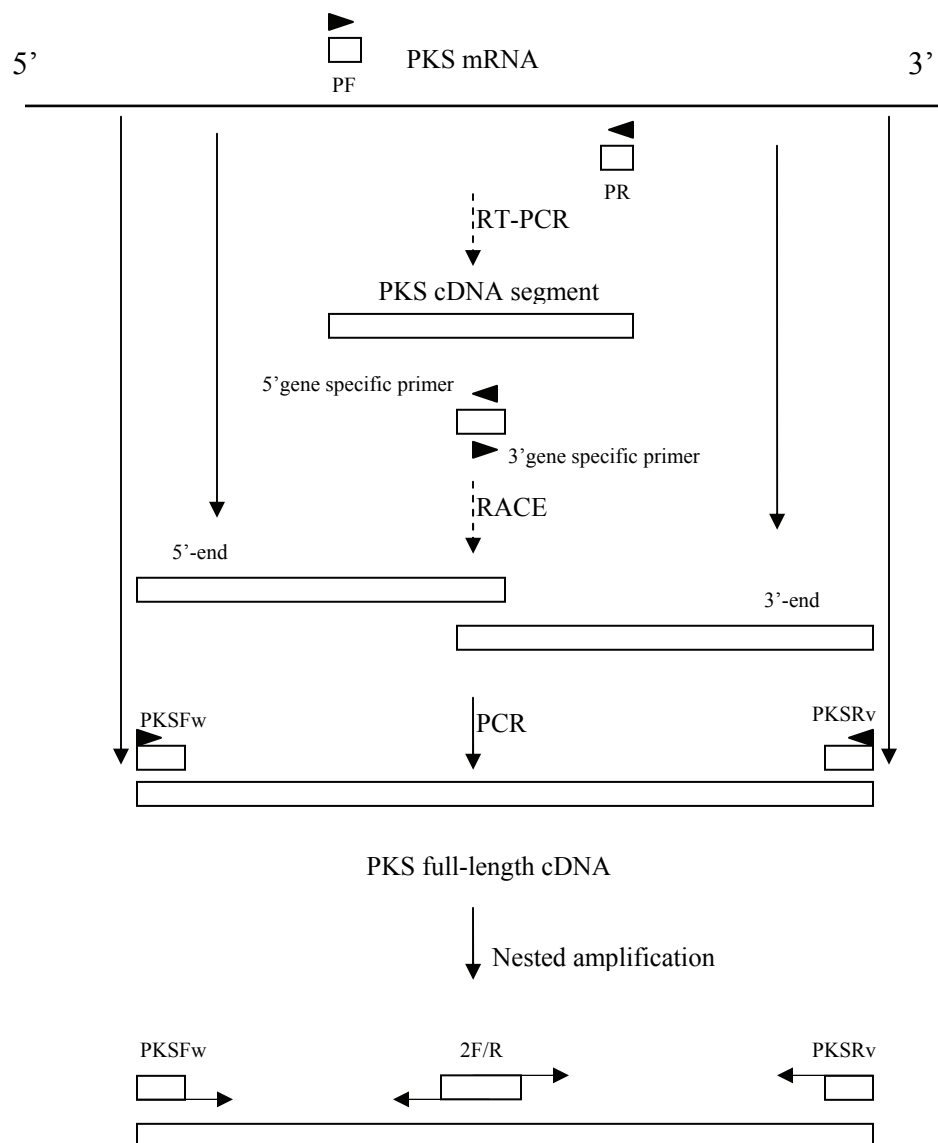


Figure 2. Outline of RT-PCR and RACE for generation of PKS full-length cDNAs. Closed arrow head indicate the sense of the primers. The 5'-, 3'-ends and full-length cDNAs were amplified from mRNA. PF, sense degenerate primer; PR, antisense degenerate primer; PKSFw and PKSRv, amplification primers. For nested amplification, the gene-specific primers and amplification primers were used as nested primers.

```

PKSG2      ATGAATCATCTTCGTGCTGAGGGTCCGGCTCCGTTCTCGCCATCGGCACCGCCAATCCG 60
           ▶ PKSFw

PKSG2      GAGAACATTTTAATACAAGATGAGTTTCCTGACTACTACTTTCGGGTCACCAAAAGTGAA 120

PKSG2      CACATGACTCAACTCAAAGAAAAGTTTCGAAAAATATGTGACAAAAGTATGATAAGGAAA 180

PKSG2      CGTAACTGTTTCTTAAATGAAGAACACCTAAAGCAAAACCAAGATTGGTGAGCACGAG 240

PKSG2      ATGCAAACTCTGGATGCACGTCAAGACATGTTGGTAGTTGAGGTTCCAAAACCTGGGAAG 300

PKSG2      GATGCTTGTGCAAAGGCCATCAAAGAATGGGGTCAACCCAAGTCTAAAATCACTCATTTA 360

PKSG2      ATCTTCACTAGCGCATCAACCACTGACATGCCCGGTGCAGACTACCATTGCGCTAAGCTT 420

PKSG2      CTCGGACTCAGTCCCTCAGTGAAGCGTGTGATGATGTATCAACTAGGCTGTTATGGTGGT 480

PKSG2      GGAACAGTTCTACGCATTGCCAAGGACATAGCAGAGAATAACAAAGGCGCACGAGTTCTC 540

PKSG2      GCCGTGTGTTGTGACATGACGGCTTGCTTGTTCGTGGGCCTTCAGATTCTAACCTCGAA 600
           Gene-specific primer 2F/R

PKSG2      TTACTAGTTGGACAAGCTATCTTTGGTGATGGGGCTGCTGCTGTCATTGTTGGAGCTGAA 660

PKSG2      CCCGATGAGTCAGTTGGGGAAGGCCGATATTTGAGTTAGTGTCAACTGGGCAGACATTC 720

PKSG2      TTACCAAACCTCGGAAGGAACATTTGGGGGACATATAAGGGAAGCAGGACTGATGTTTGAT 780

PKSG2      TTACATAAGGATGTGCCTATGTTGATCTCTAATAATATTGAGAAATGTTTGATTGAGGCA 840

PKSG2      TTTACTCCTATTGGGATTAGTGATTGGAACCTATATTTTGGATTACTCACCCAGGTGGG 900

PKSG2      AAAGCTATTTTGGACAAAGTAGAGGAGAAGTTGCATCTAAAGAGTGATAAGTTTGTGGAT 960

PKSG2      TCACGTCATGTGCTGAGTGAGCATGGGAATATGTCTAGCTCAACTGTCTTGTGTTGTTATG 1020

PKSG2      GATGAGTTGAGGAAGAGGTCGTTGGAGGAAGGGAAATCTACCACTGGAGATGGATTGAG 1080

PKSG2      TGGGGTGTTCTTTTGGGTTTGGTCCAGGTTTGACTGTCGAAAGAGTGGTCCTGCGTAGT 1140

PKSG2      GTTCCGATCAATTATTAA 1158
           PKSRv *
           ◀

```

Figure 3. Nucleotide sequence of the *PKSG2* full-length cDNA. Position of gene-specific and amplification primers are underlined; *, stop codon.

PKSG2	-----MNHLRAEGPASVLAIGTANPENILIQDEFDPDYFRVTKSEHMTQLKEKFRKIC	53
CannabisCHS	MVTVEEFRKAQRAEGPATIMAIGTATPANCVLQSEYPDYFRITNSEHKTELKEKFKRMC	60
AlfalfaCHS	MVSVSEIRKAQRAEGPATILAIGTANPANCVEQSTYPDFYFKITNSEHKTELKEKFORMC	60
PKSG2	DKSMIRKRCNFLNEEHLKQNPRLVEHEMQTLDAQDMLVVEVPKLGKDACAIAKEWGQP	113
CannabisCHS	DKSMIRKRYMHLTEELKENPNLCAYEAPSLDARQDMVVVEVPKLGKEAATKAKEWGQP	120
AlfalfaCHS	DKSMIRKRYMYLTEELKENPNVCEYMAPSLDARQDMVVVEVPRLGKEAAVKAKEWGQP	120
PKSG2	KSKITHLIFTSASTTDMPGADYHCAKLLGLSPSVKRVMMYQLGCGGGTVLRIAKDIAEN	173
CHSCannabis	KSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQCGCFAGGTVLRLAKDLAEN	180
AlfalfaCHS	KSKITHLIVCTTSGVDMPGADYQLTKLLGLRPYVKRYMMYQCGCFAGGTVLRLAKDLAEN	180
PKSG2	NKGARVLAVCCDMTACLFRGPSDSNLELLVGQAI FGDGAAAVIVGAEPDES VGERPIFEL	233
CannabisCHS	NKGARVLVVCSEITAVTFRGPN DTHLDSL VGQALFGDGS AALIVGSDPIPEV-EKPIFEL	239
AlfalfaCHS	NKGARVLVVCSEVTAVTFRGPSDTHLDSL VGQALFGDGAALIVGSDPVPEI-EKPIFEM	239
PKSG2	VSTGQTFLPNSEGTIGGHIREAGLMFDLHKDVPMLISNNIEKCLIEAFTPIGISDWSNIF	293
CannabisCHS	VSAAQTILPDSG AIDGHLREVGLTFHLLKDVPGLISK NIEKSLNEAFKPLGISDWSNLF	299
AlfalfaCHS	VWTAQT IAPDSEGAIDGHLREAGLTFHLLKDVPGLVSKNITKALVEAFEPLGISDWSNIF	299
PKSG2	WITHPGGKAILDKVEEKLHLKSDKFVDSRHVLSEHGNMSSSTVLFVMDLKRKRSLEEGKS	353
CannabisCHS	WIAHPGGPAILDQVESKLALKTEKLRATHVLSEYGNMSSACVLFILDEMRRKCVEDGLN	359
AlfalfaCHS	WIAHPGGPAILDQVEQKLALKPEKMNATREVLSEYGNMSSACVLFILDEMRRKSTQNGLK	359
PKSG2	TTGDGFEGVLF GFGPGLTVERVVLRSVPIN Y	385
CannabisCHS	TTGEGLEWGVLF GFGPGLTVETVVLH SVAI--	389
AlfalfaCHS	TTGEGLEWGVLF GFGPGLTETVVLRSVAI--	389

Figure 4. Comparison of the deduced amino acid sequences of *C. sativa* PKs and *M. sativa* CHS2. Amino acid residues from catalytic triad (Cys14, His303 and Asn 336), starter substrate-binding pocket (Ser133, Glu192, Thr194, Thr197 and Ser338), “gatekeepers” (Phe215 and Phe265) and other ones important for functional diversity (GFGPG loop, Gly256 and Met137) are marked with *. Residues that shape the geometry of the active site are marked with +. Differences on amino acid sequence are highlighted in gray (Numbering in *M. sativa* CHS2).

The replacement of Thr197 by Leu slightly reduced its catalytic efficiency to substrate *p*-coumaroyl-CoA; however, it was increased for the substrate acetyl-CoA. It was found that the change of three amino acid residues (Thr197Leu,

Gly256Leu and Ser338Ile) converts a CHS activity to 2PS activity. In PKSG2, the substrate-binding pocket could be slightly different from that of the alfalfa CHS2 by changes from polar to nonpolar amino acid residues (Thr190Leu) and from one bigger amino acid residue to a smaller one (Glu185Asp185). Although, the residues that shape the geometry of the active site (Pro131, Gly156, Gly160, Asp210, Gly256, Pro298, Gly299, Gly300, Gly329, Gly368, Pro369 and Gly370) are preserved as on alfalfa CHS2 Leu209 is replaced by the amino acid Ile.

Table 3. Homology percentage of *C. sativa* PKSG2 ORF with CHSs, STSs and STCS.

PKS (species, accession numbers)	PKSG2
CHS-type PKS1 (<i>C. sativa</i> , AAL92879)	67
CHS_1 (<i>H. lupulus</i> , CAC19808)	66
CHS2 (<i>H. lupulus</i> , BAB47195)	68
CHS3 (<i>H. lupulus</i> , BAB47196)	72
CHS4 (<i>H. lupulus</i> , CAD23044)	71
VPS (<i>H. lupulus</i> , BAA29039)	71
CHS2 (Alfalfa, AAA02824)	65
2PS (<i>G. hybrida</i> , P48391)	61
STCS (<i>H. macrophylla</i> , AAN76182)	60
STCS (<i>M. polymorpha</i> , AAW30010)	53
STS (peanut, BAA78617)	60
STS (vine, AAB19887)	62
STS (<i>P. strobes</i> , CAA87013)	61
BBS (<i>P. sylvestris</i> , CAA43165)	60
BBS (<i>B. finlaysoniana</i> , CAA10514)	57
PCS (<i>A. arborescens</i> , AAX35541)	51
OKS (<i>A. arborescens</i> , AAT48709)	53
BPS (<i>H. perforatum</i> , ABP49616))	54
BIS (<i>S. aucuparia</i> , ABB89212)	55
HKS (<i>P. indica</i> , BAF44539)	55
ACS (<i>H. serrata</i> , ABI94386)	56
ALS (<i>R. palmatum</i> , AAS87170)	60

The CHS-based homology modeling predicted that our cannabis PKS has the same three-dimensional overall fold as alfalfa CHS2 (Figure 5). A schematic representation of the residues that shape the geometry of the active site of cannabis PKSG2 is shown in figure 6.

