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Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR

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ABSTRACT

Trichomes, especially the capitate-stalked glandular hairs, are well known as the main sites of cannabinoid and essential oil production of *Cannabis sativa*. In this study the distribution and density of various types of *Cannabis sativa* L. trichomes, have been investigated by scanning electron microscopy (SEM). Furthermore, glandular trichomes were isolated over the flowering period (8 weeks) by laser microdissection (LMD) and the cannabinoid profile analyzed by LCMS. Cannabinoids were detected in extracts of 25–143 collected cells of capitate-sessile and capitate stalked trichomes and separately in the gland (head) and the stem of the latter. Δ^9-Tetrahydrocannabinolic acid [THCA (1)], cannabidiolic acid [CBDA (2)], and cannabigerolic acid [CBGA (3)] were identified as most-abundant compounds in all analyzed samples while their decarboxylated derivatives, Δ^9-tetrahydrocannabinol [THC (4)], cannabidiol [CBD (5)], and cannabigerol [CBG (6)], co-detected in all samples, were present at significantly lower levels. Cannabichromene [CBC (8)] along with cannabinol (CBN (9)) were identified as minor compounds only in the samples of intact capitate-stalked trichomes and their heads harvested from 8-week old plants. Cryogenic nuclear magnetic resonance spectroscopy (NMR) was used to confirm the occurrence of major cannabinoids, THCA (1) and CBDA (2), in capitate-stalked and capitate-sessile trichomes. Cryogenic NMR enabled the additional identification of cannabichromenic acid [CBCA (7)] in the dissected trichomes, which was not possible by LCMS as standard was not available. The hereby documented detection of metabolites in the stems of capitate-stalked trichomes indicates a complex biosynthesis and localization over the trichome cells forming the glandular secretion unit.

1. Introduction

Cannabis sativa L. is an annual, dioecious herb (Flemming et al., 2007; Pacifico et al., 2006), belonging to the family of Cannabaceae and originating from Eastern and Central Asia (Candolle, 1886; de Barge, 1860). It has long been used in traditional Asian medicine, mainly in India, before the Christian era (Zuardi, 2006). Phytocannabinoids (cannabinoids), a unique group of terpenophenolics possessing alkylresorcinol and monoterpene moieties in their molecular structure (Fig. 1) are considered the most responsible compounds for the biological activities of *C. sativa* L. More than 100 cannabinoids have been identified and structurally elucidated, including recently isolated new entities (Ahmed et al., 2008a,b; ElSohly and Slade, 2005; Radwan et al., 2009, 2008a,b). Because of their psychoactivity, Δ^9-tetrahydrocannabinol (THC (4)) and cannabidiol (CBD (5)) are the most studied and interesting compounds of the class.

Despite the recent completion of *C. sativa* genome sequencing (van Bakel et al., 2011), the metabolic pathway of cannabinoids is not fully understood so far. The biosynthesis starts with the formation of two cannabinoid precursors, namely geranyldiphosphate (GPP), originating predominantly from the non-mevalonate pathway (MEP) (Fellermeier et al., 2001), and olivetolic acid, derived from the not yet fully elucidated polyketide pathway. GPP and olivetolic acid are condensed to form cannabigerolic acid (CBGA (3)) by an enzyme predicted to be a representative of the geranyltransferase group (Fellermeier and Zenk, 1998). CBGA (3) is subsequently transformed to tetrahydrocannabinolic acid (THCA (1)), cannabidiolic acid (CBDA (2)) and cannabichromenic acid (CBCA (7)) by THCA synthase (Taura et al., 1995), CBDA synthase (Taura et al., 1996), and CBCA synthase (Morimoto et al., 1998), respectively. The protein structures and enzymatic activities of

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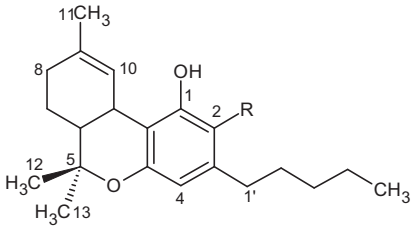
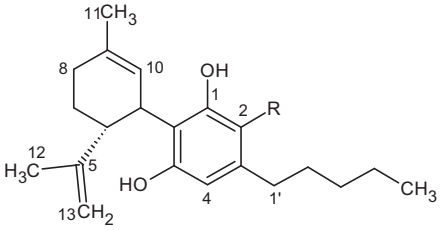
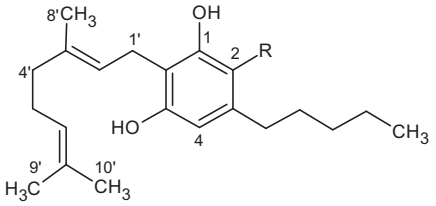


