

# The inheritance of chemical phenotype in *Cannabis sativa* L. (III): variation in cannabichromene proportion

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Received: 21 April 2008 / Accepted: 7 August 2008 / Published online: 23 August 2008  
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**Abstract** The mechanism that controls the proportion of cannabichromene (CBC), a potential pharmaceutical, in the cannabinoid fraction of *Cannabis sativa* L. is explored. As with tetrahydrocannabinol (THC) and cannabidiol (CBD), CBC is an enzymatic conversion product of the precursor cannabigerol (CBG). CBC is reported to dominate the cannabinoid fraction of juveniles and to decline with maturation. This ontogeny was confirmed in inbred lines with different mature chemotypes. A consistent CBC presence was found in early leaves from a diverse clone collection, suggesting that CBC synthase is encoded by a fixed locus. Morphological variants possessing a ‘prolonged juvenile chemotype’ (PJC), a substantial proportion of CBC persisting up to maturity, are presented. PJC is associated with a reduced presence of floral bracts, bracteoles, and capitate-stalked trichomes. Genetic factors causing these features were independent of the allelic chemotype locus *B* that was previously postulated and regulates THC and CBD synthesis and CBG accumulation. In contrast to previously described *Cannabis* chemotypes, the cannabinoid composition of PJCs showed plasticity in that reduced light levels increased the CBC proportion. The ability of PJC plants to enable the production of pharmaceutical raw material with high CBC purity is demonstrated.

**Keywords** Cannabichromene · *Cannabis* · Chemotype · Genotype · Ontogeny · Plasticity

## Introduction

Cannabinoid biogenesis

*Cannabis* plants synthesise and accumulate cannabinoids as carboxylic acids (e.g., cannabichromenic acid, CBCA). In this paper these compounds will be indicated by the abbreviations for their neutral forms. The most common cannabinoids, those with a pentyl side chain, are cannabidiol (CBD; Adams et al. 1940; Mechoulam and Shvo 1963), delta 9-tetrahydrocannabinol (THC; Gaoni and Mechoulam 1964a), cannabichromene (CBC; Gaoni and Mechoulam 1966) and cannabigerol (CBG; Gaoni and Mechoulam 1964b).

The first specific step in the pentyl cannabinoid biosynthesis is the condensation of geranylpyrophosphate (GPP) with olivetolic acid (OA) into CBG. This reaction is catalysed by the enzyme geranylpyrophosphate:olivetolate geranyltransferase (GOT; Fellermeier and Zenk 1998). CBG is the direct precursor for each of the compounds THC (Taura et al. 1995), CBD (Taura et al. 1996) and CBC (Gaoni and Mechoulam 1966; Morimoto et al. 1997, 1998). The different conversions of CBG are enzymatically catalysed, and for each reaction an enzyme has been identified: THC acid synthase (Taura et al. 1995), CBD acid synthase (Taura et al. 1996) and CBC acid synthase

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(Morimoto et al. 1997, 1998). Cannabinoids with propyl side chains, as identified by Vree et al. (1971) and de Zeeuw et al. (1972), result if GPP condenses with divarinic acid instead of OA, into cannabigerovarin (CBGV). The three cannabinoid synthase enzymes are not selective for the length of the alkyl side chain and convert CBGV into the propyl homologues of CBD, THC and CBC, which are indicated as cannabidivarin (CBDV), delta 9-tetrahydrocannabivarin (THCV) and cannabichromevarin (CBCV), respectively (Shoyama et al. 1984).

Cannabinoids are deposited in the non-cellular, secretory cavity of glandular trichomes. Sirikantaramas et al. (2005) confirmed the presence of the central precursor CBG and an exclusive THC synthase activity in the secretory cavity and concluded that this is not only the site of THC accumulation but also of its biosynthesis. As THC, CBD and CBC all result from CBG conversions, it was suggested that CBD and CBC are also synthesised in the secretory cavity. Mahlberg and Kim (2004) report that glandular trichomes are exclusively specialised to synthesise high amounts of cannabinoids and that other tissues contain only very low levels. They recognised three types of glandular trichomes: the small bulbous and the large capitate-sessile form that are both present on leaf surfaces throughout the plant and the large capitate-stalked form that develops after flower initiation on inflorescence bracts (small leaves) and bracteoles (structures enclosing the ovary). These authors report that the cannabinoid content of capitate-stalked glands is about 20 times that of capitate-sessile glands.

According to Taura et al. (1995, 1996), CBD and THC synthase are very similar in respect of their affinity for CBG ( $K_m$  values 134 and 137  $\mu\text{M}$ , respectively) and their catalytic capacity (turnover number  $k_{\text{cat}}$ , 0.19 and 0.20  $\text{s}^{-1}$ , respectively). The affinity of CBC acid synthase for the CBG substrate is higher ( $K_m = 23 \mu\text{M}$ ) than that of CBD and THC synthase but, in contrast, its catalytic capacity is lower ( $k_{\text{cat}} = 0.04 \text{ s}^{-1}$ ) (Morimoto et al. 1998).

It has been previously concluded that the inheritance of CBD and THC composed chemotypes is controlled by a monogenic, co dominant mechanism (de Meijer et al. 2003). A single locus referred to as *B*, with two alleles,  $B_D$  and  $B_T$ , encoding CBD and THC synthase respectively, was postulated. According to this model, a true-breeding, strongly CBD predominant plant has a  $B_D/B_D$  genotype at the *B* locus, a

true-breeding, strongly THC predominant plant has a  $B_T/B_T$  genotype and plants with substantial proportions of both CBD and THC are heterozygous  $B_D/B_T$ . de Meijer and Hammond (2005) concluded that plants accumulating the precursor CBG have a mutation of  $B_D$  called  $B_0$ , in the homozygous state, which encodes for a poorly functional CBD synthase. The genetic control of the CBC biosynthesis remains to be clarified. de Meijer et al. (2003) briefly addressed two options: a further allele  $B_C$  at the *B* locus, encoding CBC synthase, or the involvement of a completely different locus that may or may not be allelic.

#### The occurrence of cannabichromene in *Cannabis* chemotypes

To discriminate between quantitative and qualitative aspects of cannabinoid accumulation, de Meijer et al. (2003) considered the yield of a certain cannabinoid as a complex trait. In this view, quantitative yield components, including the total cannabinoid content, are polygenic, not related to specific metabolic pathways and are heavily affected by the environment. In contrast, the cannabinoid composition strictly depends on the metabolic pathways followed by the plant to convert common precursors into specific end products. This paper focuses on chemotype in the latter qualitative sense of the proportions of the pertinent cannabinoids within the total cannabinoid fraction.

According to several reports, chemotypes with high proportions of CBC appear to be a juvenile expression. Shoyama et al. (1975), Rowan and Fairbairn (1977) and Vogelmann et al. (1988) reported a shift from high CBC proportions, or even CBC predominance in young seedlings or cuttings towards a strong THC predominance in the same plants at maturity. Morimoto et al. (1997, 1998) reported a development from strong CBC predominance to strong CBD predominance.

The proportion of CBC in the total cannabinoid fraction may also vary under the influence of photoperiod and light quality. Valle et al. (1978) compared the cannabinoid composition of flowering plants grown under a 10 and 12 h photoperiod, respectively, and found that the 12 h treatment increased THC content and decreased CBC content. Mahlberg and Hemphill (1983) examined the effect of light on the cannabinoid composition of the vegetative leaves of a THC predominant strain. Unlike THC production,

CBC production was maintained at comparable or greater levels under light-stressed conditions than in daylight controls. As a result, the CBC/THC ratio increased significantly under light-stress. It was concluded that the processes of both synthesis and accumulation of CBC are independent of those for THC.

In mature inflorescences of drug, fibre and undomesticated strains from a wide range of origins, CBC commonly occurs as a minor constituent beside much more substantial proportions of THC and/or CBD. In reports on large-scale germplasm evaluations, mature plants are usually characterised by the contents of the major cannabinoids CBD and THC, whereas CBC is often neglected (e.g., Small and Beckstead 1973; de Meijer et al. 1992; Small and Marcus 2003). Turner and Hadley (1973) however, found that certain drug strains are devoid of CBD and have CBC as a minor component beside THC. Baker et al. (1983) reported on the minor presence of CBC in CBD-predominant plants from Sri Lanka and India. Beutler and Der Marderosian (1978) analysed samples from Mexican and Siberian plants and their cross progeny. They found a constant presence of CBC in all plants, occupying a 3–9% proportion of the total cannabinoid fraction. Hillig and Mahlberg (2004) examined the cannabinoid profile of a representative germplasm collection and classified the individual plants into three discrete chemotypes: strongly THC predominant; strongly CBD predominant and intermediate. CBC was detected as a minor compound in each category but not in all plants.

Yotoriyama et al. (1980) presented a chromatogram of a Japanese THC-predominant fibre strain showing only a trace of CBD and a CBC peak with an area similar to the THC peak. Plants reaching such substantial CBC proportions at maturity are uncommon. Holley et al. (1975), in one of the few reports on such plants, listed samples from India with CBC proportions up to 64% of the total cannabinoid fraction and with THC as the major complementary cannabinoid, although it is not explicitly specified as to whether these samples originated from mature plants. In the germplasm screening preceding our breeding programme, two accessions showing an unusual CBC proportion at maturity were identified. A clone with a CBC proportion of 58%, and a complementary cannabinoid fraction dominated by CBD, was selected from an Afghan hashish landrace. The second source was a Korean fibre landrace, which comprised mainly

THC/CBC plants with CBC proportions ranging from 7% to 58%.

#### Aim of this work

This paper aims to extend the genetic model for chemotype inheritance in *Cannabis* (de Meijer et al. 2003; de Meijer and Hammond 2005), by exploring the mechanisms that control the CBC proportion in the cannabinoid fraction. To this purpose breeding experiments were conducted with chemotypes characterised by contrasting CBC proportions at maturity. With the focus on CBC, a study of the ontogenetically and environmentally induced variation in chemotype also appeared essential.