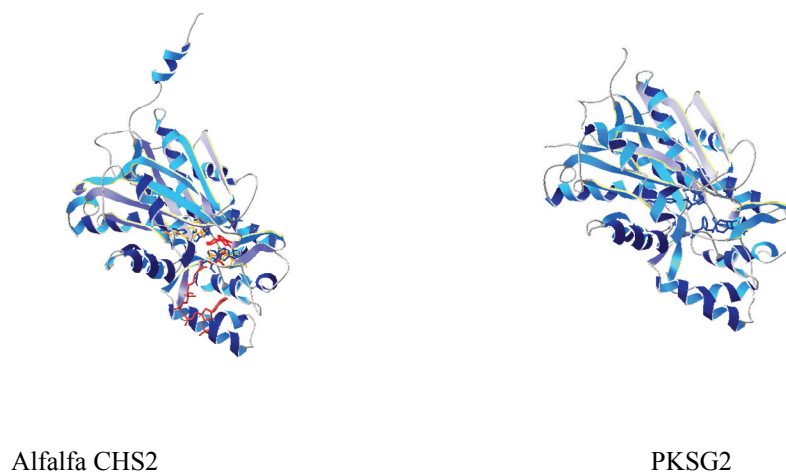


Figure 5. Structural comparison of alfalfa CHS2 crystal structure with the 3D models from the deduced amino acid sequences of cannabis PKS cDNAs. The active site residues are shown as blue backbones; in alfalfa CHS structure naringenin and malonyl-CoA are shown as red and dark red backbones.

The model could suggest small differences in the local reorientation of the residues that shape the active site of the cannabis PKSG2 and, as it was mentioned above, they could be important for steric modulation of the active-site architecture, which could also affect the substrate and product specificity of the enzyme reaction. Motif analyses (<http://www.cbs.dtu.dk/services/> ; <http://urgi.versailles.inra.fr/predator/> and http://myhits.isb-sib.ch/cgi-bin/motif_scan/) predicted PKSG2 to be a non-secretory protein with a putative cytoplasmic location. In addition, potential residues for post-translational modifications such as phosphorylation and glycosylation were also predicted. However, biochemical analyses are required to prove that PKSG2 is under post-translational control. It is known, that post-translational modifications of enzymes form part of an orchestrated regulation of metabolism at multiple levels. Usually, the nuclear and cytoplasmic proteins are modified by glycosylation, phosphorylation or both (Wilson, 2002; Well and Hart, 2003; Huber and Hardin, 2004). Phenylalanine ammonia lyase (PAL), the first enzyme of phenylpropanoid biosynthesis, is regulated by reversible phosphorylation (Allwood *et al.*, 1999; Cheng *et al.*, 2001). PAL plays an important role in the biosynthesis of flavonoids, lignins and many other compounds.

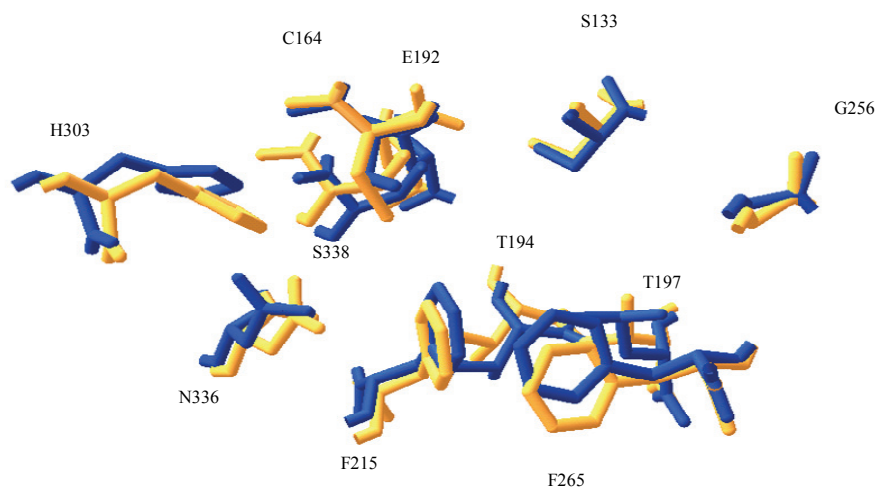


Figure 6. Relative orientation of the sidechains of the active site residues from *M. sativa* CHS with the 3D model of *C. sativa* PKS2. The corresponding sidechains in alfalfa CHS are shown in yellow backbones and are numbering.

IV.3.4 A PKS family in cannabis plants

We characterized one PKS cDNA from glandular hairs (*PKSG2*), which was also identified in leaves, by RT-PCR and sequencing. Although, a low expression of the known cannabis CHS-type PKS (*PKS1*) was reported in female flowers, glandular hairs, leaves and roots (Raharjo *et al.*, 2004b), we detected by RT-PCR that it is also expressed in male flowers. Southern blot analyses of *C. sativa* genomic DNA showed that three homologous *PKS* genes are present (Raharjo, 2004). Apparently our *PKSG2* cDNA corresponds to a second member of the *PKS* gene family in cannabis. A phylogenetic analysis (Figure 7) from our cannabis *PKSG2* revealed that it groups together with other non-chalcone and non-stilbene forming enzymes and appears to be most closely related to the CHSs 2, 3, 4 and VPS from *H. lupulus*, while the known cannabis CHS-type *PKS1* groups with chalcone forming enzymes and is most closely related with *H. lupulus*

CHS1, of which expression is highly specific in the lupulin glands during the cone maturation (Matousek *et al.*, 2002a).

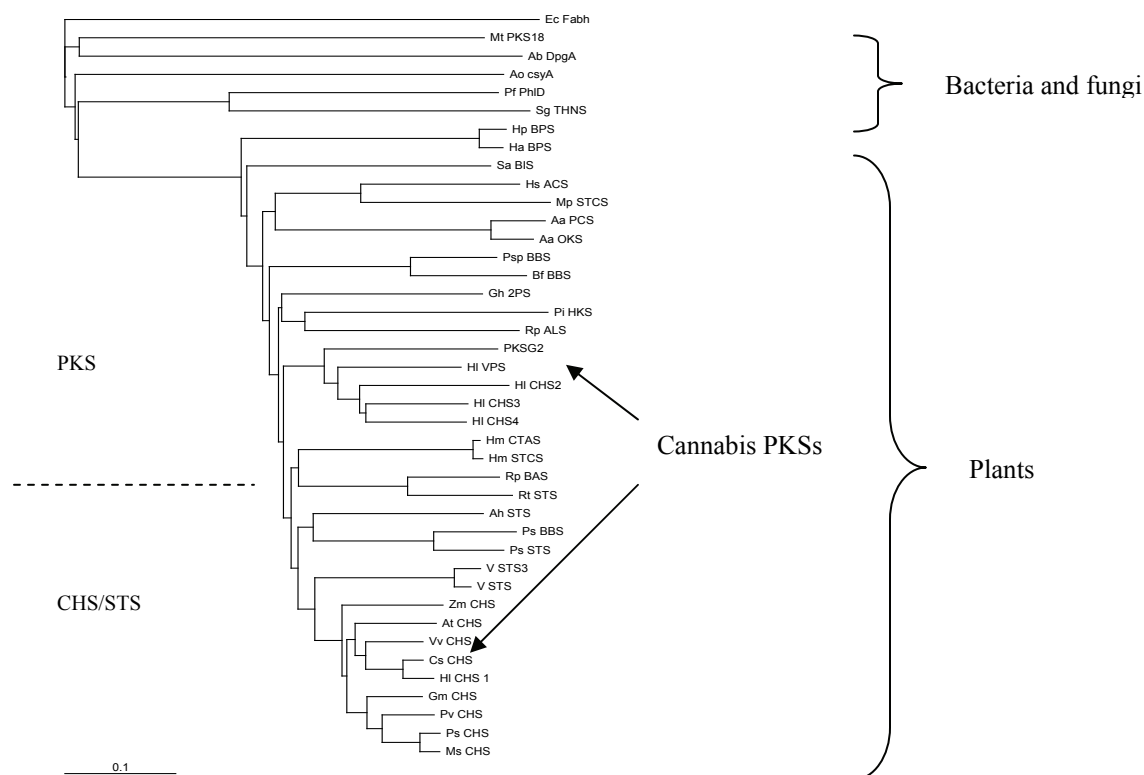


Figure 7. Relationship of *C. sativa* PKSs with plant, fungal and bacterial type III PKSs. The tree was constructed with III type PKS protein sequences. *E. coli* β -ketoacyl synthase III (*Ec_FabH*, accession number 1EBL) was used as out-group. Multiple sequence alignment was performed with CLUSTALW (1.83) program (European Bioinformatics Institute, URL <http://www.ebi.ac.uk/Tools/clustalw/index.html>) and the tree was displayed with TreeView (1.6.6) program (URL <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). The indicated scale represents 0.1 amino acid substitution per site. Abbreviations: Mt_PKS18, *Mycobacterium tuberculosis* PKS18 (AAK45681); Ab_DpgA, *Amycolatopsis balhimycina* DpgA (CAC48378); Ao_csyA, *Aspergillus oryzae* csyA (BAD97390); Pf_PhlD, *Pseudomonas fluorescens* phlD (AAB48106); Sg_THNS, *Streptomyces griseus* (BAA33495); Hp_BPS, *Hypericum perforatum* BPS (ABP49616); Ha_BPS, *Hypericum androsaemum* BPS (AAL79808); Sa_BIS, *Sorbus aucuparia* BIS (ABB89212); Hs_ACS, *Huperzia serrata* ACS (ABI94386); Mp_STCS, *Marchantia polymorpha* STCS (AAW30010); Aa_PCS, *Aloe arborescens* PCS (AAX35541); Aa_OKS, *A. arborescens* (AAT48709); Psp_BBS, *Phalaenopsis* sp. 'pSPORT1' BBS (CAA56276); Bf_BBS, *Bromheadia finlaysoniana* BBS (CAA10514); Gh_2PS, *Gerbera hybrida* 2PS (P48391); Pi_HKS, *Plumbago indica* HKS (BAF44539); Rp_ALS, *Rheum palmatum* ALS (AAS87170); Hl_VPS, *Humulus lupulus* VPS (BAA29039); Hl_CHS2, *H. lupulus* CHS2 (BAB47195); Hl_CHS3, *H. lupulus* CHS3 (BAB47196); Hl_CHS4, *H. lupulus* CHS4 (CAD23044); Hm_CTAS, *Hydrangea macrophylla* CTAS (BAA32733); Hm_STCS, *H. macrophylla* STCS (AAN76182); Rp_BAS, *R. palmatum* BAS (AAK82824); Rt_STS, *Rheum tataricum* STS (AAP13782); Ah_STS, *Arachis hypogaea* STS (BAA78617); Ps_BBS, *Pinus sylvestris* BBS (pinosilvin synthase, CAA43165); Ps_STS, *Pinus strobus* STS (CAA87013); V_STS3, *Vitis* sp. cv. 'Norton' STS3 (AAL23576); V_STS, *Vitis* spp. STS (AAB19887); Zm_CHS, *Zea mays* CHS (AAW56964); Gm_CHS, *Glycine max* CHS (CAA37909); Pv_CHS, *Phaseolus vulgaris* CHS (CAA29700); Ps_CHS, *Pisum sativum* CHS (CAA44933); Ms_CHS, *Medicago sativa* CHS (AAA02824); Vv_CHS, *Vitis vinifera* CHS (CAA53583); Cs_CHS, *Cannabis sativa* CHS-like PKS1 (AAL92879); Hl_CHS1, *H. lupulus* CHS1 (CAC19808).

