Chapter 6
Chemical and Morphological Phenotypes in Breeding of *Cannabis sativa* L.

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Abstract This chapter has two parts. The first part details five characters that contribute to phenotypic diversity in *Cannabis*. Cannabinoids can be assayed by quantity (dry weight percentage of cannabinoids in harvested material) or by quality (the THC/CBD ratio, or chemotype). Cannabinoid quality is largely genetic, possibly monogenic. We dissect the monogenic inheritance model (two alleles at a single gene locus). Essential oil is composed of volatile, aromatic terpenoids. Terpenoid content varies between different varieties. Hemp seed oil consists of polyunsaturated fatty acids, including omega-6 and omega-3 fatty acids, which are under genetic control. Protein has received less attention than oil, despite hemp's value as a protein supplement. Bast fibers are phloem (sap-conducting) cells in stalks. The second part presents the current breeding status of phenotypes for various uses. Breeding for fiber production includes monoecious cultivars, dioecious cultivars, high percentage of primary fiber, fast-retting phenotypes, and unique morphological markers in low-THC plants. Selective cross-breeding for cannabinoids includes prevalent-THC, prevalent-CBD, and cannabinoid-free plants. Relatively few cultivars have been bred specifically for seed production.

6.1 Introduction

A century ago, Italian farmers grew over 100,000 ha of industrial hemp annually (Ranalli and Casarini 1988). Seed for sowing was self-produced by the farmers. Breeding was by mass selection, where many individuals with desirable phenotypes

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were selected and their seeds harvested. Taller and thicker females were left in the field after complete fertilization because harvesting was done by hand. Local improvements gave rise to many landraces named after the province where they came from, such as Ferrara, Bologna, Modena, Rovigo, and Carmagnola.

Professional breeders began to crossbreed diverse landraces, subjected them to recurrent selection, and created the first hybrid *Cannabis* cultivars. Dewey (1928) crossed Ferrara with an inbred Chinese landraces to select 'Ferramington.' In Hungary, Fleischmann (1931) inbred landraces from Bologna and Ferrara to create 'F-hemp.' In Italy, Crescini (1934) introduced crossing and selfing, using both genders, to study morphological variants in Carmagnola and non-Italian varieties.

Hirata (1927) made the first studies on monoecious hemp derived from the 'Karafuto' landrace in Japan. In the Soviet Union, Grishko (1935) initiated work that led to monoecious hemp. And in Germany, Neuer and Sengbusch (1943) fixed the monoecious trait, and increased fiber content. Their efforts gave rise to 'Fibrimon,' a parent of modern cultivars from France ('Férimon,' 'Fédora,' 'Félina,' 'Futura,'), Ukraine ('Juso 11'), Poland ('Beniko,' 'Białobrezskie'), Hungary ('Uniko B'), and Romania ('Secuieni 1').

Plants with unique morphological traits may serve as easy-to-see markers of low-THC crops. Savelli (1932) described Ferrara plants with leaflets webbed into palmate lobes, which Crescini (1956) named the *pinnatofidofilla* mutation. Allavena (1961) isolated plants with *pinnatofidofilla* and *monofilla* ("simple leaf") while he bred 'Fibranova' from Carmagnola, Turkish, and German lines (Fig. 6.1a, b).

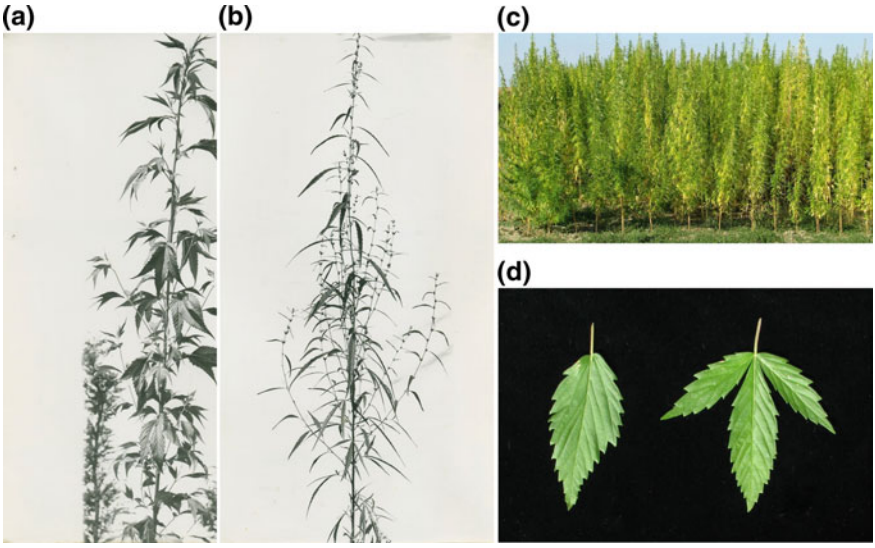

Fig. 6.1 **a** Hemp plant with *pinnatofidofilla* mophological character, **b** *Monofilla* character in Italian hemp line, photographs taken by Domenico Allavena in the 1950s, **c** First year of basic seed production for Carmaleonte in 2011, **d** Leaf variants. Simple leaf shape in 'Ermes' (on left) compared to usual tri-leafleted plant (on *right*)

de Meijer (1999) provides an excellent summary of 20th century breeding, more extensive than ours here. He describes traditional Italian cultivars, claimed by Clarke and Merlin (2013) as "practically unavailable," which is not true. Thanks to Bruno Casarini, three industrial hemp varieties are still available: 'Carmagnola,' 'C. S.' (Carmagnola Selezionata) and 'Fibranova.' Their lines remain pure and original because they have been multiplied in alternative years at the experimental station of CREA in Anzola Emilia (Bologna).

Because of space limitations, we refer the reader to other chapters in this book for prerequisite information. See Ernest Small and David Potter for basic anatomy underlying phenotypic variation. For more on genomics and molecular markers, see the chapters by Jonathan Page, Chiara Onofri and Giuseppe Mandolino.

6.2 Cannabinoids

Briosi and Tognini (1894) recognized glandular trichomes as the site of resin synthesis and accumulation. Recent work has focused on capitate stalked glandular (CSG) trichomes, which consist of two parts—a nearly-spherical resin head (gland head) atop a multicellular stalk. The resin head incorporates a rosette of secretory disk cells at its base, covered by a thin, distensible sheath or cuticle. Cannabinoids and terpenoids accumulate in a secretory cavity between the disk cells and the cuticle (Kim and Mahlberg 1997; Happyana et al. 2013). Disk cells also secrete biosynthetic enzymes, such as THCA synthase, into the secretory cavity (Sirikantaramas et al. 2005).

Cannabinoid biosynthesis requires phenol and terpenoid precursors (Taura et al. 1995, 1996, 2007, 2009). The pathway, with key chemical structures, is illustrated in Fig. 6.2. See the chapter by Supaart Sirikantaramas and Futoshi Taura for an elaboration. Cannabinoid content differs in terms of quantity and quality. Quantity and quality have different modes of inheritance (Hillig 2002). Cannabinoid quantity (dry weight percentage) is polygenic and influenced by environmental factors. Cannabinoid quality (the cannabinoid profile or chemotype) is largely genetic—possibly monogenic.

6.2.1 Cannabinoid Quantity

Cannabinoid quantity is assayed as dry weight percentage of cannabinoids in harvested material. Initially this was estimated as "percent resin," beginning with Procter (1864), who compared Indian gañjā (9% resin) with American hemp from Philadelphia (12% resin). Now we know percent resin is not a good indicator of psychoactive potency—high-CBD plants may also secrete a lot of resin.

Percent resin was abandoned after the discovery of cannabinoids. Americans searched for hemp plants with "low marihuana content" (Matchett et al. 1940;

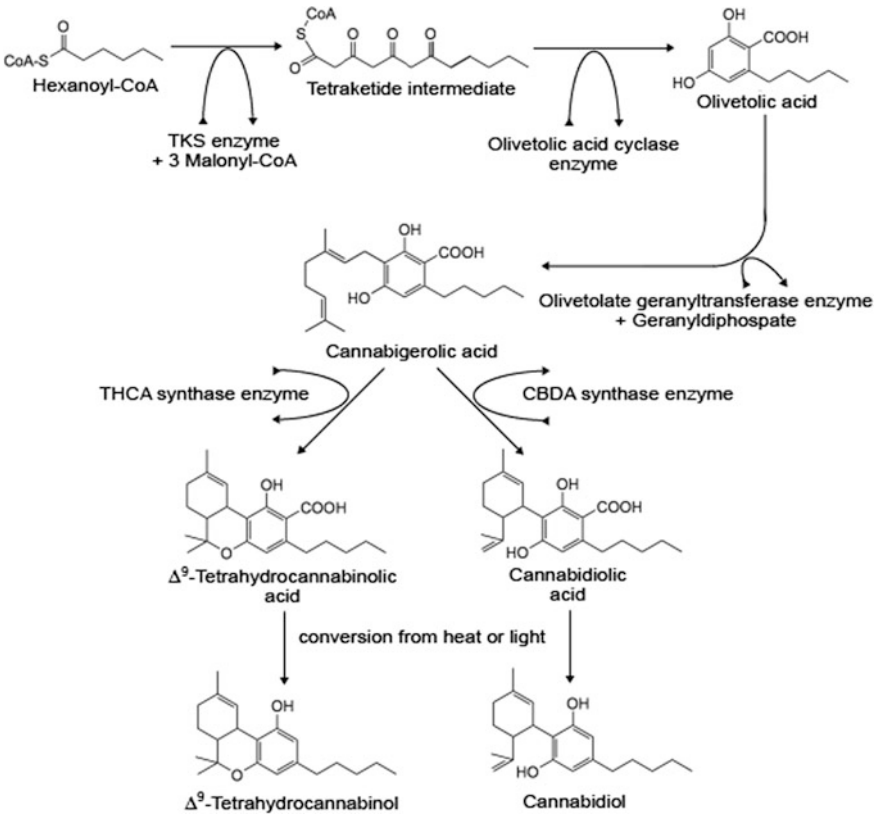

Fig. 6.2 Cannabinoid biosynthetic pathway, leading to the two major phytocannabinoids, THC and CBD (courtesy J. McPartland)

Robinson 1941). German breeders began selecting plants with "low hashīsh content" (Hitzemann 1941; Sengbusch 1956; Bredemann et al. 1956). Fournier (1981) bred low-THC plants, "this is probably the first time in the world that such action is taken." His statement's hubris is gauling [sic] because the French depended upon 'Fibrimon' developed by the aforementioned Germans.