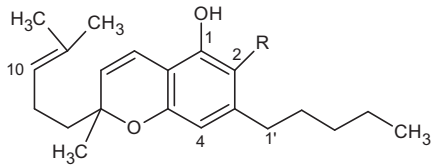
1 R=COOH Δ^9-Tetrahydrocannabinolic acid (THCA)
4 R=H Δ^9-Tetrahydrocannabinol (THC)



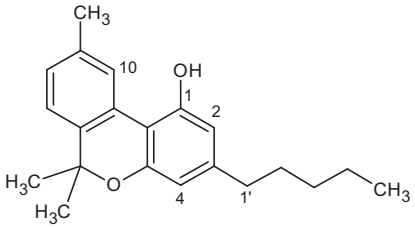
2 R=COOH Cannabidiolic acid (CBDA)
5 R=H Cannabidiol (CBD)



3 R=COOH Cannabigerolic acid (CBGA)
6 R=H Cannabigerol (CBG)



7 R=COOH Cannabichromenic acid (CBCA)
8 R=H Cannabichromene (CBC)



9 Cannabinol (CBN)

Fig. 1. Structures of identified cannabinoids.

THCA and CBDA synthases have been elucidated comprehensively (Sirikantaramas et al., 2004; Taura et al., 2007b). Although CBCA synthase was purified to homogeneity (Morimoto et al., 1998), its sequence has not yet been deposited into the public databases. Finally, THC (4), CBD (5), and cannabichromene (CBC (8)) are formed by decarboxylation of their acidic forms during storage or through interaction with heat and light (Lewis and Turner, 1978).

Recently, new insights into the cellular localization of the cannabinoids biosynthesis were revealed, focusing on the capitate-stalked trichomes (Kim and Mahlberg, 1997; Marks et al., 2009; Sirikantaramas et al., 2005) as the main site of their storage (Lanyon et al., 1981; Petri et al., 1988; Turner et al., 1978). Capitate-stalked trichomes consist of two parts, the gland (head) and the stem (Fig. 1B). The head contains disk cells which are surrounded by the storage cavity. Disk cells are presumed to be the site of cannabinoid production (Kim and Mahlberg, 1991, 1997, 2003). The stem is formed by stipe cells and basal cells (Kim and Mahlberg, 1997) and is not yet functionally characterized. Kim and Mahlberg (1997) reported that cannabinoids, represented by THC, in capitate-stalked trichomes are secreted particularly from disk cells and accumulated in the cell wall, the fibrillar matrix – a surface feature of vesicles in the storage cavity, the subcuticular wall, and the cuticle. Furthermore, Marks et al. (2009) confirmed the head of the capitate-stalked trichome to be the major site of cannabinoids production by proving the presence of cDNAs encoding for three possible polyketide (only one provided olivetol), MEP pathway, and THCA synthases.

Each tissue and cell type of plants has a specific task that is driven by its own unique transcriptome, proteome, and metabolome. Identifying the functions of specific tissues and cell types from plants requires accurate and efficient methods for collecting the populations of the material of interest. Recently, the combination of laser-assisted microdissection technique with diverse range of molecular technologies has allowed this purpose to be achieved (Day et al., 2005). Laser microdissection (LMD) and molecular biology techniques have been used successfully to localize enzymes of artemesinin biosynthesis in the apical cells of trichomes of *Artemisia annua* L. (Olsson et al., 2009). Furthermore, this alliance method has been applied for constructing a cDNA library from isolated phloem cells of rice leaf tissue (Asano et al., 2002) and comprehensive proteome analysis of specific plant tissues (Schad et al., 2005a). LMD has not only been combined with molecular biology techniques but also with metabolites analysis techniques. LMD together with GC–MS has been used to analyze a set of metabolites from vascular bundles of *Arabidopsis thaliana* (Schad et al., 2005b) and its combination with cryogenic NMR and HPLC or MS has enabled the comprehensive analysis of metabolites from the secretory cavities of *Dilantris pillansii* leaves (Schneider and Hölscher, 2007) or the identification of two phenolic compounds in the stone cells of Norway spruce respectively (Li et al., 2007).

In this study, the distribution and density of various types of *C. sativa* L. trichomes, have been determined by scanning electron microscopy (SEM) analysis. Moreover to further advance the understanding of cannabinoids production in trichomes, we hereby report on metabolite profiles as analyzed in specific cells of the glandular hairs as a secreting plant organ, particularly in the intact capitate-sessile and capitate-stalked trichomes as well as in the heads and