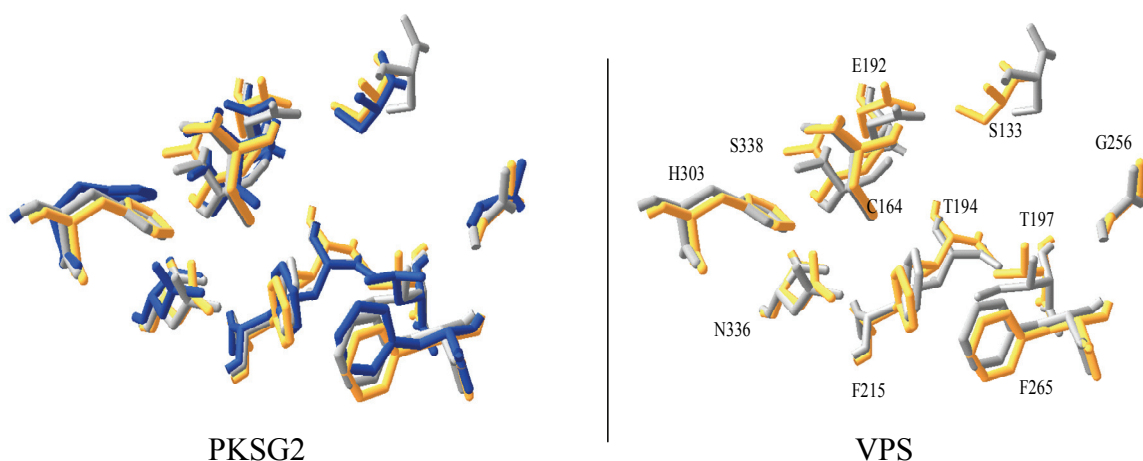


Figure 8. Relative orientation of the sidechains of the active site residues from the 3D model of *H. lupulus* VPS with the 3D model of *C. sativa* PKSG2. The corresponding sidechains in alfalfa CHS are shown in yellow and are numbering; for VPS in gray and for PKSGs in blue.

A comparison of the 3D models of PKSG2, VPS and alfalfa CHS predicted variations in the orientation of the active site residues (Figure 8) which could indicate differences in the specificity for the substrates between VPS and PKSG2.

It seems that the PKS cDNA *PKSG2* isolated from glandular trichomes could encode an olivetolic acid-forming PKS. The fact that cannabinoid biosynthesis takes place in the glandular hairs (Sirikantaramas *et al.*, 2005) and higher cannabinoid content is found in bracts together with an activity for an olivetol synthase (Chapter III) supports this hypothesis. The initial characterization of the *PKSG2* cDNA and the known cannabis *CHS-type* *PKS1* opens an opportunity to study their function and diversity, as well as to learn more about signals or factors that could control their transcription and translation.

The isolation and identification of PKSs with different enzymatic activity in one plant species has been reported, as well as the occurrence of PKS gene families within a species (Rolfs and Kindl, 1984; Zheng *et al.*, 2001; Samappito *et al.*, 2002). The CHS- and STS-type, and olivetol-forming PKS activities from crude protein extracts from *C. sativa* (Chapter III), the expression and partial

characterization of a PKS cDNA from leaves with CHS-type activities (Raharjo *et al.*, 2004b), the characterization of one PKS cDNA generated from mRNA of glandular hairs (this study) and the small gene family of PKSs detected in genomic DNA (Raharjo, 2004) suggest the participation of several PKSs in the secondary metabolism of this plant.

Recently, the crystallization of a cannabis PKS, condensing malonyl-CoA and hexanoyl-CoA to form hexanoyl triacetic acid lactone, was reported (Taguchi *et al.*, 2008). It has been proposed that pyrones or polyketide free acid intermediates undergo spontaneous cyclization to yield alkylresorcinolic acids or stilbenecarboxylic acids (Akiyama *et al.*, 1999; Schröder Group; Chapter II). The homology of this protein with our PKSG2 was 97%. Although, the differences in the amino acid residues from both sequences are small (Figure 9), probably because of the variety of cannabis plant used, a complete biochemical characterization of the protein encoded by *PKSG2* is necessary to confirm that it is a hexanoyl triacetic acid lactone forming enzyme.

HTAL	MNHLRAEGPASVLAIGTANPENIL	QDEFDPYYFRVTKSEHMTQLKEKFRKICDKSMIRK	60					
PKSG2	MNHLRAEGPASVLAIGTANPENILI	QDEFDPYYFRVTKSEHMTQLKEKFRKICDKSMIRK	60					
HTAL	RNCFLNEEHLKQNPRLVEHEMQTL	DARQDMLVVEVPKLGKDACAIAKEWGQPKSKITHL	120					
PKSG2	RNCFLNEEHLKQNPRLVEHEMQTL	DARQDMLVVEVPKLGKDACAIAKEWGQPKSKITHL	120					
HTAL	IFTSASTTDMPGADYHCAKLLGL	SPSVKRVMMYQLGCGGGTVLRIAKDIAENNKGARVL	180					
PKSG2	IFTSASTTDMPGADYHCAKLLGL	SPSVKRVMMYQLGCGGGTVLRIAKDIAENNKGARVL	180					
HTAL	AVCCD	IMACLF	RGPS	ESDLELLVGQAIFGDGAAAVIVGAEPDES	VG	GERPIFELVSTGQTI	240	
PKSG2	AVCCDM	TACLF	RGPS	SDSNLELLVGQAIFGDGAAAVIVGAEPDES	VG	GERPIFELVSTGQTF	240	
HTAL	LPNSEGTIGGHIREAGLI	FDLHKDVPMLISNNIEKCLIEAFTPIG	ISDWSNIFWITHPGG	300				
PKSG2	LPNSEGTIGGHIREAGLM	FDLHKDVPMLISNNIEKCLIEAFTPIG	ISDWSNIFWITHPGG	300				
HTAL	KAILDKVEEKLHLKSKDFVDSRHV	LSEHGNMSSSTVLFVMDLKRKSLEEKGSTTGDGFE	360					
PKSG2	KAILDKVEEKLHLKSKDFVDSRHV	LSEHGNMSSSTVLFVMDLKRKSLEEKGSTTGDGFE	360					
HTAL	WGVLF	GF	GPGLTVERVV	RSVP	IKY	385		
PKSG2	WGVLF	GF	GPGLTVERVV	LR	SP	IN	Y	385

Figure 9. Comparison of the deduced amino acid sequences of the *C. sativa* PKS2 and HTAL. Differences on amino acid sequence are highlighted in gray.

Olivetolic acid, an alkylresorcinolic acid, is the first precursor in the biosynthesis of pentyl-cannabinoids (Figure 10) and the identification of methyl- (Vree *et al.*, 1972), butyl- (Smith, 1997) and propyl-cannabinoids

(Shoyama *et al.*, 1977) in cannabis plants suggests the biosynthesis of several alkylresorcinolic acids with different lengths of side-chain moiety. It is known that the activated fatty acid units (fatty acid-CoAs) act as direct precursors forming the side-chain moiety of alkylresorcinols (Suzuki *et al.*, 2003). Probably, more than one PKS forming alkylresorcinolic acids or pyrones co-exist in cannabis plants. The detection of THCA, a pentyl-cannabinoid, and THVA, a propyl-cannabinoid, in female flowers (Chapter III) from the same variety of cannabis plants that we used for this study, emphasizes the biochemical characterization of PKSG2.

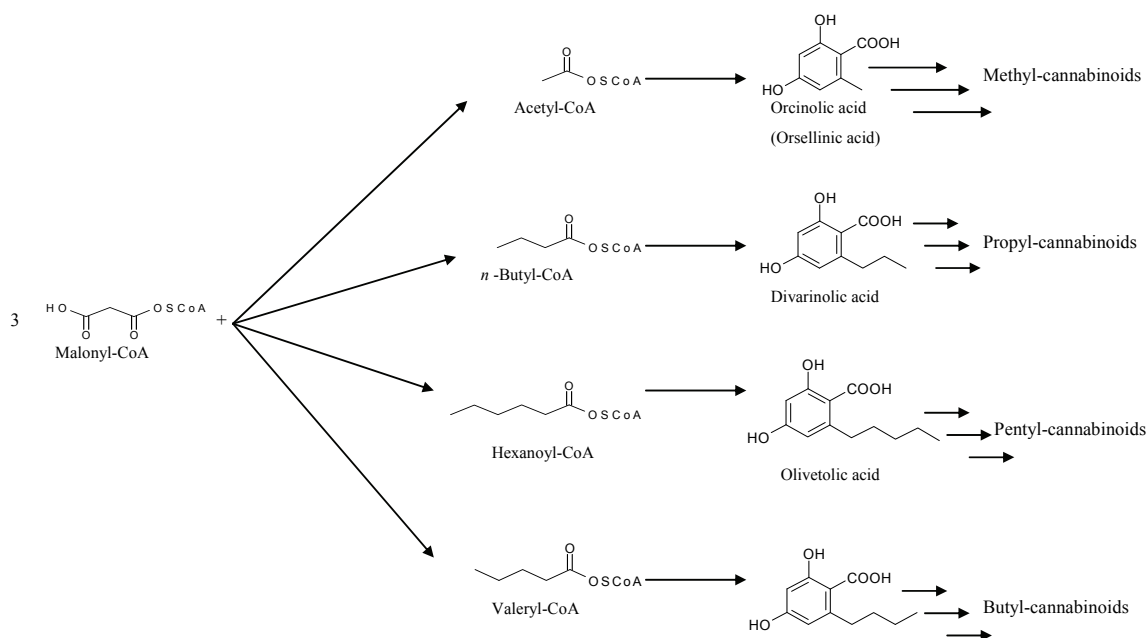


Figure 10. Proposed substrates for cannabis alkylresorcinolic acid-forming PKSs

Acknowledgements

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Chapter V

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 analyzed by ¹H-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.

V.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 70 different cannabinoids have been found so far (ElSohly and Slade, 2005). 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 and De Petrocellis, 2006; Di Marzo *et al.*, 2007). *Cannabis 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). ¹H-NMR spectroscopy is one of these platforms which is currently being explored together with principal component analysis (PCA), the most common method to analyze 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 analyzed by ¹H-NMR spectroscopy. Expression of the *THCA synthase* gene was also monitored by reverse transcription-polymerase chain reaction (RT-PCR).

V.2 Materials and methods

V.2.1 Chemicals

CDCl_3 (99.80%) and CD_3OD (99.80%) were obtained from Euriso-top (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.

V.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 under a light intensity of 1930 lux, 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 as is described in Chapter IV. As negative control, cones of *Humulus lupulus* were collected in September 2004 from the Pharmacognosy gardens (Leiden University) and stored at -80 °C.

Cannabis 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 rpm and 25 °C under a light intensity of 1000–1700 lux. Somatic embryogenesis was initiated from cell cultures maintained in hormone free medium. Cellular viability measurement was according to Widholm (1972).

V.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. *Pythium 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, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Acros Organic, Geel, Belgium) and $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (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 nm 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).

V.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 $\text{MeOH:H}_2\text{O}$ (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 $\text{MeOH:H}_2\text{O}$ 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:H₂O (1:1) and 2D-NMR (HMBC, HMQC, J-Resolved and ¹H-¹H-COSY) was used. Ten fractions were collected and the profiles were analyzed by TLC with silica gel 60F₂₅₄ thin-layer plates developed in ethyl acetate-formic acid-acetic acid-water (100 : 11 : 11 : 26) and revealed with anisaldehyde-sulfuric acid reagent. From fraction 7 tyramine and glutamyl-tyramine were identified and tryptophan was identified in fraction 9. 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 x 2.1 mm, 3.5 µm, ODS) and eluted with acetonitrile-water (10:90) at 1.0 ml/min and 254 nm. Phenylalanine was identified from subfraction 3. For LC-MS analyses, 5 µl of samples resuspended in MeOH were analyzed in an Agilent 110 Series LC/MS system (Agilent Technologies, Inc., 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 100–60% MeOH for 1 min. The optimum APCI conditions included a N₂ nebulizer pressure of 35 psi, a vaporizer temperature of 400 °C, a N₂ 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 x 4.6 mm, 5 µm, Zorbax Eclipse XDB-C18, Agilent) was used.

V.2.5 NMR Measurements, data analyses and quantitative analyses

The dried fractions were dissolved in CDCl₃ and MeOD:D₂O (1:1, pH 6), respectively. KH₂PO₄ was used as a buffering agent for MeOD:D₂O. Hexamethyldisilane (HMDS) and sodium trimethylsilyl propionate (TSP) were used as internal standards for CDCl₃ and MeOD:D₂O, 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) and Choi *et al.* (2004b).

V.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.