Cannabinoid quantity is affected by many genes, and modulated by the environment. Genes determine a plant's chemotype and the expression of cannabinoid-producing machinery (i.e., density of CSG trichomes, size of resin heads). Gender is another genetic factor; female flowers produce more cannabinoids than male flowers. Environmental factors include photoperiod, light quantity and quality, soil nutrients, and temperature. Valid quantitative comparisons between plants must minimalize environmental variables. In a common garden experiment (CGE), plants of different provenances are grown in a single location, under identical environmental conditions, and uniformly processed.

Small and Cronquist (1976) chose a specific quantity, 0.3% THC in dried female flowering tops, as the dividing point between *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica*. This quantity was adopted as the maximum allowed in industrial hemp by the European Union (EU) and Canada. In 2001 the EU tightened the restriction to 0.2%. Reducing the cut-off by a third was overkill, because 1% THC is the threshold for psychoactivity (Chait et al. 1988; Grotenhermen and Karus 1998), and the 0.2% cut-off produced dramatic consequences in term of loss of genetic variability.

Measuring minute quantities of such a notoriously labile substance has pushed analytical capabilities to the limits of precision. For example, field samples are compared to THC reference standards, supplied by chemical companies, which unfortunately vary from their stated concentrations. Poortman van der Meer and Huizer (1999) distributed identical samples to 30 European laboratories, and they reported variable THC levels, with a relative standard deviation of 29%. In other words, around one-third of the labs reported THC levels either 29% above or 29% below the true value.

Accuracy also depends upon sampling protocol. Measuring cannabinoid levels at peak, uniform plant maturity is critical. Diverse definitions of "peak maturity" have plagued the testing of registered hemp cultivars. THC levels in 'Finola' varied from 0.05 to 0.32% in plants sampled at different dates (Callaway 2008). Protocols equate the sampling of female dioecious plants with the sampling of monoecious plants (a mix of male and female flowers). Given lower THC levels in male flowers, this introduces bias in favor of monoecious crops. The EU limit of 0.2% was crafted by regulators from France and Ukraine, whose plant breeders specialize in monoecious hemp. A French institute, L'Agence de Services et de Paiement, has been charged with policing EU hemp regulations (Bertucelli 2013, 2015).

6.2.2 Cannabinoid Quality

Cannabinoid quality is assayed as the THC/CBD ratio (THC percentage dry weight divided by CBD percentage dry weight). Breeders and taxonomists refer to this as the "cannabinoid profile" or "chemotype." As a dimentionless ratio, THC/CBD cancels two quantities (THC%, CBD%), and therefore provides a more valid comparison of many studies that grew plants under many different conditions.

Fetterman et al. (1971) presented data as a quotient of THC+CBN/CBD, and assigned plants to two populations: "drug-types" with a quotient >1.0, and "fiber-types" with a quotient <1.0. Unlike individual cannabinoid quantities, the ratio remained fairly stable in plants. The chemical phenotype of nine *Cannabis* accessions stayed the same, regardless of plant age, gender, plant part (flowers, leaves), year, or place of growth.

Fairbairn and Liebmann (1974) proposed that the "qualitative picture," THC- or CBD-prevalent plants, is a genetic trait independent of environmental conditions. In

dissent, Turner et al. (1979) highlighted an accession whose cannabinoid phenotype varied depending on gender and plant age.

Hemphill et al. (1980) also found the "cannabinoid profile" remained fairly constant, whereas quantitative levels of THC and CBD varied between female and male plants and between vegetative leaves and flower bracts. They analysed 12 strains of drug- and fiber-type plants.

Small and Beckstead (1973) measured THC% and CBD%, and omitted CBN% as an artifact of aging. They parsed a Cartesian graph into three sectors, with the horizontal axis divided by a line at CBD 0.5%, and the vertical axis divided by a line at THC 0.3%. Plotting a sample's THC% and CBD% in the graph categorized it as Type I: THC >0.3%, CBD <0.5%; Type II: THC >0.3%, CBD >0.5%; or Type III: THC <0.3%, CBD >0.5%. This innovative approach regrettably blurred the concepts of quantity and quality, by defining chemotype with quantitative measures. They also recognized Type IV plants, with significant levels of cannabigerol monomethylether (CBGM).

Fournier (1981) confused matters by defining two "chemotypes" within monoecious French hemp. Type I: average THC/CBD = 0.71 (corresponding to Small's Type II); Type II: average THC/CBD = 0.05 (corresponding to Small's Type III). Subsequently, Fournier et al. (1987) recognized three chemotypes: "Fiber": THC <0.3%, CBD >0.5%, THC/CBD <0.1; "Intermediate": THC >0.5%, CBD >0.5%, THC/CBD >0.5; "Drug": THC >2.0%, CBD <0%, THC/CBD undefined. They added a fourth phenotype, CBG-dominant plants (rather than Small's CBGM plants).

de Meijer et al. (1992) analyzed chemotypes using two approaches. They employed Small and Beckstead's graph (moving one dividing line to THC 0.5%) and plotted three fiber-type accessions. Some individual plants in all three accessions strayed from the Type III sector. Then they measured cannabinoid profile as a quotient of the THC/CBD ratio in 97 accessions, each accession's ratio determined from a bulked sample of 20 individual plants. For breeding purposes, de Meijer does not measure chemotype until he has subjected a landrace to at least three or four cycles of selfing.

Hillig and Mahlberg (2004) maximized qualitative aspects. They measured individual plants, and determined the proportion of chemotype I, II, and III individuals within each accession (previous researchers quantified THC% and CBD% within each accession by mixing bulked samples). They defined chemotype as a quotient, $\log_{10}$ (THC%/CBD%), Type I with a quotient >1.0, Type II with a quotient <−0.7, and plants with intermediate values assigned to Type II.

Chemotype stability has been confirmed in 21st century studies. De Backer et al. (2012) measured THC and CBD in clones—cuttings from three drug-type plants. THC levels increased during vegetation and flowering stages, but "the chemotype of clones was recognizable at any developmental stage."

Pacifico et al. (2008) inversed the cannabinoid ratio as CBD/THC. The quotient of this ratio is easier to read for breeders of high-CBD hemp plants. They measured cannabinoid content in 116 plants at 10 time-points, from seedling to flowering stages. They plotted results as $\log_{10}$ (CBD/THC), with values <0.0 assigned to

Type I, and >0.0 assigned to Type II/III plants. Only four of the 116 plants crossed the line at isolated time points, from Type II/III to Type I.

Broséus et al. (2010) tested four ways to identify chemotype in young, month-old seedlings of Type I plants (13 drug-type strains) and Type III plants (11 fiber-type cultivars). First they measured chemotype as (THC+CBN/CBD). This misclassified 8.1% of seedlings—three fiber-types and 20 drug-types (mostly from one strain, "Afghan"). Next they used principal component analysis (PCA) with eight compounds: THC, CBD, CBN, THCV, guaiol, bulnesol, γ-eudesmol, and α-bisabolol. The PCA scatterplot illustrated that most of the plants presented important differences in their chemical composition according to the selected compounds, except for a highlighted ellipse where 14 Type I and $\sim$100 Type III plants overlapped (Type III mostly 'Kompolti' and 'Fraise Sativa'). They subjected the same data set to linear discriminant analysis (LDA), a type of canonical analysis that uses machine learning with a training set. LDA yielded a 6.0% false positive fiber rate (FPF%, the percentage of samples classified as Type III whereas they are Type I), and a 0.3% FPD (false positive drug) rate. Lastly they applied a support vector machine (SVM), a model similar to LDA, but uses non-linear hyperplane mapping. SVM yielded 1.3% FPF and 0.3% FPD.

6.2.3 Cannabinoid Genetics

de Meijer et al. (2003) proposed that chemotype is determined by two alleles at a single gene locus, termed the *B* locus. The B_T allele encodes THCA-S, and the B_D allele encodes CBDA-S. Plants prevalent in THC and with little or no CBD have B_T/B_T genotypes. Plants prevalent in CBD and with little or no THC have B_D/B_D genotypes. Plants that produce nearly equal amounts of THC and CBD have B_T/B_D genotypes (de Meijer 2014). Thus B_T and B_D alleles do not express the classical Mendelian genetic behavior of binary traits, where one allele is dominant and one is recessive. In de Meijer's model, the alleles for THCA-S and CBDA-S are codominant, because both alleles are expressed. In other words, neither phenotype is recessive—heterozygous individuals express both phenotypes.

