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Time course of cannabinoid accumulation and chemotype development during the growth of *Cannabis sativa* L

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Abstract The time course of cannabinoid accumulation in the leaves of individual plants of three *Cannabis* accessions was determined by gas-chromatographic analysis in greenhouse-grown plants. The total amounts and the concentration ratios of CBD, THC and CBG were determined; two accessions (an experimental hybrid, (21R × 15R) × NL, and plants from a seized seed lot) were found chemotypically uniform, with all plants belonging to chemotpe II (mixed) and I (high THC) respectively. The Carmagnola accession showed chemotypic heterogeneity, with a majority of plants belonging to chemotype III. The CBD/THC and CBG/CBD ratios were shown to be largely constant in the leaves, since 28 and until 103 days after sowing, and consistent with the ratios determined on mature inflorescences. CBD and THC maximum amounts in the leaves showed a peak in the leaves around 80 days from sowing, and were shown to be simultaneous during the growth period, irrespective of the chemotypes. Callus cultures were obtained from all the five different chemotypes (I, II, III, IV, V), and GC analyses were performed. Independently of the type and amount of cannabinoids in the mother plants, it was confirmed that callus cultures of *Cannabis* were not able to produce detectable amounts of any cannabinoids.

Keywords · *Cannabis* · Chemotypes · Gas-chromatography · Hemp · Callus

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Introduction

About 400 different chemical substances have been isolated from *Cannabis sativa* L. (Turner et al. 1980); many secondary metabolites like terpenes, alkanes, flavonoids, nitrogenous compounds are abundant in *Cannabis* tissues. Cannabinoids are a peculiar class of substances unique to *Cannabis* genus. Up to now 66 different cannabinoids were found in hemp, the most abundant being cannabidiol (CBD), Δ^9-tetrahydrocannabinol (Δ^9-THC or THC), cannabigerol (CBG) and cannabichromene (CBC; Holley et al. 1975; de Zeeuw et al. 1972).

The role of cannabinoids in *Cannabis* plants is not well understood, but defensive properties towards biotic (insect, bacteria and fungi) and abiotic (dessication and ultraviolet radiation) stresses are hypothesized (Pate 1994). The interest in cannabinoids is today mainly focused on the necessity to limit THC content (the only psychoactive cannabinoid) in fibre hemp, but also on cannabinoids' therapeutic activity and potential employment for pharmaceutical purposes (Mechoulam 2000; Stott and Guy 2004; Pertwee 2004). Δ^9-THC is one of the most useful

molecules that can be obtained for medical applications from *Cannabis*, as it mediates a number of therapeutic effects; the same molecule, however, is also responsible for the intoxicating effects of marijuana and hashish as well (Mechoulam 1970). As a consequence, fibre hemp cultivation still has several restraints in some countries, mainly due to its close genetic and phenotypic relationships with marijuana. EU requires, for granting issues to fibre hemp growers, that THC amount does not exceed 0.20% (dry weight of the reproductive part of the plant at flowering).

Cannabinoids are terpenophenolic substances, differing in the structure of their terpenic moiety and/or the length of the prenyl side chain attached to the phenolic portion. In vivo, they are present as acidic forms (THCA, CBDA, CBCA), that are decarboxilated in the corresponding neutral forms as consequence of heating or drying. In this paper, cannabinoids will be referred to by their neutral forms (e.g., THC, CBD). Cannabinoids are present in all the aerial parts of the *Cannabis* plant, correlated with the presence of glandular trichomes, stalked or sessile, especially present on bracts and leaves (Turner et al. 1978). The pathway and the site of biosynthesis of cannabinoids has not been completely clarified but some authors supposed that cannabinoids are synthetized in specialized disc cells, present in the glandular trichomes, accumulated in the adjacent secretory cavity and finally exuded as resin (Mahlberg and Kim 2004) or, alternatively, that the cannabinoid synthases themselves are secreted (Sirikantaramas et al. 2005). It is commonly accepted that the first cannabinoid synthetized is cannabigerol (CBG), produced by condensation of a phenol-derived olivetolic acid and a terpene-based geranyl diphosphate catalysed by GOT (geranyldiphosphate:olivetolate geranyltransferase; Fellermeier and Zenk 1998; Fellermeier et al. 2001). From CBG, Δ^9-THC, CBD and CBC are synthetized, each by a specific synthase (Sirikantaramas et al. 2004).