V.2.7 RT-PCR and PCR conditions

The degenerated primers ActF (5'-TGGGATGAIATGGAGAAGATCTGGCATCAIAC-3') and ActR (5'-TCCTTYCTIATITCCACRTCACACTTCAT-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'-GATACAACCCCAAACCACTCGTTATTGTC-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: 5 cycles of denaturation for 45 s at 94 °C, 1 min annealing at 48 °C, 1 min DNA synthesis at 72 °C; following 5 cycles with annealing at 50 °C and 5 cycles with annealing at 55 °C, and ending with 30 cycles with annealing at 56 °C. A Perkin Elmer DNA Thermal Cycler 480 and a Taq PCR Core kit (QIAGEN, Hilden, Germany) was 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.

V.3.9 Statistics

Data were analyzed by SIMCA-P 11.0 software (Umetrics Umeå, Sweden) and MultiExperiment Viewer MEV 4.0 software (Saeed *et al.*, 2003; Dana-Faber Cancer Institute, MA, USA). For analyses involving two and three or more groups paired *t*-test, ANOVA and PCA were used, respectively with $\alpha = 0.05$ for significance.

V.3 Results and discussion

V.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 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* (Chapter I) 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. As it is shown in figure 1 cellular growth was not significantly affected by the treatments. However, no signals for cannabinoids in ^1H -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 reveled similar results.

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
<i>P. aphanidermatum</i>	4 and 8 g/ml	2, 4 and 7
<i>B. cinerea</i>	4 and 8 g/ml	1, 2 and 4
Signaling compounds in plant defense		
Salicylic acid	0.3 mM, 0.5 mM 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 $\mu\text{g/ml}$	2 and 4
Cannabis pectin hydrolyzed	2 ml-aliquot	2 and 4
Pectin	0.1 mg/ml	Every 2 days
Sodium alginate	150 $\mu\text{g/ml}$	2 and 4
Abiotic:		
AgNO_3	50 and 100 μM	2 and 4
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	50 and 100 μM	2 and 4
$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$	50 and 100 μM	2 and 4
UV 302 nm	30 s	2 and 4
UV 366 nm	30 min	2 and 4

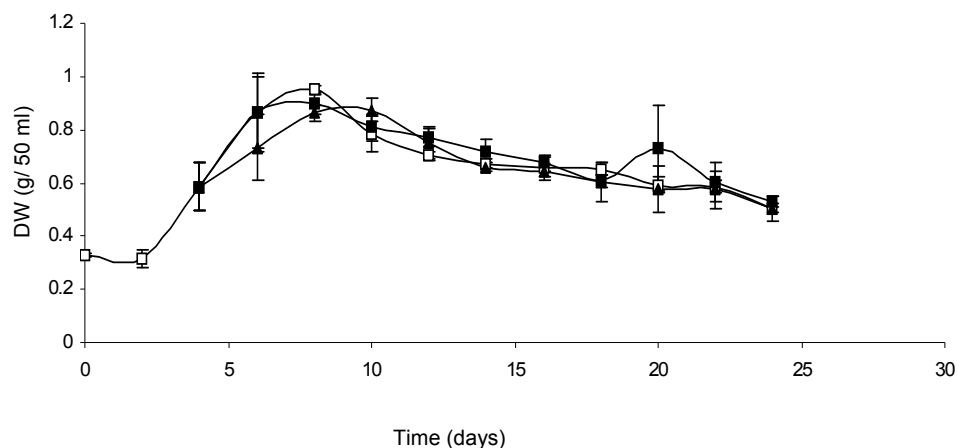


Figure 1. Accumulation of biomass of control (open symbols) and elicited (closed symbols) cannabis cell suspension cultures. Pectin-treated cell cultures (squares) and JA-treated cell cultures (triangles). Values are expressed as means of triplicates with standard deviations.

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 (Figure 2 panel A). DNA amplification of *THCA synthase* in cannabis leaf confirms that conditions and primer concentration were optimal (Figure 2 panel B). 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).

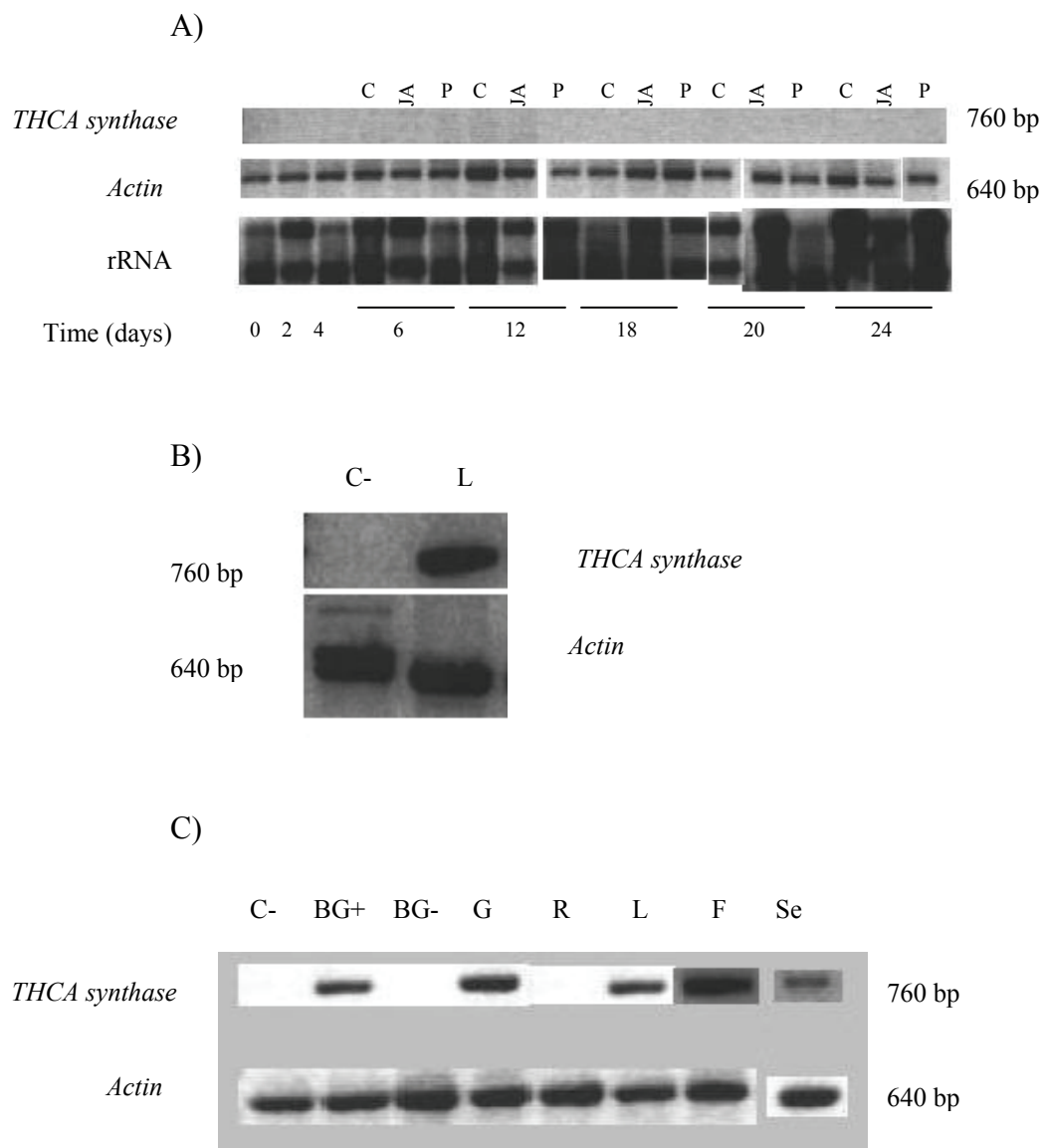


Figure 2. Expression of *THCA synthase*. In panel A *THCA synthase* and *Actin* mRNAs in cannabis cell suspension cultures; C, control; JA, JA-treated cell suspension cultures; P, pectin-treated cell suspension cultures. In panel B the *THCA synthase* and *Actin* genes; C-, negative control (*H. lupulus*); L, cannabis leaf. 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.

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 *Papaver 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 catalyzes 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 (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 accumulates 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 (Figure 2 panel C). No *THCA synthase* gene expression was found in glandular trichome-free bracts or in roots (Figure 2 panel C). Sirikantaramas *et al.* (2005) found *THCA synthase* gene expression in glandular trichomes as well. Although, seedlings did not accumulate cannabinoids (Chapter III), low expression of the *THCA synthase* gene was observed by RT-PCR (Figure 2 panel C). 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 (Figure 3). 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.

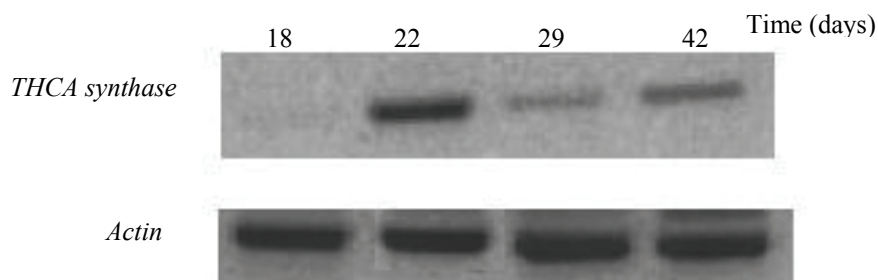


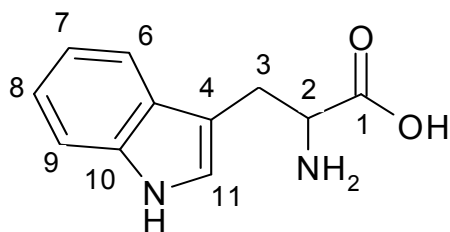
Figure 3. Expression of *THCA synthase* during the development of glandular trichomes on flowers from cannabis plants.

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

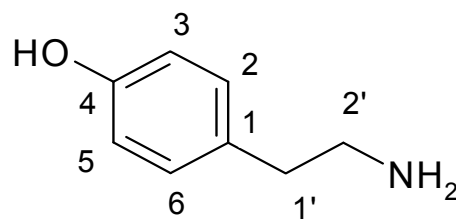
Analyses on the ^1H -NMR spectra of methanol-water extracts from elicitor-treated cell cultures showed differences with the control (Figure 4). Tryptophan (1) (Table 2), tyramine (2), glutamyl-tyramine (3) (Table 3) and phenylalanine (4) (Table 4) were isolated and identified from MeJA treated cell cultures.

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

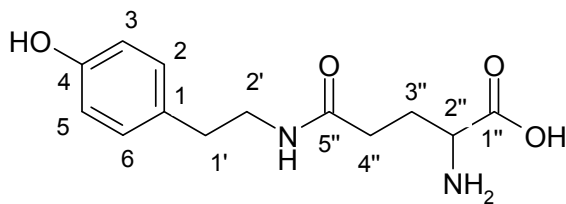
Position	^1H -NMR	^{13}C -NMR	HMBC
1		175.8	
2	3.86 (<i>dd</i> , 8.0, 4.0 Hz)	56.5	C-1,3,4
3	3.51 (<i>dd</i> , 15.9, 4.0 Hz) 3.14 (<i>dd</i> , 15.9, 8.9 Hz)	28.0	C-2,4,5,11 C-2,4,5,11
4		109.0	
5		128.5	
6	7.68 (<i>d</i> , 8.0 Hz)	118.1	C-4,8,10
7	7.03 (<i>t</i> , 8.0 Hz)	120.0	C-5,9
8	7.10 (<i>t</i>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



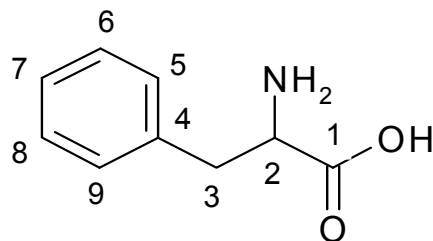
(1)



(2)



(3)



(4)