Previous breeding experiments by Yotoriyama et al. (1980) suggested codominant inheritance. They crossed THCA-dominant males with CBDA-dominant females, and the F_2 population consisted prevalent-THC plants (n = 40), mixed THC-CBD plants (n = 101), and prevalent-CBD plants (n = 58), a distribution consistent with segregation into codominant B_T/B_T, B_T/B_D, and B_D/B_D genotypes.

de Meijer's monogenic inheritance model requires further validation. There are discrepancies: THC/CBD ratios in *Cannabis* show continuous variation, and by no means segregate into 100% THC, 50:50, or 100% CBD populations. Kojoma et al. (2006) cloned THCA-S sequences from "fiber-type" plants that produced no detectable THCA—ostensibly B_D/B_D genotypes. Several THCA-S sequences were polymorphic, expressing a total of 37 amino acid substitutions. Kojoma proposed that these polymorphism decreased THCA-S activity in fiber-type plants. Thichak

et al. (2011) also showed that THC can be synthesized by B_D/B_D plants. They probed 100 Thai plants with PCR primers designed to amplify THCA-S. The allele was absent in 37 plants (B_D/B_D), yet five of them produced THC (mean 0.4%, range 0.28–0.60%).

Other models are out there. Japanese researchers reported classical Mendelian genetic behavior, rather than codominant segregation. Nishioka (in Isbell 1973) crossed a CBDA-producing strain with a THCA-producing strain, and "demonstrated that the CBDA producing strain was genetically recessive." Takashima (1982) crossed CBDA-dominant plants with THCA-dominant plants and suggested the latter trait is genetically dominant. Beutler and der Marderosian (1978) crossed a CBDA-dominant male plant with a THCA-dominant female plant, and the F_1s segregated into 2/3 high CBDA and 1/3 high THCA plants.

Cascini et al. (2013) challenged the monogenic inheritance model. They carried out bacterial cloning and real-time quantitative PCR of THCA-S in 12 *Cannabis* samples of unknown provenance. They reported a variable copy number for THCA-S in each sample, between one and four.

Weiblen et al. (2015) used the same methods to probe for THCA-S and CBDA-A genes. Drug-type "Skunk#1" yielded three polymorphic copies of THCA-S, and two copies of CBDA-S. The latter contained stop codons and frame shift mutations, thus were nonfunctional. Fiber-type 'Carmen' yielded one copy of CBDA-S and three copies of THCA-S copies; the latter were polymorphic and probably nonfunctional. Based on this and other evidence (Marks et al. 2009), Weiblen proposed that THCA- and CBDA-synthase are encoded by separate but linked regions.

Onofri et al. (2015) used the same methods to probe for THCA-S and CBDA-A in 18 strains of drug-type and fiber-type plants. They found many polymorphisms. Some strains expressed more than two transcribed sequences; the inbred hybrid "Haze" had five. They also measured THC and CBD content, and used this data to identify polymorphisms that expressed fully-functional enzymes, versus polymorphisms that expressed enzymes with less (or no) catalytic ability. Within the 18 strains, THCA-S averaged 2.9 SNPs (single nucleotide polymorphisms) per sequence, and CBDA-S averaged 5.7 SNPs per sequence.

Sequencing the *Cannabis* genome has presented more challenges to the monogenic inheritance model. Van Bakel et al. (2011) revealed the presence of more than one transcribed gene for THCA-S and for CBDA-S, as well as pseudogenes related to THCA-S and CBDA-S. McKernan et al. (2016) used Illumina (Next-Gen) genomic sequencing coupled with two different primer sets to generate amplicons for THCA-S in thirteen medicinal strains, including four high-CBD strains. Only one strain had a single THCA-S copy, the rest had multiple polymorphic copies. "Chemdog" expressed five THCA-S copies—one with a stop codon, one likely inactive, and three putatively active copies. Among the prevalent-CBD strains, "Sour Tsunami" expressed six THCA-S copies—three with frameshift mutations (stop codons), one inactive, one unknown, and one putatively active ("Sour Tsunami" does produce some THC).

6.3 Essential Oil

Cannabis essential oil gained a lot of early attention (O'Shaughnessy 1839; Bohlig 1840; Personne 1857; Valente 1880, 1881; Roux 1886; Valieri 1887; Prain 1893; Easterfield and Wood 1896). An essential oil is the volatile, aromatic liquid extracted from flowering tops by steam distillation, vaporization, or solvent extraction. The primary constituents of essential oil are terpenoids. *Cannabis* produces about 200 terpenoids, mostly monoterpenoids ($C_{10}H_{16}$ templates) and sesquiterpenoids (C_{15} H_{24} templates) (Rice and Koziel 2015).

Terpenoid biosynthesis in *Cannabis* goes through two independent but interactive pathways: The 2-methyl-D-erythritol-4-phosphate (MEP) pathway is responsible for monoterpenoids and some sesquiterpenoids. The mevalonate (MVA) pathway is responsible for most sesquiterpenoids. The MEP pathway generates geranyldiphosphate, the monoterpenoid precursor of cannabinoids.

Terpenoids are biosynthesized in glandular trichomes, and terpenoids account for up to 10% of resin head contents (Potter 2009). Günnewich et al. (2007) cloned and sequenced two *Cannabis* genes involved in monoterpenoid synthesis: limonene synthase and α-pinene synthase. Limonene smells "lemony" and α-pinene smells "piney". They can be extracted for use in perfumes and shampoos. More importantly, terpenoids modulate the effects of THC, and impart diverse medicinal benefits (McPartland and Pruitt 1999; McPartland and Mediavilla 2001; Russo 2011). This is not a new discovery: Prain (1893) extracted essential oil (terpenoids) and resin (cannabinoids) from Indian gañjā. He attributed gañjā's "*narcotic* effect" to the resin, and surmised, "It seems possible that to some extent the *exciting and exhilarating* effect of gañjā resides in an essential oil."

Hooper (1908) noted that the perceived quality and cost of three *charas* specimens correlated with their essential oil content and not with their resin content: Grade N° 1: essential oil 12.7% and resin 40.2%; Grade N° 2: essential oil 12.4% and resin 40.9%; Grade N° 3: essential oil 12.0% and resin 48.1%.

When Swiss industrial hemp cultivation restarted in the early 1990, entrepreneurs sold *Duftsäckli*, "fragrance pillows." These small cloth bags filled with flowering tops provided aromatherapy for anxiety, perfumed a bedroom, or mothproofed a closet. Scientist entrepreneurs gained federal support to study essential oils.

Mediavilla and Steinemann (1997) analyzed terpenoid profiles of 14 European fiber cultivars and five drug strains from Switzerland, Bolivia, and the USA. They also conducted scent tests with 15 volunteers, who gave high ratings to essential oils with high monoterpene percentages, and low ratings to essential oils with high sesquiterpene concentrations.

For field-cultivated plants, Mediavilla and Steinemann (1997) report an average yield of 1.3 L essential oil per ton of undried plants; equaling about 10 L ha^{-2}. Preventing pollination increases yield, Meier and Mediavilla (1998) obtained 18 L ha^{-2} from dioecious sinsemilla crops, versus 8 L ha^{-2} from pollinated crops. Mediavilla et al. (1999) ranked the suitability of cultivars as sources of essential oil,

led by 'Kompolti Hibrid TC,' 'Moldovan,' and 'Białobrezskie' (all judged suitable based on scent tests).

Growth stage and harvest date affect the monterpenoid/sesquiterpenoid ratio. Potter (2009) analyzed a prevalent-THC clone (G2 M6). The M/S ratio averaged 25.9/74.1 in young foliage (mostly sessile glandular trichomes), and flipped to 62.0/38.0 in flowering tops (mostly CSG trichomes). In flowering tops this ratio stayed fairly consistent irrespective of harvests date between weeks 9 and 13. Myrcene levels in flowering tops (47.2% of total) were three times higher than those in young foliage (14.8% of total), which dominated the flip in M/S ratios. A prevalent-CBD clone (G5 M13) resulted in similar trends across the board.

Potter (2009) found that steam-distilled fresh plant material yielded a very similar terpenoid profile to that of "enriched trichome preparation" (ice water hashīsh) made from the same plants. Potter reported very high yield rates obtained from a prevalent-CBD clone (G5 M16) grown outdoors: 7.7 ml m^{-2}. This extrapolates to 77 L ha^{-2}, seven times greater than Mediavilla. But Potter only harvested ten plants, which may have skewed yield results.

Casano et al. (2011) compared 16 proprietary hybrid accessions characterized as "mostly indica" or "mostly sativa." The two groups differed statistically in their terpenoid profiles. "Mostly indica" plants had higher levels of limonene, β-myrcene, camphene, and several unidentified peaks. "Mostly sativa" plants had higher levels of sabinene, Δ-3-carene, α-phellandrene, 1,8-cineole, cis-β-ocimene, trans-β-ocimene, α-terpinolene, and several unknowns.

Rice and Koziel (2015) analyzed odorous compounds emitted from marijuana, and showed that only a small fraction of volatiles causes its characteristic odor. To wit, compounds with high odor impact are not always the most abundant in concentration. About 11 compounds were under the detection level of the instrument but with positive odor impact. The most odorous compounds were aldehydes (e.g., benzaldehyde, decanal, meptanal) and terpenoids (β-myrcene, linalool, β-caryophyllene).

6.4 Hemp Seed Oil and Protein

Hemp seed analysis began soon after agricultural chemistry became a scientific discipline. Buchholz (1806) extracted 19.1% oil from German hemp seed. Anderson (1857) extracted 31.84% oil from Scottish hemp seed, and attributed Buchholz's results to "old and imperfect methods." The first direct comparison was made by Schaedler (1883), who measured oil content in German hemp (33.60%) and Russian hemp (31.42%). Next came Wherrell (1897), who compared Russian hemp (33.8%) and American hemp (30.3%). Kriese et al. (2004) compared oil content in 51 hemp cultivars, which ranged from 26.3% to 37.5%. They report no clear clustering according to geographic origin, although most of their accessions were European hybrid cultivars or unknowns.