Several *C. sativa* variants with different phenotypes characterized by specific cannabinoid ratios and quantities, have been described (chemotypes; Small and Beckstead 1973). Chemotype I is the "drug" type, with a THC amount over 0.30% of inflorescence dry weight, and a CBD content lower than 0.50% (i.e., with low CBD/THC ratio). Chemotype II, the intermediate type, has both CBD and THC, in a ratio around the unity (typically 0.5–2.0); chemotype III, the "fibre" type, has mainly CBD, and a level of THC lower than 0.30% (down to undetectability). Later, two other chemotypes were defined: chemotype IV has a prevalence of CBG (>0.30%), but also CBD (<0.50%; Fournier et al. 1987); and chemotype V, with amounts of all cannabinoids practically undetectable by standard gas-chromatographic analysis (Mandolino and Carboni 2004).

Recent genetic analyses demonstrated that the cannabinoid type (i.e., the chemotype) a *Cannabis* plant is endowed with, is determined by the allelic status at a single locus, *B*; as a consequence of this simple determinism, chemotype can be easily introgressed and segregates into any genetic background (de Meijer et al. 2003; Mandolino et al. 2003; Pacifico et al. 2006).

A variety of studies demonstrated that the overall cannabinoid amount is dependent upon several factors. A cool summer can reduce the cannabinoids contents of the same accession (Latta and Eaton 1975; de Mejer 1992); dry and windy conditions can raise cannabinoids content, and the THC content of leaves was reported to decrease after consistent nitrogen fertilization (Bócsa et al. 1997). According to other authors, total cannabinoid content is also dependent on plant sex and developing phase of the plant (Fetterman et al. 1971; Fairbairn and Rowan 1975). However, few studies determined the chemotype of *Cannabis* plants at different growth stages. Indeed, if the concept of the dependence of the chemotype from the environmental conditions and growth stage would be verified, a radical re-examination of the present legislation, THC analysis protocols and quality controls of the hemp cultivations would be required. In general, the quantitative component of cannabinoid content (i.e., the total amount of cannabinoids synthetized by the plant) is under strong environmental influences (Mandolino et al. 2003; Mandolino 2004). It is also known that in proximity of flowering, the cannabinoid content reaches its maximum in trichome-rich organs; on the contrary, no cannabinoids were reported in roots and seeds, and a few reports analyzing the cannabinoid production of cultured hemp cells were unable to detect any THC or CBD (reviewed in Mandolino and Ranalli 1999).

In order to investigate the dependence of cannabinoids content from the growth stage and differentiation of the hemp tissues, and the possible shifts of

chemotype during the development of the plants, in the present paper we traced the time course of the amount of the three major cannabinoids (THC, CBD and CBG) in three different *Cannabis* accessions belonging to the three main known chemotypes (I, II and III). In addition, explants from *Cannabis* plants of known chemotype were cultured, and undifferentiated tissues (calli) were obtained and analyzed for the cannabinoids content, with the aim to verify the absence of cannabinoids in cultured *Cannabis* cells, and check the possible influence of the chemotype (i.e., the genotype at the *B* locus) on the cannabinoid content.

Material and methods

Three *Cannabis* strains were used to determine the individual chemotype (ratio of amounts) in developing and fully developed flowering plants: Carmagnola, an Italian dioecious fibre variety; (21R × 15R) × NL hybrid, obtained at CRA-ISCI by crossing a drug variety, Northern Lights, with the fibre cv. Fibranova; an unknown *Cannabis* strain, provided by the Italian Police upon seizure. These three accessions were known from previous analyses to correspond to chemotypes III, II and I respectively, and from genetic and marker analysis to have a B_D/B_D, B_D/B_T and B_T/B_T genotype at the *B* locus, respectively (de Meijer et al. 2003; Pacifico et al. 2006). Plants of each strain (44 Carmagnola, 40 (21R × 15R) × NL1 and 32 plants from seized seed) were grown in a greenhouse under environmental conditions: the temperature varied during the growth of the plants between 24°C and 34°C (April-August), and the photoperiod was kept at 16:8 (light:dark) h by artificial lighting when necessary. The duration of the growth period was dependent on the earliness of the different accessions, and showed wide individual variation. By the end of august, however, i.e., about 180 days after planting (DAP), all the plants had flowered.

Starting from 28 DAP, when, under our conditions, the *Cannabis* plantlets were at 3rd leaf stage (defined as stage 1006 in the decimal code for hemp growth; Mediavilla et al. 1998), one young expanded leaf was periodically picked up from each plant of each accession, and individually analysed by GC. The last leaf sampling was made at 103 DAP (code 2101–2201, depending on sex). Because it was necessary to avoid the effects of the leaf age on cannabinoid analysis, the leaves sampled were always the most expanded ones placed at the sub-apical stem node of the plant.