#### Materials and methods

##### Chemotype monitoring

Table 1 presents five female inbred lines that were grown for periodic assessments of their cannabinoid contents. Up to five seedlings per line were evaluated under similar glasshouse conditions. Plants were kept under permanent light for the first 2 weeks after emergence. Then, to induce flowering, the 24 h photoperiod was dropped to 19 h and further gradually reduced by 15 min per day. When the photoperiod reached the level of 11 h, it was kept constant until the end of the experiment. The onset of flowering was visible in all plants by the day the 11 h photoperiod was reached. Commencing shortly after seedling emergence, at weekly intervals, and always around mid-day, samples were taken from the most recently developed tissues. These were, in order: (a) the last expanded apical stem leaves; (b) the last expanded inflorescence leaves; (c) bracteoles, bracts and leaves from inflorescences with white, immature stigma; (d) bracteoles, bracts and leaves from inflorescences with brown, mature stigma. In addition, the question of whether the same tissue shows changes in cannabinoid composition in the course of ageing was investigated. Leaflets were periodically sampled from a fixed leaf pair at the 3rd or 4th stem node. Per plant, per sampling date, the samples were individually extracted and analysed as described below. The respective cannabinoid contents were totalised and the individual cannabinoids were quantified as relative

**Table 1** Characteristics of materials used in the chemotype monitoring and the breeding experiments

Code	Generation/type	Source population	Putative genotype	Cannabinoid composition <sup>a</sup>					
				CBD	CBC	CBGM <sup>b</sup>	THC	CBG	
Lines used in chemotype monitoring experiment									
55.6.2.6.4.21	S <sub>4</sub> inbred line	'Haze', marijuana strain	B <sub>T</sub> /B <sub>T</sub>	0.5	1.7	0.4	95.6	1.9	
2001.22.6.20.14	S <sub>3</sub> inbred line	((Afghan × Skunk) × (Haze × Skunk))	B <sub>D</sub> /B <sub>D</sub>	91.2	2.9	1.0	3.7	1.2	
2002.2.4.4.2	S <sub>2</sub> inbred line	((Afghan × Skunk) × (S. Italian fibre hemp))	B <sub>0</sub> /B <sub>0</sub>	8.7	3.4	0.1	0.4	87.4	
RJ97.11.23	S <sub>2</sub> inbred line	Afghan hashish landrace	? <sup>c</sup>	61.9	30.6	4.2	2.5	0.8	
2000.577.118.3.5	S <sub>3</sub> inbred line	Korean fibre landrace	? <sup>c</sup>	0.8	22.4	7.3	69.3	0.2	
CBC rich breeding progenitors									
2000.577.118	Non-inbred seedling	Korean fibre landrace	? <sup>c</sup>		33.0	9.9	57.1		
2000.577.121	Non-inbred seedling	Korean fibre landrace			39.5	7.8	52.7		
RJ97.11	Non-inbred clone	Afghan hashish landrace	? <sup>c</sup>	33.2	57.8	6.8	2.2		

<sup>a</sup> The proportions (% w/w) of the individual cannabinoids in the total cannabinoid fraction assessed at maturity

<sup>b</sup> Cannabigerol-monomethylether

<sup>c</sup> A priori unknown

proportions of the total content. Per accession, per sampling date, mean cannabinoid proportions were calculated.

In order to determine whether a certain presence of CBC is a universal, albeit transitory, characteristic of *Cannabis*, early stem leaves from 178 vegetative cuttings from our clone library, representative of a variety of source populations, were analysed for cannabinoid content.

#### Breeding experiments

Only the high CBC parental materials for the progenies studied are presented in Table 1. The various true breeding cross parents that were THC or CBG predominant and at maturity had only trace amounts of CBC are not included. The female clone RJ97.11 was selected at HortaPharm B.V., Amsterdam, The Netherlands. The Korean fibre landrace 2000.577, from which two female seedlings were used for breeding experiments, came from the *Cannabis* collection at Plant Research International (formerly CPRO), Wageningen, The Netherlands. All progenies were produced from female parents only. In order to self-fertilise or mutually cross female plants, a partial masculinisation was chemically induced. Isolating plants in paper bags throughout the generative stage ensured the self-fertilisations. The distribution of

chemotypes in segregating progenies was determined and  $\chi^2$  values were calculated to test the conformity of observed segregation ratios to those expected on the basis of hypothesised models. Three sets of breeding experiments were performed:

- Direct inbreeding of the source materials with a high CBC proportion.
- Crossing of material with a high CBC proportion (original source material or inbred offspring directly derived from it) and various THC predominant materials (putative genotype B<sub>T</sub>/B<sub>T</sub>, de Meijer et al. 2003) and various CBG predominant materials (putative genotype B<sub>0</sub>/B<sub>0</sub>, de Meijer and Hammond 2005). Inbreeding of the resulting progenies.
- Mutual crossing of two different high CBC inbred lines, one based on the Afghan and the other on the Korean parental source. Inbreeding of the resulting progeny.

#### Study of the effect of light intensity on the CBC proportion

It was noticed that plants tended to show higher CBC proportions when, for self-fertilisation, they were grown in paper isolation bags. To investigate this effect systematically, five CBC rich female clones were grown under different levels of photosynthetically

active radiation (PAR). Two clones (M240, M271) were derived from the Afghan breeding source, one (M274) from the Korean breeding source, and two (M272, M273) were selected from Afghan  $\times$  Korean cross progenies. In M271, the cannabinoid fraction complementary to CBC was a mixture of CBD and THC in comparable amounts. In the other clones the complementary cannabinoid fraction was dominated by CBG. Initially, all cuttings were kept under identical conditions of permanent light: a 2 week rooting phase under an average PAR level of 7/57 W/m<sup>2</sup> (12/12 h) and, after transplanting to 31 pots, another 2 weeks of vegetative development under an average PAR level of 38/94 W/m<sup>2</sup> (12/12 h). For generative development and maturation, they were then subjected to a 12 h photoperiod for 60 days. During this stage the cuttings were placed under four different levels of PAR, on average of 67.4, 37.9, 23.3 and 0.9 W/m<sup>2</sup> respectively, as measured just above the canopy. Actual PAR values were automatically recorded at 5-min intervals and for the entire generative stage cumulative PAR was estimated at 174.5, 98.2, 60.3 and 2.3 MJ/m<sup>2</sup>, respectively. The areas with varying light levels were constructed in a single glasshouse compartment with horizontal and vertical shading of different densities. Fans insured sufficient air circulation. Temperature and relative air humidity were constant within the four light regimes. Per regime, five to eight cuttings per clone were fully randomised and spaced in a density of 16 plants/m<sup>2</sup>. Edging plants of similar age and size were used to avoid margin effects on the test cuttings. At maturity, the botanical raw material of each cutting (BRM: the total mass of leaves, floral leaves, bracts and bracteoles) was dried, weighed and homogenised and its cannabinoid content and cannabinoid composition were assessed. Yields of BRM and cannabinoids in g/cutting were multiplied by 16 and expressed as yields in g/m<sup>2</sup>. Per clone, treatment effects were tested (Anova *F*-test, *P* = 0.05) and treatment means were compared pair-wise by Fisher's LSD method (*P* = 0.05).

The assessment and the expression of cannabinoid composition

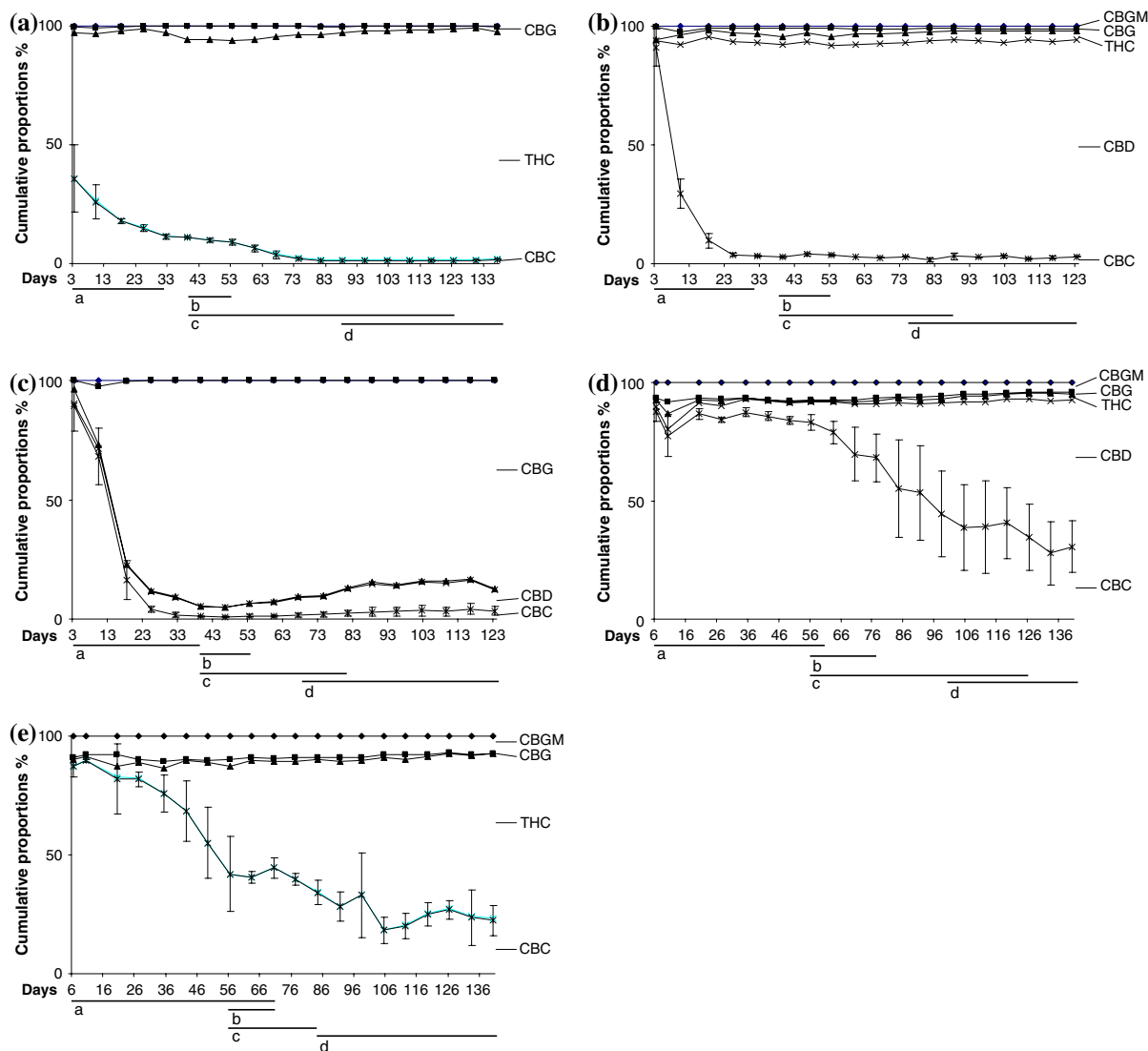
Mature floral clusters from every plant considered in the breeding experiments were sampled. For the chemotype monitoring experiment, various types of immature and mature samples were taken as specified