Hemp seed oil consists of 75–85% polyunsaturated fatty acids (PUFAs), including omega-6 and omega-3 fatty acids, which are essential for human health (Deferne and Pate 1996). The primary omega-6 is linoleic acid (LA, 18:2 Ω6), and the major omega-3 is alpha-linolenic acid (ALA, 18:3 Ω3). Hemp oil also contains gamma-linolenic acid (GLA 18:3 Ω6) and stearidonic acid (SDA 18:4 Ω3), as well as monounsaturated fatty acids (oleic acid, 18:1 Ω9), and saturated fatty acids (e.g., palmitic acid, 16:0; stearic acid, 18:0) (Callaway 2004). The first number in the biochemical shorthand indicates the number of carbon atoms in the fatty acid. The second number, following the colon, indicates the number of double bonds. The third number, following the omega symbol, indicates the location of the first double bond in relation to the terminal (omega) methyl group.

Fatty acid profiles vary amongst varieties. Theimer and Mölleken (1995) proposed a "regiospecificity of unsaturation"—plants from higher latitudes produce a higher unsaturated/saturated ratio. Their evidence is weak: They measured nine fatty acids (only two PUFAs, LA and ALA), in five poorly-provenanced samples: "West Europe, Romania, Russia, Hungary, China." The Russian sample produced less ALA than the others, "Since this variety was grown in Southern Russia with subtropic climate these data indicate a temperature dependent regulation of fatty acid desaturation." However, the Russian sample produced more LA than any of the others.

Deferne and Pate (1996) supported the hypothesis, reasoning that unsaturated lipids remain more mobile at colder winter temperatures. Callaway et al. (1996) analyzed fatty acid profiles in 'Finola' (Central Russian), 'Kompolti' (Hungarian of Italian decent), and 'Futura-77' (hybrid of Central Russian, Italian, and Turkish landraces). 'Finola' produced more SDA, GLA, and ALA than the other two. However, 'Finola' produced the least amount of LA, the other PUFA in the study. Nevertheless the authors concluded that "more unsaturated fatty acid content among high-latitude origin *Cannabis* specimens… may reflect a regional evolutionary selection pressure."

Mölleken and Theimer (1997a, b) analyzed fatty acid profiles in over 500 accessions of fiber-, drug-, and wild-type plants from around the world. They present little data and no statistics. GLA levels were highest in a sample from Ermaskovskaya (Arkhangelsk) and lowest in a sample from Jamaica, so they reiterate the temperate versus tropic argument.

Ross et al. (1996) compared five world-wide accessions and found trends between the unsaturated/saturated ratio and geographical origin. However, the ratio clearly increased with seed maturity; therefore measuring seeds at uniform maturity is critical. Kriese et al. (2004) analyzed fatty acid profiles in 51 world-wide accessions, and detected four groups by hierarchical clustering. They found no clustering according to geographic origins, although true geographic provenance would be hard to determine because most of the accessions were hybrids.

Shelenga et al. (2012) measured nine fatty acids in 20 landraces collected across Russia. Unlike observations by Callaway and colleagues, SDA content was greatest in the most southern accession (Dagestan). From their data we plotted latitude against the sum of unsaturated fatty acids (SDA+GLA+ALA), and found no

correlation ($r^2 = 0.07$, $p = 0.27$), although the range in latitude was small, 43–57° N. Longitude ranged from 53 to 127° E, but no significant correlation was seen: $r^2 = 0.15$ ($p = 0.15$).

Climate and latitude use to be considered responsible for *C. indica* and *C. sativa* cannabinoid profiles. Now we know that genetics governs chemotypes. Similarly, fatty acid profiles are also under genetic control. The Indian Hemp Drugs Commission (1894) made an indirect comparison between *C. indica* and *C. sativa*. They analyzed seed from Hyderbad, compared their results with Frankfurt (1894), and concluded that Indian seeds contained more fiber but less oil than German seeds. Anwar et al. (2006) analyzed three accessions from across Pakistan, compared their results with European data, and came to the same conclusion.

Small et al. (1976) made the first direct comparison from an explicitly taxonomic perspective. They measured percent oil in 13 drug-type accessions (mean 27.7%) and 208 "less intoxicant" accessions (mean 32.9%), a significant difference ($p < 0.05$). The aforementioned study by Kriese et al. (2004) that clustered plants by their fatty acid profiles included a Korean landrace that segregated into a cluster by itself, due to low levels of SDA and GLA. Most accessions in her study were hybrids or unknowns, as with other comparative studies (e.g., Mediavilla et al. 1999; Small and Marcus 2000; Blade et al. 2005). GLA content has been increased from 2 to 4% in the 'Ermo' cultivar, after just two cycles of half-seed selection (Grassi, personal communication, 2016).

Protein has received less attention than oil, despite hemp's value as a protein supplement. The protein is concentrated in hemp seed cake—crushed hemp seed expelled of its oil fraction. Better yet, modern technology can peel the seed of its hard, fibrous shell, yielding a protein-rich dehulled kernel.

Buchholz (1806) led with the first analysis; he extracted 24.7% *eiweißstoffe* ("albuminous stuff") from German hemp seed. Anderson (1857) measured 22.60% in Scottish hemp seed, and noted Buchholz's similar results. The first direct comparison between hemp varieties was made by Schaedler (1883), who measured *eiweißstoffe* content in German hemp (15.95%) and Russian hemp (15.00%).

Callaway (2004) and Callaway and Pate (2009) provide new comparisons: Dehulled hempseed consists of 45% protein, compared to 32% in soybean and 11% in egg white. Hemp seed protein consists of about 66% edestin and 33% albumin. Both are globular proteins, highly digestible, and contain all essential amino acids. Edestin is analogous to casein in milk; albumin is the primary protein in egg white. The amino acid profile of hempseed is comparable to that in soybean protein and egg white protein.

6.5 Bast Fiber

Bast fibers are phloem (sap-conducting) cells in stalks of dicot plants. *Cannabis* produces phloem and xylem in concentric circles within a stalk (Fig. 6.3). Directly under the epidermis lies a ring of cortex (i.e., bark)—a mix of parenchyma cells and

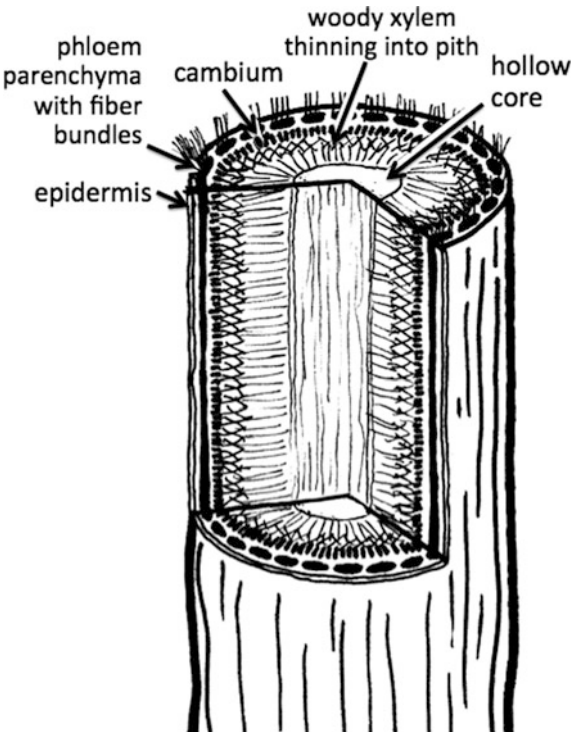

Fig. 6.3 Cross section of a hemp stalk (courtesy J. McPartland)

phloem "primary fibers." Primary fibers initiate in the growing tip of a plant, and elongate as the plant grows taller. They coalesce into bundles, with 10 to 40 primary fibers per bundle. Primary fibers constitute a small percentage of the stalk. de Meijer (1994) estimated 10–15% by weight of dry, unretted stalk in "natural" *Cannabis*; breeding in the 20th century has doubled that percentage. In one cross section of stalk, Snegireva et al. (2015) counted 6118 primary fibers.

Internal to the cortex is the ring of cambium. It consists of unspecialized meristem cells, which give rise to phloem (outwards) and xylem (inwards). Phloem cells arising from cambium are called "secondary fibers." Snegireva et al. (2015) estimated that a full-grown plant produces 700,000–800,000 primary fibers and two million secondary fibers.

Primary fiber cell length averages 25 mm (range 5–55 mm) and width averages 25 μm (range 10–50 μm). Primary fiber cell length is proportional to the length of the internode (Briosi and Tognini 1897). After an internode stops elongating, the cambium starts to form secondary fibers. Secondary fibers contribute to the girth of stalks, especially near the base. Secondary fiber cells are relatively short, and lack the tensile strength of primary fibers. Their length and width averages 7.6 mm and 7.9 μm (Snegireva et al. 2015). When hemp is processed for high-tensile yarn, the secondary fiber is separated as tow and used for other purposes.

Internal to the cambium lies a ring of xylem. Xylem fiber cells transport water. The cells are small, averaging 0.53 mm long and 32 μm wide (de Meijer 1994). Their walls are heavily lignified, and constitute the woody core of the stalk. The woody component of processed hemp is called the hurd (a.k.a., the core or shive). It thins out towards the center of the stalk, becoming pith. The center of the stalk is often hollow (Fig. 6.3).