Callus was induced from leaf explants of chemotype I, II and III plants, selected among the same three accessions described above; for chemotype IV callus induction, plants belonging to Bernabeo accession, with a preminent CBG profile (Pacifico et al. 2006), were used, while for chemotype V callus induction, plants showing a completely flat GC (belonging to the USO31 Ukrainian fibre variety, Pacifico et al. 2006), were selected. Seeds of each accession were surface-sterilized with ethanol 100% and $NaClO_4$ 1%, and placed in sterile microboxes (EC02, Micropoli, Italy) at 24 ± 2°C under a 16:8 photoperiod in a growth chamber until they germinated. Seedlings were then transferred in flasks containing B5 medium (Gamborg 1966) supplemented with sucrose 3% and agar 8% (Phytagel^{TM}, Sigma Aldrich), and placed under the same conditions as previously described. After 30 days of growth in aseptic conditions, apical leaf segments were excised and placed in 9 cm Petri dishes containing B5 medium supplemented with kinetin 5 mg/ml and NAA (1-naphtalenic acid) 0.1 mg/ml (Mandolino and Ranalli 1999). White and friable calli were obtained in 7–15 days on several explants. After explant excision, the mother plantlets were transferred in pots containing a 1:1 mixture of sand and peat, kept for 20 days under controlled conditions in a growth chamber, and placed in the greenhouse under the same conditions of the other plants. These plants were only assayed for cannabinoid type and content at flowering; in this way, the chemotype of the plants originating each of the five callus lines, and their genotype at the *B* locus was fully determined.

For GC analyses, all the samples (leaves, reproductive parts and calli) were dried at 65°C for 48 h, powdered, weighed, and 100 mg d.w. were individually analysed by gas-cromathography to quantify Δ^9-THC, CBD and CBG, using the procedures described elsewhere (Pacifico et al. 2006).

Results

In Fig. 1, the time course of the average total cannabinoid content in the leaves of the three accessions examined is shown. The average amount increased

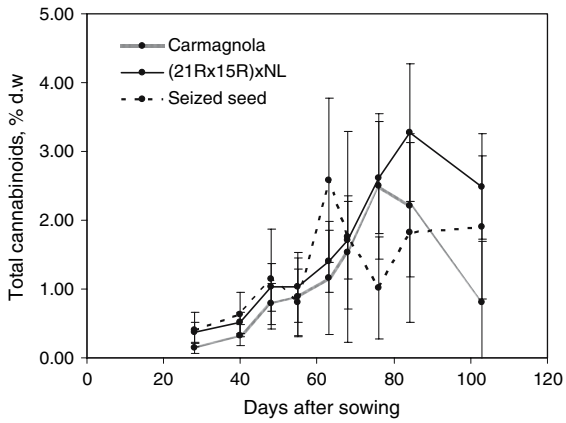

Fig. 1 Time courses of total cannabinoid content (percent of the leaf dry-weight, mean ± standard deviation) for 1044 leaves of the three accessions examined. Standard deviations (SD) are marked as error bars

similarly for all accessions until about 60 DAP, as shown by the extensive overlapping of the three curves. After 60 DAP, standard deviations greatly increased as cannabinoids are further accumulated, indicating a high variability among the leaves sampled. Starting from 80–85 DAP, a decrease in the content of the average total cannabinoid content of the leaves was observed in the hybrid and fibre accessions, but it was not equally evident in seized seed, for which variation was particularly high, and life cycle longer.

The highest total cannabinoid content recorded in an individual was 6.36 % d.w. (4.39% CBD, 1.92% THC and 0.05% CBG) in a (21R ×15R) × NL plant, 90 DAP. No plants of chemotype V (zero cannabinoids; Mandolino and Carboni 2004) were detected within these three accessions, in agreement with previously published results (Pacifico et al. 2006).

The average amount of the main cannabinoids (CBD, THC and CBG) accumulated during the growth period in the three accessions is shown in Fig. 2. Average CBG contents in the leaves were very low (0.00–0.20% d.w.). In Carmagnola leaves, and in the hybrid (21R × 15R) × NL, CBD was found to be the major cannabinoid throughout the entire growth period (Fig. 2a,b), while THC was the predominant cannabinoid at all stages in the plants from the seized seed (Fig. 2c). The time at which the cannabinoids reached the maximum levels in the leaves was, to some extent, accession-specific but, within Carmagnola and the hybrid accession, CBD and THC peaked at the same moment: 76 DAP in Carmagnola and after 80 DAP in (21R × 15R) × NL (Fig. 2a,b); the variability in the data relative to plants from seized seed did not allow to draw similar conclusions for this material (Fig. 2c), for which the highest variability in THC content was found; however, the mean content was always above 0.20% d.w., the threshold set by EU to issue subsidies to hemp growers. In Carmagnola plants, on the contrary, the average THC content (black symbols in Fig. 2a) never exceeded 0.20%. In agreement with this finding, the THC content in inflorescences also remained below 0.20% in Carmagnola and in the hybrid, and above 0.20% in the drug type.