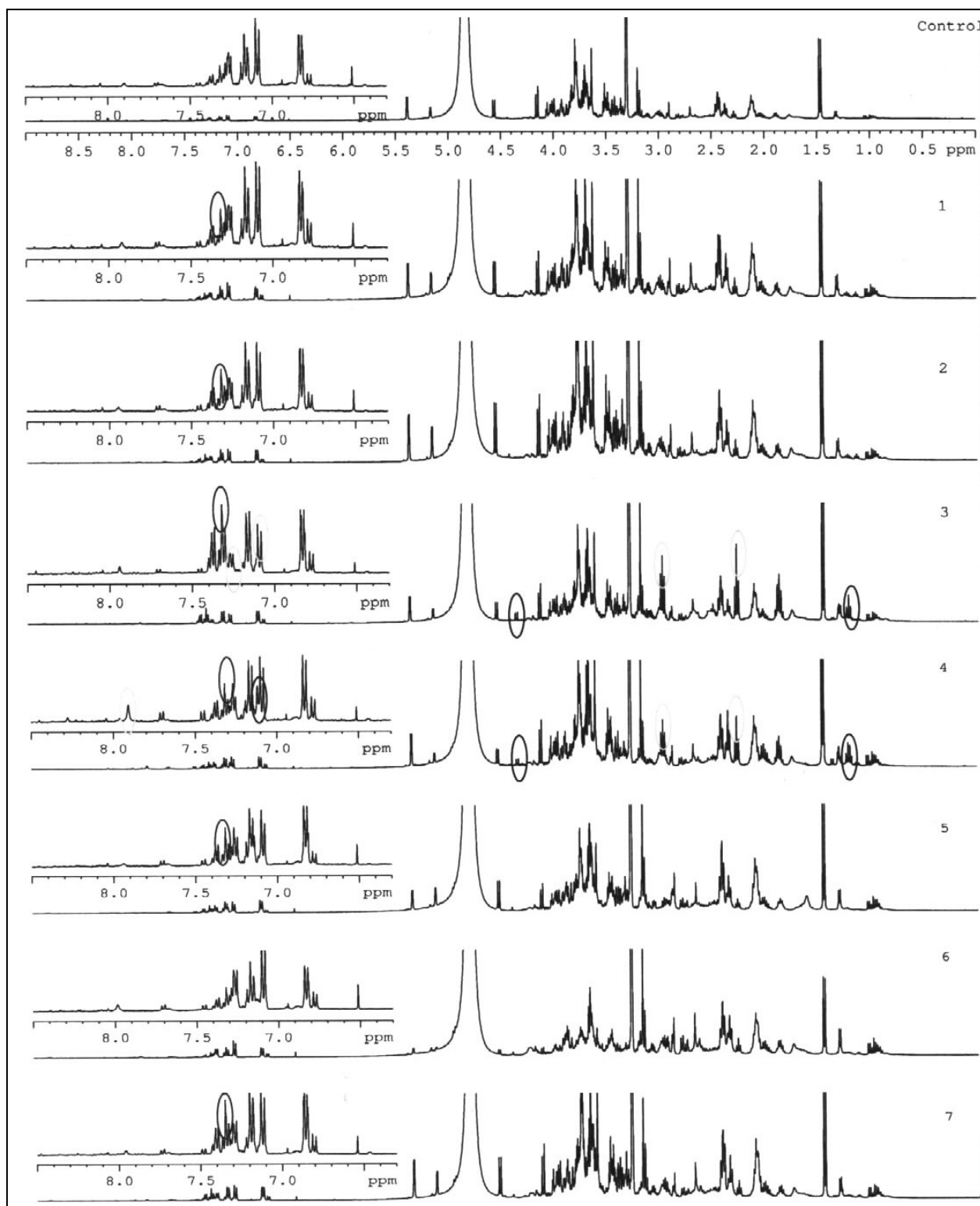


Figure 4. ^1H -NMR spectra of MeOH:Water extracts from cannabis cell suspension cultures elicited by pectin extract/hydrolyzed (1); Sodium alginate (2); Silver nitrate (3); Nickel sulfate (4); cobalt chloride (5); UV 302 nm (6); *B. cinerea* (7). Circles represent changes in peak area rate.

Table 3. ^1H -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		
	^1H -NMR	^{13}C -NMR	HMBC	^1H -NMR	^{13}C -NMR	HMBC
1		127.0			129.6	
2		129.4	C-4,6,1'	7.01 (<i>d</i> , 8.0 Hz)	129.3	C-4,6,1'
6			C-4,2,1'			C-4,2,1'
3		115.5	C-1,5	6.69 (<i>d</i> , 8.0 Hz)	115.0	C-1,5
5			C-1,3			C-1,3
4		156.5			155.5	
1'		32.2	C-1,2(6),2'	2.68 (<i>t</i> , 8.0 Hz)	34.2	C-1,2(6),2'
2'		41.0	C-1,1'	3.34 (<i>t</i> , 8.0 Hz)	41.2	C-1,1',5'
Glutamic acid moiety						
1''	-	-	-		172.5	
2''	-	-	-	3.56 (<i>dd</i> , 15.0, 7.2 Hz)	54.0	C-1'',3'',4''
3''	-	-	-	2.05 (<i>m</i>)	26.5	C-1'',2'',4'',5''
4''	-	-	-	2.38 (<i>t</i> , 7.2 Hz)	31.0	C-2'',3'',5''
5''	-	-	-		173.5	

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

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

In the others treatments with biotic and abiotic elicitors, except with UV exposure, the signal at $\delta 7.34$ was increased and corresponded to phenylalanine. An overview of ^1H -NMR spectra of methanol–water fractions of a time course from elicited cell cultures with JA and pectin is shown in Figure 5. Principal component analysis (PCA) showed that the separations (Figure 6) are based on the aromatic region (PC4) and on culture age or harvest–time (PC3). During the logarithmic growth phase alanine ($\delta 1.48$ and $\delta 3.72$; Table 5) is the predominant compound, glutamic acid and glutamine ($\delta 2.12$, $\delta 2.16$, $\delta 2.40$ and $\delta 2.44$), and valine ($\delta 0.96$, $\delta 1.00$ and $\delta 3.56$) were predominant compounds in JA–treated cells, while aspartic acid ($\delta 2.80$, $\delta 2.84$ and $\delta 3.96$) and γ -aminobutyric acid (GABA, $\delta 1.92$, $\delta 2.32$ and $\delta 3.0$) are the predominant compounds in pectin–treated and control cells. In the stationary phase of cellular growth tyrosine ($\delta 3.88$ and $\delta 3.24$), phenylalanine ($\delta 3.92$) and tryptophan ($\delta 3.48$) increased. These results are similar to those from MeJA–treated cells, where alanine ($\delta 1.49$) and tyramine ($\delta 7.12$) were predominant from 0 to 12 h after treatment; phenylalanine ($\delta 7.34$) reached a maximum concentration at 24 h (Figure 7) and tryptophan content was also induced after 12 h by elicitation with MeJA (Figure 8). Ethanol glucoside ($\delta 1.24$) was a predominant compound after 48 to 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.

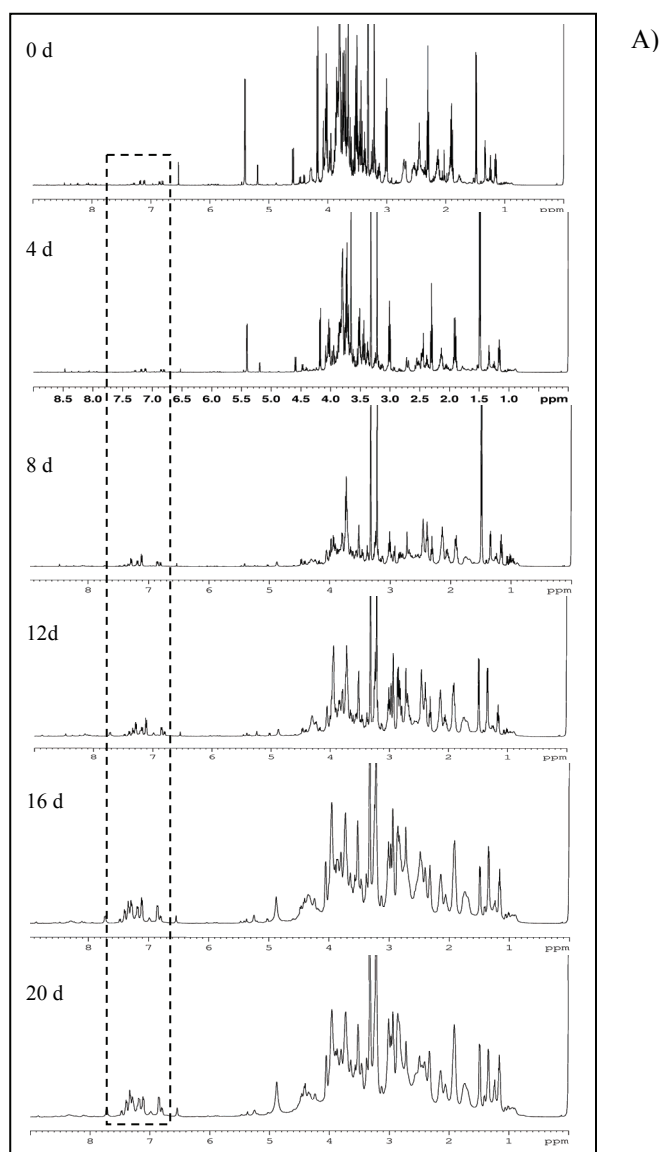
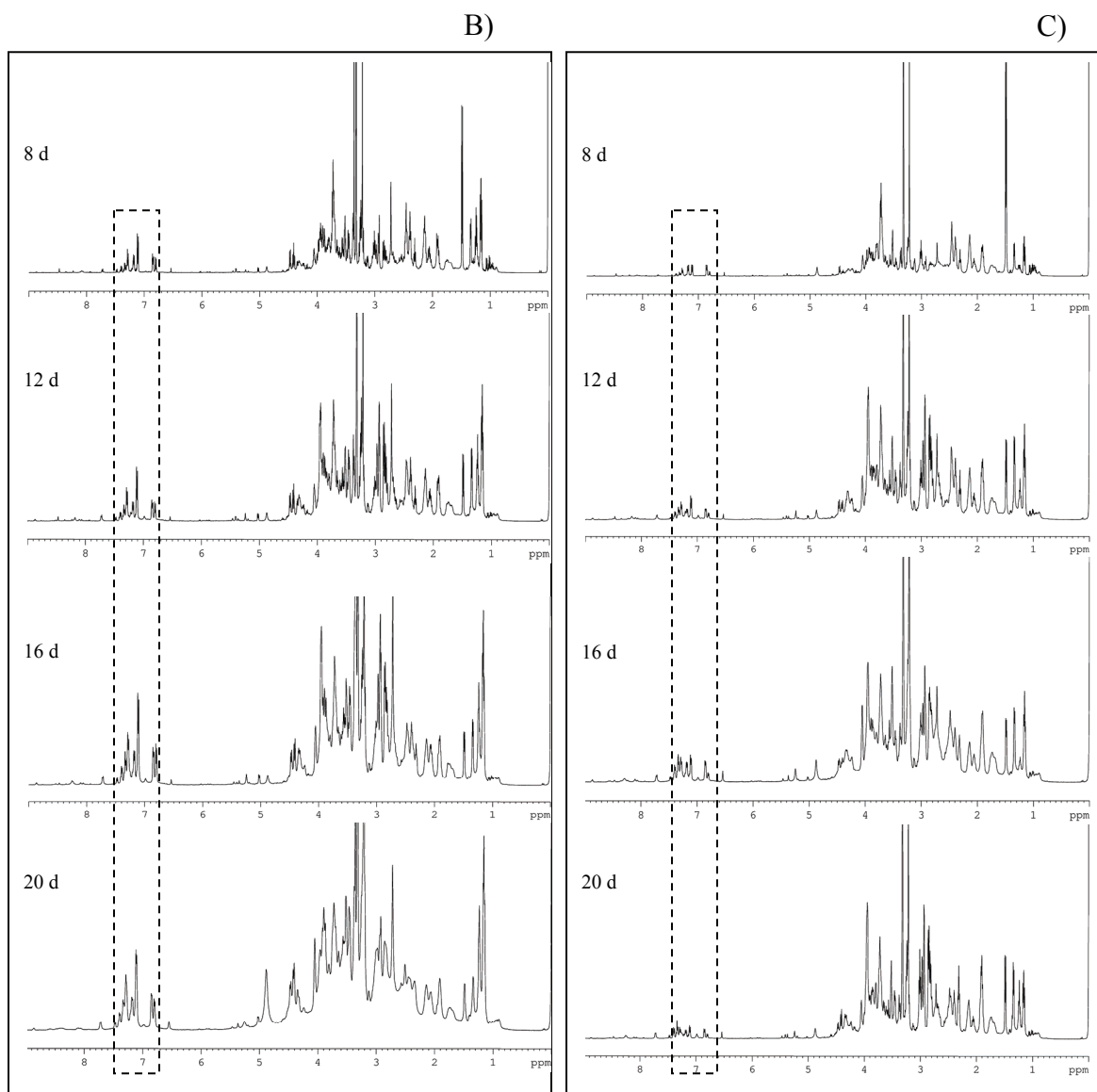


Figure 5. ^1H NMR spectra of MeOH:Water extracts from control (A), JA- (B) and pectin-treated (C) cannabis cell suspension cultures.