above. Sample extraction and GC analysis took place as described by de Meijer et al. (2003). The identities of the detected compounds were confirmed by GC-MS. Cannabinoid peak areas were converted into dry weight concentrations using a linear calibration equation obtained with a CBD standard range. The contents of the individual cannabinoids were expressed as weight percentages of the dry sample tissue. Weight proportions of the individual cannabinoids in the total cannabinoid fraction were used to characterise the cannabinoid composition.

## Results

### Chemotype monitoring

Figure 1 presents the cannabinoid composition during the life cycle as assessed in the latest developed tissues of true-breeding THC predominant-, CBD predominant-, CBG predominant and Afghan and Korean inbred lines. All the lines considered showed a strong presence of CBC shortly after emergence which declined with ageing. The plants predominant in THC at maturity had a CBC proportion in the total cannabinoid fraction ( $P_{CBC}$ ) of about 40% 3 days after emergence. This proportion gradually decreased over a 10-week period and stabilised at about 1–3% in the immature floral samples (Fig. 1a). The first true leaves of the lines predominant in CBD and CBG at maturity had a  $P_{CBC}$  of about 90%. Then the  $P_{CBC}$  rapidly reduced and after only 3 weeks, still in the stage that primary stem leaves were sampled, a level of about 1–5% was reached. This percentage remained stable for their remaining lifetime (Fig. 1b, c). The Afghan and Korean inbred lines showed a  $P_{CBC}$  of about 90% shortly after emergence which decreased more slowly than in the aforementioned materials and stabilised at the more substantial level of ca. 25% of the cannabinoid fraction of the mature floral samples (Fig. 1d, e). The true-breeding THC, CBD and CBG predominant inbred lines showed an increase in total cannabinoid content during the sampling period from ca. 0.8–11%, 0.7–10% and 0.25–4%, respectively. This parameter was therefore negatively correlated with the declining  $P_{CBC}$  value ( $r = -0.80$ ,  $-0.38$  and  $-0.57$  for the three lines respectively). In the Afghan and the Korean inbred lines, the total cannabinoid content varied between 1% and 3% throughout



**Fig. 1** The cannabinoid composition, represented as cumulative proportions of the total cannabinoid fraction, in the course of the life time of various inbred lines. *X*-axes present the sampling time in days from seedling emergence. Solid lines under the *X*-axes specify the tissue that was sampled: (a) the latest expanded apical stem leaves; (b) the latest expanded inflorescence leaves; (c) bracteoles, bracts and leaves from inflorescences with white, immature stigma; (d) bracteoles, bracts and

leaves from inflorescences with brown, mature stigma. (a) A true-breeding THC predominant inbred line (putative genotype  $B_T/B_T$ ). (b) A true-breeding CBD predominant inbred line (putative genotype  $B_D/B_D$ ). (c) A true-breeding CBG predominant inbred line (putative genotype  $B_G/B_G$ ). (d) An inbred line directly derived from the Afghan RJ97.11 source clone. (e) An inbred line directly derived from the Korean 2000.577.118 seedling

the sampling period and showed little correlation with  $P_{CBC}$ .

The samples which were periodically taken from different leaflets of a fixed primary leaf pair, preserved the same cannabinoid composition throughout the entire sampling period in all the accessions (data not shown). The early vegetative leaves from all accessions in the clone library contained CBC. It was

the major cannabinoid in 4.5% of the samples and the second in 78%.

#### Breeding experiments

Results are presented in terms of the proportions of CBG, CBD, CBC and THC. In all parental materials and progenies, these four cannabinoids together

**Table 2** The total cannabinoid content and the proportion of CBC in the cannabinoid fraction in the successive inbred generations from the source materials RJ97.11 and 2000.577

Source accession	Generation	No. of plants	Total cannabinoid content (% w/w) Mean $\pm$ SD	Proportion of CBC (%) Mean $\pm$ SD
RJ97.11	S <sub>0</sub>	1	3.88	57.8
	S <sub>1</sub>	29	2.93 $\pm$ 0.72	66.3 $\pm$ 7.4
	S <sub>2</sub>	37	2.69 $\pm$ 0.84	57.7 $\pm$ 13.4
	S <sub>3</sub>	5	3.48 $\pm$ 0.65	36.0 $\pm$ 10.1
2000.577	S <sub>0</sub>	2	1.47 $\pm$ 0.22	36.2 $\pm$ 3.3
	S <sub>1</sub>	20	1.34 $\pm$ 0.54	35.0 $\pm$ 10.5
	S <sub>2</sub>	30	3.71 $\pm$ 1.87	26.5 $\pm$ 11.7
	S <sub>3</sub>	10	2.67 $\pm$ 0.97	38.0 $\pm$ 9.2

occupied on average 95% or more of the cannabinoid fraction. The single complementary cannabinoid was cannabigerol-monomethyl-ether (CBGM), which is not discussed.

#### *Inbreeding of progenitors with a high proportion of CBC*

Within the inbred generations of RJ97.11, the absolute contents of CBC and CBD were uncorrelated:  $r = 0.17$ ,  $0.08$  and  $-0.11$  for the S<sub>1</sub>, S<sub>2</sub> and S<sub>3</sub> respectively. Table 2 gives means and standard deviations for the total cannabinoid content and P<sub>CBC</sub> of the successive inbred generations from RJ97.11. In the course of inbreeding there was no systematic trend noticeable in either the mean values or the variabilities of these characteristics. Within generations, the variation in the cannabinoid proportions was considerable, but discontinuity in the pattern of cannabinoid composition was not observed and the parental plant and the consecutive inbred generations of this line were essentially constant in respect of their CBC/CBD chemotype.

The absolute contents of CBC and THC within the inbred generations of 2000.577 showed limited correlation:  $r = 0.12$ ,  $0.21$  and  $0.66$  for the S<sub>1</sub>, S<sub>2</sub> and S<sub>3</sub>, respectively. Means and standard deviations for the total cannabinoid content and P<sub>CBC</sub> of the 2000.577 inbred generations did not show a systematic trend (Table 2). Within generations, the variation in the cannabinoid proportions was substantial but gradual and there was no segregation into discrete chemotypes. The parental mixed CBC/THC chemotype was expressed by all individuals of the generations observed.

#### *Crosses of Afghan high P<sub>CBC</sub> plants with various THC and CBG predominant materials*

All 14 F<sub>1</sub>s, irrespective of whether they resulted from crosses of Afghan derived plants with true breeding THC predominant or CBG predominant plants, were chemotypically uniform and only had a limited P<sub>CBC</sub> (Table 3). Hybrids resulting from an Afghan  $\times$  THC predominant cross (nos. 1, 2, 5, 9, 11, 14) had chemotypes predominated by CBD and THC and within an F<sub>1</sub> the absolute CBD and THC contents were strongly correlated ( $r$  values generally 0.8–0.9). All F<sub>1</sub> plants resulting from Afghan  $\times$  CBG predominant crosses (nos. 3, 4, 6, 7, 8, 10, 12, 13) were strongly CBD predominant.