The three main chemotypes (I, II and III), however, are not defined by the total amount of the different cannabinoids, but by the ratio between CBD and THC content. The time course of this ratio is shown in Fig. 3 for all the plants analysed. In this figure, log transformation of CBD/THC ratios was used, to fit all the data in the same plot, where also the ratios measured in the inflorescences are shown. The ratios characterizing the different chemotypes were very clearly distinguished throughout the entire period of leaf sampling and analysis, and remained strongly segregated during the 103 days of the analyses. In total, 1044 leaves from 116 plants were sampled and individually analysed by GC, and only in four cases a "switch" from one chemotype to another was observed (Fig. 3); in these four cases, single leaves belonging to chemotype III plants of Carmagnola variety, were transitorily placed in the group of chemotype II plants. In general, however, the chemotype was maintained by all plants during their entire life cycle, from the earliest leaves to the inflorescence (compare circles and triangles in Fig. 3), and stable and meaningful changes from one chemotype to another were never observed, irrespective of the initial chemotype.

In a previous paper (Pacifico et al. 2006), it was reported that Carmagnola variety was composed of different chemotypes. This was also observed in this work, as 4 Carmagnola plants showed CBD/THC ratios typical of chemotype II, and therefore clustered along with the hybrid (21R × 15R) × NL plants during the whole life cycle until flowering (Fig. 3, black circles and triangles in the gray cluster). The main group of 40 chemotype III Carmagnola plants of Fig. 3 (black symbols in the upper part of the Figure), despite its homogeneity as far as CBD/THC ratios

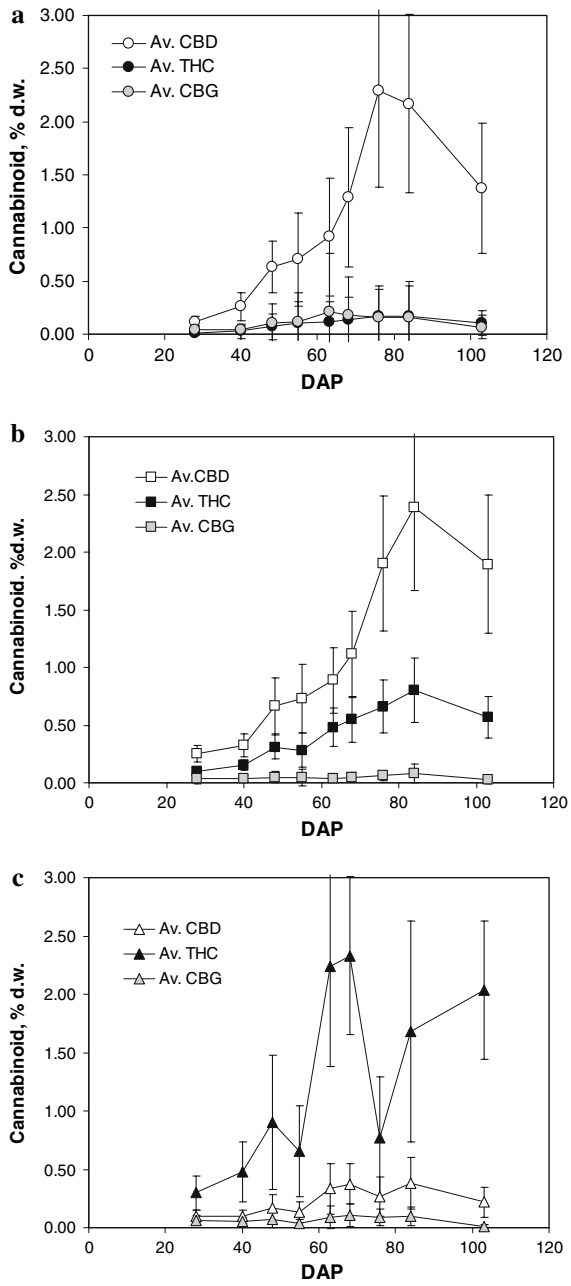

Fig. 2 Time course of the amount of the three main cannabinoids (CBD, THC and CBG) in the leaves of the Cannabis accessions: fibre cv. Carmagnola (**a**), the hybrid (21R × 15R) × NL (**b**) and the seized, putatively drug material (**c**)