Figure 5. *Continued..*

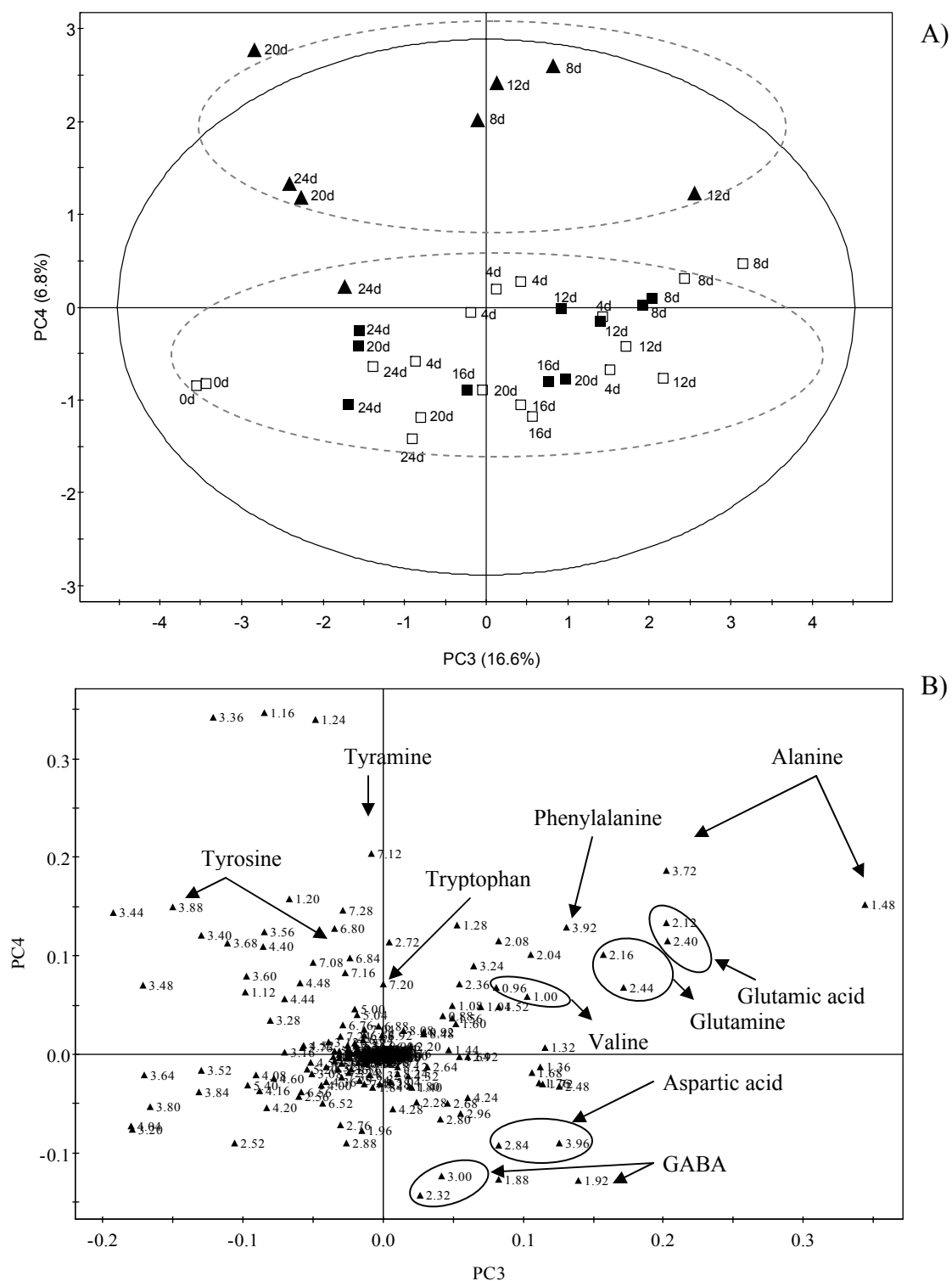
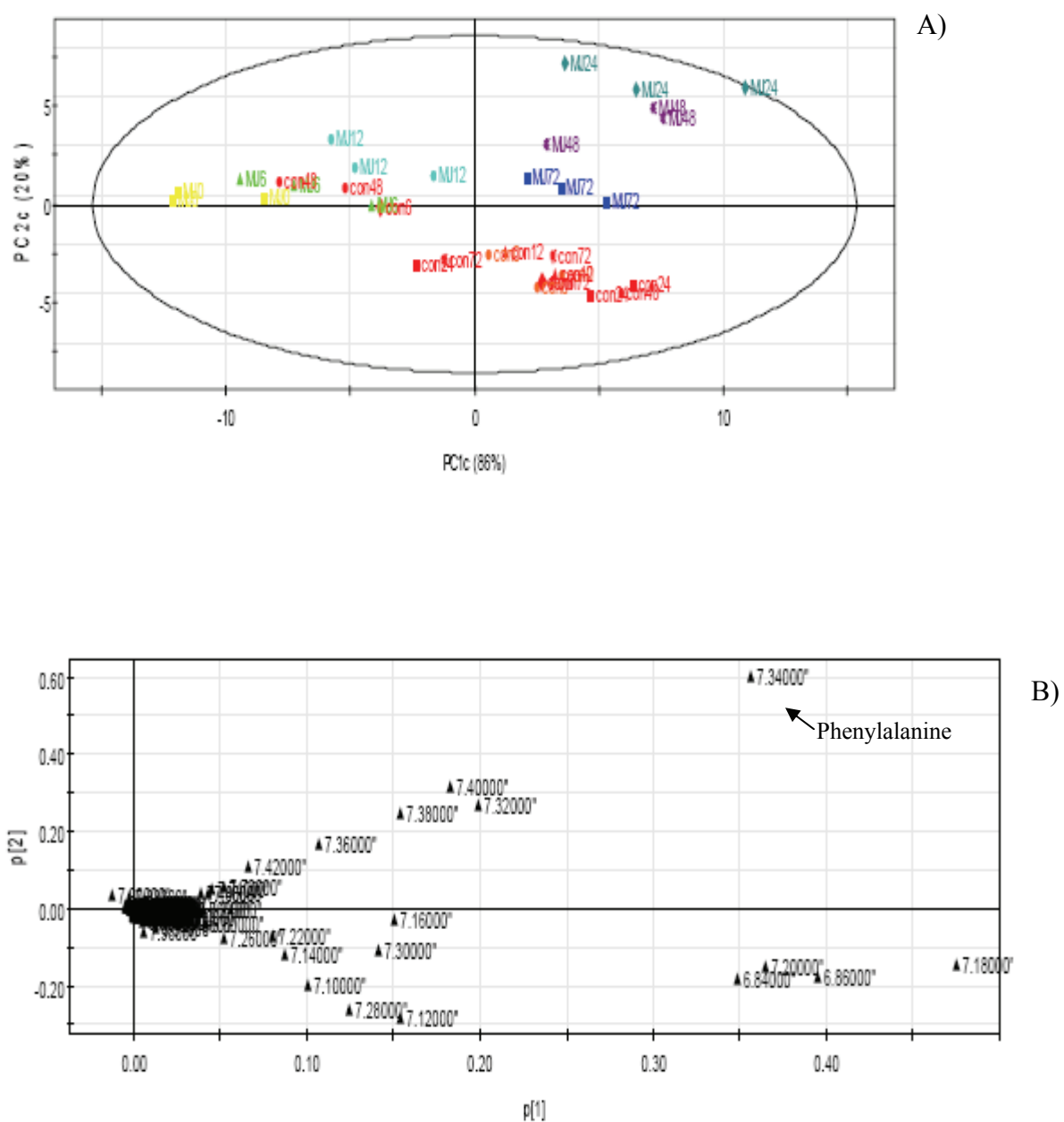


Figure 6. A) Score and B) loading plot of PCA of ^1H -NMR data of MeOH:Water fractions from cannabis cell cultures. Open squares, control cells; closed squares, and pectin-treated cell closed triangles, JA-treated cells; d, day. The ellipse represents the Hotelling T2 with 95% confidence in score plots.

Table 5. Chemical shifts (δ) of metabolites detected in $\text{CH}_3\text{OH}-d_4$ - KH_2PO_4 in $\text{H}_2\text{O}-d_2$ (pH 6.0) from ^1H -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 , <i>d</i> , 7.2), 3.73 (H- α , <i>q</i> , 7.2)
Aspartic acid	2.83 (H- β , <i>dd</i> , 17.0, 7.9), 2.94 (H- β' , <i>dd</i> , 17.0, 4.0), 3.95 (H- α , <i>dd</i> , 8.1, 4.0)
GABA	1.90 (H-3, <i>m</i> , 7.5), 2.31 (H-2, <i>t</i> , 7.5), 3.00 (H-4, <i>t</i> , 7.5)
Fumaric acid	6.54 (H-2, H-3, <i>s</i>)
Threonine	1.33 (H- γ , <i>d</i> , 6.5), 3.52 (H- α , <i>d</i> , 4.9), 4.24 (H- β , <i>m</i>)
Valine	1.00 (H- γ , <i>d</i> , 7.0), 1.05 (H- γ' , <i>d</i> , 7.0)
Tryptophan	3.27 (H-3), 3.50 (H-3'), 3.98 (H-2), 7.14 (H-8, <i>t</i> , 7.7), 7.22 (H-7, <i>t</i> , 7.7), 7.29 (H-11, <i>s</i>), 7.47 (H-9, <i>dt</i> , 8.0, 1.3), 7.72 (H-6, <i>dt</i> , 8.0, 1.3)
Tyrosine	3.01 (H- β), 3.20 (H- β'), 3.86 (H- α), 6.85 (H-3, H-5, <i>d</i> , 8.4), 7.18 (H-2, H-6, <i>d</i> , 8.4)
Phenylalanine	3.09 (H-3, <i>dd</i> , 14.4, 8.4), 3.30 (H-3', <i>dd</i> , 14.4, 9.6), 3.94 (H-2, <i>dd</i>), 7.36 (H-5, H-6, H-7, H-8, H-9, <i>m</i>)
Glutamic acid	2.05 (H- β , <i>m</i>), 2.45 (H- γ , <i>m</i>)
Glutamine	2.13 (H- β , <i>m</i>), 2.49 (H- γ , <i>m</i>),
Sucrose	4.19 (H-1', <i>d</i> , 8.5), 5.40 (H-1, <i>d</i> , 3.8)
α -glucose	5.19 (H-1, <i>d</i> , 3.8)
β -glucose	4.58 (H-1, <i>d</i> , 7.9)
Gentisic acid*	6.61 (H-3, <i>d</i> , 8.2), 6.99 (H-4, <i>dd</i> , 8.2, 2.5), 7.21 (H-6, <i>d</i> , 2.5)
Ethanol glucoside	1.24 (H-2, <i>t</i> , 6.9)

*in $\text{CH}_3\text{OH}-d_4$



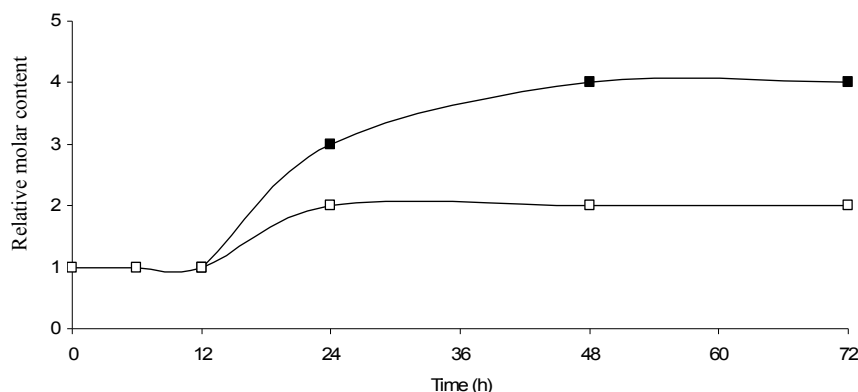


Figure 8. Time course of tryptophan accumulation in control (open symbols) and elicited (closed symbols) cultures of *C. sativa*. MeJA was used as elicitor and was added to cell cultures at the beginning of the time course.