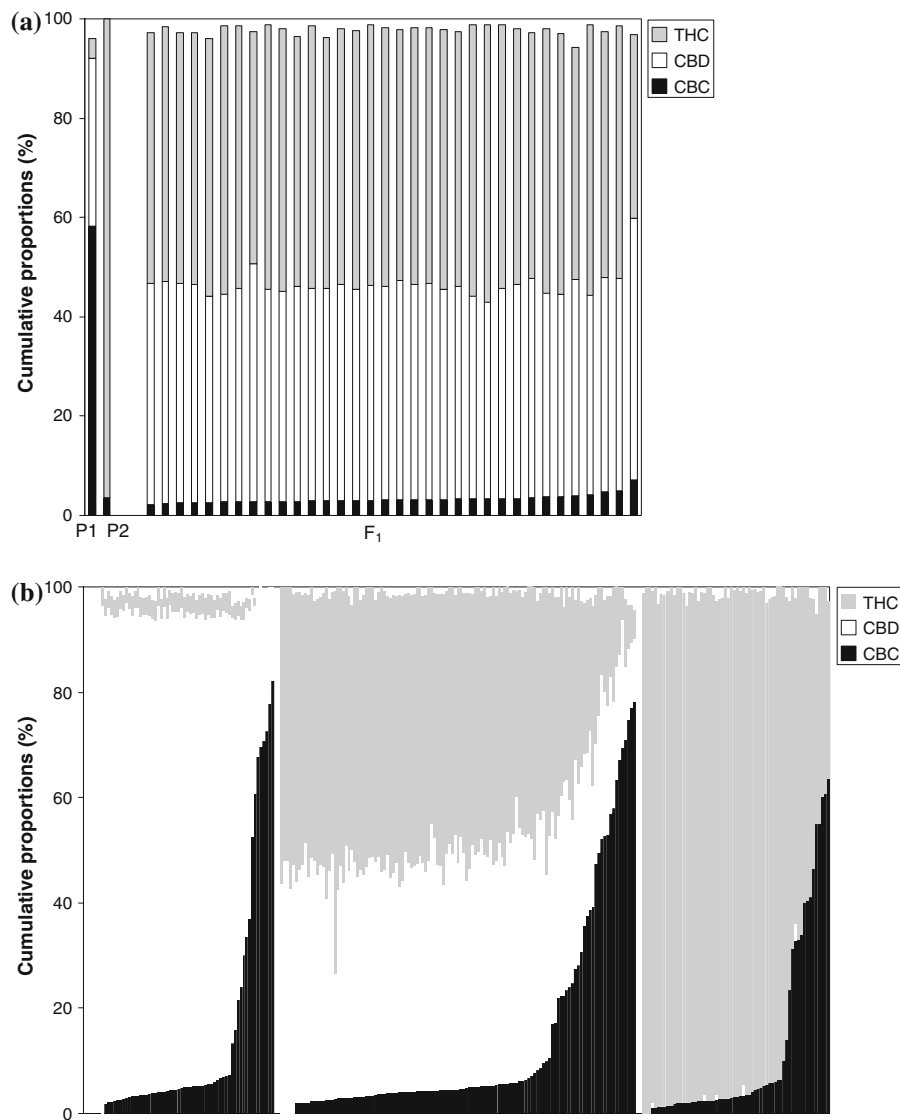
The stack bar diagram of Fig. 2a presents the chemotypes of the parental plants and the F<sub>1</sub>s of one of the Afghan  $\times$  THC predominant crosses (Table 3, cross no. 11). Figure 2b shows the distribution of chemotypes in the large pooled F<sub>2</sub> (Table 3, F<sub>2</sub>s M, N and O) that was based on three randomly chosen inbred F<sub>1</sub> plants from this cross and comprised 244 individuals. Irrespective of the CBC proportion, 59 plants with a THC/CBD content ratio ranging from 0.00 to 0.053 were CBD predominant; 121 contained both THC and CBD in a comparable proportion (THC/CBD content ratio range 0.33–3.88) and 64 plants were THC predominant (THC/CBD content ratio  $\geq 18.87$ ). With a  $\chi^2$  value of 0.22, a 1:2:1 segregation ratio is readily accepted (threshold for acceptance at  $P = 0.05$ :  $\chi^2 < 5.99$ ). Within the three discrete segregant groups based on the THC/CBD content ratios, individuals in Fig. 2b are sorted by increasing P<sub>CBC</sub>. It appears that within each group, the first  $\frac{3}{4}$  of the plants have low P<sub>CBC</sub> values up to approximately

**Table 3** Phenotypic data for F<sub>1</sub> and F<sub>2</sub> progenies resulting from crosses between Afghan plants (P1) with a high proportion of CBC (P<sub>CBC</sub>), and various true breeding THC and CBG predominant materials (P2)

Cross	P1 P <sub>CBC</sub>	P2 P <sub>CBC</sub>	F <sub>1</sub> P <sub>CBC</sub> min-avg-max	No. of F <sub>1</sub> plants	F <sub>2</sub> progeny	F <sub>2</sub> low P <sub>CBC</sub> range <sup>a</sup> (min-max)	No. of F <sub>2</sub> low P <sub>CBC</sub> plants	F <sub>2</sub> high P <sub>CBC</sub> range <sup>a</sup> (min-max)	No. of F <sub>2</sub> high P <sub>CBC</sub> plants	χ <sup>2</sup> value <sup>b</sup>	3:1 accepted P = 0.05	R-value <sup>c</sup> Ctot-P <sub>CBC</sub> (F <sub>2</sub> s)
1	71.4	1.8	3.2-4.2-7.7	32	A	0.0-5.3	5	38.9-69.5	4	1.81	Yes	-0.70
2	77.5	1.8	1.9-3.1-8.2	24	B	0.0-14.0	39	27.5-73.6	9	1.00	Yes	-0.51
3	77.5	1.5	3.1-3.5-4.0	2	C	1.3-9.0	35	30.0-60.6	12	0.01	Yes	-0.66
4	63.9	1.5	4.0-5.0-5.9	2	D	0.0-12.9	78	18.2-91.8	23	0.27	Yes	-0.62
5	71.4	2.5	3.3-4.3-5.3	9	E	0.9-6.8	10	25.2-84.5	6	1.33	Yes	-0.66
6	71.4	0.3	-7.1-	1	F	0.0-7.8	29	15.1-58.7	13	0.79	Yes	-0.70
7	71.4	1.5	-8.9-	1	G	1.3-7.4	39	17.9-69.1	6	3.27	Yes	-0.66
8	77.5	0.0	2.2-5.2-7.9	7	H	0.0-8.6	19	55.0-90.9	3	1.52	Yes	-0.48
9	63.9	2.5	2.7-2.9-3.3	4	I	2.7-12.1	27	14.5-95.4	10	0.08	Yes	-0.48
10	63.9	2.2	4.9-7.1-10.8	21	J	0.0-11.1	57	14.7-94.6	18	0.04	Yes	-0.32
11	58.3	3.5	2.2-3.3-7.2	34	K	0.0-5.8	38	22.6-37.4	5	4.10	No	-0.60
12	41.4	0.0	0.0-2.2-10.1	47	L	0.0-10.9	47	14.1-87.0	31	9.04	No	-0.61
13	39.0	0.0	0.0-1.0-3.6	22	M	0.0-10.0	40	14.1-71.1	12	0.10	Yes	-0.48
14	57.8	2.9	2.3-4.8-12.3	28	N	0.0-13.3	77	17.0-78.3	20	0.99	Yes	-0.64
					O	0.0-10.2	69	17.4-82.2	26	0.28	Yes	-0.59
					P	0.0-13.6	71	17.9-100.0	25	0.06	Yes	-0.20
					Q	0.0-13.0	52	17.6-100.0	25	2.29	Yes	-0.48
					R	0.0-12.6	13	23.1-36.7	2	1.09	Yes	-0.28
					S	0.0-4.9	10	23.2-34.9	2	0.44	Yes	-0.41
							755		252	0.00	Yes	

<sup>a</sup> Per F<sub>2</sub> the segregant groups 'low P<sub>CBC</sub>' and 'high P<sub>CBC</sub>' were discriminated on the basis of a discontinuity in the ranked P<sub>CBC</sub> values  
<sup>b</sup> χ<sup>2</sup> values were calculated to test conformity to the model of a single Mendelian locus with a recessive allele, encoding 'high P<sub>CBC</sub>' and a dominant allele encoding 'low P<sub>CBC</sub>'. The χ<sup>2</sup> threshold for acceptance at P = 0.05 is 3.84  
<sup>c</sup> The coefficient of correlation between the total cannabinoid content and P<sub>CBC</sub>





**Fig. 2** Stack bar diagrams showing the cannabinoid composition of the clone RJ97.11 (P1) and a true-breeding THC predominant plant (P2) and their hybrid F<sub>1</sub> (a) and F<sub>2</sub> (b). For the representation of the F<sub>2</sub>, the 244 plants were primarily classified

on the basis of their CBD/THC content ratio. Subsequently, within the three resultant groups, individuals were sorted by increasing proportion of CBC

8% whereas, after a sudden increase, the latter ¼ shows  $P_{\text{CBC}}$  values of 15–80%. A higher  $P_{\text{CBC}}$  was observed in individuals with relatively low total cannabinoid content. For the 244 F<sub>2</sub> plants presented in Fig. 2b, these two characteristics were negatively correlated ( $r = -0.51$ ).

Chemotypical data on  $P_{\text{CBC}}$  for all the 14 crosses between Afghan high  $P_{\text{CBC}}$  plants and various low  $P_{\text{CBC}}$ , THC or CBG predominant materials is summarised in Table 3. In all the F<sub>2</sub>s, comparable distributions

of the  $P_{\text{CBC}}$  values were found as illustrated in Fig. 2b, and there was also a consistent negative correlation between  $P_{\text{CBC}}$  and the total cannabinoid content. When ranked by  $P_{\text{CBC}}$  value, all F<sub>2</sub> progenies showed a clear discontinuity in the  $P_{\text{CBC}}$  inclination trend and some also showed a clear gap in the  $P_{\text{CBC}}$  value range (e.g. A, B, C, E). This separates ca. 75% of the individuals with a narrow range of lower values from ca. 25% with a wide range of higher values. For 17 of the 19 F<sub>2</sub>s that were considered,  $\chi^2$  tests

**Table 4** Dihybrid segregation in F<sub>2</sub> progenies resulting from crosses between Afghan high P<sub>CBC</sub> plants, with a complementary fraction of mainly CBD, and various low P<sub>CBC</sub> true breeding THC predominant materials

Progeny	P <sub>CBC</sub> low			P <sub>CBC</sub> high			Total	$\chi^2$ <sup>a</sup>	3:6:3:1:2:1 Accepted P = 0.05
	CBD	CBD/THC	THC	CBD	CBD/THC	THC			
A	3	2	0	1	1	2	9	7.30	Yes
B	10	19	10	2	7	0	48	3.78	Yes
C	8	12	15	2	6	4	47	6.90	Yes
F	10	10	9	3	7	3	42	3.52	Yes
G	14	15	10	3	2	1	45	7.68	Yes
K	10	15	13	1	3	1	43	6.74	Yes
M	8	22	10	2	5	5	52	2.41	Yes
N	21	37	19	2	13	5	97	3.45	Yes
O	16	33	20	10	11	5	95	3.64	Yes
R	5	3	5	0	2	0	15	6.51	Yes
S	3	4	3	0	2	0	12	2.22	Yes
All	108	172	114	26	59	26	505	9.64	Yes

Per progeny, per chemotype category, the number of individuals is given

<sup>a</sup>  $\chi^2$  values were calculated to test conformity to the model of two independent Mendelian loci. According to this model one locus has a recessive allele, encoding 'high P<sub>CBC</sub>', and a dominant allele encoding 'low P<sub>CBC</sub>'. The other locus has two codominant alleles, encoding either CBD or THC predominance when homozygous, and a mixed CBD/THC chemotype when heterozygous. The  $\chi^2$  threshold for acceptance at P = 0.05 is 11.07

accepted a 3:1 segregation ratio for the low P<sub>CBC</sub> versus the high P<sub>CBC</sub> group. The F<sub>2</sub>s from the Afghan × CBG predominant crosses (D, E, H, I, J, L, P, Q) consistently reached higher P<sub>CBC</sub> values than the F<sub>2</sub>s from the Afghan × THC predominant crosses.