were concerned, was heterogeneous as for other cannabinoids. This was evident considering CBG/CBD ratios, by which it is possible to identify and separate chemotype III from chemotype IV (prevalent CBG). Eight out of 40 Carmagnola plants had relatively high absolute amounts of CBG compared to the others (data not shown), but nevertheless they clustered with these latter, until flowering, in CBG/CBD plots (Fig. 4, black circles and triangles). Another single Carmagnola plant showed, on the contrary, a CBG content systematically higher than both CBD and THC, at all the growth stages except the earliest, and until flowering (Fig. 4, white circles and triangle). Therefore, this plant (with a CBG content up to 2.01% of the leaf d.w. and up to 92% of the total cannabinoid content) should be considered belonging to chemotype IV (prevalent CBG; Mandolino and Carboni 2004; Pacifico et al. 2006), whereas the other Carmagnola plants all belong to chemotype II or III. Therefore, this Italian fibre ecotype confirmed to be heterogeneous as for chemotype distribution, with 39 plants belonging to chemotype III, 4 to chemotype II and one to chemotype IV. No CBG-prevalent plants were found in the other two accessions: the hybrid (21R × 15R) × NL plants all belong to chemotype II, and the plants obtained from the seized seed all belong to chemotype I.

In the eight chemotype III Carmagnola plants for which relatively high amounts of CBG were measured during the entire growth period, the peak of CBG accumulation preceeded of 8–10 days the peak of CBD (Fig. 5a). In the single chemotype IV plant, however, it was observed that CBD and CBG peaks were almost simultaneous, and earlier than observed in the other chemotypes (67 days vs. 76 days, Fig. 5b).

In no accession or chemotype, the amount of cannabinoids, their ratio or the time course of their production in the leaves, was found to vary significantly between the male and the female plants (data not shown).

White and friable callus was obtained from leaf explants of all the accessions, irrespective of the chemotype or cannabinoid content of the mother plant. The rate of growth of the different clones obtained was very differentiated, and a different number of subculturing steps were necessary to obtain a sufficient mass of callus growing on hormone-free medium, and for subsequent GC analysis. Gas-chromatography of *C. sativa* calli, at all the stages of formation and/or culture (both grown on media supplemented with growth regulators and on hormone-free media), of all five chemotypes, showed a complete absence of any appreciable peak under the

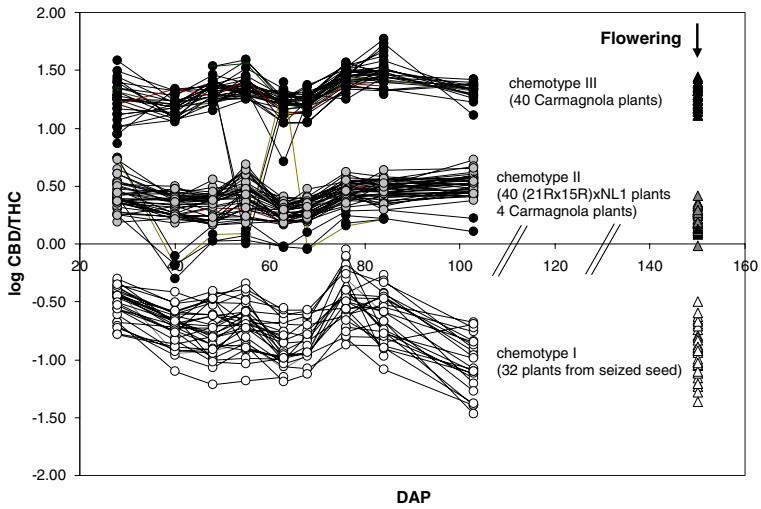

Fig. 3 Time course of the $\log_{10}$CBD/THC for the 116 plants belonging to the three accessions. Chemotypes I, II and III are clearly distinguishable throughout the entire growth period, and overlapping to the accessions, with the exception of four Carmagnola plants belonging to chemotype II instead of III (black circles in the gray cluster). Triangles on the right indicate the $\log_{10}$CBD/THC ratio of the same set of plants at flowering. The individual plants flowered at times different for each individual, but for clarity the data points are depicted as if flowering was simultaneous

standard analytical conditions used (Fig. 6). In callus tissues, no cannabinoids were detectable, irrespective of the genotype at the *B* locus, even if the original plant showed a high content of cannabinoids.