6.6 Part II: Current Breeding Status

6.6.1 Fiber Production

Breeding for fiber production is economically constrained by today's limited use of European hemp fiber for textiles. In 2004 about 12 million Euros was invested in a hemp textile plant at Comacchio in Italy. The textile plant and regional farmers utilized harvesting and processing equipment designed for flax, which meant the hemp could only be 1 m tall. This strict condition was met by growing "baby hemp"—early varieties (e.g., 'Felina 34'), sown at 80–100 kg $ha^{-1}$of seed. When plants reached 1 m tall, they were killed with herbicide (4 kg ha^{-1}), and dew retted in the field. Unfortunately, crops under this agronomic regimen yielded little straw (3.0–3.5 ton ha^{-1}), with a low percentage of clean fiber (2–4% of long combed fiber). Income for farmers was extremely low so in 2007 the plant went bankrupt.

We could write a whole book on the subject of fiber-type hemp breeding, and several have (e.g., Ranalli and Casarini 1988; Bòcsa and Karus 1997; Capasso 2001; Bouloc 2006). Here we limit the discussion to new fiber-type cultivars bred in Italy for eco-friendliness and for unique "morphological markers."

Traditional water retting of hemp stalks is a microbiological process (retting is rotting), and poses ecological problems and health risks. To mitigate these risks, breeding programs have considered fast-retting varieties. 'Carmaleonte' is a monoecious cultivar whose fiber is easily separated by dew retting (Fig. 6.1c). It does not require water retting, which is environmental harmful. 'Carmaleonte' is a cross between 'Carmono' and 'Kompolti Sárgaszárú.' Bósca bred 'Kompolti Sárgaszárú' in the 1960s by crossing 'Kompolti' with a yellow-stalked mutant by Hoffmann (1947), who selected the yellow-stalked mutant from a cross between a Finnish landrace and an Italian landrace. A Dutch seed company has introduced two new monoecious varieties with fast retting characteristics named 'Markant' and 'Invory.' In the next future the new yellow stem variety named 'Fibror-79' will be available from Federation Nationale de Producteurs de Chanvre, FNPC (Thouminot, personal communication).

Di Candilo et al. (2000) subjected pollen of 'Carmagnola' and 'Fibranova' to ^{60}Co gamma radiation, which generated new dioecious cultivars with low THC and unique morphological markers. 'Red petiole' produces THC 0.09% and anthocyanin-tinted petioles—stable and uniform characters. 'Yellow apex'

produces THC 0.17% and yellow leaflets at the top of the plant. This morphological variant was incompletely dominant, and therefore after few cycles of multiplication, the green color returned.

'Ermes,' the first new Italian monoecious cultivar, shows a unique leaf mutation (Fig. 6.1d left). Instead of usual three-fingered digitate leaflets, 'Ermes' seedlings have leaflets webbed together into a palmate-lobed shape, or even a simple leaf shape (Canapa Industriale 2010). The character is recessive, and crossing with external pollen destroys the marker, so early visual examination allows the breeder to maintain a pure variety without a need to chemically analyze the progenies (Fig. 6.1d right). 'Ermes' derives from 'Fibranova,' bred with an autochthonous variety named SiMonA, obtained from an accession (CAN-19) shared with the IPK genebank in 1984. Grassi (pers. commun. 2015) crossed 'Ermes' with a low-cannabinoid male radiated with ^{60}Co gamma radiation, and selected 'Ermo.' This monoecious cultivar produces the typical spectrum of *Cannabis* terpenoids. Sprouts of 'Ermo' seeds express significant levels of two anti-inflammatory flavonoids, cannflavin A and B (Werz et al. 2014).

6.6.1.1 Cannabinoid Content

Selective cross-breeding of drug-type *Cannabis* accelerated in the 1970s. This clandestine effort shifted from the USA to the Netherlands in the 1980s, and breeders began selling hybridized "strains" (e.g., Watson 1985). Recreational strains became the foundation of a legitimate industry after the lifting of restrictions against medicinal cannabis.

HortaPharm BV took 'Medisins,' a "Skunk#1" clone, through the Plant Breeders Rights registration procedure in the Netherlands, and received European Breeders Rights in 1996 (de Meijer 1999). Two years later, HortaPharm's germplasm collections was transferred to GW Pharmaceuticals Plc in England. GW Pharmaceuticals has obtained Plant Breeders Rights for 'Gayle,' 'Grace,' 'Gill,' 'Galina,' and 'Guinevere' (Potter 2009). Bedrocan BV in the Netherlands produces 'Bedrocan,' 'Bedrodrobinol,' 'Bedica, 'Bediol' and more recently 'Bedrolite' (Hazekamp and Fischedick 2012), but not yet registered.

Burgeoning interest in medicinal CBD has led to the selection of high-yielding CBD hybrids. Sativex®, a standardized extract with a THC/CBD ratio of 1:1, blends a prevalent-THC strain, "G1," and a prevalent-CBD strain, "G5" (Potter 2004). Bedrocan's 'Bediol' is a prevalent-CBD strain (Fischedick et al. 2010). Breeders in USA states allowing medicinal cannabis have released "Charlotte's web," "Harlequin," "Cannatonic," "AC/DC," and many others (Lee 2013).

Fournier et al. (1987) described a CBG-predominant fiber-type variety, "Plant X," bred from an unnamed French monoecious cultivar. de Meijer and Hammond (2005) describe a "southern-Italian fiber hemp" whose cannabinoid profile was "79.6% pure CBG" (proportion of CBG in total cannabinoid fraction). They determined that CBG dominance is due to a mutation in the B_D allele that normally expresses the CBDA synthase enzyme.

Plant Breeders Rights were obtained for 'Carma,' a prevalent-CBG cultivar of Italian provenance. The cultivar yields two analogs of CBG. One is named carmagerol, where the terminal double bond is replaced by two hydroxyl groups (Appendino et al. 2008a). The other is a farnesyl prenylogue of CBG, sesqui-CBG (Pollastro et al. 2011). The cultivar also yields cannabimovone, a nonpsychoactive cannabinoid with a rearranged terpenoid skeleton (Taglialatela-Scafati et al. 2010).

'Carma' was selected from 'Carmagnola,' which expresses its own unique phytochemistry, such as cannabioxepane, a tetracyclic cannabinoid (Pagani et al. 2011). Many "minor" cannabinoids show potent antibacterial activity (Appendino et al. 2008b) and anti-inflammatory activity (Tubaro et al. 2010). 'Ermo' also obtained Plant Breeders Rights. Its flowering tops have a total cannabinoid content of only 0.05% (Onofri et al. 2015).

de Meijer et al. (2009a) selected a prevalent-CBC line by crossing mutants found in Afghan and Korean landraces. The plants produce relatively few perigonal bracts with CSG trichomes, leading to an abundance of sessile glandular trichomes. The phenotype is patent protected (US20110098348).

Fiber hemp breeders have long sought cannabinoid-free *Cannabis*. German breeders identified mutants lacking glandular trichomes, and characterized them as "completely hashish-free" (Sengbusch 1956; Bredemann et al. 1956). Ukrainian breeders identified two cannabinoid-free phenotypes: plants lacking glandular trichomes and plants whose glandular trichomes had white resin heads (Gorshkova et al. 1988). Ten years later Virovets (1998) released three monoecious lines with <0.03% THC: 'USO-11,' 'USO-14,' and 'USO-31.' A new generation of Ukrainian cultivars claim to be THC-free, such as 'Zolotonosha-15' and 'Hlukhivs'ki 33' (Holoborodko et al. 2008). French breeders created 'Férimon 12' with <0.1% THC by 1987, and released 'Santhica 23' in 1997, a "THC-free plant," whose dominant cannabinoid is CBG (Holoborodko et al. 2008).

de Meijer et al. (2009b) bred a "Zero" line. They started with five 'USO-31' individuals devoid of cannabinoids, crossed and back-crossed with THC-, THCV-, and CBD-dominant lines. Zero plants did not feel sticky; they produced CSG trichomes in normal densities, although the resin heads were smaller than normal plants. They attribute the absence of cannabinoids to a "knockout" of gene(s) for TKS or OAC enzymes (Fig. 6.2). The phenotype is patent protected (US9035130).

In Italy, a national program to produce medicinal cannabis began in 2014. It is organized by the Stabilimento Chimico Farmaceutico Militare in Florence, which belongs to the Army ministry. Unique varieties for producing the (dry flowers) are being supplied by CREA-CIN in Rovigo. CINRO is the name of the first variety, with about 8% CBD and 7% THC. The CINBOL variety yields about 20% THC. Varieties with 17% CBD and other minor cannabinoid combinations are under evaluation. (Grassi, personal communication, 2016).

6.6.1.2 Seed Production

Marquart (1919) reports a taxonomic character that no one else has measured: the ratio of seed yield to stalk yield. Three Russian landraces yielded the highest ratio: 35.2, 34.2, and 33.9%. Southern varieties yielded the least: Italian 5.6% and Turkish 3.8%. A German cultivar bred from Central Russian germplasm, 'Havelländischen hanf' (later called 'Schurig hanf') yielded a lot of seed compared to its fiber yield; the cultivar was fairly short (Heuser 1927). Serebriakova-Zinserling (1928) travelled to northern Russia, where she found short plants being cultivated for their seed oil; she coined a new variety, *C. sativa* var. *praecox*.

Relatively few cultivars have been bred specifically for seed production. Most hemp seed is obtained from "dual usage" cultivars harvested for both fiber and seed, such as 'Alyssa,' 'Crag,' 'Fasamo,' 'Tiborszállási,' 'USO-14,' and 'USO-31.' Breeders in Yúnnán selected 'Yún Má N^{o} 1' for dual usage, it yields 1500 kg/hm^2 seed and 12,750 kg/hm^2 stalks (Guo et al. 2011). Two cultivars registered in Spain have been grown for birdseed, 'Delta-Llosa' and 'Delta 405' (Gorchs and Lloveras 2003).

The oilseed cultivar 'Finola' (formerly 'FIN-314') is a cross of two northern Russian landraces from the Vavilov Institute. 'Finola' is dioecious, of short stature (1.5 m tall), and early maturation, ca. 100 days (Callaway et al. 1996; Callaway 2004).