All F<sub>2</sub>s from the Afghan × THC predominant crosses segregated into fairly pure CBD plants, mixed CBD/THC plants and fairly pure THC plants in a 1:2:1 ratio (accepted by  $\chi^2$  tests, data not shown), based on discontinuities in the THC/CBD ratio of the complementary cannabinoid fraction and irrespective of P<sub>CBC</sub>. The segregation was clear-cut. General THC/CBD value ranges for the chemotype classes over all F<sub>2</sub>s of this type were: CBD predominant (0 ≤ THC/CBD ≤ 0.09), mixed THC/CBD (0.26 ≤ THC/CBD ≤ 3.88) and THC predominant (11.71 ≤ THC/CBD). Data on the dihybrid segregation of the characters, P<sub>CBC</sub> value and THC/CBD ratio are summarised in Table 4. For all F<sub>2</sub>s,  $\chi^2$  tests accepted a 3:6:3:1:2:1 segregation ratio for the variants (low P<sub>CBC</sub>-CBD predominant):(low P<sub>CBC</sub>-mixed THC/CBD):(low P<sub>CBC</sub>-THC predominant):(high P<sub>CBC</sub>-CBD predominant):(high P<sub>CBC</sub>-mixed THC/CBD):(high P<sub>CBC</sub>-THC predominant).

Based on the predominance of either CBG or CBD in the cannabinoid fraction complementary to CBC,

the F<sub>2</sub>s from the Afghan × CBG predominant crosses segregated consistently into CBD predominant versus CBG predominant plants in a 3:1 ratio (accepted by  $\chi^2$  tests, data not shown). Five plants could not be classified by this criterion (Table 5, footnote 'b'). Data on the dihybrid segregation of the characters, P<sub>CBC</sub>-value and predominance of either CBD or CBG in the complementary cannabinoid fraction, are summarised in Table 5. For seven of the eight F<sub>2</sub>s,  $\chi^2$  tests accepted a 9:3:3:1 ratio for the variants (low P<sub>CBC</sub>-CBD predominant):(low P<sub>CBC</sub>-CBG predominant):(high P<sub>CBC</sub>-CBD predominant):(high P<sub>CBC</sub>-CBG predominant).

As with the Afghan high P<sub>CBC</sub> progenitor, the high P<sub>CBC</sub> segregants did not produce the usual resinous flower clusters. Instead, they had leafy inflorescences with a few small bracteoles, and bracts that only carried sessile glandular trichomes and practically no stalked ones (Fig. 3).

#### *Cross of Korean high P<sub>CBC</sub> material with CBG predominant material*

The F<sub>1</sub> resulting from the cross of the Korean inbred (S<sub>3</sub>) line 2000.577.118.3.7 and a true breeding CBG predominant inbred line was uniform for chemotype (Fig. 4a). With a value range of 18.1–39.0%, P<sub>CBC</sub>

**Table 5** Dihybrid segregation in F<sub>2</sub> progenies resulting from crosses between Afghan high P<sub>CBC</sub> plants, with a complementary fraction of mainly CBD and various low P<sub>CBC</sub> true breeding CBG predominant materials

Progeny	P <sub>CBC</sub> low		P <sub>CBC</sub> high		Total	$\chi^2$ <sup>a</sup>	9:3:3:1 Accepted P = 0.05
	CBD predominant	CBG predominant	Cannabinoid complement CBD predominant	Cannabinoid complement CBG predominant			
D	62	16	13	10	101	4.95	Yes
E	7	3	5	1	16	1.78	Yes
H	16	3	3	0	22	3.05	Yes
I	18	9	7	3	37	1.20	Yes
J	43	14	12	6	75	0.69	Yes
L	43	4	20	11	78	17.41	No
P <sup>b</sup>	57	14	16	8	95	1.95	Yes
Q <sup>b</sup>	43	9	15	6	73	2.28	Yes
All	289	72	91	45	497	11.44	No

Per progeny, per chemotype category, the number of individuals is given

<sup>a</sup>  $\chi^2$  values were calculated to test conformity to the model of two independent Mendelian loci. According to this model one locus has a recessive allele, encoding 'high P<sub>CBC</sub>' and a dominant allele encoding 'low P<sub>CBC</sub>'. The other locus has a recessive allele, encoding CBG predominance and a dominant allele encoding CBD predominance. The  $\chi^2$  threshold for acceptance at  $P = 0.05$  is 7.82

<sup>b</sup> One high P<sub>CBC</sub> individual from progeny P, and four from Q were excluded. In these plants CBC was the single cannabinoid detected and so they could not be further classified on the basis of a complementary cannabinoid fraction

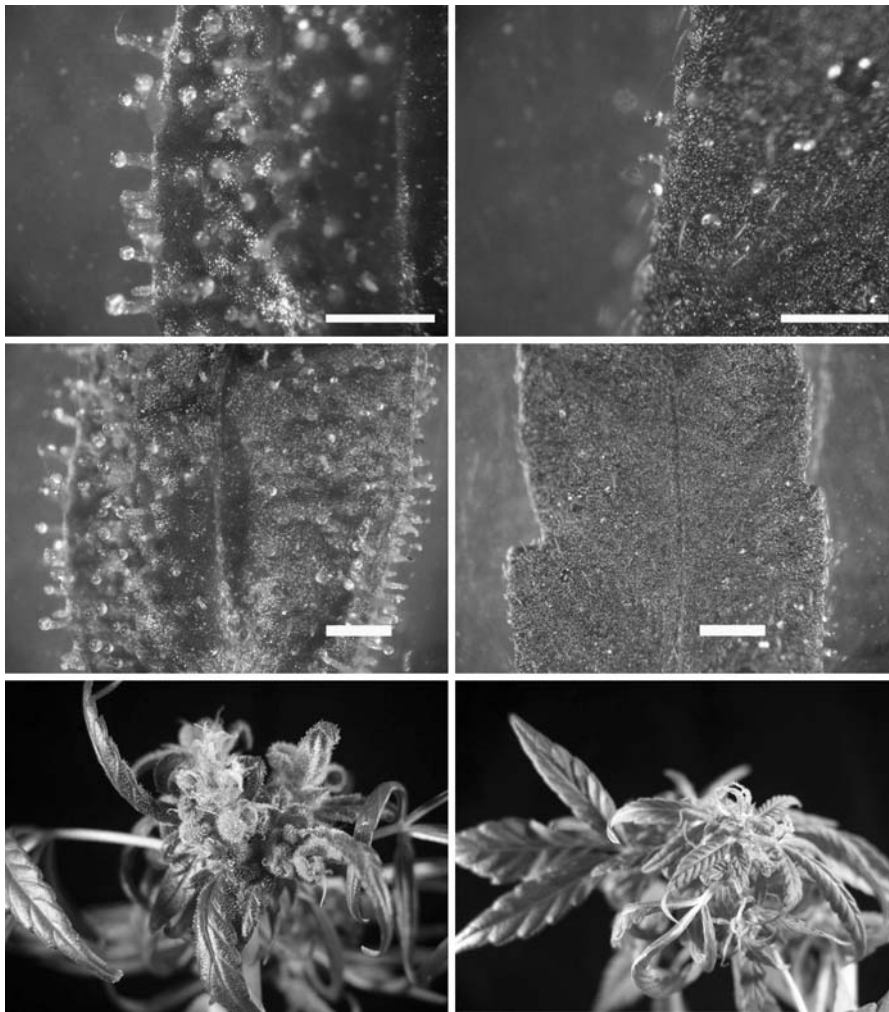
was much higher than in the F<sub>1</sub>s obtained with Afghan plants. The average P<sub>CBC</sub> value of the eight F<sub>1</sub> plants was 30.0%, which is close to the parental average P<sub>CBC</sub> value of 25.5%. Besides CBC, THC was the major cannabinoid in all F<sub>1</sub> plants and some individuals also had a minor proportion of CBD and/or CBGM. As with the Afghan based progenies, the F<sub>1</sub> individuals were self-fertilised to produce inbred F<sub>2</sub>s. The chemotypes of the pooled F<sub>2</sub> plants, sorted by P<sub>CBC</sub> are presented in Fig. 4b. The F<sub>2</sub> achieved a much wider P<sub>CBC</sub> range than the F<sub>1</sub>: 8.6–69.3%. The average P<sub>CBC</sub> of the 122 F<sub>2</sub> plants was 33.1%. In contrast with the F<sub>2</sub>s obtained with Afghan plants, the pattern of P<sub>CBC</sub> values did not show any discontinuity and the distribution of individuals over P<sub>CBC</sub> classes followed a Gaussian pattern. In alignment with the F<sub>2</sub>s obtained with Afghan progenitors, P<sub>CBC</sub> in this F<sub>2</sub> was also negatively correlated with the total cannabinoid content ( $r = -0.58$ ). All Korean based high P<sub>CBC</sub> plants had a poor plant habit in respect of drug production. The inflorescences were very open, floral bracts were virtually absent and the bracteoles were small and poorly covered with stalked glandular trichomes.