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 analyzed (Figure 9). No significant differences were found in the pools of sucrose and glucose in elicited and control cultures ($P < 0.05$). 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 zero $\mu\text{mol}/100 \text{ mg DW}$. 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 ($P < 0.05$). 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 ($P < 0.05$). 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 ($P < 0.05$).

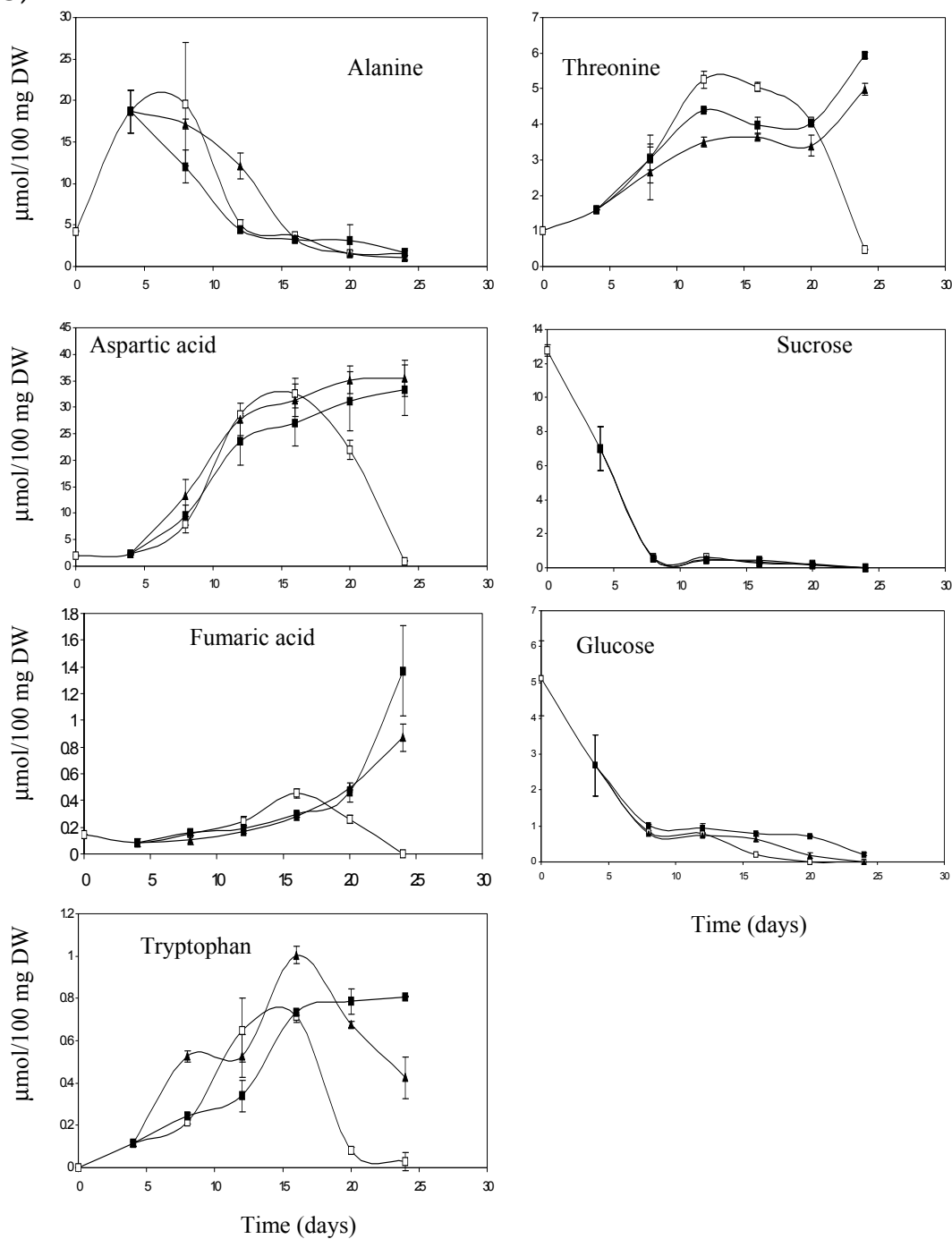


Figure 9. Time course of identified metabolite content in control (open symbols) and elicited (closed symbols) cultures of *C. sativa*. Pectin-treated cell cultures (squares) and JA-treated cell cultures (triangles). TSP was used as internal standard ($1.55 \mu\text{mol}$). Values are expressed as means of three replicates with standard deviations.

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 ($P < 0.05$). 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; Zulak *et al.*, 2008), the differences in pools of some of the metabolites analyzed were observed after elicitation treatments before day 20 (Figure 9) when the cellular viability started to decrease (Figure 10).

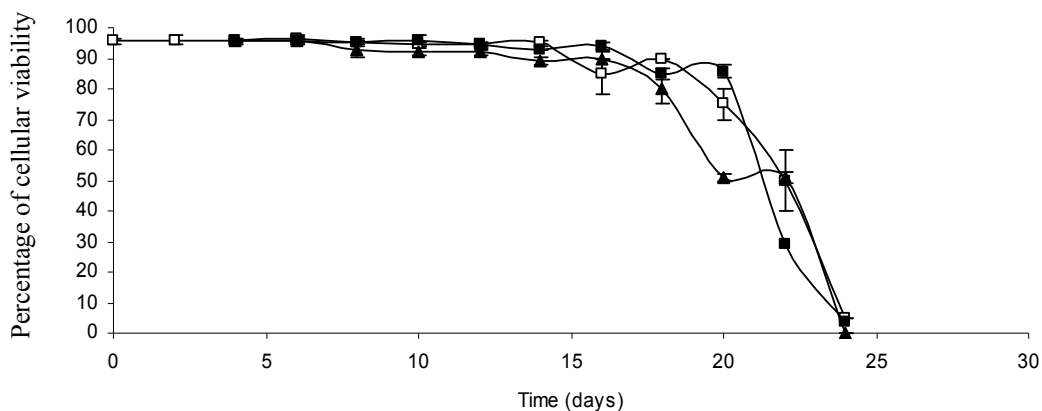


Figure 10. Cellular viability during the time course of control (open symbols) and elicited (closed symbols) cultures of *C. sativa*. Pectin-treated cell cultures (squares) and JA-treated cell cultures (triangles). Values are expressed as means of three replicates with standard deviations.

After day 20, larger differences were found in cultures with more than 95% of dead cells. Gentisic acid (2,5-dihydroxybenzoic acid, $\delta 6.61$, $\delta 6.99$ and $\delta 7.21$; Figure 11) was identified in culture medium and was not affected by the pectin- and JA-treatment.

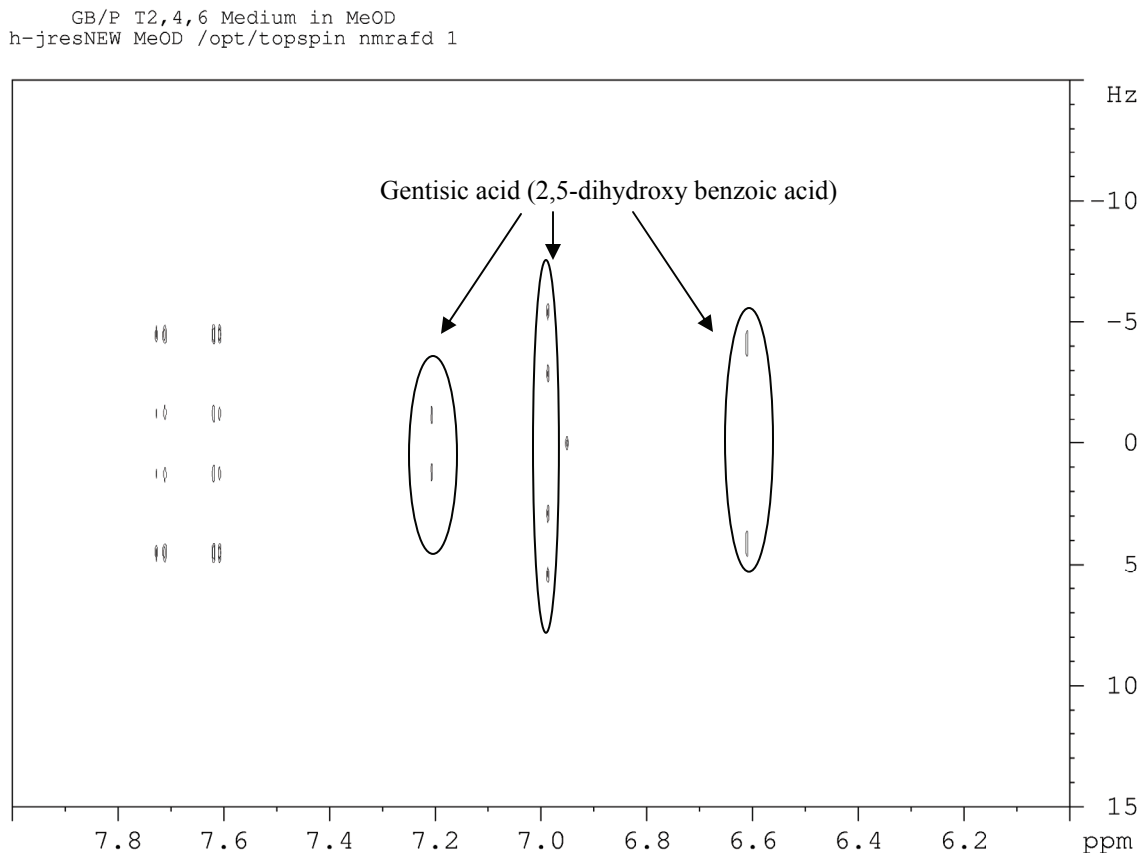


Figure 11. J -resolved ^1H -NMR spectra of medium culture from cannabis cell suspensions in the range of $\delta 6.0$ – $\delta 8.0$.

Figure 12 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 (McCaman *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) Aellen (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 (Chapter I). These secondary metabolites were not identified in the NMR spectra and further analyses using more sensitive 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.

V.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.

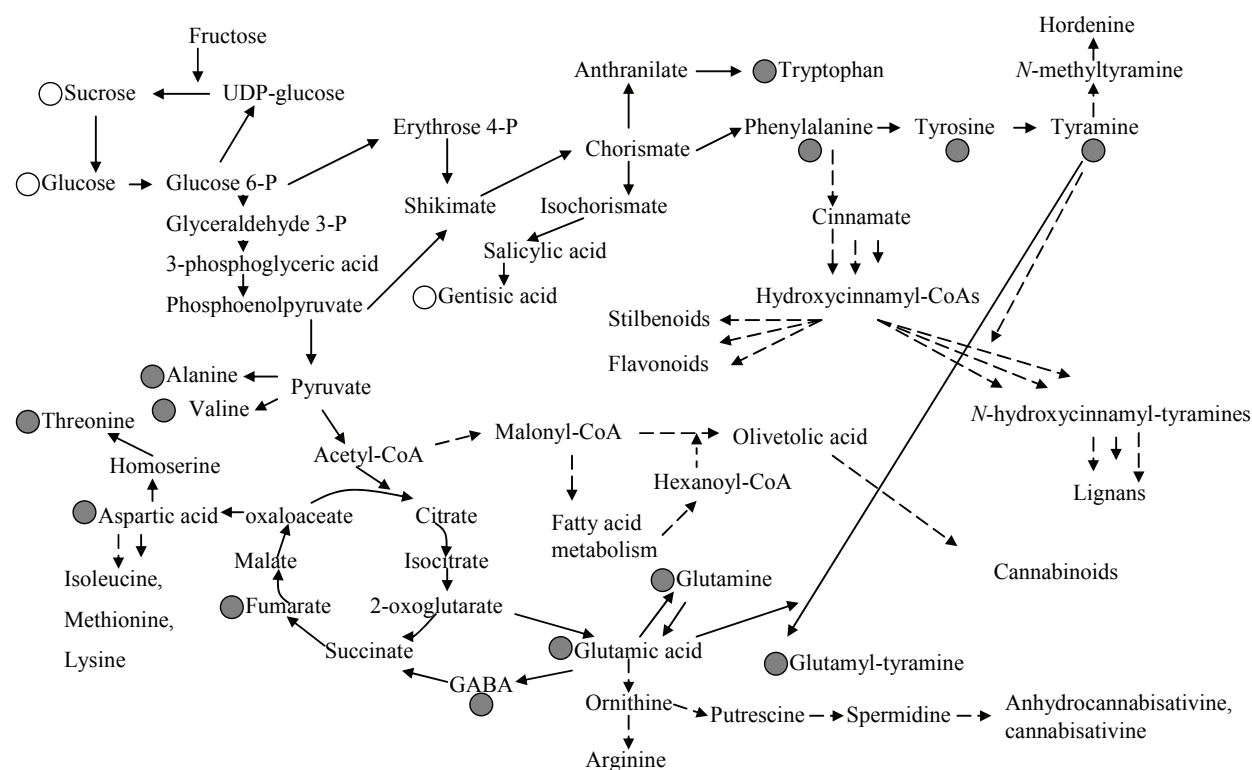


Figure 12. Proposed metabolite linkage map between primary and secondary metabolism in cannabis cell suspension cultures. Metabolites identified in this study are associated with circles. Open circles, unaffected by elicitation; closed circles, metabolites affected by elicitation; dashed line, proposed pathways for biosynthesis of metabolites in cannabis plants.