Discussion

In this paper, data about cannabinoids content ratios in the *Cannabis* leaves are discussed, as they are particularly relevant, in view of the proposed modifications of the official analysis protocol required by UE for granting subsidies to the growers: sampling of leaves instead of inflorescences, at a relatively early stage of development, and determination, for chemotype identification, of CBD/THC ratios rather than of absolute THC (Pacifico et al. 2006). Only cannabinoids commonly present in all the three accessions at detectable amounts were considered in this paper.

In Carmagnola and (21R × 15R) × NL leaves, the time courses of total and single cannabinoids accumulation are quite similar. A late decrease in total content was observed, probably related to maturation or leaf senescence phenomena, concomitant with the onset of flowering which was very heterogeneous among plants (Figs. 1 and 2).

The high variability among samples was particulary evident in plants from the seized seed, for which average THC was always above 0.20% d.w. at each growth stage while, in Carmagnola plants, the average THC content never exceeded 0.20% (Fig. 2). The current EU legislation fixed at 0.20% of the plant inflorescence's dry weight the upper content limit to issue subsidies to hemp growers. Therefore, all the plants belonging to the seized seed are not eligible for EU subsidies and could even be considered "drug material", while all Carmagnola plants, irrespective of the chemotype (II, III or IV), can be cultivated and be issued with comunitarian grants.

CBG amounts were in most cases too small to study the time course of accumulation. The only exceptions were the eight relatively high-CBG, chemotype III Carmagnola plants, and the single chemotype IV plant of the same variety. In the eight high-CBG plants, the maximum levels of CBG accumulation preceeded of about 9 days the maximum CBD accumulation in the same leaves (Fig. 5a). In the single chemotype IV plant, however, CBD and CBG peaks were earlier (67 days), and reached much more in proximity (Fig. 5b). Whether this could be due to the anomalies in the CBD biosynthetic flow postulated for chemotype IV plants (de Meijer and Hammond 2005) or to the fact that only one plant with these characteristics could be examined, it cannot be decided by these data, and deserves further investigations.

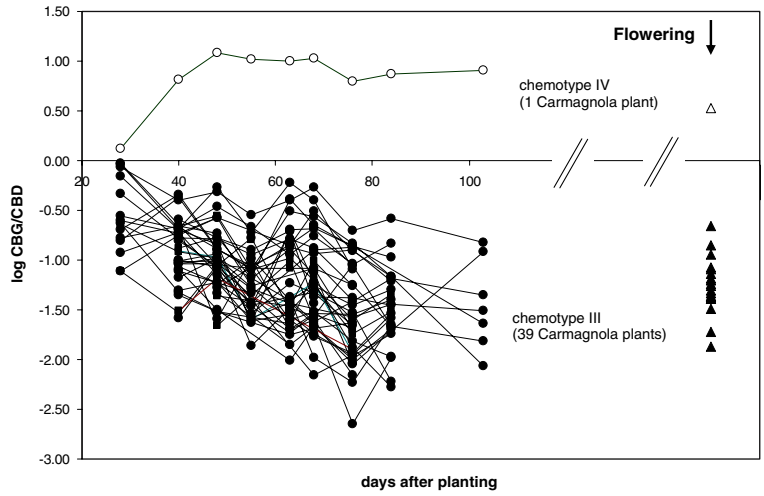

Fig. 4 Time course of the $\log_{10}$CBG/CBD for the Carmagnola plants indicated as belonging to chemotype III in Figure 3. Only one single Carmagnola plant (white circles) showed a CBG/CBD ratio typical of chemotype IV plants. Triangles on the right indicate the CBG/CBD ratio of the same set of plants at flowering. The individual plants flowered at times different for each individual, but for clarity the data points are depicted as if flowering was simultaneous

The seized material was chemotypically homogeneous, as all 32 plants examined were chemotype I (drug type, with low CBD/THC; Figs. 2c and 3); similarly, all the 40 plants of the experimental hybrid (21R × 15R) × NL analysed were chemotype II (mixed CBD and THC). Chemotype II material also showed a prevalence of CBD over THC, i.e., CBD/THC ratios exceeding the unity (Figs. 2b and 3); this was observed to be constant throughout the entire life cycle of the plants, but it cannot be considered a general feature of chemotype II plants, as in different chemotype II populations, CBD/THC ratios below 1.0 were on the contrary observed (see de Meijer et al. 2003, for a discussion on the genetic meaning of CBD/THC ratios variability in chemotype II plants).