In the F<sub>2</sub>, CBC was accompanied by a complementary cannabinoid fraction predominated by either

THC (in 90 plants) or CBG (in 32 plants). With a  $\chi^2$  value of 0.10, a 3:1 segregation ratio for THC versus CBG predominant is readily accepted (threshold for acceptance at  $P = 0.05$ :  $\chi^2 < 3.84$ ).

#### *Mutual cross of Afghan based and Korean based high P<sub>CBC</sub> inbred material*

A high P<sub>CBC</sub> inbred F<sub>2</sub> individual selected from the (Korean × CBG predominant) progeny was crossed with a selected high P<sub>CBC</sub> inbred F<sub>2</sub> individual originating from an (Afghan × CBG predominant) progeny. Figure 5 presents the chemotypes of the inbred F<sub>2</sub> parents and their mutual hybrid F<sub>1</sub>. The CBC proportion of the F<sub>1</sub> individuals is greatly reduced in comparison with the parental plants. The minimal, average and maximal P<sub>CBC</sub> levels in the F<sub>1</sub> were 3.1–5.3–7.7%. The average total cannabinoid content of the 13 F<sub>1</sub> plants was 9.2% (range 7.4–11.2%) which by far exceeds the parental total cannabinoid contents of ca. 1% (Korean based inbred F<sub>2</sub> parent) and ca. 4% (Afghan based inbred F<sub>2</sub> parent). Besides CBC, the complementary cannabinoid fraction of the F<sub>1</sub>s was consistently CBG predominant with a residual presence of CBD. In contrast with the parents, the F<sub>1</sub> individuals had fairly dense floral clusters consisting of bracteoles



**Fig. 3** Mature floral tissues of different inbred  $F_2$  segregants resulting from an Afghan (high  $P_{CBC}$ )  $\times$  (low  $P_{CBC}$ ) cross. Photographs on the left show a segregant with a negligible CBC proportion and photographs on the right show a segregant rich

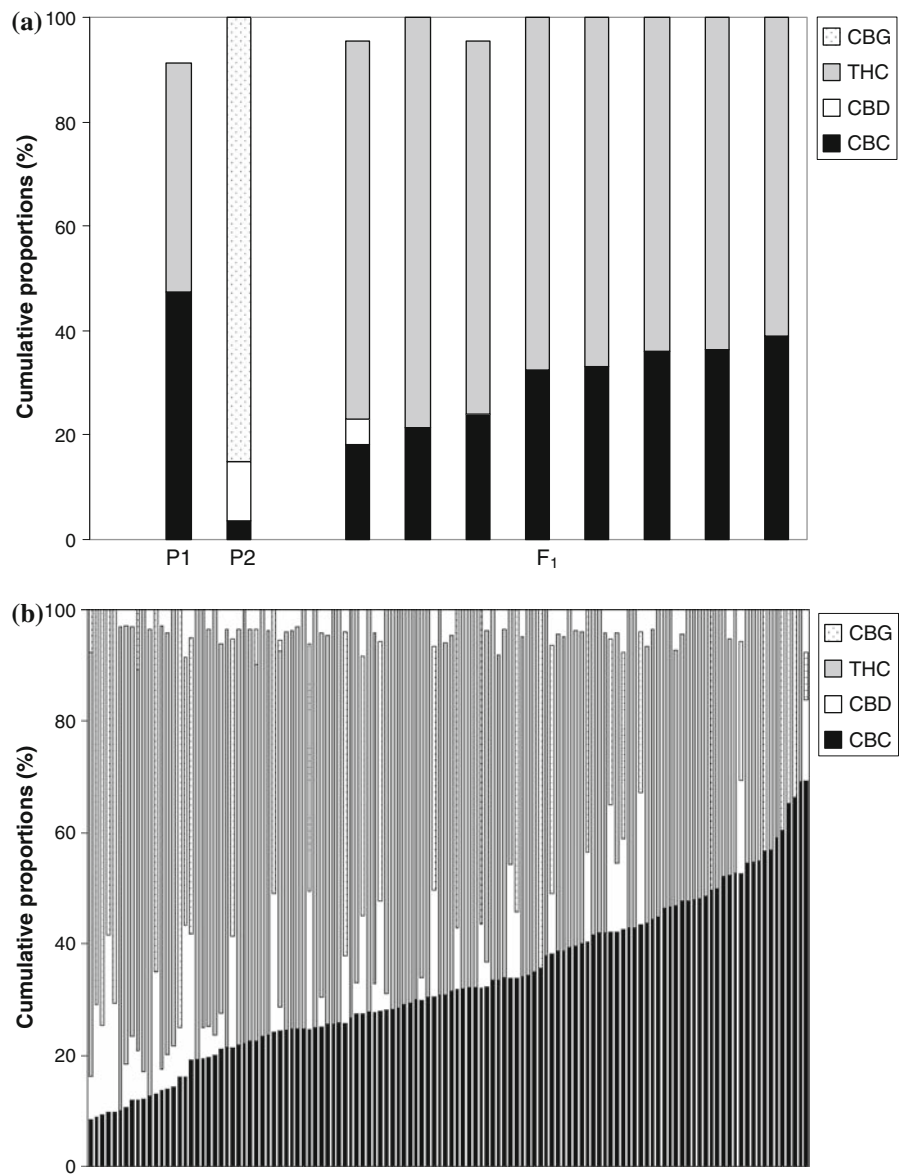
in CBC. *Top*: bract surface detail (bar represents 100  $\mu\text{m}$ ); *middle*: bract surface overview (bar represents 5 mm); *bottom*: entire floral cluster (Photos by courtesy of T.J. Wilkinson)

and bracts that were covered with normal densities of stalked glandular trichomes. A large  $F_2$  generation of 195 individuals, obtained from a single  $F_1$  plant, was evaluated. The total cannabinoid content ranged from 0.83% to 10.99% and  $P_{CBC}$  ranged from 6.23% to 100%, and both parameters were negatively correlated ( $r = -0.46$ ). The ranked  $P_{CBC}$  values showed a slow trend for the majority of the  $P_{CBC}$  values and a somewhat steeper inclination for a minority at the end (Fig. 5b).  $F_2$  individuals with high CBC proportions showed the morphological features as illustrated for such plants in Fig. 3. As in the  $F_1$ , the complementary cannabinoid fraction was consistently CBG predominant with a residual presence of CBD.

#### Study of the effect of light intensity on the CBC proportion

Five CBC rich clones were grown under four different light intensities during a 60 days generative period. Under the most reduced light level, all plants died within the first 2 weeks of the experiment. Under the remaining regimes, variable numbers of plants survived until the end of the experiment. Their physiological maturity was demonstrated by a limited seed set due to a slight monoeciousness in one of the clones. Results for these regimes are presented in Table 6. With a reduction of light, all five clones showed an upward trend in  $P_{CBC}$ . Those from the

**Fig. 4** Stack bar diagrams showing the cannabinoid composition of the inbred seedling 2000.577.188.3.7 (P1) and a true-breeding CBG predominant plant (P2) and their hybrid F<sub>1</sub> (a) and F<sub>2</sub> (b). For both generations, plants were sorted by increasing proportion of CBC

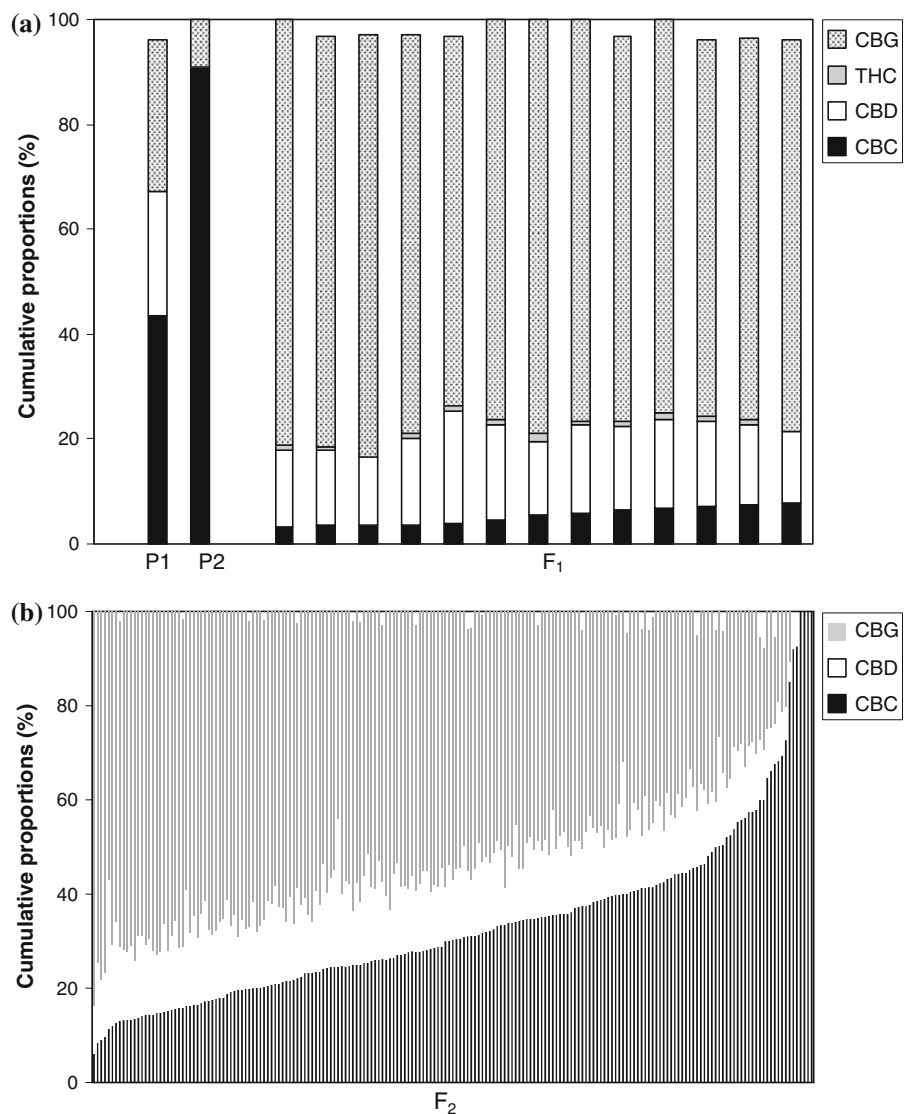


60.3 MJ/m<sup>2</sup> area had a significantly ( $P = 0.05$ ) higher  $P_{\text{CBC}}$  value than those under 174.5 MJ/m<sup>2</sup>. No significant effect of light level on the absolute CBC content was found in the dry botanical raw material of four of the clones. Only the CBC content of M271 was significantly affected, but in this case light levels and CBC contents did not show a coherent trend. In contrast, with reduced light, the total cannabinoid content decreased significantly in four clones. With the exception of clone M274, the resultant CBC yield dropped considerably with reducing light, mainly due to a decreasing yield of botanical dry matter.