'Finola' is a hemp version of Arabidopsis—the lab rat of plant genetics. Explants of 'Finola' have been grown under sterile conditions (Romocea and Grassi 2010). Its entire genome has been sequenced (Van Bakel et al. 2011). The 'Finola' transcriptome helped elucidate enzymes responsible for cannabinoid biosynthesis (Stout et al. 2012; Gagne et al. 2012). Bielecka et al. (2014) identified several enzymes responsible for unsaturated fatty acid production in 'Finola.'

The Canadian cultivar 'CanMa' is a cross of 'Finola' and 'ESTA-1.' Canadians have gone to seed in a big way. They have bred several dioecious seed varieties, such as 'ESTA-1,' a cross of ['ESTA-1' x 'Finola'] named 'CanMa,' as well as 'Petera,' 'CFX-1,' 'CFX-2,' and 'CRS-1.' However, 'Finola,' a Finnish cultivar, accounted for nearly a third of the national acreage (Alberta Agriculture and Forestry 2015).

In Italy, difficulties with fiber markets have turned attention to seed production. But as we mention above, southern (Mediterranean) seed yield is relatively low, and northern European varieties grown in the south flower too early. Breeding experiments are now underway crossing 'Finola' with 'Carmagnola'-derived varieties, with the introduction of monoecious trait (Grassi, personal communication, 2016).

House et al. (2010) analyzed seed chemistry in four cultivars, 'Crag,' 'Finola,' 'USO 14,' and 'USO 31.' They measured protein, oil, fiber, and 18 amino acids in four products: whole hemp seed, dehulled hemp seed, hemp seed cake, and hemp hulls. Furthermore they accessed protein digestibility in an in vivo (rat) assay, calculated an amino acid score based on a World Health Organization formula, and summed all this into a protein digestibility-corrected amino acid score (PDCAAS).

Table 6.1 Crude comparisons derived from data in House et al. (2010)

	'Crag'	'Finola'	'USO 31'	'USO 14'
Seed oil %	32.65	30.33	27.80	28.40
Seed protein %	25.53	22.97	23.70	22.65
NDF fiber %[a]	30.28	32.33	33.60	34.05
Amino acid score	'Crag' > 'USO 31' > 'Finola' ≈ 'USO 14'			
Digestibility	'Finola' > 'USO 14' > 'Crag' ≈ 'USO 31'			
PDCAAS	'Crag' > 'USO 31' > 'Finola' ≈ 'USO 14'			

[a]Neutral detergent fiber = less digestible fiber (i.e., cellulose, hemicellulose, lignin)

House and colleagues compared hemp with other foodstuffs. They did not directly compare the four cultivars. Rehashing their data is dicey (e.g., unequal sample sizes in whole seed comparisons, no dehulled data for 'Finola,' no seed cake data for 'USO 14'). No statistical inferences can be derived from these crude comparisons; they may not be statistical significant; nevertheless, see Table 6.1.

6.7 Conclusions

A 1938 article in Popular Mechanics Magazine famously claimed that hemp "can be used to produce more than 25,000 products" (Windsor 1938). Here we have focused upon cannabinoids, terpenoids, hemp seed oil and protein, and bast fiber. Hemp breeders are busy optimizing plants for these many products.

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References

Allavena D (1961) Fibranova, nuova varietà di canapa ad alto contenuto di fibra. Sementi Elette 5:34–44

Anderson T (1857) On the composition of hemp-seed. Trans Highland Agric Soc Scotland (Ser III) 7:128–130

Anwar F, Latif S, Ashraf M (2006) Analytical characterization of hemp (*Cannabis sativa*) seed oil from different agro-ecological zones of Pakistan. J Am Oil Chem Soc 83:323–399

Appendino G, Giana A, Gibbons S, Maffei M, Gnavi G, Grassi G, Sterna O (2008a) A polar cannabinoid from *Cannabis sativa* var. Carma. Nat Prod Commun 3:1977–1800

Appendino G, Gibbons S, Giana A, Pagani A, Grassi G, Stavri M, Smith E, Rahman MM (2008b) Antibacterial cannabinoids from *Cannabis sativa*: a structure-activity study. J Nat Prod 71:1427–1430

Bertucelli S (2013) Legislative controls on the cultivation of hemp. In: Bouloc P (ed) Hemp: industrial production and uses. CABI, Wallingford, UK, pp 125–127
Bertucelli S (2015) La filière du chanvre industriel, éléments de compréhension macroéconomiques. OCL 22(6):D602
Beutler JA, der Marderosian AH (1978) Chemotaxonomy of *Cannabis*. I. crossbreeding between *Cannabis sativa* and *C. ruderalis,* with analysis of cannabinoid content. Econ Bot 32:387–394
Bielecka M, Kaminski F, Adams I, Poulson H, et al (5 additional authors) (2014) Targeted mutation of D12 and D15 desaturase genes in hemp produce major alterations in seed fatty acid composition including a high oleic hemp oil. Plant Biotechnol J 12:613–623
Blade SF, Ampong-Nyarko K, Przybylski R (2005) Fatty acid and tocopherol profiles of industrial hemp cultivars grown in the high latitude prairie region of Canada. J Ind Hemp 10(2):33–43
Bòcsa I, Karus M (1997) Der Hanfanbau: Botanik, Sorten. Anbau und Ernte. C.F, Müller, Heildelberg
Bohlig JF (1840) *Cannabis sativa* und *Urtica dioica* chemisch analysiert. Jahrbuch für praktische Pharmacie und verwandte Fächer 3:1–58
Bouloc P (ed) (2006) Le chanvre industriel, production et utilisation. Éditions France Agricole, Paris
Bredemann G, Schwanitz F, Sengbusch RV (1956) Problems of modern hemp breeding, with particular reference to the breeding of varieties with little or no hashish. Bull Narc 8(3):31–35
Briosi G, Tognini F (1894) Intorno alla anatomia della canapa (*Cannabis sativa* L.) parte prima—organi sessuali. Atti dell'Instituto Botanico di Pavia (Serie II) 3:91–209
Briosi G, Tognini F (1897) Intorno all'anatomia della canapa (*Cannabis sativa* L.) parte seconda—organi vegetativi. Atti dell'Istituto Botanico di Pavia (Serie II) 4:167–329
Broséus J, Anglada F, Esseiva P (2010) The differentiation of fibre- and drug type *Cannabis* seedlings by gas chromatography/mass spectrometry and chemometric tools. Forensic Sci Int 200:87–92
Buchholz CF (1806) Beiträge zur pflanzenchemie. Analyse des hanfsamens. Neues allgemeines J der Chem 6:615–630
Callaway JC, Tennilä T, Pate DW (1996) Occurrence of "omega-3" stearidonic acid (cis-6,9,12,15-octadecatetraenoic acid) in hemp (*Cannabis sativa* L.) seed. J Int Hemp Assoc 3(2):61–64
Callaway JC (2004) Hempseed as a nutritional resource: an overview. Euphytica 140:65–72
Callaway JC (2008) A more reliable evaluation of hemp THC levels is necessary and possible. J Ind Hemp 13:117–144
Callaway JC, Pate DW (2009) Hempseed oil. In: Moreau RA, Kamal-Eldin A (eds) Gourmet and Health-Promoting Specialty Oils. American Oil Chemists Society Press, Urbana IL, pp 185–213
Canapa Industriale (2010) *Applicazioni biomediche di canapa.* Available at: http://www.isci.it/Rovigodocument/Linee_di_ricerca/Biomedical.html
Capasso S (2001) Canapicoltura: passato, presente e futuro. Istituto di Studi Atellani, Napoli
Casano S, Grassi G, Martini V, Michelozzi M (2011) Variations in terpene profiles of different strains of *Cannabis sativa* L. Acta Horticulturae 925:115–121
Cascini F, Passerotti S, Boschi I (2013) Analysis of THCA synthase gene expression in *Cannabis*: a preliminary study by real-time quantitative PCR. Forensic Sci Int 231:208–212
Chait LD, Evans SM, Grant KA, Kamien JB, Johanson CE, Schuster CR (1988) Discriminative stimulus and subjective effects of smoked marijuana in humans. Psychopharmacology 94:206–212
Clarke RC, Merlin MD (2013) Cannabis evolution and ethnobotany. University of California Press, Berkeley USA
Crescini F (1934) Indagine intorno all'eredità dei caratteri in *Cannabis sativa* L. L'Italia Agricola 71(3):1–26
Crescini F (1956) La fecondazione incestuosa processo mutageno in *Cannabis sativa* L. *Caryologia* 9(l):82–92

Di Candilo M, Di Bari V, Giordano I, Grassi G, Pentagelo A, Ranalli P (2000) Due nuovi genotipi di canapa da fibra: descrizione morfo-produttiva. Sementi Elette 46:25–31

De Backer B, Maebe K, Werstraete AG, Charlier C (2012) Evolution of the content of THC and other major cannabinoids in drug-type *Cannabis* cuttings and seedling during growth of plants. J Forensic Sci 57:918–922