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Concluding remarks and perspectives

In the phytochemistry from *Cannabis sativa* L., six secondary metabolite groups (cannabinoids, flavonoids, stilbenoids, terpenoids, alkaloids and lignans) have been identified. Pharmacological aspects of the best known group of the secondary metabolism of this plant, cannabinoids, have been extensively studied. Other studies have been focused on the elucidation of the cannabinoid biosynthetic pathway. Although, it has not been completely elucidated, and the same applies for other secondary group biosynthetic pathways in the plant, it has been suggested that a polyketide synthase (PKS) catalyzes the synthesis of the first precursor of the cannabinoid pathway, the olivetolic acid. However, the identification of flavonoids and stilbenoids in the plant involve the presence of more than one PKS.

In this study, the interest was focused on PKSs, their functions in the cannabinoid and flavonoid biosynthesis and the identification of *PKS* genes. Activity of an olivetol-forming PKS and activities of PKSs type CHS and STS were identified from plant tissues. These activities showed to be different in plant tissues. Olivetol-forming PKS activity seemed to be related to the growth and development of the glandular trichomes (hairs) on the female flowers and cannabinoid biosynthesis, a higher cannabinoid accumulation in the bracts than other cannabis plant tissues was shown. Although, type-CHS activity preceded the accumulation of flavonoids in the female flowers and it seemed to be also related to the growth and development of the glandular trichomes on female flowers CHS activity was lower than olivetol-forming PKS activity. The biosynthetic fluxes from cannabinoid and flavonoid pathways seemed to be differentially regulated; differences in the accumulation of these two compounds during the growth and development of the glandular trichomes on the female flowers were observed. Significant activity of type-CHS PKS in roots could not be correlated with flavonoid biosynthesis. Metabolic profilings during development and growth of the cannabis roots to identify the main secondary metabolite groups should be performed to correlate the PKS activities identified in roots. It seemed that stilbenoid accumulation depends on the STS activity, the basal activity of type-STS PKS detected during the growth and development

of the glandular trichomes on female flowers was related to the absence of stilbenoids.

One PKS cDNA (*PKSG2*) was characterized and identified in leaves and glandular trichomes, according to expression analyses by RT-PCR. The expression of the known cannabis CHS-type PKS (*PKS1*) was not tissue-specific, as it was identified in flowers (female and male) and glandular hairs; and from previous studies in leaves and roots by Northern blot. *PKSG2* seems to be a non-chalcone and non-stilbene forming enzyme and *PKS1* a chalcone forming enzyme, according to the phylogenetic analysis. Furthermore, the substrate specificity of *PKS2* is different from CHS and VPS, according to the homology modeling analysis. Although, *PKSG2* is 97% similar to cannabis PKS (*PKS-1*) recently identified, which biosynthesizes hexanoyl triacetic acid lactones, according to the homology analyses the biochemical characterization of the protein encoded by *PKSG2* needs to be carried out. As cannabinoids with different side-chain moiety lengths have been identified in cannabis plants and the detection of THCA, a pentyl-cannabinoid, and THVA, a propyl-cannabinoid, in a same plant tissue, as it was shown on the cannabinoid profile from female flowers highlights the necessity to analyze the biochemical characteristics of *PKSG2*.

No cannabinoids were produced by cannabis cell suspension, calli or embryo cultures; neither did elicited cannabis cell cultures, as it was shown by LC-MS and ¹H-NMR spectroscopy. During a time course the *THCA synthase* gene expression was not detected in the cell cultures corroborating no cannabinoid biosynthesis. In cannabis plants, cannabinoid pathway seemed to be linked to tissue-specificity and/or developmental controls, as it was shown only in cannabis plant tissues containing glandular trichomes such as leaves and flowers the expression of *THCA synthase* gene was observed and it was linked to the development and growth of glandular trichomes on flowers. As cannabinoids are cytotoxic compounds they should be biosynthesized and stored into the glandular trichomes, studies about the development and metabolism of glandular tissues should be considered to increase product yield. Knowledge about the regulatory control of secondary metabolite biosynthetic pathways and gland differentiation may be required to generate successfully these compounds in cell or tissue cultures. Cannabis glandular tissue should be considered as a model system for research.

Summary

Cannabis sativa L. plants produce a diverse array of secondary metabolites, which have been grouped in cannabinoids, flavonoids, stilbenoids, terpenoids, alkaloids and lignans; the cannabinoids are the best known group of natural products from this plant. The pharmacological aspects of this secondary metabolite group have been extensively studied and the cannabinoid biosynthetic pathway has been partially elucidated. Although, it is known that the geranyl diphosphate (GPP) and the olivetolic acid are initial precursors in this route the biosynthesis of the olivetolic acid has not been found yet. It has been suggested that the olivetolic acid biosynthesis could be initiated by a polyketide synthase (PKS). This thesis was focused on the characterization of PKSs in cannabis plants.

More than 480 compounds have been identified from *C. sativa* but only 247 are considered as secondary metabolites. These latter are grouped into cannabinoids, flavonoids, stilbenoids, terpenoids, alkaloids and lignans. However, what do we know about their biosynthesis and role in the plant? **Chapter 1** summarizes the natural compounds in cannabis from a biosynthetic view. It seems that enzymes belonging to the polyketide synthase group could be involved in the biosynthesis of the initial precursors from the cannabinoid, flavonoid and stilbenoid biosynthetic pathways.

The Polyketide Synthases (PKSs) are condensing enzymes which form a myriad of polyketide compounds. In plants several PKSs have been identified and studied. Aspects such as specificity, reaction mechanisms, structure, as well as evolution are reviewed in **Chapter 2**.

In **Chapter 3** polyketide synthase (PKS) enzymatic activities were analyzed in crude protein extracts from cannabis plant tissues. Differences in activities of chalcone synthase (CHS), stilbene synthase (STS) and olivetol-forming PKS were observed during the development and growth of glandular trichomes on the female flowers. Although, cannabinoid biosynthesis and accumulation take place in glandular trichomes no activity for an olivetolic acid-forming PKS was

detected in this tissue. Content analyses of cannabinoids and flavonoids from different tissues revealed differences in their distribution, suggesting a diverse regulatory control on the biosynthetic fluxes of their biosynthetic pathways in the plant.

Chapter 4 reports *in silico* expression analysis of a *PKS* gene isolated from glandular trichomes. The deduced amino acid sequence showed 51–72% identity to other CHS/STS type sequences of the PKS family. Further phylogenetic analysis revealed that this PKS (PKSG2) grouped with other non-chalcone and stilbene-producing PKSs. Homology modeling analyses of this cannabis PKS predicts a 3D overall fold similar to alfalfa CHS2 with small steric differences on the residues that shape the active site of the cannabis PKSG2.

Cannabis sativa cell culture induction has been reported for several purposes. However, cannabinoids have not been detected in cell cultures so far. Although, elicitation has been employed in the cell cultures for inducing and/or improving secondary metabolites there are no reports concerning elicitation effect on secondary metabolite production in *C. sativa* cell cultures. In **Chapter 5** the effect of elicitation on secondary metabolism of the plant cell cultures is reported. Metabolic profiles analyzed by ¹H-NMR spectroscopy and principal component analyses (PCA) showed variations in some of the metabolite pools. However, no cannabinoids were found in both control and elicited cannabis cell cultures. *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.

Samenvatting

Cannabis sativa L. planten produceren een breed spectrum aan secundaire metabolieten. Deze kunnen worden onderverdeeld in cannabinoïden, flavonoïden, stilbenoïden, terpenoïden, alkaloiden en lignanen. De meest bekende groep van de natuurlijke componenten van deze plant zijn de cannabinoïden. De farmacologische aspecten van deze secundaire metabolieten groep zijn zeer uitgebreid onderzocht en de biosynthese route van de cannabinoïden is gedeeltelijk bekend. Hoewel het bekend is dat geranyl difosfaat (GPP) en olivetolzuur de eerste precursors zijn in deze biosynthese route, is de biosynthese van olivetolzuur nog niet aangetoond. Er is gesuggereerd dat de olivetol biosynthese geïnitieerd kan worden door een polyketide synthase (PKS). Dit proefschrift gaat over de karakterisering van polyketide synthases in cannabis planten.

Van *C. sativa* zijn er meer dan 480 verbindingen geïdentificeerd waarvan er waarschijnlijk slechts 247 secundaire metabolieten zijn. Deze groep kan onderverdeeld worden in cannabinoïden, flavonoïden, stilbenoïden, terpenoïden, alkaloiden en lignanen. Maar, wat weten we over de biosynthese en over de functie van deze verbindingen in de plant? **Hoofdstuk 1** is een samenvatting waarin de natuurlijke verbindingen uit cannabis worden beschreven vanuit een biosynthese perspectief. Het blijkt dat enzymen die tot de polyketide synthase groep behoren betrokken kunnen zijn bij de biosynthese van de initiële precursors van de cannabinoïd, flavonoïd en stilbenoïd biosynthese routes.

De polyketide synthases (PKSs) zijn compacte enzymen welke een zeer groot aantal polyketide verbindingen maken. In planten zijn er verschillende PKSs geïdentificeerd en bestudeerd. Een overzicht van aspecten zoals specificiteit, reactiemechanisme, structuur als ook de evolutie wordt gegeven in **Hoofdstuk 2**.

In **Hoofdstuk 3** staat het onderzoek beschreven van ruwe eiwitextracten van cannabis plantweefsels naar de enzymactiviteiten van polyketide synthase. Er

zijn verschillen in activiteit van chalcone synthase (CHS), stilbeen synthase (STS) en olivetol-vormende PKSs waargenomen tijdens de ontwikkeling en groei van de klierhaartjes op de vrouwelijke bloemen. Hoewel de biosynthese en ophoping van cannabinoïden plaats vindt in de klierhaartjes, is er in dit weefsel geen activiteit van een olivetolzuur-vormend PKS waargenomen. Analyse van verschillende weefsels toonde verschillen aan in de concentraties van cannabinoïden en flavonoïden. Dit suggereert dat er een complexe regulatie is op de fluxen van de verschillende biosyntheseroutes in de plant.

In **Hoofdstuk 4** wordt *in silicio* de genexpressie beschreven van een PKS gen geïsoleerd uit de klierhaartjes. De verkregen aminozuursequentie vertoonde 51–72% identiteit met andere CHS/STS type sequenties van de PKS familie. Fylogenetisch onderzoek toonde aan dat deze PKS (PKSG2) overeen kwam met andere niet-chalcone en stilbeen-producerende PKSs. Model analyses op basis van de homologie van deze cannabis PKS voorspelde een “3D-overall” vouwing van het eiwit, vergelijkbaar met het lucerne CHS2, met kleine sterische verschillen van de residuen die de “active site” vormen van het PKSG2.

Het induceren van *Cannabis sativa* celcultures is beschreven voor verschillende doeleinden. Maar tot nog toe zijn in celcultures de cannabinoïden nog niet aangetoond. Hoewel bij celcultures elicitering wel is toegepast voor het induceren en/of verbeteren van de secundaire metaboliet productie, zijn er geen gegevens beschikbaar betreffende het elicitering effect op de secundaire metaboliet productie in *C. sativa* celcultures. In **Hoofdstuk 5** wordt het effect van elicitering op het secundaire metabolisme van de celcultures beschreven. Metabolietprofielen, geanalyseerd met behulp van ¹H-NMR spectroscopie en “principal component analysis (PCA)” vertoonde variaties in enkele van de metabolietgroepen. Echter zowel in de controle als in de geëliciteerde cannabis celcultures zijn er geen cannabinoïden gevonden in. Met behulp van een tijdreeks werd de genexpressie van THCA-synthase gevolgd. De resultaten suggereren dat andere verbindingen uit de signaalroute de cannabinoïd biosynthese route kunnen reguleren

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