## Discussion

All seedlings representing the previously postulated genotypes  $B_T/B_T$ ,  $B_D/B_D$  and  $B_0/B_0$ , showed a gradual development from a major presence of CBC in juvenile tissues into a minor one in the generative tissues. The Afghan and Korean materials also showed a gradual sequential reduction of the CBC proportion during tissue development though the decline was slower and the CBC proportion stabilised at a higher level. In addition, a consistent and, generally, significant presence of CBC was found in early vegetative

**Fig. 5** (a) Stack bar diagram showing the cannabinoid composition of a high  $P_{CBC}$  inbred individual P1 selected from a (Korean  $\times$  CBG predominant) progeny, a high  $P_{CBC}$  inbred clone P2 originating from an (Afghan  $\times$  CBG predominant) progeny and their hybrid  $F_1$ . (b) The cannabinoid composition of an  $F_2$  obtained from a self-fertilised  $F_1$  plant



leaves from a large and diverse clone library. It appears that juvenile *Cannabis* tissue invariably shows a substantial proportion of CBC in the cannabinoid fraction, and that CBC-synthase activity is therefore a fixed feature. This finding is in agreement with Shoyama et al. (1975), Vogelmann et al. (1988) and Morimoto et al. (1997). If, as Hillig and Mahlberg (2004) report, CBC is not detected in certain mature flower samples, it is quite likely that its presence can be confirmed in earlier leaves of the corresponding plants.

The monitoring experiment showed that once a leaf is fully formed, its cannabinoid composition remains stable (also in agreement with Pacifico et al. 2008), so

the decline of the CBC proportion of subsequent tissues cannot be attributed to cannabinoid re-allocation and degradation. One possible cause could be organ-specific CBC synthase activity. It may affect the relative rates of the respective, competing CBG conversions in the subsequently formed tissues. Capitate-sessile trichomes occur in low densities on all aerial surfaces throughout the life cycle of the plant, whereas capitate-stalked trichomes occur in high density and exclusively on the floral bracts and bracteoles. Had CBC synthase activity been restricted to, or more prominent in, sessile trichomes, it would have explained the decline in CBC proportion once the inflorescences start to develop. Although the

**Table 6** Means for the yield of dry botanical raw material (BRM), the total cannabinoid (Ctot) and CBC content in the homogenised BRM, the proportion of CBC in the total cannabi-noid fraction ( $P_{\text{CBC}}$ ) and the resulting CBC yield, for five clones grown under three different light levels during a 60 days generative period

Clone	Cumulative PAR (MJ/m <sup>2</sup> )	No. of cuttings tested	No. of surviving cuttings	BRM yield (g/m <sup>2</sup> )	Ctot (% w/w)	CBC content <sup>a</sup> (% w/w)	$P_{\text{CBC}}$ <sup>a</sup> (% w/w)	CBC yield <sup>a</sup> (g/m <sup>2</sup> )
M240	174.5	7	7	356 a	2.46 a	1.18 a	49.0 a	4.27 a
	98.2	7	7	174 b	2.55 a	1.40 a	56.3 a	2.50 b
	60.3	7	4	144 b	1.50 b	1.21 a	82.5 b	1.76 b
M271	174.5	8	8	821 a	2.75 a	1.88 b	68.2 a	15.46 a
	98.2	8	8	483 b	2.98 a	2.10 a	70.3 ab	10.20 b
	60.3	8	8	248 c	2.14 b	1.53 c	71.6 b	3.87 c
M272	174.5	5	5	258 a	2.04 a	1.85 a	90.7 a	4.79 a
	98.2	5	5	120 b	1.92 a	1.85 a	96.7 b	2.18 b
	60.3	5	1	53 b	1.61 a	1.61 a	100.0 b	0.85 b
M273	174.5	6	6	257 a	3.61 a	0.53 a	14.7 a	1.35 a
	98.2	6	5	172 ab	2.58 b	0.59 a	24.0 b	0.95 ab
	60.3	6	2	109 b	1.36 c	0.45 a	33.5 b	0.49 b
M274	174.5	5	5	203 a	1.28 a	0.45 a	35.5 a	0.91 a
	98.2	5	5	126 a	0.92 b	0.39 a	42.8 b	0.48 a
	60.3	5	2	226 a	0.69 b	0.30 a	43.8 b	0.68 a

Light levels are indicated by the cumulative PAR estimated for the entire generative period. Means are based on the cuttings that survived until the end of the experiment. Per column, per clone, means showing a common letter are not different at  $P = 0.05$

<sup>a</sup> ‘CBC’ refers to the in total detected CBC alkyl homologues and degradants (CBC, CBCV, CBL)

cannabinoid contents of individual gland types have been assessed (e.g., Mahlberg and Kim 2004), no statements on compartmentalised CBC synthase activity in sessile trichomes are available.

The fact that a relatively prominent  $\text{CBG} \rightarrow \text{CBC}$  conversion coincides with a low cannabinoid metabolism, suggests that enzyme kinetics may also play a role in the observed profile changes. Taura et al. (1995, 1996) and Morimoto et al. (1998) reported different kinetic properties for CBC synthase versus CBD and/or THC synthase so it is conceivable that in  $B_{\text{T}}/B_{\text{T}}$ ,  $B_{\text{D}}/B_{\text{D}}$  and  $B_{\text{D}}/B_{\text{T}}$ -genotypes the competitive relationship of the reactions  $\text{CBG} \rightarrow \text{THC}$ ,  $\text{CBG} \rightarrow \text{CBD}$  and  $\text{CBG} \rightarrow \text{CBC}$  is affected by the substrate concentration [CBG]. However, the decline of CBC in the  $B_{\text{D}}/B_{\text{D}}$  inbred line, where CBC synthase is only competing with a weak CBD synthase (de Meijer and Hammond 2005), cannot be explained by this proposition. Perhaps, during the maturation of these plants, the rate at which CBG is produced by the enzyme GOT (Fellermeier and Zenk 1998) exceeds the combined turnover of CBC synthase and the weak CBD synthase, resulting in CBG accumulation.

A third possibility is that in ordinary low  $P_{\text{CBC}}$  plants, the CBC synthase gene is only expressed in the juvenile state and that the Afghan and Korean plants differ in the sense that, due to an inheritable factor, this gene expression is maintained throughout the adult stage.

To a certain degree, the juvenile high  $P_{\text{CBC}}$  cannabinoid composition persists as a relict in the inflorescences of the adult Afghan and Korean materials. To signify this phenotype as opposed to the ordinary low  $P_{\text{CBC}}$  chemotype, we propose the term ‘prolonged juvenile chemotype’ (PJC). Progenies obtained through self-fertilisation of PJC plants invariably expressed the chemotype of the parent involved. The variation in cannabinoid proportions among individual offspring was gradual and segregation into discrete chemotypes was not observed. This is quite distinct from what was found when mixed CBD/THC individuals were inbred (de Meijer et al. 2003): their offspring showed a clear-cut segregation pattern and comprised pure CBD-, mixed CBD/THC and pure THC chemotypes in a 1:2:1 ratio. Plants with mixed CBD/THC chemotypes were therefore considered to be heterozygous at

a single chemotype locus. The absence of discrete chemotype segregation in their inbred progenies suggests that individuals with substantial amounts of either CBC and CBD (Afghan breeding source), or CBC and THC (Korean) are homozygous, or else that PJC has a polygenic background. Had CBC synthase been encoded by an allele  $B_C$ , of the same locus  $B$  where the alleles  $B_T$ ,  $B_D$  and  $B_0$  encode the other synthases, the inbred offspring of a CBD/CBC plant ( $B_D/B_C$ ) and a THC/CBC plant ( $B_T/B_C$ ) should have segregated into different chemotypes in a 1:2:1 ratio. As this was not observed, the encoding of CBC synthase by a further allele  $B_C$ , at locus  $B$  can be excluded. The crosses of the Afghan CBD/CBC plants with true breeding THC predominant material ( $B_T/B_T$ ) provide additional evidence against this model. The inbred  $F_2$ s of these crosses contained segregants with substantial proportions of each of the three compounds CBD, CBC and THC (Fig. 2b). As diploids only allow for the concurrent and substantial synthesis of two of these cannabinoids per locus and per genotype, the expression of such a triple compound chemotype precludes the possibility that CBC synthase is also encoded by a  $B$  allele.