Deferne JL, Pate DW (1996) Hemp seed oil: a source of valuable essential fatty acid. J Int Hemp Assoc 3(1):1–7

de Meijer EPM, van der Kamp HJ, van Eeuwijk FA (1992) Characterisation of *Cannabis* accessions with regard to cannabinoid content in relation to other plant characters. Euphytica 62:187–200

de Meijer EPM (1994) Diversity in Cannabis. Doctoral thesis, Wageningen Agricultural University, Wageningen, The Netherlands

de Meijer EPM (1999) *Cannabis* germplasm resources. In: Ranalli P (ed) Advances in Hemp research, pp 133–151. Haworth Press, New York

de Meijer EPM, Bagatta M, Carboni A, Crucitti P, Cristiana Moliterni VM, Ranalli P, Mandolino G (2003) The inheritance of chemical phenotype in *Cannabis sativa* L. Genetics 163:335–346

de Meijer EPM, Hammond KM (2005) The inheritance of chemical phenotype in *Cannabis sativa* L. (II): cannabigerol predominant plants. Euphytica 145:189–198

de Meijer EPM, Hammond KM, Micheler M (2009a) The inheritance of chemical phenotype in *Cannabis sativa* L. (III): variation in cannabichromene production. Euphytica 165:293–311

de Meijer EPM, Hammond KM, Sutton A (2009b) The inheritance of chemical phenotype in *Cannabis sativa* L. (IV): cannabinoid-free plants. Euphytica 168:95–112

de Meijer EPM (2014) The chemical phytotypes (chemotypes) of *Cannabis*. In: Pertwee RG (ed) Handbook of Cannabis. Oxford University Press, Oxford, UK, pp 89–110

Dewey LH (1928) Hemp varieties of improved type are result of selection. In: USDA Yearbook 1927, pp 358–361. United States Department of Agriculture, Washington DC

Easterfield TH, Wood TB (1896) The constituents of Indian hemp resin. Proc Camb Phil Soc 9:144–148

Fairbairn JW, Liebmann JA (1974) The cannabinoid content of *Cannabis sativa* L grown in England. J Pharm Pharmacol 26:413–419

Fetterman PS, Seith ES, Waller CW, Guerrero O, Doorenbos NJ, Quimby MW (1971) Mississippi-grown *Cannabis sativa* L.: preliminary observation on chemical definition of phenotype and variations in tetrahydrocannabinol content versus age, sex, and plant part. J Pharm Sci 60:1246–1249

Fischedick J, Van Der Kooy F, Verpoorte R (2010) Cannabinoid receptor 1 binding activity and quantitative analysis of *Cannabis sativa* L. smoke and vapor. Chem Pharm Bull 58:201–207

Fleischmann R (1931) Hanf- und Flachskultur in Ungarn. Faserforschung 9:143–149

Fournier G (1981) Les chimiotypes du chanvre (*Cannabis sativa* L.) Intérêt pour un programme de selection. Agronomie 1:679–688

Fournier G, Richez-Dumanois C, Duvezin J, Mathieu JP, Paris M (1987) Identification of a new chemotype in *Cannabis sativa*: cannabigerol-dominant plants, biogenetic and agronomic prospects. Planta Med 53(3):277–280

Frankfurt S (1894) Über die Zusammensetzung der Samen und der etiolierten Keimpflanzen von *Cannabis sativa* und *Helianthus annuus*. Landwirtschaftlichen versuchs-stationen 43:143–182

Gagne SJ, Stout JM, Liu E, Boubakir Z, Clark SM, Page JE (2012) Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. Proc Natl Acad Sci U S A 109:12811–12816

Gorchs G, Lloveras J (2003) Current status of hemp production and transformation in Spain. J Ind Hemp 8(1):45–64

Gorshkova LM, Senchenko GI, Virovets VG (1988) Способ оценки растений конопли на содержание каннабиноидных соединений [Method of evaluating hemp plants for content of cannabinoid compounds]. Реферативный журнал 12(65):322. See also patent SU1380687

Grishko NN (1935) Биология конопли (*The biology of cannabis*). State Publishing House of Collective and State Farms Literature, Kiev-Kharkov
Grotenhermen F, Karus M (1998) Indusrial hemp is not marijuana: comments on the drug potential of fiber Cannabis. J Int Hemp Assoc 5:96–101
Günnewich N, Page JE, Köllner TG, Degenhardt J, Kutchan TM (2007) Functional expression and characterization of trichome-specific (-)-limonene synthase and (+)-a-pinene synthase from *Cannabis sativa*. Nat Prod Commun 2:223–232
Guo HY, Guo MB, Hu XI, Xu YP, Wu JX, Zhang QY, Chen X, Yang M (2011) Industrial hemp variety 'Yúnmá N^{o} 1' seed and stalk high yield cultivation model. Southwest China J Agric Sci 24(3):888–895
Happyana N, Agnolet S, Muntendam R, van Dam A, Schneider B, Kayser O (2013) Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR. Phytochemistry 87:51–59
Hazekamp A, Fischedick JT (2012) *Cannabis*—from cultivar to chemovar. *Drug Testing and Analysis*. wileyonlinelibrary.com, doi:10.1002/dta.407
Hemphill JK, Turner JC, Mahlberg PG (1980) Cannabinoid content of individual plant organs from different geographical strains of *Cannabis sativa* L. J Nat Prod 43:112–122
Heuser O (1927) Die Hanfpflanze. In: Herzog RO (ed) Technologie der Textilfasern, Band 5, Teil 2: Hanf und Hartfasem. Julius Springer, Berlin, pp 1–102
Hillig KW (2002) Letter to the editor. J Ind Hemp 7(1):5–7
Hillig KW, Mahlberg PG (2004) A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). Am J Bot 91(6):966–975
Hirata K (1927) Sex determination in hemp (*Cannabis sativa* L.). J Genet 19(1):65–79
Hitzemann W (1941) Untersuchungen auf "Haschisch" bei verschiedenen Hanfsorten eigenen Anbaues in Deutschland. Arch Pharm 279:353–387
Hoffmann W (1947) Helle Stengel—eine wertvolle Mutation des Hanfes (*Cannabis sativa* L.). Der Züchter 17/18(2):56–59
Holoborodko P, Virovets V, Laiko I, Bertucelli S, Beherec O, Fournier G (2008) Results of efforts by French and Ukrainian breeders to reduce cannabinoid levels in industrial hemp (*Cannabis sativa* L.). Available at:www.interchanvre.com/docs/article-Laiko.pdf
Hooper D (1908) Charas of Indian hemp. Year-Book Pharm 1908:435–444
House JD, Neufeld J, Leson G (2010) Evaluating the quality of protein from hemp seed (*Cannabis sativa* L.) products through the use of the protein digestibility-corrected amino acid score method. J Agric Food Chem 58:11801–11807
Indian Hemp Drugs Commission (1894) Report of the Indian hemp drugs commission, 1893–1894. Government Central Printing Office, Simla
Isbell H (ed) (1973) Research on cannabis (marihuana). Bull Narc 25:37–48
Kim ES, Mahlberg PG (1997) Immunochemical localization of tetrahydrocannabinol (THC) in cryofixed glandular trichomes of *Cannabis* (Cannabaceae). Am J Bot 84:336–342
Kojoma M, Seki H, Yoshida S, Muranaka T (2006) DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" *Cannabis sativa* L. Forensic Sci Int 159(2–3):132–140
Kriese U, Schumann E, Weber WE, Beyer M, Brühl L, Matthäus B (2004) Oil content, tocopherol composition and fatty acid patterns of the seeds of 51 *Cannabis sativa* L. genotypes. Euphytica 137:339–351
Lee MA (2013) Project CBD update: the tango of supply and demand. O'Shaughnessy's Winter/Spring 2013:22–23
Marks MD, Tian L, Wenger JP, Omburo SN, Soto-Fuentes W, He J, Gang DR, Weiblen GD, Dixon RA (2009) Identification of candidate genes affecting delta-9-tetrahydrocannabinol biosynthesis in *Cannabis sativa*. J Exp Bot 60:3715–3726
Marquart B (1919) Der Hanfbau. Paul Parey, Berlin
Matchett JR, Levine J, Benjamin L, Robinson BB, Pope OA (1940) Marihuana investigations. II, the effect of variety, maturity, fertilizer treatment and sex on the intensity of response to the Beam tests. J Am Pharm Assoc (Sci Edn) 29:399–404

McKernan KJ, Helbert Y, Tadigotla V, McLaughlin S, Spangler J, Zhang L, Smith D (2016) Single molecule sequencing of THCA synthase reveals copy number variation in modern drug-type *Cannabis sativa* L. BioRxiv. doi:10.1101/028654

McPartland JM, Pruitt PL (1999) Side effects of pharmaceuticals not elicited by comparable herbal medicines: the case of tetrahydrocannabinol and marijuana. Altern Ther Health Med 5:57–62

McPartland JM, Mediavilla V (2001) Nichcannabinoide Inhaltsstoffe von *Cannabis*. In: Grotenhermen F (ed) Cannabis und Cannabinoide. Verlag Hans Huber, Bern, Switzerland, pp 429–436

Mediavilla V, Steinemann S (1997) Essential oil of *Cannabis sativa* L. strains. J Int Hemp Assoc 4 (2):82–84

Mediavilla V, Bassetti P, Leupin M, Mosimann E (1999) Agronomic characteristics of some hemp genotypes. J Int Hemp Assoc 6(45):48–53

Meier C, Mediavilla V (1998) Factors influencing the yield and the quality of hemp (*Cannabis sativa* L.) essential oil. J Ind Hemp Assoc 5(1):16–20

Mölleken H, Theimer RR (1997a) Survey of minor fatty acids in *Cannabis sativa* L. fruits of various origins. J Ind Hemp Assoc 4(1):13–17

Mölleken H, Theimer RR (1997b) Evaluierung von *C. sativa* saatgutherkünften im Hinblick auf ein verbesserte Ölqualität, pp. 485–499. In: Bioresource Hemp 97, Proceedings of the Symposium, Frankfurt, Germany. nova-Institute, Köln, Germany

Neuer HV, Sengbusch RV (1943) Die Geschlechtsvererbung bei Hanf und die Züchtung eines monöcischen Hanfes. Der Züchter (Zeitschrift für theoretische und angewandte Genetik) 15 (3):49–62