Chemotype of each plant was found to be very constant throughout all the stages analyzed, perfectly coherent when detemined on either leaves or inflorescences, and clearly clustered in a way largely corresponding to the three accessions (Figs. 3 and 4; see below for the Carmagnola exception). The demonstration of the constancy of chemotype during the growth of hemp plants has important implications for the sampling operations and analysis protocols for the determination of cannabinoids; it is clear from our data that a chemotype III plant (e.g., a fibre hemp) will continue to belong to this chemotype, and will not develop the low CBD/THC ratios typical of drug plants, whatever the moment of the analysis. Conversely, no plant born for the efficient production of THC will develop the high CBD/THC ratios typical of the fibre hemp, irrespective of growth stage. Therefore, any confusion between chemotypes appeared from these data impossible, both for the genetic constitution of the plants, and for the remarkable constancy with which such genetic determinants are expressed since very early developmental stages, and until flowering (de Meijer et al. 2003; Mandolino et al. 2003; Mandolino and Carboni 2004; Mandolino 2004; Pacifico et al. 2006). Therefore, the overall data set presented here shows that, even if CBD, THC and CBG amounts were not constant at the different ages of the plants, the chemotype did not change.

Carmagnola variety was the only of the three accessions examined that showed chemotype heterogeneity. In fact, four Carmagnola plants clustered in Fig. 3 among the chemotype II plants, consistently showing, throughout all the growth, a mixed cannabinoid profile. Carmagnola plants were initially assigned to chemotype III (40 plants) and chemotype II (4 plants). However, if absolute amounts of CBG, and CBG/CBD ratios in the single plants are considered, further levels of heterogeneity were evidenced. In fact, while in all the plants from seized seed and in all the hybrid plants, CBG was absent or present at very low levels, in Carmagnola variety nine out of the 40 plants assigned to chemotype III showed CBG contents comparatively high. Eight of these plants clustered along the other Carmagnola plants in CBG/CBD plots (Fig. 4), as their higher CBG content is balanced by a correspondingly higher CBD amount. One of the nine Carmagnola plants, on the contrary, showed a CBG/CBD ratio constantly and significantly higher than the others. This single plant can

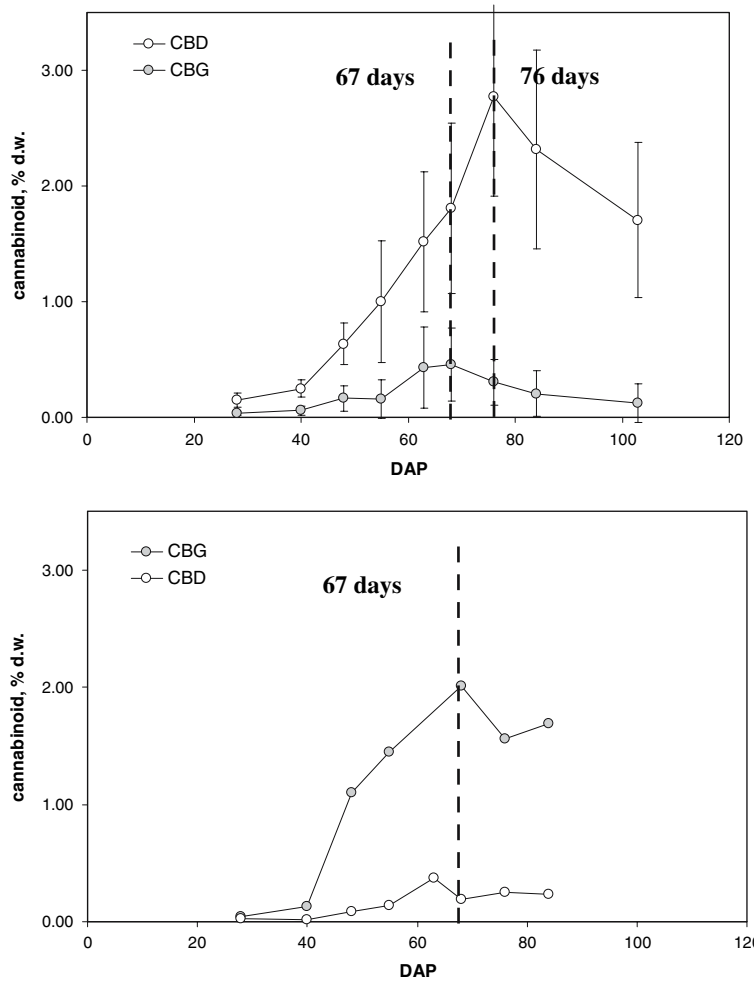

Fig. 5 Time course of average CBD and CBG accumulation in the leaves of the eight high-CBG producing chemotype III plants (**a**) and in the single plant of chemotype IV (**b**) of Carmagnola variety

therefore be assigned to chemotype IV, as defined by Fournier et al. (1987) and, Mandolino and Carboni (2004), and as genetically characterised by de Meijer and Hammond (2005) and Pacifico et al. (2006). Therefore, according to this analysis, Carmagnola variety confirmed previous reports of its chemotypic heterogeneity. However, it should be pointed out that chemotype III and IV plants from Carmagnola variety could not be distinguished at the first sampling (about 28 days after sowing; Fig. 4), when on the contrary the other main chemotypes were already perfectly differentiated (Fig. 3).