The crosses between plants with contrasting CBC proportions demonstrated that the genetic factor responsible for PJC has a monogenic, recessive nature as far as the Afghan lineage (based on RJ97.11) is concerned. The dihybrid segregation data indicates that this factor is inherited independently from locus  $B$ . A cross involving a Korean high  $P_{CBC}$  parent yielded an  $F_1$  with a gradual range of intermediate  $P_{CBC}$  values and an  $F_2$  that did not segregate for  $P_{CBC}$ . This suggests a different, polygenic background for PJC in the Korean material. The mutual cross between Afghan and Korean based high  $P_{CBC}$  materials further supports the hypothesis that the shared feature of PJC is attributable to different genetic factors. The  $F_1$  did not show PJC and its associated morphological features. This result is explicable if, in the case of the monogenic factor, the low  $P_{CBC}$  allele from the Korean parent dominates the PJC allele from the Afghan parent and if, vice versa for the polygenic factor, the Afghan low  $P_{CBC}$  genotype ‘dilutes’ the Korean PJC genotype. In the  $F_2$  the PJC phenotype reappeared. The CBC proportion showed a wide range from 6% to 100% and the distribution of CBC proportions was intermediate between a discontinuous monogenic pattern and a Gaussian polygenic one.

This is also in agreement with the hypothesis of different genetic backgrounds for PJC in the Afghan and Korean breeding sources.

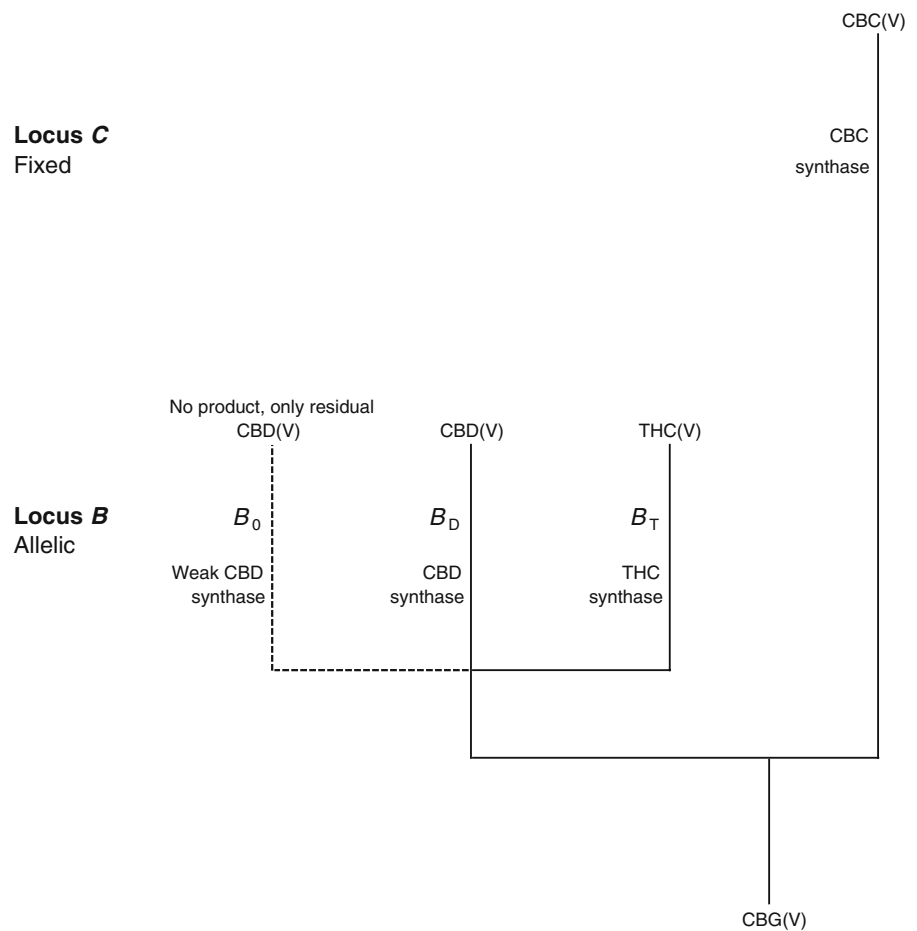
It is unlikely that variation in  $P_{CBC}$  at maturity is due to an allelic locus encoding differential CBC synthase activity. The finding that heterozygous individuals obtained through crosses with the Afghan plants show a low  $P_{CBC}$  chemotype, conflicts with such a hypothesis. It implies that a minimally active synthase suppresses the activity of a functional synthase. In a comparable circumstance, the opposite was found in the interaction of the minimally functional allele  $B_0$  with the functional alleles  $B_D$  and  $B_T$ . In the heterozygous genotypes  $B_D/B_0$  and  $B_T/B_0$ , the functional allele dominates the expression of the minimally functional one (de Meijer and Hammond 2005). The apparently polygenic background of PJC in the Korean material is also counter to the hypothesis of an allelic chemotype locus. It appears more likely that the genetic factors responsible for PJC, primarily affect floral morphology. In particular, they reduce the formation of capitate-stalked trichomes that have a high cannabinoid metabolism. Consequently, in the generative stage, PJC plants will retain a relatively large proportion of sessile trichomes and a low cannabinoid content. Under such circumstances, as in juvenile plants, CBC synthase appears to be put in a stronger competitive position. As has been already argued, this may be due to a variety of reasons: CBC synthase activity that is relatively high in sessile trichomes; differential kinetic parameters of the synthases involved; or a prolonged juvenile CBC synthase gene expression.

The  $F_2$ s from the Afghan  $\times$  CBG predominant crosses reached higher  $P_{CBC}$  values than the  $F_2$ s from the Afghan  $\times$  THC predominant crosses. Apparently, the  $B_0/B_0$  genotype, in which the pathway  $CBG \rightarrow THC$  is completely obstructed, and the pathway  $CBG \rightarrow CBD$  largely so, offers the best opportunities for breeding plants with a high purity of CBG’s third conversion product, CBC. In such plants, CBC synthase is only competing against a weak CBD synthase.

With the experiment where five clones were grown under different light levels, we confirmed the finding of Mahlberg and Hemphill (1983) that light stress increases the CBC proportion in the total cannabinoid fraction. The CBC proportion increased because the absolute CBC content in the botanical raw material remains more or less stable under low light levels,



**Fig. 6** Updated model for the regulation of the different conversions of CBG(V) by the independent loci *B* and *C*. Locus *B* has two common alleles,  $B_D$  and  $B_T$ , responsible for the conversion of CBG(V) into CBD(V) and THC(V) respectively. A mutation of  $B_D$ , called  $B_0$ , encodes a weak CBD synthase and leads, in the homozygous state, to CBG(V) accumulation and residual CBD(V) production. In heterozygous genotypes,  $B_D$  and  $B_T$  dominate  $B_0$ , whereas  $B_D$  and  $B_T$  are mutually codominant. Locus *C* is fixed and invariably encodes CBC synthase which converts CBG(V) into CBC(V). The activity of CBC synthase relative to CBD and/or THC synthase is variable. It is high in juveniles and in morphological variants lacking stalked glandular trichomes



whereas the content of the complementary cannabinoids CBD, THC and CBG decreases. The effect of light levels on the cannabinoid composition appears to be general and direct. The physiological development was not slowed down by reduced light, which could have led to an immature crop at harvest and an ontogenetic increase of the CBC proportion. In clone M272, a product of inbreeding an Afghan  $\times$  Korean,  $B_0/B_0$  cross progeny, CBC approached absolute purity under reduced light. This demonstrates that by further optimisation of the PJC genotype and its growing conditions, pure CBC pharmaceutical raw material production will be feasible, albeit that CBC yields will be relatively low.

The statement that the polygenic, quantitative cannabinoid yield components, such as the total cannabinoid content, are not related to specific metabolic pathways and are therefore independent from the cannabinoid composition, has been previously made (de Meijer et al. 2003; de Meijer and Hammond 2005).

This concept fully applies for the mutual ratios of the cannabinoids CBD, THC and CBG in mature chemotypes. Still, a nuance needs to be introduced with regard to CBC. Obviously, the features of morphological juvenility, low total cannabinoid content and high  $P_{CBC}$ , are inherited and segregate as a causally linked complex. It is also apparent that environment, specifically light intensity, has an effect on the cannabinoid composition of PJC plants, which is a deviation from the previous concept.

This paper adds the regulation of the pathway  $CBG \rightarrow CBC$  to the model for chemotype inheritance that was previously presented by de Meijer et al. (2003) and de Meijer and Hammond (2005). The extended model, presented in Fig. 6, covers the conversions of CBG into CBD, THC and CBC, as well as the accumulation of CBG. Although variation in the CBC proportion of the cannabinoid fraction can be large, CBC synthase activity appears to be a fixed trait and is not, like CBD and THC synthase activity, under the control

of the allelic chemotype locus ‘B’. It is proposed to indicate the fixed CBC synthase locus as ‘C’. The major variation in the CBC proportion is ontogenetic. It is characterised by a substantial CBC proportion shortly after seedling emergence, a rapid decline during the juvenile development and a stabilisation at minor levels in mature inflorescences. Two inheritable variations that primarily affect floral development, resulting in a reduced presence of capitate-stalked glandular trichomes, are presented. A low total cannabinoid content and a substantial CBC proportion at maturity are associated with this morphological feature, indicated here as a ‘prolonged juvenile chemotype’. Irrespective of CBC, the distribution of various compositions of CBD, THC and CBG, as observed in the current experiments, is fully in agreement with the previously published model for chemotype inheritance. However, the inverse relationship of CBC proportion and total cannabinoid content as observed in progenies from PJC plants, and the plasticity of the cannabinoid composition of PJC plants under different light levels are at variance with our preceding model.

**Acknowledgements** We are grateful to Giuseppe Mandolino for his useful comments on a primary version of the manuscript and to David Potter for introducing the possibility of a more prominent CBC biosynthesis in the sessile trichomes.

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