Onofri C, de Meijer EPM, Mandolino G (2015) Sequence heterogeneity of cannabidiolic- and tetrahydrocannabinolic acid-synthase in *Cannabis sativa* L. and its relationship with chemical phenotype. Phytochemistry 116:57–68

O'Shaughnessy WB (1839) Extract from a memoir on the preparation of the Indian hemp, or gunjah, *(Cannabis indica)* their effects on the animal system in health, and their utility in the treatment of tetanus and other convulsive diseases. J Asiatic Soc Bengal 8(732–744):838–851

Pacifico D, Miselli F, Micheler M, Carboni A, Moschella A, Mandolino G (2008) Time course of cannabinoid accumulation and chemotype development during the growth of *Cannabis sativa* L. Euphytica 160:231–240

Pagani A, Scala F, Chianese G, Grassi G, Appendino G, Taglialatela-Scafati O (2011) Cannabioxepane, a novel tetracyclic cannabinoid from hemp, *Cannabis sativa* L. Tetrahedron 67:3369–3373

Personne J (Robiquet E, ed.) (1857) Rapport sur le concours relatif à l'analyse du chanvre présente au nom de la Société de Pharmacie. J Pharm Chim (Ser 3) 31:46–51

Pollastro F, Taglialatela-Scafati O, Allarà M, Muñoz E, Di Marzo V, De Petrocellis L, Appendino G (2011) Bioactive prenylogous cannabinoid from fiber hemp (Cannabis sativa). J Nat Prod 74:2019–2022

Poortman van der Meer AJ, Huizer H (1999) A contribution to the improvement of accuracy in the quantitation of THC. Forensic Sci Int 101:1–8

Potter D (2004) Growth and morphology of medical cannabis. In: Guy G, Robson R, Strong K, Whittle B (eds) The medicinal use of Cannabis. Royal Society of Pharmacists, London, pp 17–54

Potter D (2009) The propagation, characterisation and optimisation of *Cannabis sativa* L. as a phytopharmaceutical. Doctoral thesis, King's College, London

Prain D (1893) Report on the cultivation and use of gánjá. Bengal Secretariat Press, Calcutta

Procter W (1864) On a test for the resin of *Cannabis indica*. Proc Am Pharm Assoc 12:244–248

Rice S, Koziel JA (2015) Characterizing the smell of marijuana by odor impact of volatile compounds: an application of simultaneous chemical and sensory analysis. PLoS ONE 10(12): e0144160

Ranalli P, Casarini B (1988) Canapa: il ritorno di una coltura prestigiosa. Avenue Media Press, Bologna

Robinson BB (1941) Marihuana investigations. IV. A study of marihuana toxicity on goldfish applied to hemp breeding. J Am Pharm Assoc (Sci Edn) 30:616–619
Romocea JE, Grassi G (2010) In vivo culture of hemp culture for textile and pharmaceutical industry. In: Conference Proceedings, Innovative solutions for sustainable development of textiles industry. University of Oradea, Romania
Roux F (1886) Etude sur la cannabine. Bulletin general de Thérapeutique Médicale et Chirugicale 111:492–514
Ross SA, ElSohly HN, ElKashoury EA, ElSohly MA (1996) Fatty acids of cannabis seeds. Phytochem Anal 7:279–283
Russo EB (2011) Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol 163:1344–1364
Savelli R (1932) Studien über den Ferrarischen Hanf. Der Züchter 4:286–290
Schaedler C (1883) Die Technologie der Fette und Oele: Technologie der Fette und Oele des Pflanzen- und Thierreichs. Polytechnische Buchhandlung, Berlin
Serebriakova-Zinserling TY (1928) Ранняя конопли (Early hemp). Trudy po Prikladnoi Botanike, Genetike i Selektsii 18(1):407–410
Sengbusch RV (1956) Le chanvre "Fibrimon" et "Fibridia." In: Proceedings of the second international Flax and Hemp Congress, Courtrai, June 5–9 1956, pp 16-24. Berichte des Instituts für Bastfaserforschung, Wageningen, The Netherlands
Shelenga TV, Grigory'ev SV, Illarionova KV (2012) Биохимическая характеристика семян конопли (*Cannabis sativa* L.) из различных регионов России [Biochemical characterization of hemp seed (*Cannabis sativa* L.) from different regions of Russia]. Труды по прикладной ботанике, генетике и селекции 170:212–219
Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y (2005) Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes. Plant Cell Physiol 46:1578–1582
Small E, Beckstead HD (1973) Common cannabinoid phenotypes in 350 stocks of *Cannabis*. Lloydia 36:144–165
Small E, Cronquist A (1976) A practical and natural taxonomy for *Cannabis*. Taxon 25(4):405–435
Small E, Marcus D (2000) Hemp germplasm trials in Canada. Bioresource Hemp, 3rd International Symposium, Wolfsburg, Germany. Online proceedings: www.hemphasis.com/files/publications/biorpap.htm
Small E, Jui P, Lefkovitch LP (1976) A numerical taxonomic analysis of *Cannabis* with special reference to species delimitation. Syst Bot 1:67–84
Snegireva A, Chernova T, Ageeva M, Lev-Yadun S, Gorshkova T (2015) Intrusive growth of primary and secondary phloem fibres in hemp stem determines fibre-bundle formation and structure. AoB Plants 7: plv061
Stout JM, Boubakir Z, Ambrose SJ, Purves RW, Page JE (2012) The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in *Cannabis sativa* trichomes. Plant J 71:353–365
Taglialatela-Scafati O, Pagani A, Scala F, De Petrocellis L, Di Marzo V, Grassi G, Appendino G (2010) Cannabimovone, a cannabinoid with a rearranged terpenoid skeleton from hemp. Eur J Org Chem 11:2067–2072
Takashima D (1982) On the development of non-toxic hemp 'White Tochigi' (in Japanese). Tochigi Prefectural Agric Exp Stat Rep 28:47–54
Taura F, Morimoto S, Shoyama Y, Mechoulam R (1995) First direct evidence for the mechanism of Δ^1-tetrahydrocannabinolic acid biosynthesis. J Am Chem Soc 117:9766–9767
Taura F, Morimoto S, Shoyama Y (1996) Purification and characterization of cannabidiolic-acid synthase from *Cannabis sativa* L. Biochemical analysis of a novel enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid. J Biol Chem 271:17411–17416
Taura F, Sirikantaramas S, Shoyama Y, Yoshikai K, Shoyama Y, Morimoto S (2007) Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type *Cannabis sativa* L. FEBS Lett 581:2929–2934

Taura F, Tanaka S, Taguchi C, Fukamizu T, Tanaka H, Shoyama Y, Morimoto S (2009) Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. FEBS Lett 583(12):2061–2066

Theimer RR, Mölleken H (1995) "Analysis of the oil from different hemp (*Cannabis sativa* L.) cultivars—perspectives for economical utilization," pages 536–543 in *Bioresource Hemp*, *Proceedings of the Symposium, Frankfurt, Germany.* nova-Institute, Köln, Germany

Thichak S, Natakankitkul S, Chansakaow S, Chutipongvivate S (2011) Identification of drug-type and fiber-type of hemp (*Cannabis sativa* L.) by multiplex PCR. Chiang Mai J Sci 38(4):608–618

Tubaro A, Giangaspero A, Sosa S, Negri R, Grassi G, Casano S, Della Loggia R, Appendino G (2010) Comparative topical anti-inflammatory activity of cannabinoids and cannabivarins. Fitoterapia 81:816–819

Turner CE, Elsohly MA, Cheng PC, Lewis G (1979) Constituents of *Cannabis sativa* L.; XIV: intrinsic problems in classifying *Cannabis* based on a single cannabinoid analysis. J Nat Prod 42:317–319

Valente L (1880) Sull'essenza di canapa. Gazzetta Chimica Italiana 10:479–481

Valente L (1881) Studi sull'essenza di canapa. *Atti della Reale Accademia dei Lincei* (Series 3) 5: 126–128 (reprinted as "Sull'idrocarburo estratto dalla canapa". Gazzetta Chimica Italiana 11 (1881):196-198)

Valieri R (1887) Sulla canapa nostrana e suoi preparati in sostituzione della *Cannabis indica*. Stabilimento tipografico dell'unione, Naples

Van Bakel H, Stout JM, Cote AG, Tallon CM, Sharpe AG, Hughes TR, Page JE (2011) The draft genome and transcriptome of *Cannabis sativa* L. Genome Biol 12(10):R102

Virovets VG (1998) Interview. J Int Hemp Assoc 5:32–34

Watson DP (1985) Cultivator's choice catalog #4. Self-published, Amsterdam, Holland

Weiblen GD, Wenger JP, Craft KJ, ElSohly MA, Mehmedic Z, Treiber EL, Marks MD (2015) Gene duplication and divergence affecting drug content in *Cannabis sativa*. New Phytol 208:1241–1250

Werz O, Seegers J, Schaible AM, Weinigel C, Barz D, Koeberle A, Allegrone G, Pollastro F, Zampieri L, Grassi G, Appendino G (2014) Cannflavins from hemp sprouts, a novel cannabinoid-free hemp food product, target microsomal prostaglandin E2 synthase-1 and 5-lipoxygenase. Pharma Nutrition 2:53–60

Wherrell O (1897) Hemp-seed and hemp-seed oil. Bull Pharm 11:340–342

Windsor HH (ed) (1938) New billion-dollar crop. Popular Mech Mag 69(2):238–239, 144A

Yotoriyama M, Ito I, Takashima D, Shoyama Y, Nishioka I (1980) Plant breeding of *Cannabis*. Determination of cannabinoids by high-pressure liquid chromatography. Yakugaku Zasshi 100:611–614