Irrespective of the chemotypes of the mother plant, GC analysis of *Cannabis* callus cultures confirmed a complete absence of any cannabinoid, with no difference depending on the period spent in vitro, or the presence of growth regulators in the culture medium. GC of callus and leaf tissue, with the same genotype at the *B* locus, were completely different, as shown in Fig. 6, where the flat chromatogram indicated a complete absence of detectable amount of any compound in the retention time range of cannabinoids. The lack of capacity of undifferentiated cell cultures to produce detectable amounts of cannabinoids, was already reported by other authors (reviewed by Mandolino and Ranalli 1999), and in this paper it has been fully confirmed for all chemotypes, i.e., for all genotypes at the *B* locus. The molecular or biochemical reasons of this complete metabolic block are not known. Indeed it's unclear whether the cannabinoids' synthesis is strictly connected with the formation of glandular trichomes and needs a cell-specific activation, similarly to other species. For example, the production of hypericins and hyperforins in *Hypericum perforatum* needs the formation of small glandular structures (Pasqua et al. 2003; Gadzovska et al. 2007) and the

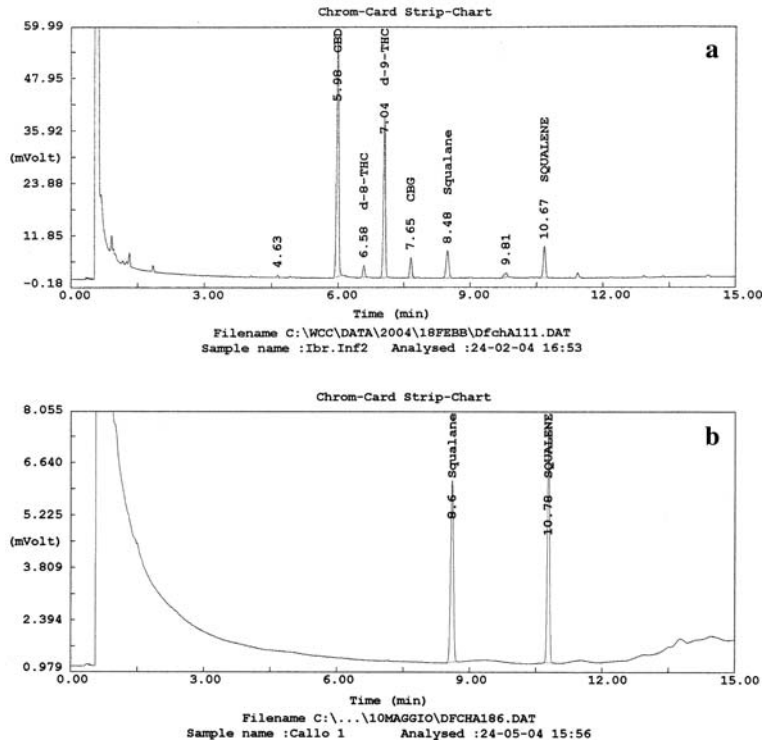

Fig. 6 Gas-chromatographic profiles of a chemotype II hybrid plant belonging to the accession (21R × 15R) × NL1 (**a**), and of the callus cultures deriving from explants of the same plant (**b**)

production of nicotine in *Nicotiana tabacum* needs organ differentiation (Peters et al. 1974). Often elicitors are widely used to enhance the production of metabolites of interest in cell cultures, otherwise very limited. Only further analysis will clarify the basis of the absence of detectable cannabinoids in callus cultures of *C. sativa*.

In the study presented here, GC comparison of the individual cannabinoids produced by single plants during the development was reported. It was shown that THC in chemotype I, and CBD in chemotype III plants, become the predominant cannabinoids since the earliest samplings (28–40 days after sowing), and that chemotype does not change in plants at different ages or in different sexes, as demonstrated by the consistency of CBD/THC or CBG/CBD ratios throughout the entire life cycle until flowering. For these reasons, we propose here that it is not necessary to wait anthesis, and to sample the flowering portion of the plants, to get safe and reliable informations about the chemotype, not only for assessing eligibility for fibre hemp subsidies, but for forensic and legal purposes as well.

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